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Maria Diakonova *Editor*

Recent Advances in Prolactin Research

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Preface

This book is devoted to some aspects of the prolactin action. Although prolactin (PRL) was discovered more than 80 years ago, our understanding of the roles of PRL in the human physiology is still very incomplete. Thus, PRL is not only a pituitary hormone with an important role in the reproduction but also acts as a cytokine, eliciting a wide variety of actions. Data gathered during the last decade have evidently demonstrated that locally produced PRL acts as the autocrine/paracrine factor and plays a role in breast cancer. Following the reestablishing a contributory role for PRL during breast oncogenesis, the scientific and clinical communities have held great hope that manipulation of the PRL axis may lead to the successful treatment of breast cancer. This hope is not yet dashed, however the role of the PRL axis is now being shown to be more complex than was first envisaged. The first aim of this book is to overview major advances in the field.

Secondly, this book presents information on the role of PRL in non-mammary tissues in physiological and patho-physiological conditions. About 100–300 functions or targets have been identified for PRL in various species. This is true for the prostate, the skin, the decidua, the brain, some immune cells, adipocytes, and several others. The book discusses the role of PRL in adipocytes, immune response, angiogenesis, as well as in prolactinomas and prostate tumorigenesis.

This book also aims to summarize current knowledge about PRL and its receptor, plasticity of the PRL axis, PRL signaling pathways, and PRL crosstalk with other oncogenic factors.

Overall, the goal of this book is to identify and review new experimental findings that have provided further insight into the role of PRL in human physiology and patho-physiology. Thus, this book will bridge between new research results, as published in journal articles, and a contextual literature review.

M. Diakonova

Contents

Chapter 1
Prolactin (PRL) in Adipose Tissue: Regulation and Functions

Nira Ben-Jonathan and Eric Hugo

Abstract New information concerning the effects of prolactin (PRL) on metabolic processes warrants reevaluation of its overall metabolic actions. PRL affects metabolic homeostasis by regulating key enzymes and transporters associated with glucose and lipid metabolism in several target organs. In the lactating mammary gland, PRL increases the production of milk proteins, lactose, and lipids. In adipose tissue, PRL generally suppresses lipid storage and adipokine release and affect adipogenesis. A specific case is made for PRL in the human breast and adipose tissues, where it acts as a circulating hormone and an autocrine/paracrine factor. Although its overall effects on body composition are both modest and species-specific, PRL may be involved in the manifestation of insulin resistance.

1.1 Introduction

Metabolic homeostasis of an individual is finely regulated by the nutritional status, energy expenditure, and hormonal signals. Peripheral organs such as the pancreas, liver, and adipose tissue, as well as many centers within the brain, respond to these changes and act coordinately to maintain metabolic stability. Prolactin (PRL) is a multifunctional pituitary hormone with more actions than all other pituitary hormones combined. These functions are broadly classified as reproductive, metabolic, osmoregulatory, and immunoregulatory. The actions of PRL are mediated by the PRL receptor (PRLR), which is expressed in all organs associated with metabolic regulation. Unique to humans, PRL is also produced at multiple extrapituitary sites,

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categorizing it as a classical circulating hormone as well as an autocrine/paracrine cytokine.

Whereas humans are the one species we wish to know most about, they are also the least accessible to experimental manipulations. Although some properties of PRL in humans are well documented, for example, the effects of drugs, prolactinoma formation, and variants of PRL and the PRLR, others remain obscure. By necessity, then, information derived from laboratory animals is essential for the understanding of PRL in human health and disease. Nonetheless, extrapolation from data obtained with rodents to humans should be done selectively and judiciously. Given that both PRL homeostasis and adipose tissue properties differ greatly among species, each section in this chapter contains an extensive comparison of these parameters in humans vs. rodents. To fully evaluate the role of PRL as a metabolic hormone with a focus on adipose tissue, the following topics are covered: (1) characteristics of PRL, (2) selected features of the PRLR, (3) adipose tissue properties, (4) expression and regulation of adipocyte PRL, and (5) metabolic actions of PRL.

1.2 Characteristics of PRL and Lactogens

1.2.1 General Features of Lactogens

Human PRL (hRPL), growth hormone (hGH), and placental lactogen (hPL), commonly referred to as lactogens, are members of the cytokine superfamily which includes over 20 proteins. Members of this family are defined by two criteria: (1) a tertiary structure of four antiparallel α helices in an up–up–down–down configuration (Fig. 1.1), and (2) binding to a nontyrosine kinase, single-pass transmembrane receptor [1]. Lactogens are made of a single polypeptide chain of 190–200 residues with 2–3 disulfide bridges. hPRL has three disulfide bridges, while hGH and hPL lack the N-terminal disulfide loop. hGH and hPL share 85% sequence homology, but only 21–22% homology with hPRL. In spite of the low homology at the primary amino acid sequence, the three-dimensional topology of the three human lactogens enables their binding to the hPRLR, as discussed below. Figure 1.1 also demonstrates that all three lactogens equally stimulate proliferation of Nb2 rat lymphocytes.

1.2.2 The PRL Protein

Humans express a single PRL gene, located on chromosome 6. In addition to the pituitary, hPRL is independently and differentially expressed at multiple sites that include the endometrium, myometrium, decidua, immune cells, brain, breast, prostate, skin, and adipose tissue [2]. Consequently, even when pituitary PRL release is severely impaired, humans are not deprived of locally produced PRL. As judged

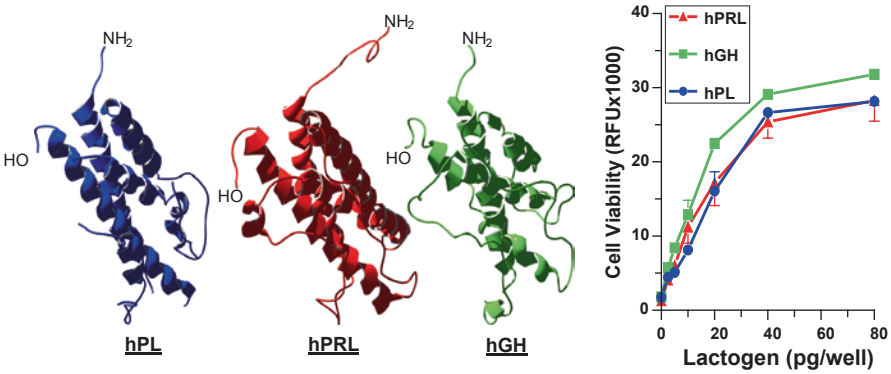

Fig. 1.1 *Left panel*: Structural similarities of the three human lactogens: *hPRL*, *hPL*, and *hGH*. *Right panel*: The three human lactogens similarly increase proliferation of Nb2 rat lymphocytes, which have long served as the most sensitive bioassay for PRL

by structural, biochemical, and functional criteria, extrapituitary and pituitary PRL proteins are identical, albeit their transcriptional regulation is dissimilar, as detailed in Sect. 1.5.

At extrapituitary sites, PRL is produced at much smaller amounts than at the pituitary, and likely remains in the vicinity of the producing cells via its association with heparin-binding proteins. Two motifs in hPRL are implicated in heparin binding [3]. These are absent in hGH and hPL, rendering them incapable of binding to heparin. The heparin-binding properties of hPRL enhance its efficacy as a cytokine by enriching its local concentration in tissues with high content of glycosaminoglycans such as adipose tissue.

PRL in rodents is primarily expressed in the pituitary, although some PRL is detectable in the decidua [4] and the lactating mammary gland [5, 6]. Unlike humans, rodents express multiple PRL-related genes which are clustered on chromosome 13 in mice and chromosome 17 in rats. These have variable degrees of sequence homology and are expressed in the uterus and placenta [7]. Some of the PRL-like proteins play important roles during late pregnancy in rodents, when they compensate for the markedly reduced pituitary PRL release. Nonetheless, under nonpregnant, nonlactating conditions, the rodent pituitary is the sole source of PRL.

1.2.3 Growth Hormone and Placental Lactogens

Humans have five GH/PL-related genes clustered on chromosome 17 [8]. These include GH-N (normal GH), primarily expressed in the pituitary, and four GH/PL-related proteins: GH-V (variant GH), PL-A, PL-B, and variant PL, all of which are expressed in the placental syncytiotrophoblast [9]. Although hPRL and hGH show little sequence homology at the amino acid level, hGH binds not only

to its cognate receptor (hGHR), but also to hPRLR, and can mimic some of PRL actions. In contrast, nonprimates' GH binds only to GHR, while PRL binds only to the PRLR. In spite of the higher sequence homology of hPLs to hGH than to hPRL, and their GH-like metabolic functions, hPLs bind to the hPRLR but not to hGHR, reviewed in [10]. Attempts to identify a unique receptor that binds hPL have not been successful. Unlike the multiplicity of GH/PL proteins in humans, mice and rats have only a single GH gene on chromosomes 11 and 10, respectively, which is primarily expressed in the pituitary.

1.2.4 Structural Diversity of PRL Proteins

The PRL protein can undergo a number of posttranslational modifications which include polymerization; proteolytic cleavage; glycosylation and phosphorylation; and impact on its stability, half-life, receptor binding, and bioactivity [11]. In addition to the 23 kDa PRL, human serum contains macroprolactin (> 100 kDa) and big PRL (40–60 kDa). Macroprolactin, a complex of monomeric PRL with IgG [12], is often elevated in hyperprolactinemic patients. Big PRL represents dimerized PRL, with an unclear relationship to macroprolactin. Proteolytic cleavage of PRL generates smaller fragments with different biological properties than the parent molecule. The N-terminal fragment, named 16 K PRL, has been best studied [13]. Recently, a family of N-terminal fragments of PRL, GH, and PL, named vasoinhibins, has been identified [14]. These peptides act on endothelial cells to suppress angiogenesis and promote vascular regression, and also play a role in tumorigenesis. Yet, the receptor(s) that mediates their action is unknown, and it is also unclear whether PRL produced at extrapituitary sites undergoes cleavage.

hPRL is N-glycosylated on Asn 34, comprising ~30% of total pituitary PRL content. Glycosylated PRL is also abundant in serum, milk, and the amniotic fluid [11]. Glycosylation reduces the binding affinity of PRL to the receptor, and affects its proteolytic cleavage, tissue distribution, and clearance. Unlike nonmodified pituitary PRL, which is stored and released from secretory vesicles, glycosylated PRL is constitutively secreted [15]. Constitutive secretion is particularly applicable to adipose tissue which does not have secretory granules. Rat PRL lacks a consensus sequence for N-glycosylation and is O-glycosylated [16]. It constitutes > 50% of serum PRL in rats, but only a minor component in the pituitary, indicating differential release rates or a longer half-life.

Phosphorylated PRL has been identified in many species, including rodents and humans [17]. PRL is phosphorylated on Ser 179 within the secretory granules of the rat pituitary, and its levels are altered under many physiological states. Studies with a molecular mimic of phosphorylated PRL (S179D) revealed that it acts as an agonist for cell differentiation and apoptosis, but as an antagonist for cell proliferation [18]. There is no information on whether extrapituitary PRL undergoes phosphorylation.

1.3 Selected Features of the PRLR

1.3.1 Cytokine-Type 1 Receptors

The cytokine-type receptors are single pass transmembrane proteins, devoid of intrinsic tyrosine kinase activity. Upon activation they are phosphorylated by a variety of cytoplasmic proteins. The receptors are subdivided into type I or type II, based on the number and spacing of cysteine and proline residues in their extracellular domain (ECD). The PRLR belongs to the type I subfamily which includes receptors for GH, leptin, a few interleukins, erythropoietin, leukemia inhibiting factor, and others [19]. Ligand binding to these receptors activates the Janus kinase-signal transducer and activator of transcription (Jak-Stat), as well as the mitogen-activated protein kinase (MAPK), and the phosphoinositide 3 kinase (PI3 K) signaling pathways.

1.3.2 Regulation of hPRLR Expression

The hPRLR gene is located on chromosome 5 in close proximity to the hGHR sequence. It is > 100 kb long and has 11 exons. Exons 1, 2, and part of exon 3 comprise the 5′-untranslated region (5′-UTR), while the rest of the exons comprise the coding region [20]. The 5′-UTR has six alternative first exons that are expressed in a tissue-specific manner. Regardless of which first exon is utilized, all are spliced into a noncoding exon 2. Transcription of the hPRLR gene is regulated at different sites by alternative promoters, each driving a specific first exon [20]. Alternative splicing within the coding region yields several isoforms that differ in length of the cytoplasmic domain, as discussed below.

The PRLR is expressed in most tissues, with the highest expression in the liver, mammary gland, adrenal, and hypothalamus [21]. Receptor expression is altered in response to changes in circulating PRL and steroid hormones, which can increase or suppress receptor expression, depending on the cell context and the physiological conditions. PRL also induces proteolytic degradation of the PRLR through ubiquitination [22]. Impairment of this process in some malignant PRLR-expressing cells (e.g., breast cancer), results in increased stability of the PRLR, enhanced responsiveness to PRL, and increased tumorigenicity.

1.3.3 Structural Elements of hPRLR Protein

The PRLR protein consists of three distinct components: an ECD which binds the ligands, a short transmembrane domain™, and a variable intracellular domain (ICD) which links to second messengers [23, 24]. The ECD is ~200 amino acids long and contains two subdomains: an amino-terminal region called D1, and a membrane-proximal region called D2, both of which have type III fibronectin-like motifs. Two

pairs of disulfide bonds in the D1 domain, and a "WS-motif" (Trp-Ser-X-Trp-Ser) in the D2 domain are critical for receptor folding and trafficking [25]. Within each species, the ECDs of all PRLR isoforms are identical. The two disulfide bonds in D1 are preserved in all species, but the WSxWS domain is seen in humans and rats but not in mice, which have a WSxWG. The ECD of the rat and mouse is 95% homologous, differing only by 11 residues. The human ECD has 71 and 74% homology to those in mice and rats, respectively [10].

1.3.4 PRLR Isoforms

Alternative splicing during transcription generates several PRLR isoforms, classified by the length of their ICD as "long," "intermediate," or "short." The long PRLR is the major form through which PRL transmits its signals. It has an apparent mass of 90 kDa and is composed of 588 residues, 364 of which are in the ICD. The ICD contains ten tyrosine residues (only nine in rodents) whose location and adjacent residues determine whether they become phosphorylated following receptor activation [24].

Humans have more PRLR isoforms than rats and mice combined. Six isoforms of hPRLR have been identified [26]: long (85–90 kDa), intermediate (50 kDa), ΔS1 (70 kDa), short 1a (56 kDa), and short 1b (42 kDa). There is also a soluble PRL binding protein which contains only the ECD (32 kDa), and is generated by proteolysis and is present in the circulation. The isoforms are expressed at variable ratios in normal and malignant cells, and some have independent, site-specific biological activities [27]. When co-expressed with the long isoform, several short isoforms inhibit transcriptional responses to PRL, suggesting that they can act as dominant negatives and may provide protection against overstimulation by PRL ([28]; Fig. 1.2).

A special case is an intermediate PRLR isoform that is expressed in rat Nb2 cells. Nb2 cells are a pre-T lymphocyte cell line which has served as the most sensitive bioassay for PRL for many decades. These cells encode a mutant receptor protein of 393 amino acids which lacks 198 residues in the cytoplasmic domain [29]. The mechanism by which PRL acts as a very potent mitogen for Nb2 cells, but as a rather weak mitogen in most other cells is unknown.

1.3.5 Ligand Binding

The secondary and tertiary configuration of both PRL and the PRLR, including specific residues in critical topological locations, determine receptor binding and activation. Yet, a structure-based explanation for the activation of the PRLR by different lactogens remains a major challenge. The rodent PRLR is activated by hPRL and hPL but not by hGH, while the hPRLR is activated by rPRL but not by mPRL [30]. The fact that human xenografts in nude mice do not respond to circulating

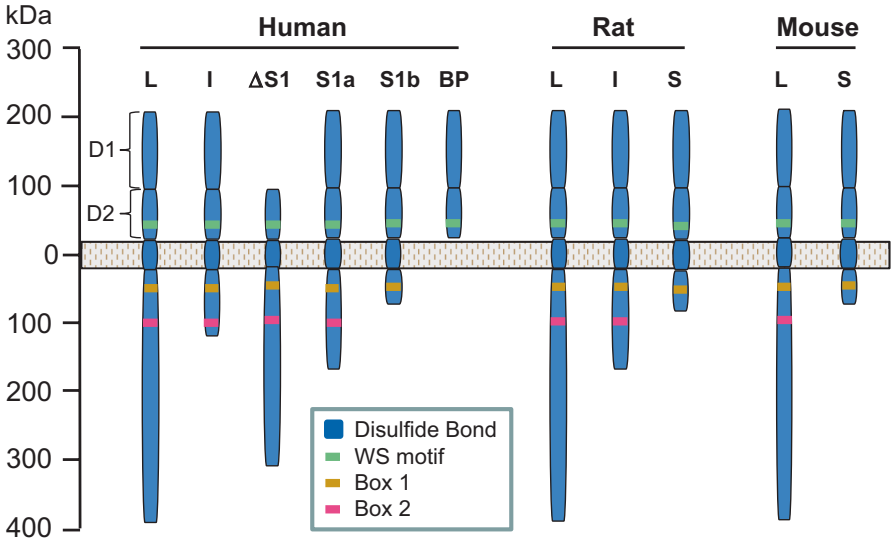

Fig. 1.2 Comparison of PRLR isoforms in rodents vs. humans. The similar extracellular domain is designated by two subdomains, *D1* and *D2*, which contain *disulfide bonds* and *WS motif*, respectively. Receptors are classified by the length of the intracellular domains as long (*L*), intermediate (*I*), and short (*S*). In addition, there is a receptor missing the D1 domain in the ECD (ΔS1), and another which contains only the extracellular domain (binding protein or BP). The intracellular domain contain two conserved regions, designated *Box 1* and *Box 2*, which link the receptor to signaling molecules

mouse PRL is overlooked by many investigators. Such unresponsiveness hinders the ability to extrapolate data from mice to humans vis-à-vis the role of PRL in tumorigenesis and metabolic regulation. The species-dependent incompatibilities may be overcome with the recent generation of humanized rats [31] and mice [32] that express the hPRL gene, and the eventual crossing of hPRL-expressing mice to immune-deficient mice.

PRLR dimerization is obligatory for signal transmission, but whether PRL induces sequential dimerization or binds to predimerized receptors is controversial [21, 25, 33]. While the former represents a long-held view, the latter has gained support, based on the identification of preformed dimers of other cytokine type 1 receptors. Two sites on PRL with different affinities are necessary for binding to the two receptors. The use of combinations of various constructs of the PRLR revealed that the TM domain is sufficient for dimerization in a ligand-independent fashion, but the interaction is strengthened by both the ECD and ICD [34]. A comprehensive cover of the different concepts of PRLR activation by its ligands can be found in a recent review [25].

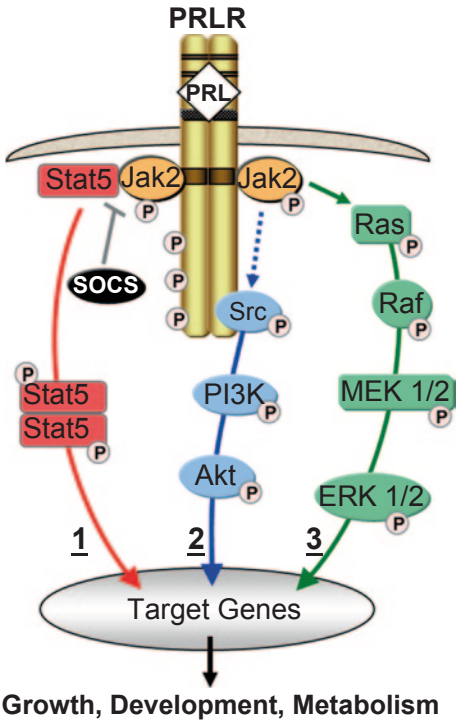

Fig. 1.3 The three major signaling transduction pathways of the long *PRLR* isoform: *Jak2-Stat5a/b*, *PI3K/Akt*, and *MAPK* which are activated by *PRL* in a cell-selective manner. See text for other explanations

1.3.6 Signal Transduction

The PRLR is devoid of intrinsic tyrosine kinase activity and utilizes the Jak2-Stat pathway as its main signaling cascade. The receptor-associated Jak2 is rapidly phosphorylated upon PRL binding and induces the phosphorylation of the receptor itself, associated kinases, and Stat proteins [23, 35]. Of the seven known Stat proteins, Stats 1, 3, and 5 can be activated by PRL, with Stats 5a and 5b especially important for mammary gland development and functions. Upon phosphorylation, Stat proteins hetero- or homodimerize via SH2–phosphotyrosine interactions. The activated dimers are translocated to the nucleus and bind to GAS (γ-interferon activation sequence) consensus elements on target genes. Milk proteins, that is, β-casein, lactalbumin, and whey acidic protein, are the best characterized PRLR-regulated genes downstream of the Jak-Stat pathway.

The Ras–Raf–MAPK pathway is also activated by PRL in many cells. Phosphorylation of Jak2 can recruit adaptor proteins such as Shc, Grb2, and SOS to the PRLR, resulting in the binding and activation of Ras and Raf. This leads to activation of the MAP kinase pathway, which is often associated with increased cell proliferation [36]. Activation of the PRLR also facilitates docking of Src family kinases, which in turn activates the PI3K/Akt pathway that mediates some of the antiapoptotic and metabolic actions of PRL [37] (Fig. 1.3).

Both the strength and duration of the PRL-induced signaling are regulated by members of the suppressors of cytokine signaling (SOCS) proteins, whose expression is rapidly induced following receptor activation [38]. The SOCS proteins interact either with the PRLR or with Jak2 and inhibit further signal activation. In sum, the signal transduction activated by the PRLR does not represent a linear progression but involves several interacting pathways that differ in predominance among the various PRLR-expressing cells.

1.4 Adipose Tissue Properties

1.4.1 Distinct Features of Adipose Tissue

Adipose tissue is an active organ that plays a pivotal role in metabolic, physiologic, and endocrine homeostasis [39, 40]. This highly specialized tissue of mesenchymal origin is comprised of multiple cell types held loosely together in a collagen matrix. The predominant cell is the terminally differentiated adipocyte, a very large cell whose size can be dramatically altered under various nutritional states. The stroma, often referred to as the stromal vascular fraction (SVF), contains pleuripotent stem cells, preadipocytes, endothelial cells, pericytes, mast cells, fibroblasts, and hematopoietic cells, primarily macrophages.

Obesity results from adipocyte enlargement through enhanced lipid accumulation (hypertrophy), as well as increased cell number (hyperplasia). The latter begins with recruitment of stem cells to the adipocyte lineage [41], followed by adipogenesis, which converts committed preadipocytes to mature adipocytes [42]. Approximately 10% of fat cells are renewed annually irrespective of body mass index, without an increase in their overall number in adults. Death of adipocytes occurs by necrosis or apoptosis. Whether apoptosis is a critical factor which determines the number of adipocytes has been debated [43]. A therapeutic induction of apoptosis in adipocytes could become a valuable approach for treating obesity [44]. Obesity is also associated with recruitment of macrophages into adipose tissue, leading to increased production of proinflammatory cytokines such as TNFα and IL-6, and development of low-level inflammation [45].

1.4.2 White and Brown Adipocytes

Two types of adipose tissue are recognized: white adipose tissue (WAT) and brown adipose tissue (BAT). White adipocytes are characterized by a single, large lipid droplet occupying up to 90% of the cell volume. The droplet consists of triacylglycerols (TAG) and cholesterol and is coated with specialized proteins, for example perilipins, which mediate interactions between the droplet and regulators of lipid metabolism. WAT secretes numerous important hormones/adipocytokines that

include leptin, adiponectin, resistin, adipsin, TNFα and angiotensinogen [46, 47], as well as PRL [48], as detailed below. After menopause, WAT also becomes a significant source of estrogen, which is produced from circulating androgen precursors by the cytochrome P_{450} enzyme aromatase. In sum, the two main functions of WAT are storage and release of lipids, and production and secretion of adipokines. Together, they affect food intake, energy balance, insulin sensitivity, lipid and glucose metabolism, and cardiovascular functions [49].

Brown adipocytes contain multilocular lipid droplets and have a high density of mitochondria. They express uncoupling protein 1 (UCP-1) which generates heat by uncoupling electron transport from oxidative phosphorylation. BAT is specialized for nonshivering heat production [50]. It was recently discovered that functional BAT, once thought to exist mainly in infants, is also present in adults [51]. Brown adipocytes are detectable during cold exposure, and are associated with decreased adiposity. There is evidence for transdifferentiation between white and brown adipocytes. When stimulated, brown adipocytes enhance energy expenditure and increase glucose and fatty acid uptake. Thermogenesis in BAT is recognized as an important determinant in energy balance, and its therapeutic manipulation could be exploited in the treatment of obesity.

1.4.3 Fat Depots: Morphological and Functional Aspects

Human WAT is found in specific anatomical depots, which differ in morphology and functions and are classified as abdominal visceral (vis) and subcutaneous (sc) fat [52, 53]. The relative distribution of these depots determines body shape. There is sex dimorphism in the regional distribution of adipose tissue, with vis fat accounting for 6% of body fat in women but 20% in men, reflecting their greater propensity to accumulate excess abdominal fat. After menopause, fat distribution in women is closer to that of men, suggesting a role for sex steroids in anatomical deposition of fat [54]. The cellular composition of the depots varies, with vis fat having more macrophages and fewer preadipocytes than sc fat, whereas sc adipocytes in obese subjects are larger than their vis counterparts. The depots also differ in the secretion of adipokine, with vis fat secreting more IL-6 but less leptin and adiponectin than sc fat.

In humans, increased abdominal vis fat is a critical factor in the manifestation of the metabolic syndrome, which has become alarmingly pervasive in recent years. The metabolic syndrome is defined by glucose intolerance, hyperinsulinemia, hypertriglyceremia, altered serum lipoprotein levels, and hypertension. Vis fat is more sensitive to β-adrenergic agonists and less responsive to insulin than sc fat, making it more lipolytically active [52, 55]. As the output of vis fat drains into the hepatic portal blood, increased influx of free fatty acids (FFA) inhibits hepatic insulin clearance and contributes to hyperinsulinemia. Chronic elevation of FFA also impairs glucose metabolism and insulin sensitivity in liver and muscle, and reduces pancreatic β cell function [56]. Other factors, for example, increased production of some adipokines and inflammatory cytokines, and altered insulin receptor expression, link obesity to the metabolic syndrome [56].

Adipose depots in mice are present in several distinct locations: (1) gonadal fat surrounding the uterus and ovaries in females and the epididymis and testes in males; (2) abdominal fat, further subdivided into mesenteric, omental, and pericardial, (3) sc fat which also includes the mammary fat pads in females, and (4) brown adipose tissue, primarily seen in neonates in the neck and thoracic regions [57]. Of all fat depots in the mouse, the gonadal depots are the largest, comprising about 30% of dissectible fat.

The mammary fat pad is especially relevant to any discussion on PRL and adipose tissue. The mammary gland contains myoepithelial and luminal epithelial cells that comprise the milk ducts and alveoli. These are embedded in a stroma composed of fibroblasts and adipocytes. Studies with rodents showed that the stroma provides signals to the epithelial cells that are critical for postnatal morphogenesis of the mammary gland [58]. Mammary adipocytes are especially active during the transitions from pregnancy to lactation, and into involution, and also contribute to tumorigenesis [59]. There are major differences in the distribution of mammary fat between humans and rodents. In rodents, stromal adipocytes are in close proximity to the epithelium, while human breast adipose tissue is interlaced with a network of fibroblasts and connective tissue, and a fibrous layer separates the epithelium from the adipocytes, reviewed in [10].

1.4.4 The Process of Adipogenesis

Adipocytes are derived from pluripotent mesenchymal stem cells (MSC), that can differentiate into adipocytes, myocytes, chondrocytes, and osteocytes [41]. Induction of MSC into the preadipocyte lineage occurs in response to stimulation/inhibition by bone morphogenic protein (BMP) family members, Wnt/β-catenin, and hedgehog signaling. White and brown adipocytes originate from different progenitors, with brown adipocytes characterized by the expression of Myf5, while white adipocytes are Myf5-negative [60].

Once committed, conversion of preadipocytes into mature adipocytes progresses through a well-coordinated, sequential activation/inactivation of a cascade of genes. This results in substantial structural, morphological, and functional changes (Fig. 1.4). Studies with murine preadipocytes, primarily 3T3-L1 cells, with supportive data from primary human preadipocytes, provided most of our knowledge of the molecular basis of adipogenesis. Under *in vitro* conditions, adipogenesis is initiated by exposure of preadipocytes to adipogenic-inducing hormones such as glucocorticoids, insulin/IGF-1, and cAMP activators [41, 61]. Although the time-course differs among the cellular models, most critical steps are common. These include an initial growth arrest, followed by clonal expansion, and a secondary arrest. Preadipocytes first withdraw from the cell cycle and become arrested at the G1/S stage. They then reenter the cell cycle and undergo 2–3 rounds of division, referred to as mitotic clonal expansion. The latter, however, may be a permissive, but not an absolute, requirement for differentiation of human preadipocytes.

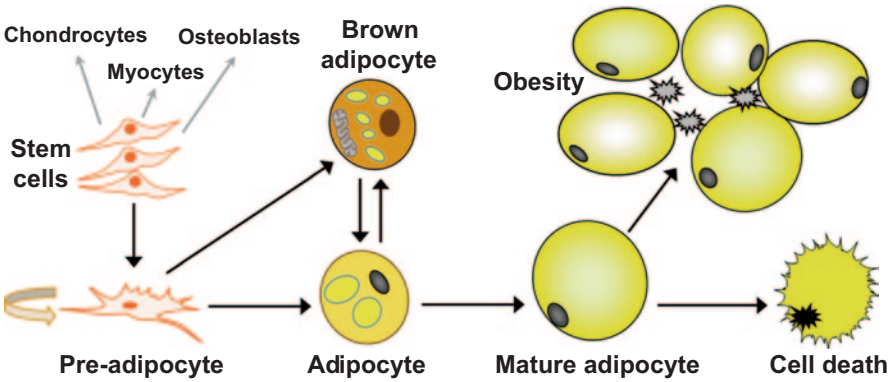

Fig. 1.4 Formation of mature adipocytes. Stem cells can differentiate into *preadipocytes*, *chondrocytes*, *myoblasts*, and *osteoblasts*. Preadipocytes undergo mitotic expansion, followed by cell cycle arrest and adipogenesis, which results in the formation of terminally-differentiated mature adipocytes. *Brown adipocytes* develop from another cell lineage, with trans-differentiation occurring between white and brown adipocytes. *Obesity* results primarily from hypertrophy by enhanced lipid accumulation, and is characterized by macrophage infiltration. *Cell death* occurs by necrosis or apoptosis

Adipogenesis progresses in well-orchestrated waves of genetic events [62]. Several transcription factors: peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBP α, β, and γ), are central to the control of adipogenesis, while other transcription factors play supportive roles [41]. During early adipogenesis, the cells irreversibly exit the cell cycle and start losing their fibroblast-like morphology. As adipogenesis progresses, the cells accumulate TAGs in response to the increased expression of lipid synthesizing enzymes. The insulin receptor, the glucose transporter GLUT4, and key adipokines such as leptin and adiponectin, are also coordinately expressed. Adipogenesis is modulated by a plethora of hormones and local factors such as glucocorticoids, GH, IGF-1, TNFα, and IL-6 [62].

1.4.5 Lipid Metabolism: Lipogenesis vs. Lipolysis

Adipose tissue is the major site of lipid metabolism, which also occurs, to a lesser extent, in the liver [63, 64]. Based on weight, fat contains twice as many calories as proteins or carbohydrates, making energy storage in the form of TAGs highly efficient. Synthesis of TAGs requires assembly of three moieties of fatty acids with a molecule of glycerol. Fatty acids are available to the adipocytes from the circulation or from local synthesis [65], and glycerol is available as glycerol-3-phosphate from glycolysis and glyceroneogenesis [66]. The overall process of lipogenesis is affected by the frequency and composition of the diet and serum glucose and lipid levels, and is regulated by insulin, which increases lipogenesis, and GH and glucagon which decrease it.

Adipocytes store TAGs when energy supply is abundant, while during periods of caloric deficits, they hydrolyze them to FFA, which serve as fuel and metabolic precursors for other tissues [46]. Stored TAGs originate from two sources: (1) dietary fat in the form of chylomicrons from the intestines, very low-density lipoproteins (VLDLs) from the liver, and nonesterified fatty acids bound to albumin, and (2) de novo lipogenesis from carbohydrates [46, 64]. Under a typical western diet, stored lipids are primarily derived from dietary triglycerides, while de novo lipogenesis predominates under excess carbohydrate intake [63]. In human adipocytes, de novo lipogenesis is significantly less active than in rats because of differences in the expression of sterol regulatory element binding protein (SREBP-1), or the composition of diet in the two species [66].

Several key enzymes, some of which are affected by PRL (see below), are involved in TAG production. One is lipoprotein lipase (LPL), which is synthetized in adipocytes and secreted into adjacent endothelial cells. LPL hydrolyzes TAGs from circulating chylomicrons and lipoproteins into FFA, which are taken up by the adipocytes via membrane transport proteins [65]. Two other enzymes are pyruvate dehydrogenase (PDH), which generates acetyl-CoA, and acetyl-CoA carboxylase (ACC), which generates malonyl CoA, an essential intermediate in fatty acid biosynthesis. A critical lipogenic enzyme is fatty acid synthase (FAS), a multienzyme complex which catalyzes the production of palmitate from acetyl Co-A and malonyl Co-A into long chain fatty acids.

Lipolysis entails a stepwise breaking down of TAGs, first to into diacylglycerols (DAG) and then to monoacylglycerols (MAG), eventually yielding three molecules of FFAs and one molecule of glycerol. Hormone sensitive lipase (HSL), so named because of its high sensitivity to insulin and catecholamines, has long been considered the rate-limiting step in lipolysis. Following the discovery of adipocytes triglyceride lipase (ATGL) by three independent groups [67], it is now recognized that both enzymes act coordinately to regulate lipolysis.

Catecholamines and insulin increase and decrease lipolysis, respectively, by altering the cAMP/PKA signaling pathway in opposite directions [65, 68, 69]. Norepinephrine and epinephrine originate from the adrenal medulla and sympathetic nerve endings. They bind to α- and β-adrenergic receptors, which are classical seven-transmembrane receptors coupled to inhibitory (Gi) or stimulatory (Gs) G-proteins, respectively. Upon activation, Gs proteins stimulate adenylyl cyclase, increase cAMP and activate protein kinase A. Once activated, PKA phosphorylates both HSL, which translocates from the cytosol to the lipid droplet, and perillipin-1, which is displaced from the surface of the droplet into the cytosol. This coordinated event provides access of HSL to stored TAGs and enables their hydrolysis. In rodents, the β3-adrenergic receptor is the primary lipolytic mediator, while in humans, β1, β2, and α2 (an inhibitor of lipolysis) receptors, play more decisive regulatory roles [69].

Inhibition of lipolysis by insulin involves cAMP-dependent and cAMP-independent mechanisms [39, 65]. Insulin binds to its receptor and activates the insulin receptor substrate (IRS). This is followed by activation of PKB/Akt and phosphorylation of phosphodiesterase 3B (PDE3B), which degrades cAMP and deactivates PKA. Hence, the suppression of lipolysis by insulin results from reduced

phosphorylation-mediated activation of HSL and perilipin. Insulin also inhibits lipolysis by stimulating phosphatase-1, which rapidly dephosphorylates and deactivates HSL. In humans, but not in rodents, natriuretic peptides stimulate lipolysis via a cGMP-dependent pathway that does not involve PDE-3B inhibition or cAMP production [70].

1.4.6 Selected Adipokines

Adipose tissue is an important endocrine organ whose hormones, the adipokines, regulate food intake, energy balance, insulin resistance, inflammatory responses, and blood pressure. In turn, the release of adipokines is influenced by the nutritional status, hormonal signals, and energy expenditure [39, 46, 71]. Since the discovery of leptin in 1994, a multitude of adipokines and adipocytokines has been identified. Some of these are exclusively produced by adipocytes, few are secreted by adipose stromal cells, and several are also produced to a variable degree by other organs. Here we focus only on leptin and adiponectin, which are affected by PRL.

Leptin is a 16-Kda protein produced primarily by mature adipocytes, and at low levels in the GI tract, muscle, mammary epithelium, placenta, and brain. Serum leptin levels increase in proportion to weight gain and decrease with weight loss, designating leptin as a "signal" of adiposity [72, 73]. The leptin receptor (Ob-R or LR) and the PRLR belong to the cytokine type 1 receptor family and share many features. Leptin utilizes Jak2/Stat 3 as its main signaling pathway, and also cross-talk with the insulin receptor through activation of IRS-1. Similar to the PRLR, the LR is alternatively spliced into secreted, short and long isoforms, with the long isoform serving as the primary mediator of leptin action. However, unlike the promiscuity of the PRLR which binds several lactogens, the LR responds only to leptin.

Leptin is a multifunctional hormone which acts on many peripheral organs and the brain. Much attention has been given to its ability to suppress appetite. Leptin can cross the blood–brain barrier through a saturable mechanism, and binds to receptors that are expressed in many sites within the brain [46]. Elevated leptin directly suppresses the orexigenic peptides neuropeptide Y and agouti-related peptide in the arcuate nucleus, while indirectly inhibiting melanin concentrating hormone and orexin in the lateral hypothalamus. Leptin also increases the proopiomelanocortin-derived anorectic peptide α-MSH. These coordinated actions ultimately lead to a reduction in food intake, increased energy expenditure, and increased thermogenesis.

The initial expectation that leptin could be used therapeutically to suppress appetite in obese patients has not materialized for several reasons. First, the short half-life of circulating leptin requires a very frequent delivery or a long-acting leptin formulation. More importantly, a prolonged rise in serum leptin levels in obesity induces leptin resistance, which results from lower leptin transport into the brain as well as reduced leptin signaling. Thus, leptin has diminished effects on food intake in obese patients [72]. Unlike rodents, in whom leptin is a major suppressor of appetite, the control of appetite in humans is more dominated by GI-derived hormones such as ghrelin, cholecystokinin, pancreatic polypeptide, peptide YY, and glucagon-like peptide [74].

Adiponectin is a major adipocytes-derived hormone which is not produced elsewhere. It circulates at very high levels, comprising as much as 0.01 % of total plasma proteins [75, 76]. Adiponectin is a 30-kDa protein with a complex structure, with some homology to collagen VIII. It circulates in many forms, from trimers to high-molecular weight decamers. In contrast to leptin, serum adiponectin is negatively correlated with fat mass, being low in obesity and high after weight loss. Adiponectin binds to two receptors (AdipoR1 and AdipoR2) which contain 7-transmembrane domains but are structurally and functionally distinct from the G-protein-coupled receptors [77]. AdipoR1 is primarily expressed in muscle and signals through AMP kinase, while AdipoR2 is expressed in liver and activates PPARα.

Adiponectin acts as an insulin sensitizer, and is classified as an antidiabetic, antiinflammatory, and antiatherogenic hormone [77]. In liver, adiponectin decreases FFA influx and reduces glucose output, while in muscle, it stimulates glucose utilization and fatty acid oxidation. Adiponectin has beneficial effects on the cardiovascular system, where it prevents atherosclerotic formation by inhibiting monocyte adhesion to the endothelium, and by suppressing transformation of macrophages into foam cells. The actions of adiponectin on the brain are not well defined. In rodents, intracerebral administration of adiponectin results in decreased body weight by increasing energy expenditure without altering food intake. In sum, low serum adiponectin is associated with insulin resistance and type 2 diabetes, while its elevation has the opposite effects. The ability of adiponectin to enhance insulin sensitivity and promote vascular health raises the prospect of its therapeutic use in the treatment of diabetes and cardiovascular diseases. However, biologically active recombinant adiponectin proteins are unstable and difficult to make, presenting a challenge for both research and clinical applications.

1.5 Expression and Regulation of Adipose PRL

1.5.1 The Discovery of Adipose PRL

PRL production in human adipose tissue was serendipitously discovered upon studying the potential role of local PRL in breast carcinogenesis [78]. Surgical specimens of normal and malignant breast tissue were separated into adipose and glandular explants and incubated for 10 days in serum-free media. Explants were analyzed for PRL gene expression by RT-PCR, and media were analyzed for secreted PRL by the Nb2 bioassay. Unexpectedly, breast adipose explants, intended to serve as negative controls, expressed and released 10–15 times more PRL than their glandular counterparts. To verify local synthesis rather than release from reuptake, explants were incubated with ^{35}S-methionine, followed by immunoprecipitation, electrophoresis, and autoradiography [78]. The presence of metabolically-labeled PRL in both tissue extracts and conditioned media strongly supported de novo synthesis of PRL.

Another unexpected observation was a progressive rise in PRL release from adipose explants up to 7 days in culture, suggesting removal from inhibitory controls. PRL release from glandular explants was suppressed by progesterone, but neither estrogen nor progesterone altered its release from adipose explants [78], indicating dissimilar regulation of PRL in the two adjacent tissues. These findings raised several intriguing questions: (1) Is PRL synthesized in other adipose depots and, if so, is it affected by obesity? (2) Which cells synthesize PRL? (3) What is the nature of the inhibitor? and (4) What are the functions of local PRL?

To address these questions, vis and sc adipose tissue explants from morbidly obese and nonobese patients were placed in culture. Similar to the profile of PRL release from breast adipose tissue, PRL release from both types of explants showed time-dependent increases [79]. PRL release from sc explants from obese patients was significantly lower than that from lean patients, with no apparent difference between men and women. Isolated mature adipocytes had an identical pattern of PRL release to that from explants. Collectively, these data showed depot-specific control of PRL production which is markedly affected by obesity. The mechanism by which obesity causes a reduction in adipocyte PRL release and its functional consequences, remain to be determined. Adipocytes are the primary source of PRL in adipose tissue, although infiltrating macrophages which express PRL [80], could add to the overall adipose PRL output in obesity. PRL expression was undetectable in adipose tissue from rats, mice or 3T3-L1 and 3T3–442A murine preadipocyte cell lines, confirming the notion that adipocyte-derived PRL is unique to humans. Yet, infiltrating macrophages may carry out some PRL production in adipose tissue in obese rodents [80].

When compared on per cell basis, PRL release from a single adipocyte is many orders of magnitude lower than that from a pituitary lactotroph. However, the human pituitary weighs 1 g, while the weight of adipose tissue in obese individuals can exceed 100 kg. Consequently, the overall PRL production by adipose tissue could approach that of the pituitary. A relevant question is whether adipose PRL affects serum PRL levels. Given that hPRL binds heparin [3], most of the PRL secreted by adipocytes is presumably retained locally by proteoglycans, which are abundant in adipose tissue but low in the pituitary, making adipose PRL a true autocrine/paracrine factor. A recent study compared serum PRL levels in obese and lean patients and found higher basal serum PRL in women than men, but no effect of obesity [81]. Serum PRL levels did not correlate with BMI, and were unchanged after massive weight loss. Another study found lower serum PRL in obese than lean children [82]. Collectively, these data demonstrate that adipose-derived PRL has little, if any, effects, on circulating PRL levels.

1.5.2 Regulation of PRL Gene Expression

The human PRL gene consists of five coding exons. It is transcribed in the pituitary from a proximal promoter which depends on Pit-1 transcription factor for

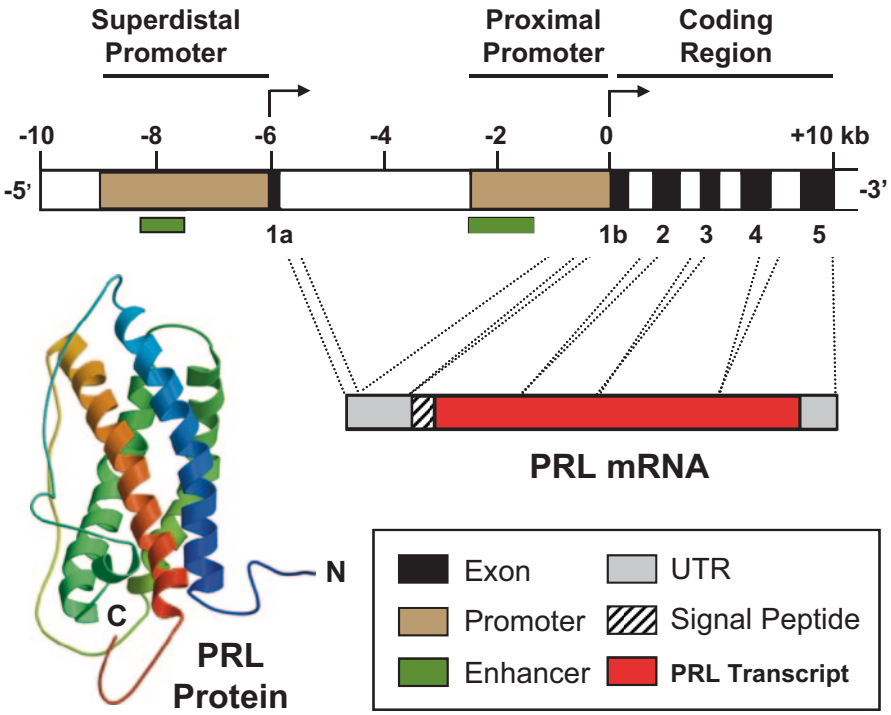

Fig. 1.5 The regulation of pituitary and extrapituitary PRL gene expression by the *proximal* and *superdistal promoters*, respectively. The PRL transcripts are identical except for a longer 5′ untranslated region (*UTR*) in the extrapituitary transcript. See text for other explanations

activation, and is regulated by dopamine, estrogen, neuropeptides, and some growth factors [83]. In contrast, expression of extrapituitary PRL is driven by a superdistal promoter, located 5.8 kb upstream of the pituitary start site. This promoter is silenced in the pituitary and does not depend on Pit-1. Exon 1a, serving as an alternative transcriptional start site (named decidual start site), is spliced into exon 1b, yielding an identical transcript to that of the pituitary except for a longer 5′ UTR (Fig. 1.5). The superdistal promoter extends −3000 bp upstream of the decidual start site and is composed of a proximal region between −350 and −60 and a distal enhancer between −2000 and −1500 [84, 85]. The dissimilar control of the PRL gene in various tissues is exemplified by progesterone, which increases PRL expression in the endometrium, decreases it in the myometrium and breast, and has no effect on pituitary PRL.

To map the active elements within the superdistal promoter, primary preadipocytes were transiently transfected with a luciferase reporter driven by a full-length decidual PRL promoter (−3000/+66), or with progressively deleted mutants [86]. Transfection with either the full length promoter or the −317 construct resulted in a 25-fold increase in luciferase activity above vector control. On the other hand,

the −1556 and −675 constructs caused only five- to eightfold increases, suggesting presence of inhibitory elements between the proximal promoter and the distal enhancer. The two positive regulatory domains correspond to those in decidual cells [87], while the inhibitory region appears to be unique to adipocytes.

1.5.3 Factors Which Affect PRL Release

Knowledge of the control of PRL release in extrapituitary sites lags behind that of pituitary PRL for several reasons. First, unlike those of rodents, human tissues are not as readily available and show high variability among tissue donors. Second, PRL release from these sites is several orders of magnitude lower than pituitary PRL, requiring the use of more sensitive, but often less specific, bioassays. Third, there is no uniform mechanism for the control of PRL release, as each cell type utilizes different regulators. Fourth, there are no storage granules in most extrapituitary sites, implying constitutive PRL release rather than calcium-dependent exocytosis as in pituitary lactotrophs. Without vesicular storage, the main control of nonpituitary PRL is transcriptional, as is the case for most cytokines. In spite of the dissimilar regulation of pituitary and nonpituitary PRL, both are under inhibitory controls, reviewed in [10]. As detailed in Sect. 1.5.5., similar to the pituitary, dopamine serves as a physiological inhibitor of adipocyte PRL [88].

Both preadipocytes and mature adipocytes express and release PRL [86]. PRL release from freshly isolated preadipocytes was low and transiently increased during early adipogenesis. PRL expression was stimulated by many agents that elevate cAMP, including epinephrine, IBMX (3-isobutyl-1-methylxanthine), a phosphodiesterase inhibitor, isoproterenol, a β-adrenergic receptor agonist, PACAP (pituitary adenylate cyclase activating peptide), and vasoactive intestinal peptide (VIP). To identify the signaling pathways involved, preadipocytes were co-incubated with ligands and inhibitors of PKA, PI3K or MEK. All inhibitors blocked isoproterenol-stimulated PRL release, while the PKA inhibitor did not affect stimulation by PACAP [86]. These data indicate that PRL production in preadipocytes is stimulated by catecholamines and other cAMP activators via interacting signaling pathways.

1.5.4 LS14 Human Adipocyte Cell Line

Primary rodent adipocytes as well as 3T3-L1 and 3T3-F442A murine adipocyte cell lines express the PRLR and can be used to study PRL actions, but they do not produce PRL. To elucidate the control of PRL production, human adipocytes must be employed. Given the scarcity of human adipose tissue, the large variability among specimens, and the short life span of primary adipocytes, we sought a source of human adipocytes that meets the following criteria: immortality, inducible terminal differentiation, PRL release, and PRL response. After obtaining a surgically removed metastatic liposarcoma, we cloned a spontaneously immortalized adipocyte

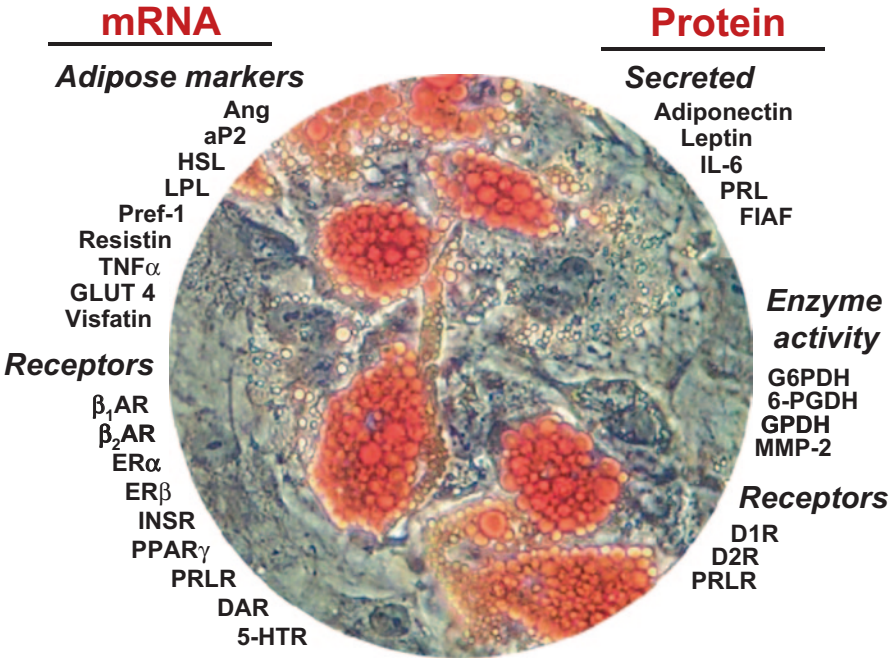

Fig. 1.6 Photograph of a lipid filled, fully differentiated LS14 human adipocyte. The various *adipose markers* and *receptors*, determined by RT-PCR are shown on the *left*, while secreted adipokines, *enzyme activities* and expression of *receptor* proteins are shown on the *right* side

cell line which was named LS14 [89]. This cell line has been in extensive use since 2005.

To characterize the adipogenic nature of LS14 cells, expression of multiple genes was compared in LS14 and primary vis adipocytes before and after differentiation. Expression of aP2, GLUT4, HSL, LPL, and angiotensinogen was similarly induced during differentiation in both cell types. PPARγ was robustly expressed, Pref-1 was low, and UCP-1 was seen only in differentiated primary cells. Expression of adiponectin and leptin was seen in both LS14 and primary cells after differentiation, while IL-6 and TNFα were barely detected in differentiated LS14 cells. LS14 cells also express visfatin, resistin, and FIAF. Of the β-adrenergic receptors, only β2 was detected in LS14 cells, as well as insulin, estrogen (both ERα and ERβ), and dopamine receptors (Fig. 1.6).

The ability of LS14 cells to release of leptin, adiponectin, and IL-6 was confirmed by respective ELISAs. The release of FIAF was detected by Western blotting, while MMP-2 activity was verified by zymography. The use of fluorimetric enzyme assays confirmed that LS14 cells have functional lipid metabolizing enzymes. These complementary approaches validated the adipogenic nature of LS14 cells, established their resemblance, with few exceptions, to primary vis adipocytes, and verified their capacity not only to express, but also to release, key adipokines.

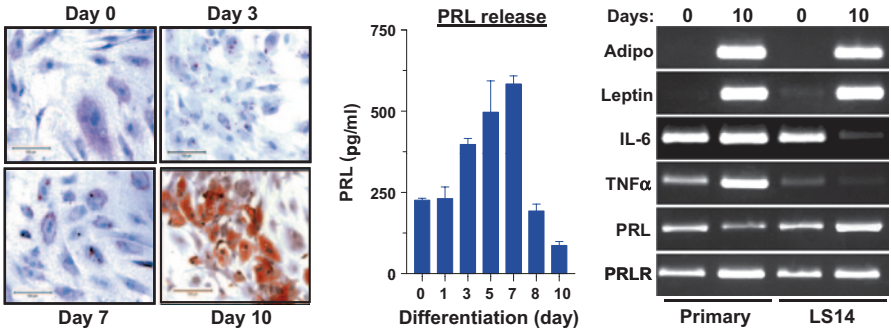

Fig. 1.7 *Left panel*: induced differentiation of primary visceral preadipocytes into mature adipocytes over a 10-day period. *Middle panel*: PRL release from *LS14* cells during adipogenesis, as determined by the Nb2 bioassay. *Right panel*: comparison of the expression of selected genes before (*day 0*) and after (*day 10*) differentiation in primary visceral adipocytes and *LS14* cells

Like primary adipocytes, LS14 cells produce PRL and respond to PRL via the PRLR. PRL expression and release in both cell types increased markedly during early adipogenesis and peaked on days 5–7, follows by a decline (Fig. 1.7). Incubation of LS14 cells with exogenous PRL caused a dose-dependent inhibition of IL-6 [89]. Unlike many cells immortalized by genetic manipulation which often resist induced differentiation, LS14 cells undergo considerable morphological and functional differentiation under the appropriate culture conditions. The availability of LS14 cells opens up new avenues for research on human adipocyte biology, and adds to the small repertoire of non-pituitary PRL-producing human cell lines.

1.5.5 Dopamine: A Physiological Inhibitor of Adipocyte PRL

The time-dependent increase in adipocyte PRL release [79] resembled the progressive rise in PRL release from freshly incubated pituitary cells, which results from the removal of tonic inhibition by hypothalamic dopamine [90]. Yet, dopamine was initially ruled out as a putative inhibitor of adipocyte PRL because a ready source of dopamine to the adipocytes was not apparent, and there was no information whether dopamine receptors (DAR) are expressed in human adipose tissue. In addition, previous studies showed no effects of dopamine on PRL release from human decidual explants [91]. This was interpreted as insensitivity of the superdistal PRL promoter to dopamine rather than as a possible absence of DAR in this tissue. Dopamine binds to five 7-transmembrane, G-protein-coupled receptors, named D1R-D5R. D1R and D5R are classified by their ability to increase cAMP, while D2R, D3R, and D4R inhibit cAMP. The inhibition of pituitary PRL by dopamine occurs through activation of D2R [90].

Unlike well-studied peripheral norepinephrine and epinephrine, the presence of dopamine in the general circulation has been overlooked by most investigators. The blood–brain barrier prevents transport of dopamine from the brain to the periphery, but small amounts of dopamine are produced by, and released from, the GI tract, adrenal medulla, and sympathetic nerve endings [92]. Little known is the fact that the major form of circulating dopamine in humans is the biologically inactive dopamine sulfate (DA-S). Sulfoconjugation is done in the GI tract by SULT1A3 sulfotransferase which is not expressed in rodents [93]. Basal serum DA-S levels at ≈10 nM exceeds by fivefold the combined levels of free dopamine, norepinephrine, or epinephrine. DA-S has a half-life of 3–4 h, compared with few minutes for unmodified dopamine [94]. Most importantly, unlike dopamine inactivation by deamination, O-methylation or glucuronidation, sulfoconjugation is reversible, and DA-S can be converted back to bioactive dopamine by arylsulfatase A (ARSA), a releasable lysosomal enzyme [95].

While pursuing the inhibitor of adipocyte PRL, our major assumption was that if human adipocytes express DAR and possess an active ARSA, circulating DA-S could serve as a readily available reservoir of dopamine for the adipocytes. Therefore, the objectives were to: (1) examine whether human adipocytes express DAR, (2) determine whether they have an active ARSA, and (3) examine if dopamine and DA-S affect adipose PRL expression and release [88]. A comprehensive approach was undertaken which included multiple analytical approaches as well as complementary cellular models: adipose tissue explants, primary adipocytes and two human adipocyte cell lines: LS14 and SW872, another liposarcoma-derived cell line from the ATCC.

Except for D3R, all other DAR are variably expressed at both the mRNA and protein levels in adipose tissue and adipocytes [88]. Expression of D1R decreases, while that of D2R increases during the first 3 days of adipogenesis. ARSA is expressed in adipocytes, and its enzymatic activity increases following adipogenesis. Dopamine at low nM concentrations suppresses cAMP, stimulates cGMP, and activates MAPK in adipocytes. Acting via D2R, both dopamine and DA-S inhibit PRL gene expression and release (Fig. 1.8). Dopamine shows a nonmonotonic dose-dependent inhibition of PRL, suggesting that the effects of inhibitory D2R at low dopamine doses is counteracted by stimulatory DAR at higher doses. The cAMP and/or MAPK signaling appear to be involved in mediating dopamine actions in adipocytes. Indeed, the superdistal PRL promoter has several cAMP responsive elements such as CREB and C/EBP, and two AP-1 sites which can respond to MAPK activation [96]. These data established dopamine as a suppressor adipocyte PRL via D2R through inhibition of cAMP and PKA. In addition to the suppression of adipocyte PRL, dopamine inhibits leptin and stimulates adiponectin and IL-6 release by binding to D1R and activating the cGMP/MAPK signaling (Fig. 1.8).

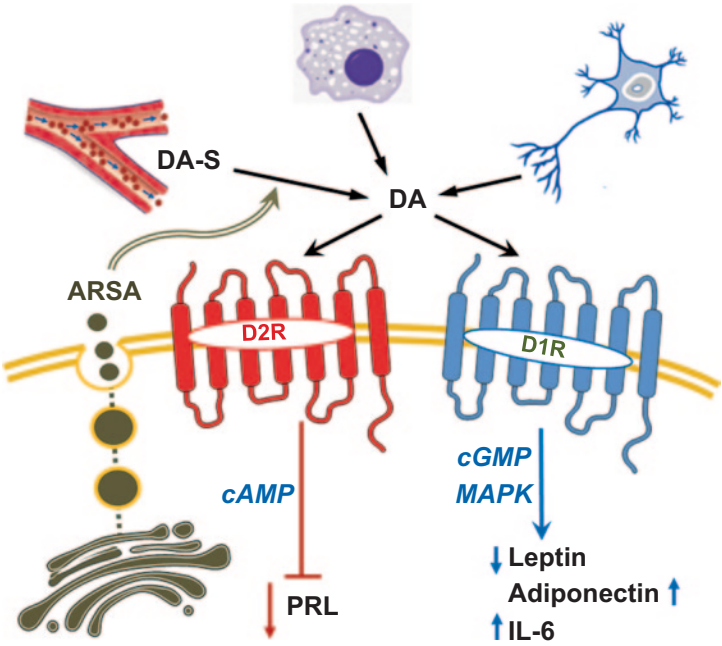

Fig. 1.8 Sources of dopamine (*DA*) and activation of *D1R* and *D2R* in human adipocytes. *DA* reaches the adipocytes from macrophages and nerve endings within adipose tissue. It is also available as dopamine sulfate (*DA-S*) which can be converted to bioactive *DA* by arylsulfatase A (*ARSA*). Acting via *D2R* and suppressing *cAMP*, *DA* inhibits *PRL* gene expression and release. Acting via *D1R* and activating both *cGMP* and/or *MAPK*, dopamine inhibits leptin and stimulates adiponectin and IL-6 release

1.6 Metabolic Functions of PRL

1.6.1 Global Actions of PRL on Body Weight and Adiposity

Chronic elevation of PRL in rats is associated with increases in food intake but inconsistent changes in body weight. Suppression of PRL results in the opposite outcome, being most effective in lactating rats and least effective in males, reviewed in [48]. Injections of PRL into the paraventricular nucleus increased food intake, suggesting interaction with hypothalamic neurons that regulate appetite [48]. A more recent study reported that chronic intracerebral infusion of PRL increased food intake without altering body weight or estrous cyclicity [97]. The complex outcome was explained by an induction of leptin resistance via activated central PRLR. Many studies on PRL-leptin interactions took advantage of pregnant and lactating rats which are hyperphagic in adaptation for increased metabolic demands by fetuses and suckling young [98, 99]. High levels of PRL (early pregnancy and lactation) or placental lactogens (mid to late pregnancy) induced central leptin resistance by

blocking its transport into the brain, and by reducing expression and signaling of its receptor, thus facilitating increased food intake.

Early studies with mice generated conflicting data. For example, elevated serum PRL, achieved by surgical or pharmacological manipulations, caused small increases in body weight and food intake with a slight decline in fat mass in males, but not females. A small decrease in retroperitoneal fat mass, but no change in body weight, was seen in PRL-overexpressing female, reviewed in [48]. Only minor changes in the overall metabolic phenotype were observed in our study with PRL-knockout mice [100]. PRL-deficiency did not affect the rate of weight gain, body composition, serum lipids, or adiponectin levels in either sex on low fat (LF) or high fat (HF) diets. Glucose tolerance was slightly impaired in very young PRL-knockout males, but not in females. Leptin was elevated only in males on LF diet. A different metabolic profile was seen in PRLR-knockout mice. The first report on a substantial decrease in weight gain and abdominal fat mass in old mice [101], was not confirmed in later studies with younger animals, attributing the weight loss in aging PRLR-deficient mice to the development of pituitary tumors.

More recent studies utilizing transgenic mice with altered PRLR, have clarified some of the above discrepancies. In one study, total PRLR deficiency was associated with resistance to HF-induced obesity due to enhanced energy expenditure and increased metabolic rate; these were attributed to the induction of brown adipocytes in several fat depots [102]. PRLR inactivation was associated with increased expression of genes that regulate brown adipocytes, suggesting that PRL suppresses transdifferentiation of white adipocytes into metabolically active brown adipocytes. Another study used mice that express only the long form of the PRLR [103]. These mice showed increased accumulation of visceral fat in older males without a significant change in body weight. The increased epididymal fat was attributed to suppression of leptin and diminished lipolysis. However, explanations for the sex-dependent changes, as well as evidence for lack of production of short PRLR isoforms were not provided.

In humans, sustained PRL elevation, caused by antipsychotic drugs [104] or prolactinomas [105], leads to increased weight, which can be ameliorated by normalization of serum PRL. Unexpectedly, the reduction in body weight in response to bromocriptine was more effective in men than women [106]. However, weight loss was not seen in all patients, was modest and delayed, and did not correlate well with the rapid and marked suppression of serum PRL levels. At present, there is no strong evidence that PRL at normal circulating levels is a major factor in human obesity. This still leaves open the possibility that certain individuals are more responsive to PRL due to variations in PRLR expression, presence of PRLR isoforms, and/or altered PRL signaling. Polymorphism in a site adjacent to the PRL gene was associated with increased risk of obesity in men but not women [107], but the relevance of this finding to the role of PRL in obesity is unclear.

1.6.2 Mammary Gland Metabolism

PRL is expressed and released by both adipose and glandular compartments of the normal human breast [78]. Recent evidence shows that activation of the PI3K/Akt pathway in the mouse mammary epithelium induced expression of autocrine PRL which is required for the initiation of lactation [6, 108]. In rats, PRL expression, determined by RT-PCR and in situ hybridization, was seen in alveolar and ductal epithelial cells in late pregnancy and throughout lactation [109]. PRLR expression in the rat mammary gland is low during most of pregnancy, increases on day 21, just before parturition, and continues to rise during lactation [110]. Both long and short PRLR isoforms are detectable in ducts and alveoli of the lactating mouse mammary gland, with some PRLR immunostaining seen near lipid droplets, suggesting expression by adipocytes [111]. In the same study, a strong PRLR immunostaining was seen in ductal epithelium of breast tissue from normal, nonpregnant, nonlactating women, and a lower staining in myoepithelial cells. Unfortunately, virtually nothing is known about expression of PRL or PRLR in the human breast during pregnancy or lactation.

Studies with rodents and ruminants were instrumental in developing the concept that during lactation, PRL acts as a physiological sensor which responds to high metabolic demands for milk production by partitioning nutrients away from adipose tissue into the mammary gland [112]. In the lactating mammary gland, PRL affects the synthesis of all milk constituents: proteins, lactose, and lipids. Here, we focus on lipids only. Compared to adipose tissue, lipid metabolism in the nonlactating mammary gland is negligible. However, at the onset of lactation, lipid production is blunted in adipose tissue and increases manyfold in the mammary gland, which produces TAGs from dietary fatty acids and de novo synthesis. The epithelial cells sequester fatty acids from adjacent adipocytes for de-novo lipogenesis [58]. As detailed in a previous review [48], PRL strongly enhances mammary lipid production by affecting the activities of many lipid biosynthetic enzymes: lipoprotein lipase (LPL), pyruvate dehydrogenase (PDH), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS).

Several issues that are relevant to PRL and the mammary gland should be considered. One is local PRL production in the lactating mammary gland in rodents [5, 6] and human breast [78]. It is unknown whether local PRL emulates circulating PRL or fulfills distinct roles, because of selective phosphorylation, glycosylation, or cleavage. Another issue is the presence of large amounts of heavily glycosylated PRL in human milk [113]. Future studies should examine in more detail the synthesis, bioactivity, and transport of PRL into milk. Notably, the developing human fetus is exposed to very high levels of PRL from the amniotic fluid and the fetal pituitary, reviewed in [114], but the biological significance of high PRL availability to the fetus remains elusive. Milk PRL may represent a continuum of PRL availability to the newborn, who can absorb intact proteins through the GI tract for several days after birth [115].

1.6.3 Adipogenesis

Based on the belief that the PRLR is not expressed in adipose tissue, it was initially proposed that PRL does not directly regulate adipocyte functions [112]. As reviewed previously [10, 48], this concept has been revised following the reports that PRLR is expressed in both brown and white adipose tissue in all species examined. Expression of long and short PRLR isoforms increases manyfolds during differentiation of rat epididymal preadipocytes [116]. In human breast preadipocyte, PRLR shows an initial decrease, followed by an increase during adipogenesis [86]. PRLR, but not GHR, was markedly induced following differentiation of 3T3-L1 cells [117], which temporally coincided with a robust activation of Stat5a and 5b [118]. PRL upregulates the expression of its receptor in epididymal adipocytes [116], and increases Stat5a and 5b activity in differentiated 3T3-L1 cells [117].

Fetal bovine serum, which contains large quantities of lactogenic hormones and is required for efficient differentiation of 3T3-L1 cells, can be replaced by either GH or PRL [119]. PRL enhances the expression of C/EBPβ and PPARγ, two key transcription factors in adipogenesis. Furthermore, ectopic expression of the PRLR in NIH-3T3 cells increases the efficacy of adipocyte conversion when stimulated with PRL and a PPARγ ligand [120]. Studies with PRLR-deficient mice are also supportive. Receptor deficiency results in reduced size of fat depots, which was due to a lower adipocyte number rather than to a change in their volume [121]. PRL also plays a role in the differentiation, or transdifferentiation, of brown adipocytes [122].

Stat5 appears to be particularly critical for adipogenesis. Stat5 activation increases early in adipogenesis, and induces both the expression and activation of PPARγ, while targeting multiple genes that are associated with lipid and glucose metabolism as well as insulin signaling in mature adipocytes [123]. It is difficult, however, to assign a commanding role for PRL in Stat5 activation because it is equally induced by GH.

1.6.4 Lipid Metabolism and Adipokine Release

There is only sparse and inconsistent information on the involvement of PRL in lipid metabolism in adipose tissue under nonlactating conditions. Various rodent models with altered PRL/PRLR provide indirect, and often weak, support to this effect [60, 100, 103]. Studies with human subjects with hyperprolactinemia have not produced compelling evidence either. As illustrated in Fig. 1.9, PRL suppressed lipogenesis by inhibiting LPL activity [124], reducing GLUT4 expression, and lowering malonyl-CoA concentrations [125]. In fully differentiated 3T3-L1 adipocytes, PRL downregulated FAS expression [126].

A confounding problem in many *in vitro* studies is the use of supraphysiological doses of PRL. For example, PRL inhibited lipolysis in rat epididymal adipose explants in a dose-dependent manner within a narrow physiological range, while a higher dose of PRL resulted in a nonmonotonic curve [100]. Loss of linear

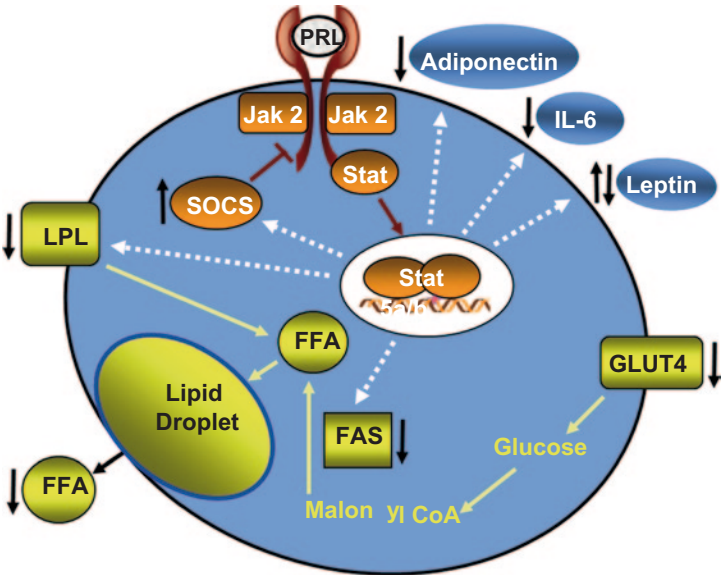

Fig. 1.9 Overall actions of *PRL* on lipid metabolism and adipokine release from adipose tissue. *PRL* inhibits lipid synthesis by suppressing Glut4, lipoprotein lipase (*LPL*), and fatty acid synthase (*FAS*), but it also inhibits lipolysis and the release of free fatty acids (*FFA*). Both *adiponectin* and *IL-6* are inhibited by *PRL* while both stimulatory and inhibitory effects of *PRL* on *leptin* have been reported

dose–response relationships at high doses has been observed in some PRL target tissues and can lead to erroneous interpretation if only a single high dose is used. At high concentrations, PRL can downregulate the receptor, hinders receptor dimerization, activates dominant negative short receptors, or induces SOCS.

Direct effects of PRL on lipolysis vary among species, showing inhibition of isoproterenol-stimulated lipolysis in rat and human adipose tissues, but having no effects on lipolysis in mouse adipose explants [100, 116]. The anti-lipolytic effect of PRL in rat epididymal adipose explants takes several hours, suggesting transcriptional regulation rather than altered cAMP levels or phosphorylation of HSL and/or perilipin, as is the case with catecholamines and insulin.

Data on the effects of PRL on adipokines vary with the species and the experimental model, i.e., whether conducted *in vivo* or *in vitro,* representing indirect vs. direct effects, respectively. Such considerations are well illustrated by the variable data on the effects of PRL on leptin (Fig. 1.9). For example, serum leptin levels are lower in PRLR-deficient mice [101, 103], and are elevated in PRL-overexpressing mice [127]. However, an inhibitory effect of PRL is suggested by higher serum leptin levels in male PRL-knockout mice [100]. In rats, elevated serum PRL levels, achieved by pituitary grafts or PRL injections, increased serum leptin levels [128], while hyperprolactinemic patients had higher [129] or unchanged [130] serum leptin.

Data on leptin, based on *in vitro* studies are either inconsistent or have inherent limitations. PRL inhibits insulin-stimulated leptin release in mouse white adipocytes [127], but potentiates the effect of insulin in brown adipocytes [131]. Incubation of rat adipose tissue explants with PRL caused dose-dependent inhibition of leptin release [116]. Unfortunately, leptin expression in 3T3-L1 cells is severely downregulated, while the presence of autocrine PRL in human adipocytes confounds studies on its effect on leptin release. Presently, it is difficult to reach a clear conclusion to what extent PRL contributes to the control of leptin release.

Adiponectin is also affected by PRL. An inhibitory effect of PRL on adiponectin release is supported by the reduced serum adiponectin levels in both PRL transgenic and PRL-treated mice [132, 133]. PRL, however, is unlikely a major regulator of adiponectin in mice since deficiencies in either PRLR [133] or PRL [100] have no effect on serum adiponectin levels. Studies with human adipose tissue explants and isolated mature adipocytes show direct inhibitory effect of PRL on adiponectin release [133, 134]. However, a similar inhibitory relationship has not been observed in hyperprolactinemia patients.

1.7 Conclusions and Future Directions

PRL should be recognized as a metabolic hormone whose actions are not confined to the lactating mammary gland. Globally, excess PRL correlates with changes in food intake and body weight in some species, with marginal effects on fat deposition. Emerging data suggest that PRL plays a role in whole body insulin sensitivity through its stimulatory effect on insulin release and regulation of adipokine release. The recent finding of lower PRL release from human sc adipose tissue in obese vs. lean individuals suggests that adipose PRL may be involved in obesity-related complications, and should be further explored (Fig. 1.10).

After being overlooked for a long time, the metabolic aspects of PRL have recently come into focus, in tune with the growing interest in obesity and diabetes. The rat may be a better model than the mouse for analyzing some metabolic aspects of PRL in live animals. On the other hand, the large repertoire of murine and human primary adipocytes and cell lines that express the PRLR provide an excellent opportunity to study interactions between PRL and metabolic hormones such as insulin, glucocorticoids, and catecholamines which affect adipogenesis, glucose, and lipid metabolism. Comparisons should also be made between the actions of PRL and GH, its sister molecule.

Being an emerging field with little fundamental knowledge, there are multiple challenges for future research. These include examination of PRL action on insulin release and β-cell functions in males and nonpregnant females, and explorations of PRL effects on the liver, a key organ in metabolic homeostasis which expresses high levels of the PRLR. Another issue of great interest is whether PRL is involved in human obesity and insulin resistance via its capacity to alter the production and release of adipokines, such as leptin and adiponectin.

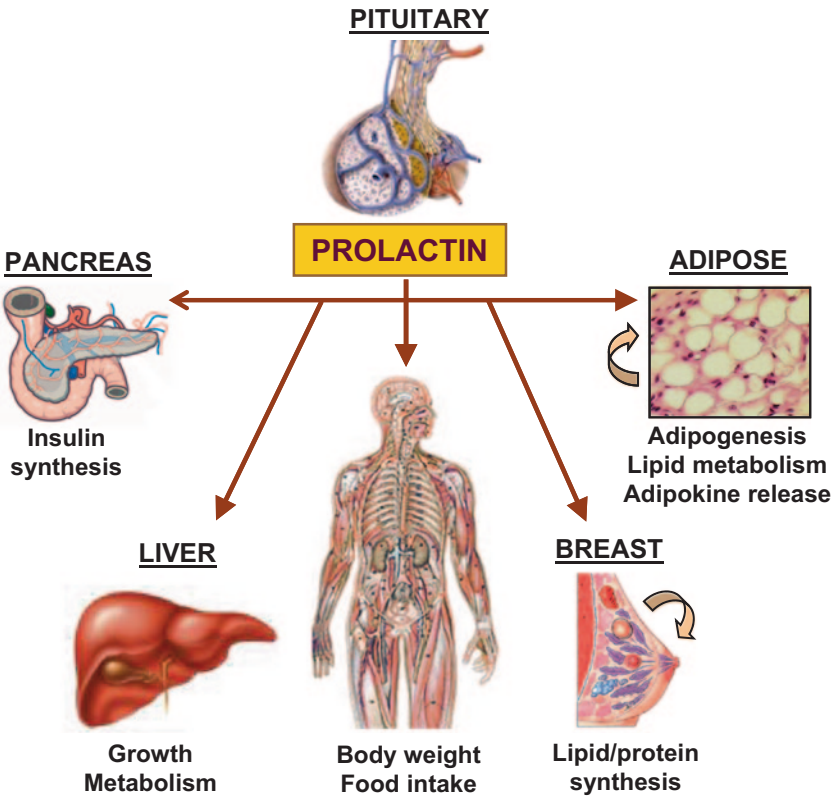

Fig. 1.10 The overall metabolic actions of PRL. PRL can reach target organs through the blood from the pituitary or through local production in adipose tissue and breast adipocytes

To better establish whether PRL plays a role in lipid metabolism more comprehensive and methodological studies are urgently needed. Notably, available data reveal that PRL affects adipocyte functions in males, indicating that its impact on metabolic homeostasis is broader than previously appreciated. Although the PRLR is highly expressed in the liver, surprisingly little work has focused on potential actions of PRL in this tissue, which is so central to metabolic homeostasis. Given that PRL regulates enzymes and transporters associated with glucose and lipid metabolism in other target organs, future studies should examine its direct effects on hepatic tissue.

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Chapter 2
Signaling Pathways Regulating Pituitary Lactotrope Homeostasis and Tumorigenesis

Allyson K. Booth and Arthur Gutierrez-Hartmann

Abstract Dysregulation of the signaling pathways that govern lactotrope biology contributes to tumorigenesis of prolactin (PRL)-secreting adenomas, or prolactinomas, leading to a state of pathological hyperprolactinemia. Prolactinomas cause hypogonadism, infertility, osteoporosis, and tumor mass effects, and are the most common type of neuroendocrine tumor. In this review, we highlight signaling pathways involved in lactotrope development, homeostasis, and physiology of pregnancy, as well as implications for signaling pathways in pathophysiology of prolactinoma. We also review mutations found in human prolactinoma and briefly discuss animal models that are useful in studying pituitary adenoma, many of which emphasize the fact that alterations in signaling pathways are common in prolactinomas. Although individual mutations have been proposed as possible driving forces for prolactinoma tumorigenesis in humans, no single mutation has been clinically identified as a causative factor for the majority of prolactinomas. A better understanding of lactotrope-specific responses to intracellular signaling pathways is needed to explain the mechanism of tumorigenesis in prolactinoma.

2.1 Introduction

Prolactin (PRL) is a 23 kDa polypeptide hormone that is a member of the growth hormone (GH) family and is primarily synthesized and secreted from lactotrope cells of the anterior pituitary gland. In mammals, PRL acts at the mammary gland to promote growth and development, milk synthesis, and maintenance of milk secretion [1]. Knockout of PRL or PRL-receptor genes in mice results in impaired growth and development of the mammary gland and absence of milk production [2, 3]. The strongest stimulus for PRL secretion from lactotrope cells is suckling,

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with the duration and intensity of the stimulus corresponding to the amount of PRL secreted into the blood [1, 4, 5].

In addition to its classical actions on the mammary gland, PRL also influences many other physiological systems. The PRL receptor is expressed in the mammary gland, gonads, uterus, brain, pituitary gland, adrenal gland, lung, heart, liver, skeletal muscle, skin, and lymphocytes. Elevated PRL levels act at the gonads to decrease the sensitivity of follicle stimulating hormone (FSH) and luteinizing hormone (LH) receptors. Furthermore, circulating PRL attenuates pulsatile secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus, reducing LH and FSH secretion from the anterior pituitary gland [6]. As a result, increased levels of PRL cause reduced secretion of and sensitivity to LH and FSH, leading to suppression of ovulation. During pregnancy, elevated serum PRL has effects that extend beyond the reproductive system. At the adrenal gland, PRL increases androgen and dihydroepiandrosterone (DHEA) steroidogenesis, and also reduces cortisol and aldosterone secretion [6]. In the liver, PRL increases lipoprotein lipase activity in hepatocytes and increases bile secretion. PRL has osmoregulatory effects in the kidney, reducing renal sodium and potassium excretion, and also increases sodium and chloride excretion in sweat and salt and water absorption in the intestine. Lastly, PRL influences the immune system by inducing proliferation of lymphocytes [6].

As PRL is involved in various different physiological systems, signaling pathways are critical for regulating lactotrope biology from humans to rodents. Pituitary lactotropes have a high-basal PRL secretory activity. To maintain PRL homeostasis, tonic inhibition by dopamine acting via the D2 receptor (D_2R) is required to limit PRL production and secretion, lactotrope proliferation, and growth of PRL-secreting adenomas [7–13]. During pregnancy and lactation, dopaminergic inhibition is diminished by estradiol, allowing local growth factors from folliculostellate support cells to stimulate lactotropes, promoting lactotrope hyperplasia and doubling in pituitary size [7, 14–16]. Circulating PRL levels are elevated during pregnancy and lactation, creating a state of physiological hyperprolactinemia. Dysregulation of the signaling pathways that govern lactotrope biology contributes to tumorigenesis of PRL-secreting adenomas, or prolactinomas [16–18], leading to a state of pathological hyperprolactinemia. Prolactinomas cause hypogonadism, infertility, osteoporosis, and tumor mass effects, and are the most common type of neuroendocrine tumors [19, 20].

In this review, we highlight signaling pathways involved in lactotrope development, homeostasis, and physiology of pregnancy, as well as implications for signaling pathways in pathophysiology of prolactinoma. We review mutations found in human prolactinoma and discuss how such mutations influence signal transduction in lactotrope cells. Lastly, we present a brief review of animal models that are useful in studying pituitary adenoma.

2.2 Signaling Pathways Regulating Pituitary Stem/Progenitor Cells Leading to Lactotrope Development/Ontogeny

During embryogenesis, the pituitary first develops from the anterior neural ridge (ANR) of the neural plate. The actual pituitary organogenesis begins at embryonic day 8.5 (E8.5) with the formation of Rathke's pouch. The ventral diencephalon, which will ultimately become the hypothalamus, develops from neural plate cells posterior to the ANR [21]. The process of pituitary development is dependent upon the homeobox gene Tift1, as well as fibroblast growth factor 8 (FGF8) and bone morphogenic protein 4 (BMP4) signaling from the ventral diencephalon. Knockout of Titf1 results in pituitary aplasia [22]. FGF8 signaling and the resulting expression of the LIM homeodomain transcription factor Lhx3 is required for pituitary development to progress beyond the formation of Rathke's pouch [21]. Without BMP signaling from the ventral diencephalon, pituitary development does not progress beyond E10. Sonic hedgehog (Shh) signaling is required for pituitary patterning and proliferating after E10. Shh works in unison with FGF8 to maintain Lhx3 expression, and it also induces BMP2 expression in the ventral pouch ([21]; Fig. 2.1).

Transient, intrinsic BMP2 and Wnt4 signaling gradients in the developing pituitary gland promote proliferation and establish a pattern that determines localization of specific pituitary cell types [21]. Somatotrope and lactotrope cells arise within the caudomedial region of the developing pituitary gland. Before each cell type can progress beyond initial proliferation and localization, expression of cell-fate-specific transcription factors is required. For lactotropes, somatotropes, and thyrotropes, expression of paired-like homeodomain factor 1 (Prop1) and Pit-1 POU homeodomain protein is required for terminal differentiation (Fig. 2.1). Prop1 is required for Pit-1 activation, and is expressed only in the developing pituitary gland. Deficiency of Prop1 leads to near complete loss of somatotrope, lactotrope, and thyrotrope cells [23]. After E17.5, cells in the Pit-1 lineage exhibit permanent cell-autonomous commitment and cannot be converted to alternative fates [21]. Hormone secretion from differentiated thyrotropes, somatotropes, and lactotropes is regulated by hypothalamic thyroid-releasing hormone (TRH), GH-releasing hormone (GHRH), and dopamine, respectively (Fig. 2.1).

The Pit-1 transcription factor binds to promoter regions of GH and PRL genes, and is required for their activation. Pit-1 can associate with coactivators and corepressors, and the Pit-1 binding partners required to activate PRL versus GH gene transcription are involved in activation of signaling pathways. Ras-dependent activation of Ets/Pit-1 synergy results in PRL gene transcription [24–26]. Pit-1 is necessary for cell-specific determination, but it is not sufficient; for lactotropes, estrogen receptor (ER), and Ets transcription factors are also required [25].

Until recently, the dogma was that the embryonic ontogeny pathways were also responsible for facultative responses to meet increased pituitary hormonal demand during periods of physiological stress, including lactotrope expansion during pregnancy. However, the identification of pituitary postnatal stem/progenitor

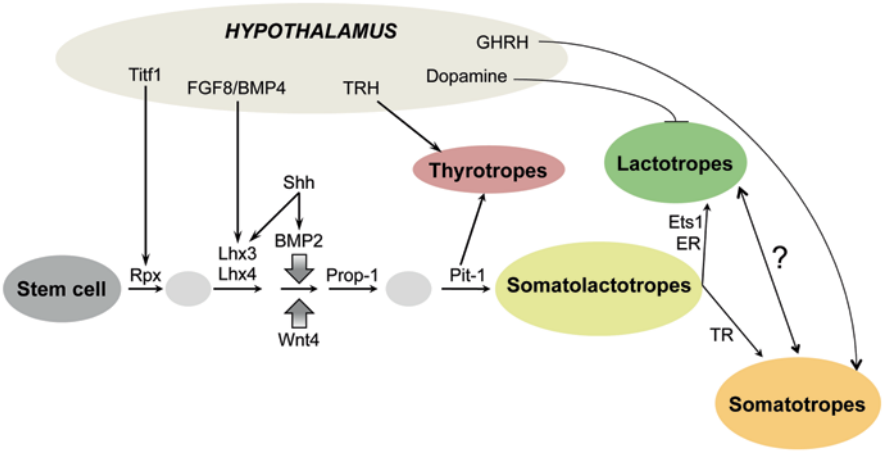

Fig. 2.1 Embryonic ontogeny and *Pit-1* pituitary cell lineage. All hormone-secreting cells in the anterior pituitary gland originate from pituitary stem cells. During embryonic development, *FGF8* and *BMP4* from the hypothalamus stimulate LIM homeodomain transcription factors (Lhx) 3 and 4. Intrinsic gradient signaling of *Wnt4* and *BMP2*, and expression of the *Prop-1* transcription factor, play key roles in determination of pituitary cell fate and localization. *Thyrotropes*, *somatotropes*, and *lactotropes* are derived from the *Pit-1* lineage. Hormone secretion from thyrotropes, somatotropes, and lactotropes is regulated in part by hypothalamic *TRH*, *GHRH*, and *dopamine*, respectively, and the *Pit-1* transcription factor is required cell-specific determination. In lactotropes, *Ets1* and *ER* are also required for prolactin (PRL) production. In *somatotropes*, the thyroid hormone receptor (*TR*) is required for growth hormone (GH) secretion. In rats, a somatolactotrope precursor cell gives rise to PRL secreting lactotropes and GH secreting somatotropes. The contribution of such a precursor cell is well described in rats, but has less of contribution in mice. The existence of a somatolactotrope cell in humans, as well as the possibility that lactotropes and somatotropes may give rise to one another in response to physiological demand, has yet to be confirmed in humans

cells (pSPCs) within the past decade has challenged this dogma. A niche containing pSPCs exists into adulthood in the pituitary gland and is the likely source of facultative organ expansions driven by upstream endocrine tropic hormones and stromal growth factors in response to increased physiological demand (Fig. 2.2). Cells from the anterior pituitary gland are capable of forming "pituispheres," and these cells segregate into the "side population." This side population contains 1–5 % of total pituitary cells, and is a FACS cell fraction known to harbor bona fide stem cells [27]. Further analysis of cells in the side population fraction revealed high expression levels of Sca1, as well as expression of other stem cell markers such as Oct-4, nanog, nestin, CD133, and Bmi-1 [27]. A few years later, three separate studies reported the existence of stem cells in the pituitary gland [28–30]. Together, these studies reveal that the periluminal pSPCs express SSEA-4, Oct4, Sox2, GFRa2, Sca1, nestin, Prop-1, Lhx-3, E-cadherin, and cytokeratins 8 and 18. Importantly, pSPC cells do not express embryonic pituitary stem cell makers Hesx-1 and Lhx-4, distinguishing these cells from embryonic pituitary stem cells. Notch signaling

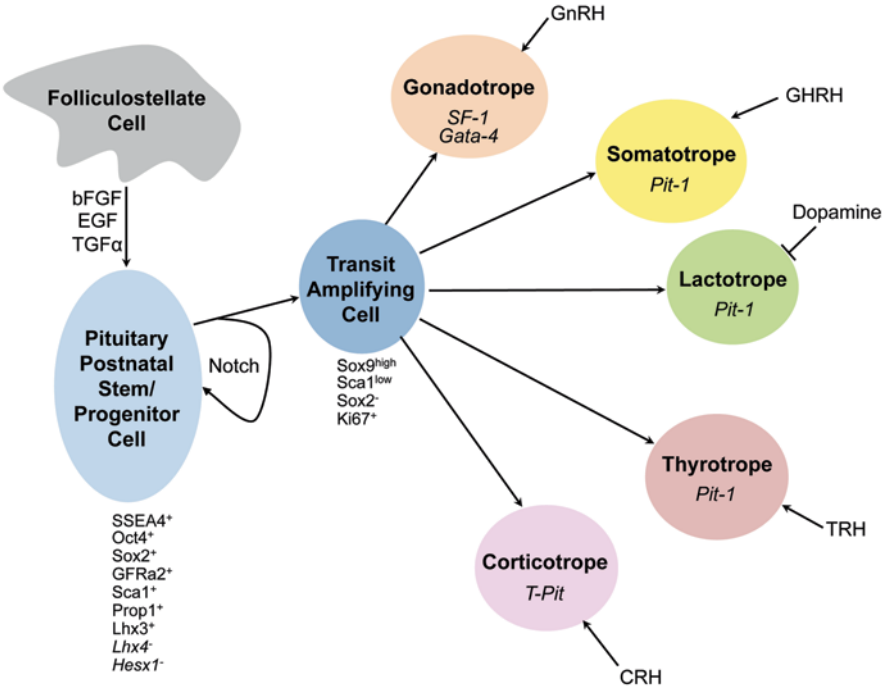

Fig. 2.2 Adult pituitary stem cells, facultative cell expansion, and pituitary tumorigenesis. A niche containing pituitary postnatal stem/progenitor cells (pSPCs) exists into adulthood in the pituitary gland and is the likely source of facultative organ expansions that occur in response to increased physiological demand. Folliculostellate support cells provide growth factors to stimulate pSPCs, and Notch signaling regulates stem cell homeostasis. The pSPCs express *SSEA-4*, *Oct4*, *Sox2*, *GFRa2*, *Sca1*, nestin, *Prop-1*, and *Lhx-3*, but do not express embryonic pituitary stem cell makers Hesx-1 and *Lhx-4*. Transit-amplifying (TAC) cells express *Sox-9* and *Sca1*, but not *Sox-2*, and proliferate more rapidly than pSPCs to allow for prompt cellular expansions in response to physiological demand. The precise signaling events that regulate these expansions remain unknown. Expression of cell-specific transcription factors is required for hormone secretion from each cell type. Hormone secretion from differentiated gonadotropes, somatotropes, lactotropes, thyrotropes, and corticotropes is regulated by gonadotropin releasing hormone (*GnRH*), *GHRH*, *dopamine*, *TRH*, and corticotropin releasing hormone (*CRH*), respectively

functions in pSPC homeostasis [31]. One study also identified putative transit-amplifying (TAC) cells, which express Sox-9, low levels of Sca1, and do not express Sox-2 [28]. The TAC cells are considered to be capable of rapid proliferation, compared to the slow asymmetric doubling of pSPCs, suggesting a role as an important precursor allowing for cellular expansions into differentiated cell types as needed to meet adaptive responses (Fig. 2.2). However, the signaling mechanisms governing these neuroendocrine expansions, the precise role of pSPCs in these adaptive responses, and whether a perturbation in the expansion process leads to prolactinoma tumor formation, all remain unknown [32].

2.3 Signaling Pathways Regulating Lactotrope Homeostasis, Physiological Expansion, and Tumorigenesis

During pregnancy, the mammalian pituitary gland doubles in size, primarily due to expansion of PRL-producing lactotrope cells. However, there is a great deal of debate as to whether this doubling in size is a result of lactotrope hypertrophy or hyperplasia. For obvious reasons, the availability of human pituitary tissue from pregnant women is scarce, and as such many questions remain concerning the morphological changes in the human pituitary gland during pregnancy. Studies in rodents are useful, but are also challenging because human and rodent pituitary physiology is not entirely analogous. In rats, bi-hormonal somatolactotrope precursor cells retain plasticity, allowing for rapid cell differentiation and expansion in response to hormonal need. Somatolactotropes differentiate into lactotropes during pregnancy and into somatotropes in response to exercise [33–36]. No such precursor cell has been identified in humans, and therefore the use of rodent models to study the pituitary during pregnancy becomes convoluted. Additionally, our understanding of the mechanism whereby expanded lactotropes return to the prepregnant state remains unclear. The role of apoptosis, senescence, or simply diminished cell synthesis activity in this process is not understood.

There is an immense capacity for expansion within the lactotrope cell population. During pregnancy, the lactotrope cell population doubles in size. As such, signaling pathways within lactotrope cells are primed to induce rapid cellular expansion. With so much capacity for expansion, there is an increased risk that problems may occur and result in uncontrolled growth. It is very likely that the signaling pathways that are in place to allow lactotropes to undergo recurrent expansions also prime the cell for tumorigenic responses, if one or more oncogenic mutations are present. Here, we will discuss the role of these signaling pathways, and will focus on the pathways that are also known to be involved in mechanisms of tumorigenesis.

2.3.1 Cyclic 3'-5'-Adenosine Monophosphate (cAMP) and Protein Kinase A (PKA) Signaling

cAMP is a second messenger that regulates a diverse set of cellular events. Upon stimulation from an extracellular ligand, G-protein-coupled receptors (GPCRs) become activated and stimulate an associated G-protein. The resulting downstream signaling events depend upon the alpha subunit of the G-protein. $G_{\alpha s}$ proteins activate adenylate cyclase, an enzyme that catalyzes the conversion of ATP to cAMP, leading to a rapid increase in intracellular cAMP and activation of cAMP-dependent PKA. Activation of the cAMP/PKA pathway stimulates the rPRL promoter via the Pit-1 binding sites of FPI and FPIII [37–39]. $G_{\alpha i}$ proteins inhibit adenylate cyclase

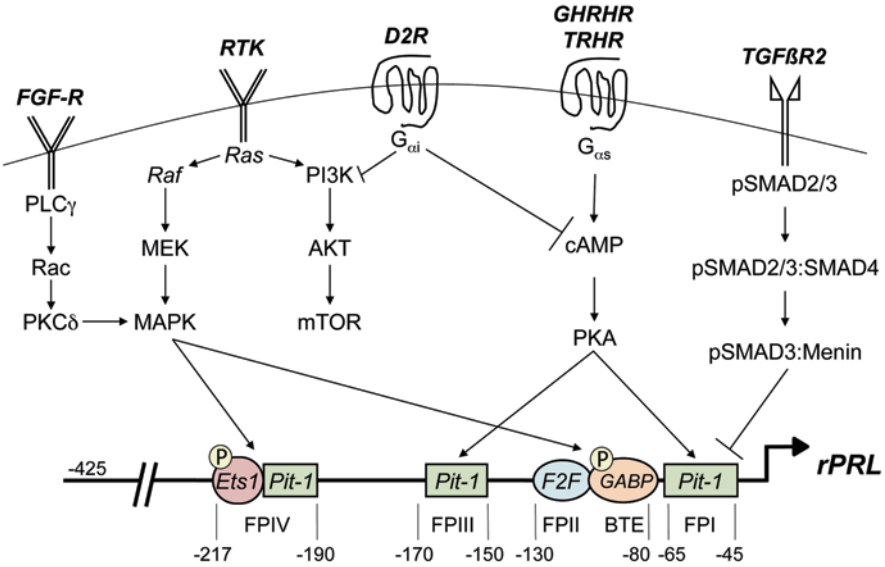

Fig. 2.3 Lactotrope signaling pathways central to pituitary cell proliferation, tumorigenesis, and proximal rat prolactin (PRL) promoter activation. Growth factor receptor tyrosine kinase (*RTK*) and GPCR signaling pathways regulating lactotrope homeostasis and rat PRL (*rPRL*) promoter activation are depicted here. The proximal *rPRL* promoter, with Pit-1 binding sites (*FPI*, *III*, *IV*), *Ets-1* and *GABP* binding sites, and the *F2F* ubiquitous factor binding site are also shown. For further details, see the review by Gutierrez-Hartmann, et al. [39]

activity, resulting in diminished intracellular cAMP levels and reduced PKA activity ([40]; Fig. 2.3).

One of the most studied in the classic regulatory pathways of lactotrope homeostasis is dopaminergic inhibition of lactotrope expansion and PRL secretion. In homeostatic conditions, the secretion of PRL from pituitary lactotropes is inhibited by dopamine. Dopamine binds to the D_2R receptor, which is coupled to a $G_{\alpha i}$ protein, and thus inhibits intracellular cAMP accumulation [7]. Without cAMP, the catalytic subunit of PKA remains sequestered by the regulatory subunit, and cytoplasmic and nuclear target proteins are not phosphorylated, preventing activation of PRL gene transcription and PRL release from the lactotrope cell (Fig. 2.3). GPCR kinases (GRKs) function to desensitize GPCRs that are involved in chemotaxis, and have been shown to play a critical role in cell motility [41]. Another level of homeostatic regulation exists within a short feedback loop between the pituitary and hypothalamus. PRL can bind at the prolactin receptor (PRL-R) on hypothalamic TIDA neurons, increasing dopaminergic release in response to both acute and chronic increases in PRL [42], and further inhibiting cAMP and PKA signaling in lactotrope cells. However, TIDA neurons become refractory when exposed to prolonged hyperprolactinemia during pregnancy or with prolactinoma.

During pregnancy, placental human chorionic gonadotropin (hCG) stimulates production of ovarian estradiol. In response to estradiol, hypothalamic tyrosine hydroxylase, the enzyme that catalyzes the hydroxylation of tyrosine to produce dopamine, is dephosphorylated and inactivated [7, 43, 44]. Similarly, when a suckling stimulus occurs in a lactating mother, dopaminergic inhibition is relieved and PRL is secreted into the blood [45, 46].

Clinically, dopamine agonists such as cabergoline and bromocriptine are used to treat hyperprolactinemia [19]. In many patients, dopamine agonists are successful in halting lactotrope cell proliferation, shrinking prolactinoma size, and reducing PRL secretion. However, a subset of patients are resistant to dopamine agonist therapy [19], likely due to dysfunctional dopamine receptors. Indeed, if dopamine signaling is abolished by dysfunction or knockout of the D_2R receptor in mice, lactotrope homeostasis is lost, resulting in prolactinoma formation [47].

2.3.2 Mitogen-Activated Protein Kinase (MAPK) Signaling

The MAPK signaling pathways connect a wide variety of extracellular signals to intracellular outcomes, including proliferation, differentiation, and apoptosis. The MAPK pathways consist of a three-level kinase cascade, where a MAPK is phosphorylated by a mitogen-activated protein kinase kinase (MAPKK), which must first be phosphorylated by a mitogen-activated protein kinase kinase kinase (MAPKKK). The ERK pathway is the best studied of the MAPK signaling pathways, as dysregulation of ERK signaling is associated with many human cancers. In the ERK signaling pathway, extracellular growth factors and mitogens bind to receptor tyrosine kinases, activating the GTPase Ras, which leads to recruitment and activation the MAPKKK Raf, phosphorylation of the MAPKK Mek, and stimulation of ERK, which ultimately results in phosphorylation of a wide variety of effector proteins including other kinases, phosphatases, and transcription factors ([48]; Fig. 2.3).

The D_2S receptor, the short isoform of D_2R, functions to upregulate MAPK signaling in lactotropes upon stimulation by dopamine [47], suggesting that basal activation of the MAPK pathway does not promote proliferation, but instead maintains lactotrope homeostasis. Furthermore, key regulators of lactotrope biology, such as thyrotropin releasing hormone (TRH) and vasoactive intestinal peptide (VIP), act via Ras to activate MAPK in somatolactotrope cells [49–51].

The duration of MAPK signaling is critical in dictating cellular response [52]. In this review, we will use the following terms: short-term (minutes to hours), long-term (hours to days), and persistent (many days or constitutive activation). Estrogen-induced PRL expression is MAPK-regulated [53], and importantly, the estrogenic effect on lactotropes during pregnancy is persistent, lasting for many months. Estrogen stimulates folliculostellate support cells to produce growth factors such as fibroblast growth factor (FGF) that act via the MAPK pathway [14]. The Ras/MAPK pathway regulates the PRL promoter via a composite Ets1/Pit-1 site [24, 25, 39, 54], and via a BTE ([55, 56]; Fig. 2.3). The precise role of MAPK signaling

in lactotrope proliferation versus differentiation has been somewhat controversial. In vitro studies using rat pituitary somatolactotrope or lactotrope cell lines have shown that short-term (24–96 h) MAPK pathway activation mediates cellular proliferation [14, 57, 58]. By contrast, long-term treatment of GH3 or GH4 rat pituitary tumor cells over 4–7 days with epidermal growth factor (EGF), fibroblast growth factor-4 (FGF4), or thyrotropin-releasing hormone (TRH) result in decreased GH4 cell proliferation and enhanced differentiation to the lactotrope phenotype [59–63]. A persistent pattern of pMAPK activation has been shown to play a pivotal role in cellular differentiation in other endocrine tumors including thyroid carcinoma and pheochromocytoma [64, 65]. The inconsistency in the reported effects of MAPK on lactotrope proliferation or differentiation suggests that the duration of MAPK activation is also critical in dictating the response of lactotrope cells.

The specific role of MAPK signaling in durable lactotrope proliferation and differentiation, and whether activated pMAPK is sufficient for lactotrope proliferation and tumor formation remains unknown. Ras mutations and persistently activated pMAPK are found in human tumors [66, 67], including prolactinomas and other pituitary tumors [18, 68, 69]. Uncontrolled activation of growth factor signaling pathways, such as the Ras/MAPK pathway, results in lactotrope hyperplasia with very delayed adenoma formation in transgenic mice [17, 70]. Transforming growth factor α (TGFα) activates the epidermal growth factor receptor (EGFR) to stimulate the Ras/Raf/MAPK pathway. TGFα is expressed in lactotropes, and upon overexpression promotes proliferation, suggesting a role for TGFα and MAPK signaling in prolactinoma formation [71].

2.3.3 Phosphatidylinositol-3-Kinase (PI3K) Signaling

The PI3K family of lipid kinases functions to activate signaling cascades that regulate diverse intracellular processes such as cell survival, cell cycle progression, and cell growth. Extracellular growth factors bind to receptor tyrosine kinases, which are associated with an intracellular PI3K. When growth factor binds, the receptor is autophosphorylated and PI3K binds to the receptor. The catalytic subunit of PI3K is allosterically activated, resulting in the conversion of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2 or PIP2) to the second messenger phosphatidylinositol-4,5-trisphosphate (PI-4,5-P3 or PIP3). PIP3 anchors Akt near the membrane via its plekstrin homology (PH) domain, where Akt is phosphorylated by 3′phosphoinositide-dependent kinase 1 (PDK1), which also has a PH domain. Akt is also phosphorylated by the mammalian target of rapamycin (mTOR) 2 complex, mTORC2. Once phosphorylated, Akt activates and inhibits several targets to ultimately influence cell survival, growth, and proliferation. PTEN, a PI-3,4,5-P3 phosphatase, can dephosphorylate PIP3 to negatively regulate PI3K/Akt signaling [72].

The D_2L receptor, a long isoform of D_2R, inhibits PI3K/Akt signaling in lactotropes upon activation by dopamine ([47]; Fig. 2.3), suggesting that inhibition of the PI3K pathway is necessary to inhibit lactotrope proliferation. Inhibition of Akt

results in decreased GH3 somatolactotrope cell viability, likely due to decreased NF-κB activity [73]. Further studies revealed that the proliferative effects of constitutively activated Akt were diminished by the mTOR inhibitor rapamycin as a result of G1 growth arrest [74]. Pharmacological inhibition of PI3K or AKT in GH4C1 somatolactotrope cells results in increased phosphorylation of ERK1/2, as well as Raf1 kinase activity [75]. However, these effects of PI3K/AKT inhibition were diminished upon cotreatment with IGF-1 [75], suggesting that the MAPK and PI3K pathways regulate lactotrope physiology through a delicate balance of intracellular signaling. Preclinical data suggest that increased Ras/MAPK and/or increased PI3K/Akt pathway activity may contribute to pituitary tumorigenesis [76].

As discussed previously, activating mutations in the Ras/MAPK signaling pathway are not sufficient to promote tumorigenesis of lactotrope cells. Transgenic mice studies targeting growth factors (nerve growth factor, TGFα, and FGF-R4) to pituitary lactotropes resulted in early hyperplasia, occurring within approximately 4 months, followed by delayed adenoma formation at approximately 10 months, but these pituitary cells were resistant to true carcinogenesis [71, 77–79]. Activating mutations in an additional pathway, often PI3K, must also occur to promote tumorigenesis [70, 80–82]. Transgenic mice studies targeting oncogenic Ras to thyroid and ovarian endocrine cells show that activated MAPK is necessary, but not sufficient, to mediate proliferative and tumorigenic responses, and that the PI3K pathway is essential [83–86]. These findings support the notion that the MAPK and PI3K signaling pathways work in unison to drive lactotrope differentiation and hyperplasia during pregnancy or prolactinoma formation.

2.3.4 Transforming Growth Factor β (TGFβ) Signaling

TGFβ signaling is important in a wide variety of cellular events, including proliferation, differentiation, and apoptosis. The TGFβ ligand binds to the heterodimerized TGFβ receptor (TGFβ-R), consisting of type I and type II receptor serine/threonine kinases. Upon dimerization, the type II receptor phosphorylates the kinase domain of the type I receptor, ultimately resulting in the phosphorylation of Smad effector proteins. Activated Smad protein complexes are translocated to the nucleus and regulate transcription of target genes [87].

Under basal conditions, TGFβ1 acts on lactotropes to inhibit the effects of estradiol on cell proliferation [12, 88]. Dopamine stimulates TGFβ1 secretion and mRNA expression, resulting in inhibited cell proliferation, suggesting that TGFβ1 mediates the inhibitory action of dopamine on lactotropes [13]. TGFβ1 also inhibits activity of the rat PRL promoter in GH4 cells [89]. Lactotropes do not express the TGFβ2 isoform, and the effect of TGFβ3 on lactotrope proliferation is negligible in the absence of high levels of estrogen [15]. Activin, a member of the TGFβ family, negatively regulates PRL production in lactotropes by repressing transcription of Pit-1. Activin also stimulates phosphorylation of Smad3, which interacts with the tumor suppressor menin to inhibit PRL transcription ([90]; Fig. 2.3).

However, upon exposure to increased estrogen concentration, TGFβ3 indirectly increases lactotrope proliferation by simulating production of growth factors from folliculostellate cells, suggesting that TGFβ3 mediates the mitogenic effects of estrogen [15]. Furthermore, this reveals that TGFβ1 and TGFβ3 have opposing actions on lactotrope cell proliferation [88]. Together these data suggest that a balance of TGFβ signaling is required for lactotrope homeostasis, and a substantial shift in this balance in favor of TGFβ3 is required for physiological lactotrope proliferation in pregnancy and lactation.

2.3.5 Hippo Signaling

The Hippo signaling pathway regulates the growth of tissues during development and regeneration, and also plays a role in cancer. The core kinase cassette of the Hippo pathway consists of mammalian sterile 20 (STE-20) like protein kinases MST1 and MST2, large tumor suppressor proteins LATS1 and LATS2, and adaptor proteins Salvador homologue 1 (SAV1), and MOB kinase activator proteins MOB1A and MOB1B. In the absence of upstream signaling, LATS1 and LATS2 phosphorylate Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), repressing the activity of YAP and TAZ by stimulating ubiquitin-mediated proteolysis. In the presence of upstream signaling, the activity of the core kinase cassette is altered and YAP and TAZ are no longer degraded. Ultimately, Hippo signaling promotes tissue growth and cell viability by regulating the activity of transcription factors such as SMADs and TEADs [91].

In mice, YAP1 activation results in increased liver size [92]. Together with data from human colorectal cancers overexpressing YAP1, it appears that activation of Hippo signaling results in dysplastic growth that promotes increased organ size. Specifically, YAP1 acts to expand multipotent undifferentiated progenitor cells, promoting organ growth in cancer [92]. Activated Hippo signaling is required for mammary gland expansion during pregnancy [93]. Transgenic mice deficient in LATS1 are infertile, have severely impaired mammary gland development, and pituitary hyperplasia [94].

The doubling of pituitary size during pregnancy presents a potential role for the Hippo pathway, although this pathway has yet to be specifically described in the pituitary gland during pregnancy or in prolactinoma.

2.3.6 Casein Kinase 2 (CK2) Signaling

CK2, a serine/threonine protein kinase, is activated by Wnt signaling and is involved in cell cycle control as well as DNA repair. Expression of CK2 is positively correlated with tumor phenotype in various cancers [95]. As of yet, little is known regarding CK2, but it embodies a good candidate for altered regulation of lactotrope cell proliferation in physiological and/or pathological conditions.

2.4 Mutations in Signaling Pathways Associated with Prolactinoma and Useful Mouse Models of Pituitary Adenoma

Neuroendocrine tumors are characterized by excessive secretion of tumor-derived hormone(s), which then inhibit upstream tropic hormones. Despite reductions in tropic hormone levels, the tumor continues to secrete hormone, creating a severely blunted endocrine-feedback mechanism. Prolactinomas are the most common type of neuroendocrine tumor. These tumors secrete excessive amounts of PRL, leading to hypogonadism, infertility, as well as tumor mass effects [19, 20].

In this section we will review mutations in signaling pathways that have been clinically identified in prolactinoma. While each of these genetic mutations accounts for only small proportion of clinical prolactinomas, they provide valuable insight into which signaling pathways contribute to prolactinoma formation, as well as those that are most important in regulation of lactotrope homeostasis. We will also provide a brief review of animal models that are useful in studying prolactinoma. A great deal has been learned from rodent models and can be applied to human pituitary physiology with awareness that not all facets are equivalent. Prolactinoma is a malady of signal transduction, and attempts to identify a single-key oncogene responsible for lactotrope tumorigenesis have been unsuccessful. Nevertheless, mutations that are identified clinically, as well as mutations that yield prolactinoma in rodents, highlight the fact that alterations in signaling pathways are common in prolactinomas.

2.4.1 Ras

Ras is a small GTPase protein that activates signaling pathways that regulate cellular processes such as proliferation, differentiation, and survival, including the MAPK and PI3K signaling pathways. In humans, there are three Ras genes: HRAS, NRAS, and KRAS. Oncogenic mutations allow Ras to remain in its GTP-bound state, resulting in constitutive activation of Ras signaling. These oncogenic Ras mutations are commonly found in human cancers, with mutations most commonly occurring in *KRAS*. An unusually invasive human prolactinoma was identified to have an HRAS G12V point mutation, and was lethal [96]. Despite the frequency with which Ras is mutated in human cancer, Ras mutations are rare in pituitary adenomas [97–100].

2.4.2 Menin

Multiple endocrine neoplasia type I (MEN1; menin) is a tumor suppressor protein that regulates transcription of cyclin kinase inhibitors such as p27 and p18 by promoting histone methylation [101]. Menin serves to regulate pregnancy-

associated islet β-cell expansion [102], suggesting a pivotal role of menin in regulating pSPC-mediated expansions during pregnancy or prolactinoma tumorigenesis. Menin-null mice develop late-onset pituitary and β-cell tumorigenesis [103, 104]. An inactivating mutation on chromosome 11q13, the site of the MEN1 gene, has been reported in sporadic human prolactinoma [97], and 60 % of MEN1-associated pituitary tumors secrete PRL [105]. However, a separate study reported that somatic MEN1 mutations do not significantly contribute to prolactinoma tumorigenesis [106], suggesting that mutations in other genes may be necessary for prolactinoma formation.

2.4.3 Heparin Secretory Transforming (hst) Gene

The hst gene was originally identified to function as a transforming gene in malignant stomach cancers [107], and encodes for fibroblast growth factor 4 (FGF4). Expression of *hst* mRNA was later identified in human prolactinomas [108], and has been shown to be a marker of invasive prolactinoma [109]. Overexpression of *hst* in rat lactotropes results in increased FGF4 production, as well as increased cell proliferation [109].

2.4.4 Pituitary Tumor Transforming Gene (PTTG)

PTTG is found in all classes of human pituitary adenomas, including prolactinoma. PTTG is expressed at low levels in normal human tissues, but shows increased expression in some human tumors and malignant cell lines. PTTG functions to regulate the separation of sister chromatids during mitosis [99], and has been shown to regulate cell division and survival in endocrine tumors [18]. PTTG was first isolated from rat GH-secreting adenoma cells, and has been shown to be induced by estrogen and stimulate FGF2 signaling, resulting in prolactinoma tumor formation and progression in rats [18]. Expression of PTTG is associated with lactotrope hyperplasia, angiogenesis, and prolactinoma development [99], and increased expression level correlates with tumor invasiveness [110]. However, as of yet, a clear correlation between PTTG and tumorigenesis in human adenoma remains unclear [99].

2.4.5 Aryl Hydrocarbon Interacting Protein (AIP)

AIP associates with the cytoplasmic aryl hydrocarbon receptor (AHR), which is a transcription factor that interacts with cell cycle regulators such as retinoblastoma protein (Rb). AIP directly interacts with AHR to regulate its subcellular localization and nuclear cytoplasmic shuttling. AIP also regulates the localization and activity of phosphodiesterase 4A5 (PDE4A5), an enzyme responsible for the hydrolysis of intracellular cAMP. Mutations in AIP can alter the interactions with AHR and

PDE4A5, providing a potential role for AIP to regulate signaling pathways that control tumorigenesis [111]. However, the precise mechanisms by which AIP acts as a tumor suppressor in pituitary tumorigenesis have not been specifically identified. Germ-line mutations in AIP have been reported in some familial types of pituitary adenoma, including prolactinomas [111–113]. AIP is considered a pituitary adenoma predisposition (PAP) gene [111]. Many patients with mutations in AIP have pituitary adenomas that secrete both GH and PRL [111], underscoring the shared ontogeny of pituitary lactotropes and somatotropes.

2.4.6 Guanine Nucleotide Activating Subunit (GNAS)

Gain-of-function somatic mutations typically occur in GPCR genes expressed in a tissue-restricted manner, and can lead to neuroendocrine adenoma formation and glandular hyperfunction. The stimulatory G protein, $G_{\alpha s}$, is a product of the *GNAS* gene and regulates activation of adenylate cyclase to produce intracellular cAMP. Activating mutations in *GNAS*, resulting in the expression of the *gsp* oncogene, are associated with somatotrope growth as well as the development of PRL and GH cosecreting adenomas in McCune–Albright syndrome [114]. An invasive prolactinoma that was resistant to dopamine agonists was observed to transition into a GH-secreting adenoma while simultaneously acquiring a de novo mutation in *GNAS* [115].

2.4.7 Unknown/Unidentified Mutations

The aforementioned mutations have been identified clinically in humans. There are many more candidate genes that have been shown to have the potential to promote prolactinoma tumorigenesis, but that have yet to be identified clinically. The majority of patients that present with prolactinoma can be successfully treated with medical therapy, thus surgical resection of tumor tissue is not necessary. As such, prolactinoma tissue is not abundantly available for genetic and molecular analyses. Unfortunately, from the tissue that is available, state-of-the-art immunohistochemical, microarray, and proteomic expression analysis, oncogenic mutation studies, and DNA epigenetic approaches have been mostly unproductive. Novel candidate oncogenes are frequently proposed for tumorigenesis of prolactinomas and other neuroendocrine tumors, but minimal progress has been made to implicate a specific oncogene or tumor suppressor, or markers of proliferation, senescence, dormancy, or antiapoptosis, in pituitary tumorigenesis [116]. The difficulty in identifying candidate oncogenes may be a result of a transient phosphorylation event that cannot be detected with traditional proteomics. Correlative studies have provided only modest information and have failed to give insights as to cause. To date, the best clues about the mechanism of pituitary tumorigenesis come from familial pituitary tumor disorders and mouse models, where mutations in conserved signaling pathways and factors that govern the cell cycle are critical in pituitary tumor formation [112, 117].

Table 2.1 Clinically identified mutations in human prolactinoma

Gene	Defect	Signaling abnormality	Phenotype	Reference
RAS	G12V;GOF mutation	Persistent MAPK, PI3K signaling	Invasive prolactinoma	[96]
MENIN	LOF mutation	Fails to induce p18 and $p27^{kip1}$	Multiple endocrine neoplasia type 1	[101]
HST	Overexpression	Induces FGF4 signaling	Invasive prolactionoma	[99]
PTTG	Overexpression	Estrogen-induced; Stimulates FGF2 signaling	Lactotrope hyperplasia; angiogenesis	[18, 99]
AIP	LOF mutation	Decreased PDE4A5 activity resulting in persistent cAMP signaling	Benign adenoma (GH and PRL cosecretion)	[111]
GNAS	GOF mutation (Gsp oncogene)	Persistent $G_{\alpha s}$ signaling	McCune–Albright syndrome (GH and PRL cosecretion)	[18]

GOF gain of function, *LOF* loss of function

2.4.8 *Useful Animal Models of Pituitary Adenoma (Table 2.2)*

In Table 2.2, we have assembled a list of animal models that have proven useful for studying pituitary adenoma; for more details, see the following references: [17, 71, 104, 118–123]. While rodent models have understandable limitations, a great deal

Table 2.2 Animal models of prolactinoma

Gene	Mutation or altered expression	Phenotype	Reference
Drd2(D2R)	KO	Delayed lactotrope hyperplasia (after 8 months); prolactinoma formation (after 16 months)	[118]
$p27^{kip1}$	KO	Spontaneous anterior pituitary tumor formation	[119]
Retinoblastoma (Rb)	+/–	Pituitary tumor formation in intermediate and posterior lobes (after 8 months)	[120, 121]
Men1	+/–	Anterior pituitary adenoma or carcinoma (after 16 months)	[104, 122]
TGFα	Targeted overexpression in lactotropes via PRL promoter	Lactotrope hyperplasia; prolactinoma formation (after 6 months)	[71]
Estrogen (treatment)	Long-term elevation of serum estrogen in Fischer-344 rats	Lactotrope hyperplasia; Prolactinoma formation	[17]
CDK4	KO	Lactotrope hypoplasia; Diminished serum PRL	[123]

KO knockout

has been learned from these models and they provide significant insights into the intracellular pathways that may be altered in abnormal human pituitary and lactotrope physiology. It is important to emphasize that although certain genetic alterations can yield PRL-secreting pituitary tumors in mice, adenoma formation is very delayed and thus is not fully accurate in representing the human disease state. This demonstrates that a single gene mutation or deletion is not sufficient, and that an additional mutation is likely required for true prolactinoma tumorigenesis.

2.5 Discussion

Although individual mutations have been proposed as possible driving forces for prolactinoma tumorigenesis in humans, no single mutation has been clinically identified as a causative factor for the majority of prolactinomas. Data collected from individual cases, genomic sequencing, and molecular arrays provide valuable insights into which signaling pathways contribute to prolactinoma formation, as well as those that are most important in the regulation of lactotrope homeostasis. The clinically identified oncogenic V12Ras mutation has been reported in one human prolactinoma that was particularly invasive. However, the same oncogene has antiproliferative and antitumorigenic properties when expressed in GH4 somatolactotrope cells (Booth and Gutierrez-Hartmann, unpublished data), suggesting that Ras signaling is antagonized in lactotrope cells, allowing for evasion of oncogenic Ras signaling and tumorigenesis. It is possible that the invasive prolactinoma with the V12Ras mutation also had an additional mutation in another protein or signaling pathway that resulted in loss of the antagonistic signal, thus allowing Ras signaling to proceed and contribute to the invasiveness of the tumor. Furthermore, recent data demonstrate that the dopamine receptor, D_2R, oppositely regulates MAPK and PI3K signaling [47], indicating that a delicate balance of these signaling pathways may be required to maintain lactotrope homeostasis. The PI3K and MAPK signaling pathways have been shown to act synergistically to promote tumorigenesis in other cancers [70, 81, 82]. Thus, it may be that deregulated MAPK signaling in lactotrope cells that results from oncogenic V12Ras is not tumorigenic as long as PI3K signaling remains in check. Concurrent mutations in MAPK and PI3K pathways may be required for full prolactinoma tumorigenesis. A better understanding of lactotrope-specific responses to Ras/MAPK and PI3K signaling is needed to explain the mechanism of tumorigenesis in prolactinoma. As such, as we move forward in our attempts to elucidate the mechanism(s) of prolactinoma tumorigenesis, it is important to consider the malady of signal transduction that occurs within lactotrope cells. It is unlikely that one sole oncogene responsible for prolactinoma will be identified; instead we must use our knowledge of signaling pathways and the interplay of signals from a cell-specific perspective to make sense of the data we acquire from arrays and clinically identified mutations.

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Chapter 3
The Many Faces of Prolactin in Breast Cancer

Wen Y Chen

Abstract Prolactin (PRL) is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs in the anterior pituitary gland of all vertebrates. The physiological role of PRL in mammary glands is relatively certain while its role in breast tumor has been a topic of debate for over 20 years. In this review, the author attempts to briefly summarize the data coming from his laboratory in the past years, focusing on G129R, a PRL receptor (PRLR) antagonist developed by introducing a single amino acid substitution mutation into human PRL (hPRL) at position 129, and a variety of G129R derivatives. The author has proposed two novel ideas for potential use of PRL, not anti-PRL agents, as an adjuvant agent for breast cancer, making it a hormone of many faces.

Abbreviations

ASC	Adult stem cell
CSC	Cancer stem cell
ELISA	Enzyme-linked immunosorbent assay
GH	Growth hormone
GHR	Growth hormone receptor
hPRL	Human prolactin
HUVEC	Human umbilical vein endothelial cell
IL-2	Interleukin-2
KO	Knockout
MT	Metallothionein promoter
NRL	Neu-related-lipocalin
PRL	Prolactin
PRLR	Prolactin receptor
qPCR	Quantitative polymerase chain reaction

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3.1 Introduction

Human breast cancer is the predominant malignancy and the leading cause of cancer death in women [1–2]. According to a recent estimation by the American Cancer Society, the lifetime cumulative risk of developing breast cancer is one in every seven women. The cause of breast cancer is still unknown, but its rarity among males indicates an etiological role for the female sex hormones, while varying geographic distribution also points to the importance of lifestyle and the environmental factors [2].

Prolactin (PRL) is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs in the anterior pituitary gland of all vertebrates. The biological activities of PRL are mediated by specific membrane receptors, namely, PRL receptors (PRLRs) [3]. On the basis of several conserved features (a single transmembrane domain and conserved amino acid sequences in the extracellular domain), PRLR together with growth hormone receptor (GHR) have been categorized into the cytokine receptor superfamily [3]. The best characterized action of PRL is on the mammary gland. In this organ, PRL plays a decisive role in the stimulation of DNA synthesis, epithelial cell proliferation, and the promotion of milk production [4]. The generation of PRL [4] and PRLR [5] gene knockout (KO) mice has unambiguously demonstrated that PRL and PRLR pathways are the key regulators in mammary development. The physiological role of PRL in mammary glands is relatively certain while its role in breast tumor has been a topic of debate for over 20 years [6–9]. In this review, the author attempts to briefly summarize the data coming from his laboratory in the past years, focusing on G129R, a PRLR antagonist developed by introducing a single amino acid substitution mutation into human PRL (hPRL) at position 129, and a variety of G129R derivatives. The author will also propose two novel ideas for potential uses of PRL as an adjuvant agent for breast cancer, making it a hormone of many faces.

3.1.1 PRL and PRLR Pathway as a Therapeutic Target in Breast Cancer

(1) Human PRLR Antagonist, G129R

At the beginning of 1990s, several lines of evidence indicated that PRL may be involved in breast cancer development. First, it was reported that female human GH (hGH) transgenic mice had a high incidence of mammary tumors in contrast to sporadic cases found in bovine GH (bGH) transgenics [10]. The high incidence of breast cancer in hGH transgenic mice was believed to be due to the lactogenic activity of hGH, which is a unique feature of primate GHs. A later report of hPRL transgenic mice confirmed the role of PRL in the stimulation of mammary tumor in the murine model [11–12]. The detection of PRL mRNA in mammary tissues [13–15] and biologically active PRL in human breast cancer cells [6] lead to the

proposal that PRL not only was a classic endocrine hormone but also an autocrine/paracrine growth factor produced locally within the mammary glands. Although this extrapituitary production of hPRL may not cause detectable systemic changes in serum PRL, it could exert significant local stimulatory effects [16]. In support of this concept, it has been reported that the expression levels of PRLR were significantly higher in human breast cancer cells and in surgically removed breast cancer tissues than in normal breast epithelial tissues [17–19]. The high levels of PRLR in malignant breast tissue make these cells highly sensitive to stimulation by PRL [19].

After the proposal that the PRL/PRLR pathway could play an etiological role in breast cancer, the race began to search for an effective PRLR blocker. In our earlier studies, we have demonstrated that the third α-helix of GH is the key for its growth promoting activities [20–25]. We further demonstrated that Gly 119 of bGH [22] or 120 of hGH [24] plays a critical role in the molecular action of GH. A detailed molecular mechanism of the GHR antagonists was later revealed [26, 27]. It is generally accepted that GH transduces its signal via a one hormone–two receptor complex either through hormone-induced sequential receptor dimerization [26–29] or binding to a preexisting receptor dimer [30–33]. Any amino acid substitution (other than Ala), especially one with a bulky side chain such as Arg, at position 120 of hGH will prevent two receptors to form a functional unit, thereby, resulting in a GHR antagonist [16–21]. As a member of the GH family, hPRL is believed to share a signal transduction mechanism similar to GH. It was, therefore, reasonable to expect that if an equivalent amino acid substitution within the third α-helix of hPRL, at Gly residue 129, would generate a human PRLR-specific antagonist [34–36].

It has been more than 15 years since the first publication concerning the G129R-based PRLR antagonist [34–36]. There were also a few proposed modifications of G129R, including the so-called pure PRLR antagonists (see review 37), as well as several G129R-based fusion proteins (see below). After years of investigation with various in vitro and in vivo assays, scientific research has reached a consensus as the following: (a) a single amino acid substitution mutation at position 129 of hPRL is required to generate a binding site 2-based potent PRLR antagonist. (b) There seems a disconnection between the results obtained using two-dimensional cell-based assays and those in animals. (c) G129R as an anti-PRLR monotherapy for breast cancer, like most other monotherapies is not effective. (d) The true role of PRL in breast cancer etiology remains elusive, which directly undermines the application of PRLR antagonists.

There is little doubt that G129R is very effective in inhibiting PRL-induced Jak/Stat signaling, especially in T-47D human breast cancer cells. As a matter of fact, the most convincing data to date regarding the antagonism of G129R on PRLR were from T-47D cells [36, 37]. The T-47D cell line is commonly used to study effects of PRL in vitro due to its robust ligand-induced signaling response. However, it is also true that PRL fails to induce similar action in many other breast cancer cells, even those with high PRLR [38]. Furthermore, most breast cancer cells, including T-47D, do not show substantial proliferative response to PRL stimulation (generally less than 30% over basal level). Likewise, there was never a substantial inhibitory effect from G129R reported in any breast cancer cell proliferation in vitro even

with doses as high as mg/ml. These results strongly suggest that the proliferation of most established breast cancer cell lines are not PRL- dependent. A recent study showed that while exogenous recombinant PRL stimulated T-47D growth, proliferation was reduced upon ectopic PRL expression [39]. It is still not clear that the ectopic expression of PRL resulting in the reduced cell proliferation is due to constant excessive proliferative signaling triggers cell senescence [40].

In our earlier studies, we have demonstrated that G129R was moderately effective in a few breast tumor models both in vitro as well as in vivo [36, 41–44]. We have also generated a transgenic mouse line, MT-G129R, in which G129R expression is driven by the methallothionein promoter (MT) (unpublished data). This line of transgenic mice expressed serum G129R at around 5 ng/ml. There was no noticeable phenotypic change in both male and female throughout their life span, except in the mammary gland. The ductal network of mammary glands from nulliparous female mice at 6 month was shown to have far less side-branches and lobuloalveoli as compared to their wild-type littermates (Fig. 3.1). These phenotypic changes were reminiscent to that of PRLR gene knockout (KO) mice [5]. Interestingly, this constant expression of G129R had little effect on Her2/*neu*-initiated breast tumor when we crossbred MT-G129R mice to HER2/*neu* mice to produce G129R/*neu* bitransgenic mice. There was virtually no change in tumor incidence curve in female bitransgenic mice as compared to their HER2/*neu* littermates (Fig. 3.1c). We anticipated that early and prolonged exposure to a PRLR antagonist would at least delay the onset of HER2/*neu*-induced mammary tumors. It is possible that the level of G129R in serum was too low in this case to exert any protective effect in the mammary glands over a powerful oncogene like HER2. Nonetheless, it does reflect the ineffectiveness of a partially blocked PRLR pathway in the process of HER2 tumorigenesis.

These observations casted significant doubt over the original idea that PRL played a major role in breast cancer tumorigenesis serving as an autocrine/paracrine growth factor that promotes breast cancer cell growth. A recent study specifically focusing on the role of autocrine/paracrine PRL in breast cancer was published [39]. It was found that a low/very low level of PRL mRNA expression in breast cancer cell lines including T-47D cells when maintained under standard growth conditions by the qPCR analysis. Also, the PRL protein secretion by these cells was below detection limit of ELISA or a very sensitive Nb2 bioassay [39]. It also pointed out that there was no correlation between the PRL mRNA expression and immunehistochemical localization of the PRL protein, indicating that the presence of PRL mRNA does not necessarily point to the presence of the PRL protein. The authors concluded that autocrine PRL signaling is not a general mechanism promoting breast cancer cell growth [39].

In light of multistep tumorigenesis in cancer development process (initiation, promotion, and progression) [40] and the intimate relationship between PRL and breast adult stem cells (ASCs) (see sections below), we speculate that the role of PRL in breast cancer etiology may be involved in the early tumor initiation phase. The ineffectiveness of blocking the PRL/PRLR pathway in well-developed breast cancer may also be due to the fact that most cancers, including breast cancer, are heterogeneous in nature and are characterized by numerous somatic mutations,

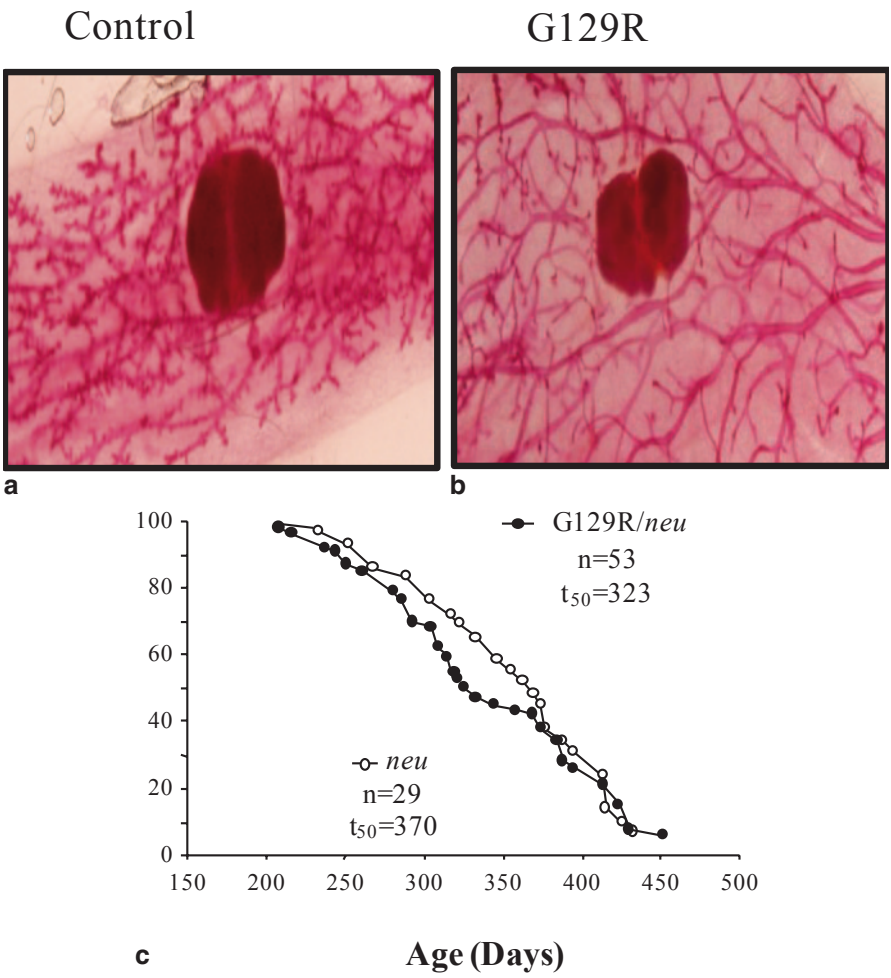

Fig. 3.1 G129R and G129R/neu bitransgenic mice. Panel **a** and **b**. Impaired mammary gland development in mice expressing G129R. The fourth inguinal mammary glands were dissected, stained in carmine alum stain and digitally photographed. The lymph node was used as a reference point to the edge of the mammary gland. Representative whole mount images from wild-type littermate (**a**) and G129R/neu (**b**) are presented. Panel **c**, the comparison of the tumor-free percentage over time between neu transgenic (*open circles*) and G129R/neu bitransgenic mice (*closed circles*). There was no statistical difference between the two groups

of which only a subset contributes to the tumor's progression known as "driver" mutations. The rest of the heterogeneous population of cancer cells is considered as neutral "passenger" mutations [40, 45]. Based on data collected so far, PRLR-positive cells in most late stage breast cancer are likely passenger cells and the PRL/PRLR pathways may serve as one of the neutral pathways, which may explain why there were rare PRLR mutations found in breast cancer serving as classic

gain-of-function mutation (except for one, [46]). With this notion, researchers need to reevaluate how to deal with PRLR-positive cells in breast cancer.

(2) G129R Fusion Proteins

To further increase the potency of G129R, we have attempted to generate G129R-based bifunctional fusion proteins including G129R fusions with an angiogenesis inhibitor (endostatin) [47], an immune system modulator (interleukin 2, (IL-2)) [48], and a modified truncated cytotoxin (PE40KDEL) [49]. Each fusion protein was designed to target the PRLR-positive cells via the G129R moiety and at the same time attack a hallmark common to cancer cells via the second moiety serving as targeted breast cancer therapeutics.

(A) G129R-endostatin [47]: It has been established that tumor angiogenesis is one of the hallmarks of cancer [40]. The disruption of angiogenesis has been proven to be an effective strategy to cause regression of certain tumors [50]. One of the early well-studied angiogenesis inhibitors was endostatin, which acts through the inhibition of endothelial cells [50]. We reported that the fusion protein (G129R-endostatin) was able to retain G129R activities in T-47D-based assays. At the same time, G129R-endostatin inhibited human umbilical vein endothelial cell (HUVEC) proliferation and disrupted the formation of endothelial tube structures with potency similar to that of endostatin. More importantly, the therapeutic efficacy of G129R-endostatin was confirmed using a mouse breast cancer cell line 4T1 in vivo. G129R-endostatin has a significantly prolonged serum half-life as compared with that of G129R or endostatin alone, and exhibited greater tumor inhibitory effects than G129R and endostatin individually or in combination.

(B) G129R-IL-2 [48]: This fusion protein was designed to target another cancer hallmarks, immune evasion [40]. Again, the novel fusion protein was bifunctional, i.e., it was able to block signal transduction induced by hPRL as well as to activate T lymphocytes near the tumor site. The antitumor activities of G129R-IL-2 were demonstrated in vivo using a syngeneic model system with BALB/c mice and EMT6-hPRLR breast cancer cells.

(C) G129R-exotoxin [49]: To increase the potency of G129R, we also fused G129R to a truncated form of *Pseudomonas* exotoxin A (PE40). We demonstrate that the fusion toxin, like the other fusion proteins, retained the binding ability to hPRLR on T-47D human breast cancer cells and inhibit STAT5 phosphorylation induced by hPRL. In addition, we show that G129R-PE40-KDEL is selectively cytotoxic to breast cancer cell lines expressing the hPRLR and that cell death is associated with the inhibition of protein synthesis and does not involve caspase mediated apoptosis.

We further tested the efficacy of the three fusion proteins as a combination therapy in an aggressive but clinically relevant mouse tumor model [51]. To test the feasibility and to optimize a treatment regimen, allografts of a mammary carcinoma cell line (McNeuA) derived from an MMTV-*neu* transgenic mouse were used. Growth of the allografts was significantly retarded by regimens, which combined all three

fusion proteins. After establishing the dosing regimen, two doses of cocktail treatment (low and high doses administered twice weekly) along with individual component controls were administered to female MMTV-*neu* transgenic mice after surgical removal of a naturally occurring tumor. The average tumor recurrence time was significantly delayed in both low and high combination treatment groups in comparison to the no treatment control group. The total number of lung metastases was also significantly decreased in both combination treatment groups.

Despite these promising results, the relatively difficult production process of these fusion proteins and the potential immunogenicity problem of these proteins in the clinical settings as therapeutics hampered the enthusiasm of further development of these promising potential drugs.

(3) The Role of Anti-PRLR in Breast Cancer Combination Therapy

It is now believed that any single agent designed to treat a heterogeneous cancer mass is bound to fail simply because it eventually incubates drug-resistant cancer cells [40]. With this guideline, anti-PRL/PRLR pathway may serve as a valuable combinational option. Several studies have been conducted in our laboratory in the past.

(A) G129R combination with antiestrogen receptor agents. It has been reported that there was a potential interplay between PRL and E_2 in regulation of gene expression and cell growth [52]. PRL alone induced either a weak or nonproliferative response in T-47D and BT-483 cells, respectively, while it drastically enhanced cell proliferation in E_2-stimulated cultures [52]. In our earlier studies, we have shown that G129R was effective in enhancing tamoxifen's inhibitory effect in breast cancer cell lines [36]. Further studies are needed in this subject.

(B) G129R combination with anti-HER2 agents. We have also conducted a more detailed study regarding G129R and Herceptin combinational approach [53]. The results demonstrated that anti-PRLR was beneficial when used in combination with Herceptin [53]. We confirmed that there was a cross talk between PRL and HER2 in two breast cancer cell lines (T-47D and BT-474) as previously reported [54]. Constitutively activate signaling via HER2 and the PRLR was apparent in BT-474 cells as indicated by the high levels of p-HER2 and p-STAT5 in the absence of exogenous stimuli. As a consequence, BT-474 cells responded poorly to Herceptin or G129R when administered as a monotherapy. This refractory state of the cells not only reflects dissociation between the PRLR and STAT5 but also suggests constitutive transactivation of HER2 via the PRLR since combination of Herceptin and G129R resulted in a significant additive inhibition of p-HER2 in BT-474 cells. In this regard, combination of the two inhibitors not only provided opportunity to inhibit the converging pathways, such as p-MAPK, but also two distinct and parallel signaling pathways (p-STAT and p-AKT), as best illustrated in the in vivo xenograft studies [53]. The combination of G129R and Herceptin treatment exhibited a significant additive effect on inhibition of the tumor growth in T-47D xenografts as

compared to either agent alone, highlighting the therapeutic potential of the combination treatment. In a separate study, G129R was also demonstrated to have additive effects in ex vivo and in vivo models with lapatinib, a small molecule HER1 and HER2/*neu* tyrosine kinase inhibitor [55].

(C) G129R combination with conventional chemotherapeutics. In our earlier studies, we have demonstrated that G129R was more effective when in combination with cisplatin [43]. In a recent study [56], the authors demonstrated that G129R was more effective in combination with paclitaxel in inhibition of tumor growth in orthotopic models of human ovarian cancer. It showed that prolonged treatment with G129R induced the accumulation of redundant autolysosomes in 3D cancer spheroids, leading to a type II programmed cell death [56].

(D) Role of breast cancer microenvironment in PRL/PRLR signaling. It is clear that other than cancer cell heterogeneity, cancer microenvironment, especially cancer-associated fibroblasts (CAFs) also influence the response of cancer cells to various stimuli and inhibitors. Tumor epithelial cells maintain 3D structure in tumor stroma and they interact with soluble factors secreted by stromal cells such as CAFs or directly with the extracellular matrix (ECM). We have demonstrated that CAFs play a critical roll in bridging the cross talk between PRL and HER2/*neu* in both mouse and human models of breast cancer [55]. To compare drug response to G129R between tumors and primary cultured cells, mammary tumors were resected and cultured as small tumor chunks (≈ 3 mm^3) or were cultured in monolayer. G129R reduced p-Neu in a dose-dependent manner ($IC_{50} \approx 10$ μg/ml) in tumor chunks, but had little effect upon primary tumor epithelial cells grown in monolayer. Similar to that observed in mouse tumor chunks, direct coculture of mouse tumor epithelial cells with CAFs restored the response of epithelial cells to G129R. The addition of PRL, as expected, induced p-Neu in both the tumor chunk and direct coculture models. The inhibitory effect of G129R was absent when CAFs were physically separated from mouse tumor epithelial cells using a transwell system, or when CAFs were replaced with normal fibroblasts in direct coculture with human or mouse tumor epithelial cells. We concluded that the inhibitory effects of G129R on p-HER2/Neu are dependent, at least in part, upon interactions of tumor epithelium with stroma [55].

In conclusion, breast cancer is a complex disease. The development of breast cancer involves a multistep process with multiple mutations. Cumulative somatic mutations in late cancer often result in a heterogeneous mixture of cell mass, of which only a subset contributes to the tumor's progression known as "driver" mutations. The ineffectiveness of blocking PRL/PRLR pathway influences the breast cancer growth calls upon a reevaluation of the role of PRL/PRLR as a therapeutic target in breast cancer.

3.1.2 The Effects of hPRL on Breast Cancer Stem Cells as a Potential Chemotherapeutic Enhancer to Improve the Outcome of Chemotherapy

According to the NCI, chemotherapy remains to be one of the standard treatment options for breast cancer. Despite drastic improvement in recent years, the main frustration related to conventional chemotherapies is that while they are usually effective initially in controlling tumor growth, many patients relapse over time [57–59]. There are two possible explanations for these observations. The first is that all cancer cells acquire resistance under treatment, resulting in decreased overall sensitivity to therapy over time. In this case, the relative proportion of cells in residual tumors with tumorigenic properties would be expected to be similar before and after treatment. The second explanation is that a rare subpopulation of cells with tumorigenic potential is intrinsically resistant to therapy [60–62]. In this case, the relative proportion of cells inresidual tumors with tumorigenic properties would be expected to increase after treatment. Evidence emerging from recent research strongly suggests that it is likely the latter—an intrinsic, preexisting small subpopulation of cells is responsible for the chemoresistance [61, 62]. These cells have self-renewal capacity and tumor-initiating ability known as cancer stem cells (CSCs) or tumor initiating cells. The CSC theory states that tumors are organized in a cellular hierarchy, in which CSCs are the only cells with unlimited proliferation potential and the capability to drive tumor growth and progression [62]. The main features of CSC are twofold (a) CSCs are very rare within tumors and (b) CSCs are mostly in a nonproliferative dormant state, which makes them insensitive to most cytotoxic agents [60]. The lack of responsiveness of CSCs to chemotherapy may explain why clinically, tumor sizes often shrink in response to chemotherapeutic drugs but usually relapse [63, 64]. Therefore, efforts in developing future therapeutic regimens should focus on how to eliminate CSCs, which is essential to stop tumor regrowth and relapse.

The origins of CSCs within a solid tumor have not been clarified and indeed may vary from one tumor type to another [40]. In some tumors, normal tissue stem cells may serve as the cells-of-origin that undergo oncogenic transformation to yield CSCs [40]. Mammary gland, like other organs, is derived from tissue ASCs. However, unlike most organs, the female mammary tissue begins its development after adolescence. More importantly, the mammary gland is able to go through waves of rapid proliferation, differentiation, and involution (apoptosis) cycles during normal reproductive age, suggesting that ASC in mammary gland is readily responsive to various regulators including PRL. Therefore, any types of dysregulation of the self-renewal pathway of breast stem cells may be prone to tumor development [65–67].

Human breast CSC was first identified about 10 years ago [68–69]. Breast CSCs are characterized as CD44+/CD24−/lin-cells by flow cytometry [70, 71]. Another breast CSC marker, aldehydrogenase-1 (ALDH1), measured by ALDEFLUOR assay, was also identified [72]. High level of ALDH1 was found in progenitor cells but not in differentiated luminal epithelial/myoepithelial cells [72]. Ample efforts

have been placed at searching for novel cytotoxic agents that have the ability to specifically target CSCs [73]. For example, Salinomycin, a 751 Da monocarboxylic polyether antibiotic isolated from *Streptomyces albus* has been shown to be able to selectively deplete breast CSCs from tumorspheres and to inhibit tumor growth in mice [74]. Another example is that Metformin, a standard drug for diabetes, has been shown to be able to selectively kill CSC in breast cancer [75].

Considering the intimate relationship between PRL and breast ASC, we reasoned that hPRL could probably activate CSC the way it stimulates ASC. By stimulating CSC proliferation and differentiation, PRL could literally "wake up" the breast CSC from its dormancy state, i.e., changing CSC from mitotic quiescent state to active state, thus sensitizing the CSC to conventional chemotherapy. We further hypothesize that by simultaneous administration of PRL with chemotherapeutic agents it will increase the cytotoxic effects of chemotherapeutic drugs toward CSCs.

In our recent studies (manuscript in preparation), we have generated some promising preliminary data that in principle support the notion that PRL in combination with a conventional chemotherapeutic (cisplatin) could improve the outcome of chemotherapy. To confirm the effect of PRL on breast CSCs, we used tumorsphere assay to demonstrate that when cells were treated with cisplatin alone, the number of tumorspheres is marginally lower compared to control, which agrees with previous findings that breast CSCs are resistant to chemotherapeutics [63, 64]. However, when cisplatin treatment was combined with PRL, the tumorsphere count was significantly decreased in a concentration-dependent manner in both MCF-7 and HCC1954 cells measured either by total tumorsphere count or IC_{50}value (Fig. 3.2a and b).

To further test the role of PRL in breast CSC, we used in vivo tumorigenicity assay and the tumor growth assay. Our results showed that pretreatment of mouse breast cancer McNeuA cells with cisplatin and the low dose of PRL delayed tumor growth (Fig. 3.2c), suggesting the combination treatment effectively reduced tumor initiation cells or CSCs. In addition, we also treated naturally developed tumor from HER2/*neu* transgenic mice with cisplatin or cisplatin and PRL combination. Our results showed that continuous intraperitoneal administration of PRL and cisplatin significantly delayed tumor growth (Fig. 3.2d).

It is noteworthy to point out that a previous study by LaPensee et al. [76] has demonstrated that the treatment of breast cancer cells with PRL prevented cisplatin-induced G2/M cell cycle arrest and apoptosis [76]. The paper concluded that PRL confers resistance against cisplatin by activating a detoxification enzyme, thereby reducing drug entry into the nucleus. We believe that the difference in conclusion between our results and the results from LaPensee et al. lies upon the assay system. Studies in LaPensee et al. utilized traditional 2D culturing system [76], in contrast, our in vitro study was performed in 3D tumorspheres. The major difference between the two culturing systems being that the culturing conditions used for 3D tumorspheres limits proliferation and differentiation to stem/early progenitor cells. Recent studies have demonstrated that cells cultured in 3D exhibit a different drug response than cells cultured in 2D monolayer [77–79].

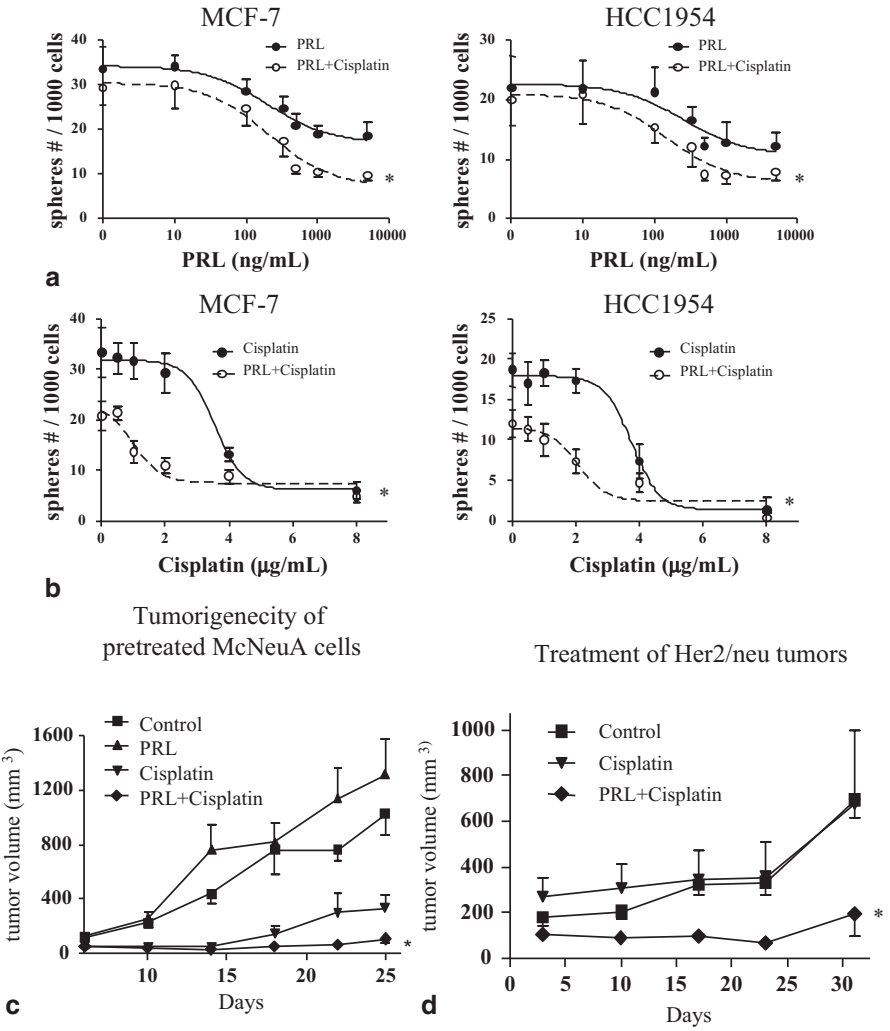

Fig. 3.2 The effects of PRL and cisplastin combination treatment in breast cancer cell tumorsphere formation and tumor growth. The effects of PRL on the secondary tumorsphere formation in MCF-7 and HCC1954 human breast cancer cell lines were shown in panels **a** and **b**. The concentration-dependent response curves of PRL, with or without fixed dose of cisplatin (2 μg/mL), and the concentration-dependent response curves cisplatin, with or without fixed dose of PRL (500 ng/mL), were summarized in panels **a** and **b**. All data represented mean ± s.e.m. of five separate experiments. Panel **c** was the summary comparison of mouse breast cancer cell (McNeuA) allograft tumor growth after cisplatin, PRL or cisplatin and PRL combination treatment**.** McNeuA cells that were pretreated with cisplatin, PRL or combination in vitro for 3 days were harvested and inoculated into recipient Her2/*neu* female transgenic mice (n-6/group). Tumor growth curves from each group were plotted for comparison. Panel **d** was the summary of the comparison of naturally developed mammary tumors in *neu* transgenic mice in response to cispaltin or cisplatin and PRL combination treatment. Tumor bearing *neu* transgenic mice (at ~0.5 cm in diameter) were assigned randomly into three groups, control ($n=9$); cisplatin (5 mg/kg, weekly, $n=9$); or PRL

It is obvious that much more work needs to be done before any conclusion can be drawn regarding this hypothesis. It becomes clear in recent years, however, that the role of PRL in breast cancer is not as a classic oncogene, such as HER2/*neu*. PRL does not possess a robust stimulatory potential in most cultured breast cancer cells in vitro and/or in vivo. The effects of hPRL in fully transformed and actively proliferating breast cancer cells are limited. In agreement with these assessments, it was reported recently by Rui's group that PRL is able to suppress a progestin-induced CK5-positive cell population in luminal breast cancer through inhibition of progestin-driven BCL6 expression [80, 81]. It was found that the PRL-STAT5a pathway is frequently lost in invasive and metastatic breast cancer. The authors proposed a working model suggesting that hPRL probably promotes tumor initiation in early phase of tumorigenesis but maintains tumor cell differentiation and suppresses progression of established breast cancer [80, 81].

Taken together, there is indisputable evidence that hPRL plays a key role in breast ASC regulation. Therefore, it is reasonable to speculate that hPRL might play a role in breast CSC as a modulator. The preliminary data presented here support the hypothesis that PRL may be able to induce CSC differentiation. The activation of CSC makes them become sensitive to conventional cytotoxic agents. Thus, the combination of PRL with cytotoxic agents may be an alternative to targeting breast CSCs, and deserves further investigation.

3.2 The Effects of hPRL on Breast ASCs as a Potential Chemopreventive Agent for Breast Cancer

Despite recent advances in early breast cancer detection and novel therapeutics, the magnitude of approximately 200,000 new cases per year demands a shift of emphasis in breast cancer research from treatment to prevention. It is well known that early pregnancy and breast-feeding are among a few factors associated with decreased risk of breast cancer. The breast tissue of normally cycling women contains three identifiable types of lobules: undifferentiated type (Lob1) and the more developed or differentiated lobules types, Lob 2, and Lob 3. [82]. The lobule structures in the lactating breast are called Lob 4, which after postlactational involution, regress to Lob3 and remain present as the predominant structure in the breast of parous women. In contrast to the breast tissue of parous women, the nulliparous breast contains mainly undifferentiated lobules (Lob1), which are highly proliferative and therefore susceptible to stimuli. As a matter of a fact, Lob1 has been identified as the site of origin of the most common breast malignancy, the ductal carcinoma [82]. On the other hand, more differentiated lobular structures have been found to be less susceptible to carcinogenesis [82]. These findings suggest that the sensitivity of the

(50 μg, daily), and cisplatin (5 mg/kg, weekly) combination ($n = 10$) for 31 days. Tumor growth curves from each group were plotted for comparison. These data demonstrated that cisplatin and PRL combination treatment was the most effective way to eliminate tumor-initiating (CSC) cells in both **c** and **d** experiments

human breast to carcinogenic insults is determined by the degree of its differentiation status.

With more and more American women delaying the birth of their first child to a later age, it is important to develop a simple and effective means of mimicking this well-defined physiological condition and thereby reducing the risk of breast cancer [82–86]. One such attempt is proposed by Russo et al. with short-term treatment with human chorionic gonadotropin (hCG), a hormone secreted by the placenta during pregnancy [83]. While it has been shown that short-term treatment with hCG induces gene alterations in the breast similar to those of pregnancy [83], it still remains to be seen whether those genetic alterations will translate to preventive effects in women against breast cancer. A phase III study is currently on-going (http://www.clinicaltrials.gov). While Russo's breakthrough concept makes a great deal of sense, it may be the time to consider hPRL, which in many ways plays more important role in mammary gland development, especially during pregnancy, as an ASCs differentiation regulator.

To investigate the role of hPRL in the process of HER2/*neu*-initiated breast tumorigenesis, we generated hPRL/*neu* bitransgenic mice by crossbreeding an hPRL transgenic mouse lines (MT-hPRL) with HER2/*neu* mice. To our great surprise, there was a significant delay and overall reduced total tumor in the hPRL/*neu* cross line when compared to their respective HER2/*neu* heterozygote littermates (Fig. 3.3) (a manuscript in preparation). This unexpected, yet very important finding, points out the potential benefit of early exposure of mammary gland to PRL. The results were more validated when it was in contrast to the results from G129R/*neu* bitransgenic data, which were generated at same time, as shown in Fig. 3.1).

A possible explanation (our working hypothesis) for this obvious protective effect in tumor incidence in the hPRL/*neu* bitransgenics could be that early exposure of the mammary gland to transgenic product hPRL induced lobule differentiation (more Lob 3, less Lob 1), which mimics the effects of early pregnancy in human, thus leading to a relatively refractory state of the mammary epithelium to the HER2/*neu* oncogene. This hypothesis is supported by our observations of mammary gland whole mounts from the transgenic mice. We found that the mammary gland of hPRL/*neu* female mice displayed a well-differentiated ductal structure (more side branches and lobuloalveoli) at both 3 and 6 months of age as compared to their littermates or age-matched nontransgenic mice (Fig. 3.3). These results are consistent with previous studies in rats, it which it has been demonstrated that early exposure to steroids offers protection from mammary carcinogenesis by inducing differentiation of terminal end buds and ducts [84–86].

Other studies have reported a significant alteration in HER2/*neu*-initiated tumorigenesis in Her2/*neu* transgenic mice conferred through various hormonal treatments. For example, using the activated HER2/*neu* transgenic mouse model, it was found that short-term treatment with estradiol or estradiol plus progesterone before tumor initiation decreased mammary tumor incidence by more than 60 % at 8–9 months of age, supporting the concept that timing is a critical factor in hormonal treatment in mammary tumorigenesis [84–86]. Recently, a bitransgenic mouse line was established in which coexpression of HER2/*neu* and aromatase

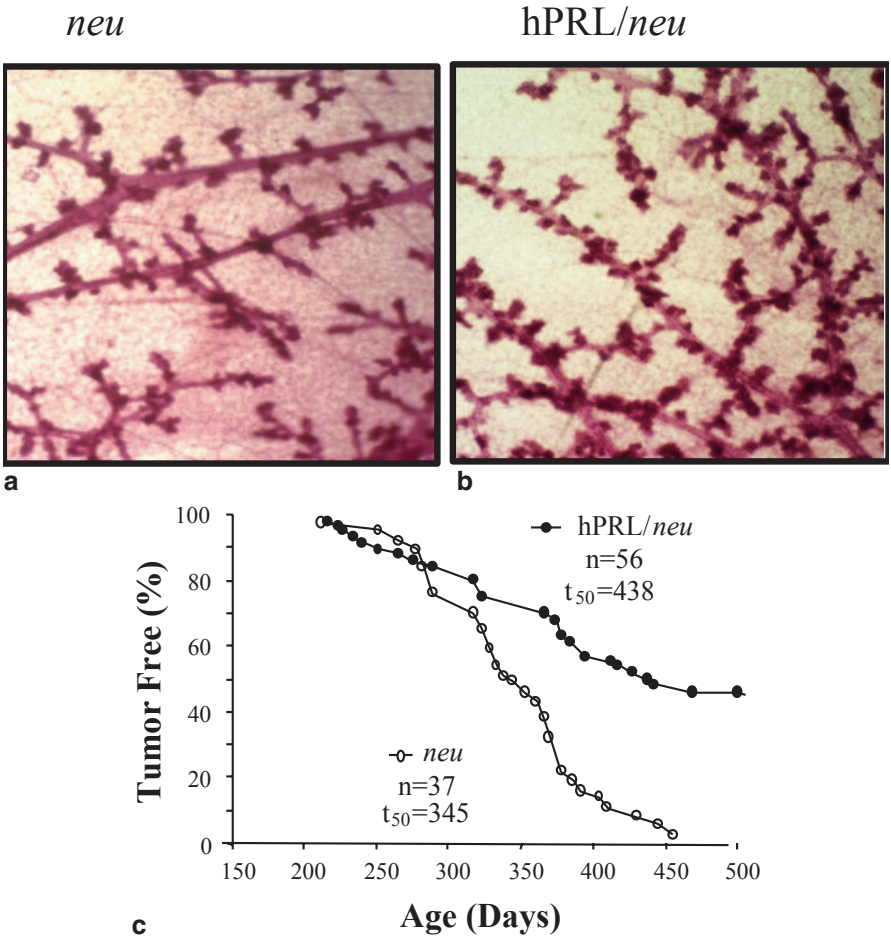

Fig. 3.3 hPRL and hPRL/neu bitransgenic mice. Panel **a** and **b**. representative whole mount images of the mammary gland in hPRL transgenic (**a**) and hPRL/neu bitransgenic mouse (**b**). The fourth inguinal mammary glands were dissected, stained in carmine alum stain and digitally photographed. The lymph node was used as a reference point to the edge of the mammary gland. Panel **c**, the comparison of the tumor free percentage over time between neu transgenic (*open circles*) and hPRL/neu bitransgenic mice (*closed circles*). The T50 of hPRL/neu bitransgenic mice was significantly delayed in hPRL/neu bitransgenic mice ($p < 0.05$)

(Aromatase/*neu*) also significantly reduced HER2/*neu*-initiated tumorigenesis [87]. In view of the close relationships among PRLR, estrogen receptor and HER2/*neu* [53–55], it is possible that these hormones influence the mammary tumorigenesis in the HER2/*neu* transgenic model via a similar mechanism.

Previous reports have demonstrated that overexpression of PRL in transgenic mice has been linked to breast tumor formation [88, 89]; however, the MT-hPRL

transgenic mouse line used in this study showed no significant increase in the incidence of palpable tumors (data not shown). One explanation for this discrepancy, albeit less likely, is that rat PRL (rPRL) was used in those two studies, whereas hPRL was used in ours. Despite the fact that rat and hPRL share only a 62% amino acid identity, it is generally accepted that hPRL activates mouse PRLRs as effectively as rPRL [89, 90]. Alternatively, it could be the differences in the expression levels of the PRL transgene. In one study, both transgenic lines of the female mice, expressing high (150 ng/ml) or low (13 ng/ml) levels of the rPRL transgene under the control of the MT promoter, developed nonmetastatic mammary tumors at 11–15 months of age [90]. Similarly, female mice expressing high (253 ng/ml) or low (45 ng/ml) levels of the rPRL transgene under the control of the hormone nonresponsive neu-related-lipocalin (NRL) promoter, which is expressed selectively in the mammary epithelia, developed invasive mammary tumors at age ~16 months [90]. The hPRL level in the serum of our female bitransgenic mice was between 3 and 10 ng/ml, which is at the lower end of the physiological level. It is noteworthy that coexpression of G129R at similar level in MMTV-*neu* female mice had little influence on breast tumor incidence (Fig. 3.1). Nonetheless, the lack of an effect in G129R/*neu* bitransgenic mice further verifies the specificity of hPRL-induced resistance to Her2/*neu*-induced breast tumorigenesis.

We have conducted a short-term treatment experiment in mice using recombinant hPRL and SA-20-hPRL, a long serum half-life derivative of hPRL in which a serum albumin-binding peptide (SA20) was fused to its amino-terminus to see if a short-term treatment could stimulate breast ASC proliferation and differentiation [91]. We found that mammary gland lobuloalveolar development in 8-week-old female mice was greater in all treated groups (daily, every 2 days, or every 3rd day over a 12-day period). The effects were more obvious in mice treated with SA20-hPRL (Fig. 3.4). These findings indicate that a relatively short-term treatment could exert significant physiological response in the mammary gland. Whether these responses elicited by hPRL will translate into protective benefit against tumorigenesis remained to be seen.

Taken together, the results presented here provide new insight into the role of hPRL in HER2/Neu-initiated breast tumorigenesis and contribute to a current discussion of the potential role of PRL in preventing breast cancer in some settings [92]. However, caution should be used to interpret this data since it is from a transgenic model where the breast tumor was initiated by marked overexpression of a single oncogene, whereas human tumors are genetically more complex. Nonetheless, this data together with that of others [83–87], support the notion that the particular differentiated state of a cell provides the critical epigenetic context that dictates the process of tumorigenesis [93]. It is conceivable, at least in mice, that the mammary tumor incidence can be influenced drastically by manipulating the time at which differentiation of the mammary epithelium occurs. Further studies are urgently needed to determine the critical factors, that lead to the prevention of mammary tumerigenesis such as timing and dosing of the hormone treatment and the hormones to use such as hPRL, estrodial/progesterone, and/or hCG or in combination.

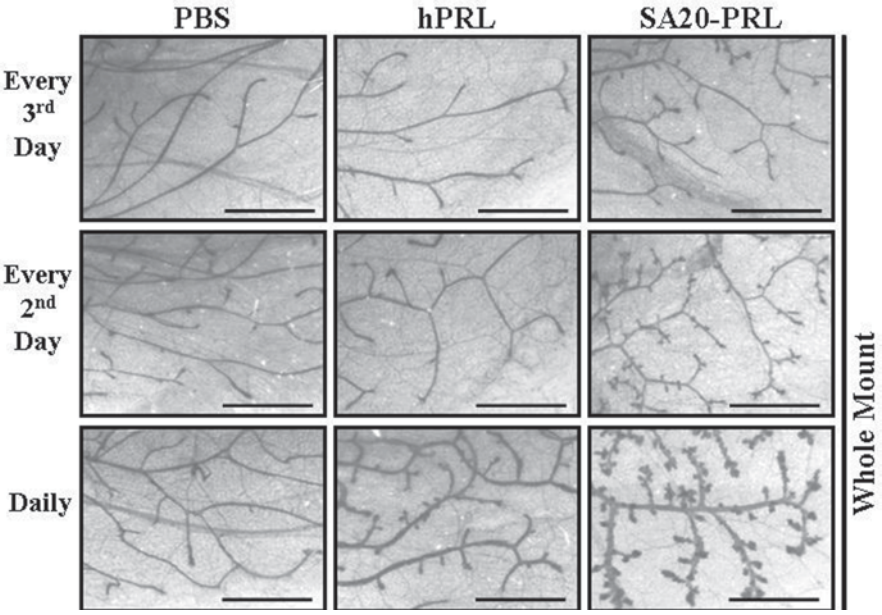

Fig. 3.4 Representative whole mount imaging comparison of the fourth inguinal mammary gland isolated from mature nulliparous mice treated with PBS, hPRL, or SA20-hPRL. Groups of 8-week-old nulliparous FVB/N female mice ($n=6$) were treated (i.p.) with hPRL (3.59 mg/kg), SA20-hPRL (4 mg/kg) or PBS everyday, every other day, or every 3rd day over a period of 12 days. Representative whole mount staining of the mammary gland used to score for alveolar development and ductal dilation as described [91]

3.3 Concluding Remarks

After years of studying the role of PRL in breast cancer using multiple breast cancer cell lines and various mouse tumor models, it becomes clear that the involvement of PRL in breast cancer is probably not as a classic oncogene. New data are emerging that suggest that the role of PRL as an autocrine/paracrine growth factor should also be reevaluated. However, since PRL has a critical role in breast ASC, or possibly in CSC regulation, and the majority of breast cancer cells are PRLR-positive. The role of PRL in breast cancer etiology, therapy, and even prevention will remain an interesting area of research in the coming years.

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Chapter 4
Regulation of Blood Vessels by Prolactin and Vasoinhibins

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Abstract Prolactin (PRL) stimulates the growth of new blood vessels (angiogenesis) either directly through actions on endothelial cells or indirectly by upregulating pro-angiogenic factors like vascular endothelial growth factor (VEGF). Moreover, PRL acquires antiangiogenic properties after undergoing proteolytic cleavage to vasoinhibins, a family of PRL fragments (including 16 kDa PRL) with potent antiangiogenic, vasoconstrictive, and antivasopermeability effects. In view of the opposing actions of PRL and vasoinhibins, the regulation of the proteases responsible for specific PRL cleavage represents an efficient mechanism for controlling blood vessel growth and function. This review briefly describes the vascular actions of PRL and vasoinhibins, and addresses how their interplay could help drive biological effects of PRL in the context of health and disease.

4.1 Introduction

Prolactin (PRL) is remarkably versatile, as it regulates various events in reproduction, osmoregulation, growth, energy metabolism, immune response, brain function, and behavior [1–3]. Blood vessels are emerging as PRL targets contributing to these actions [4]. By transporting fluid, nutrients, oxygen, hormones, growth factors, cytokines, immune cells, and waste material, the vascular system helps regulate most if not all body functions including growth, energy homeostasis, inflammation, and brain activity. PRL stimulates or inhibits the proliferation, dilation, permeability, and regression of blood vessels. These opposing effects reside within the PRL molecule as the full-length protein promotes angiogenesis, but after proteolytic processing the resulting PRL fragments, vasoinhibins, exert antiangiogenic, vasoconstrictive, and antivasopermeability effects (Fig. 4.1). The combination of these stimulatory and inhibitory properties can lead to differences in the perfusion of target tissues, thereby influencing their growth and function. In this review, we

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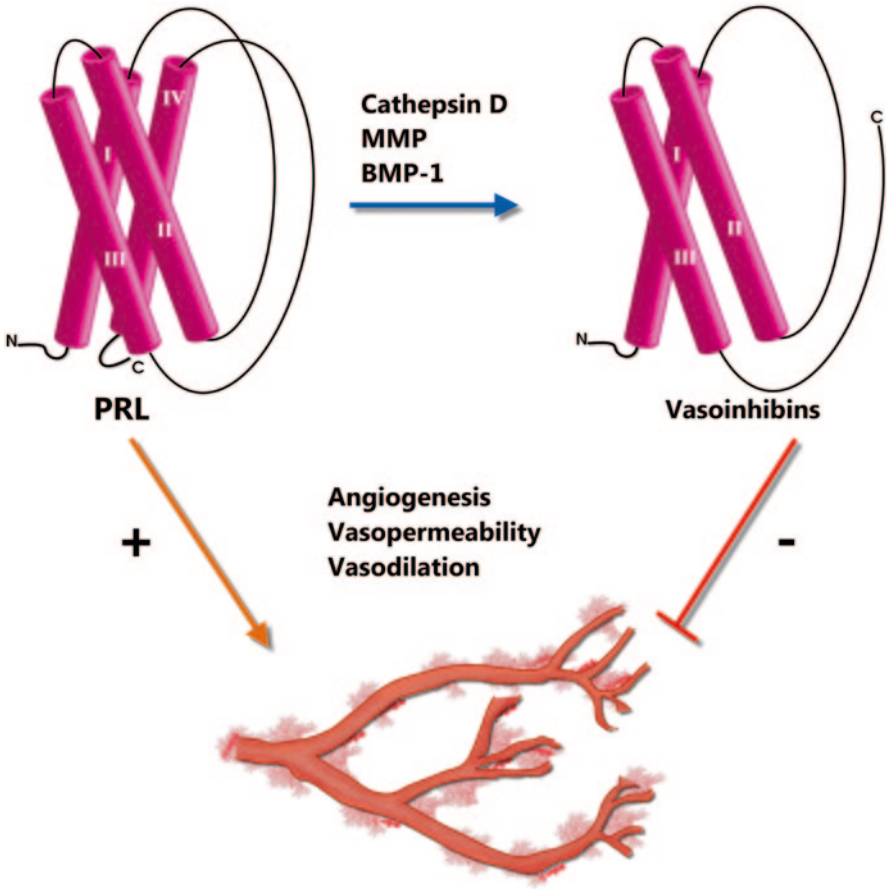

Fig. 4.1 *PRL* stimulates blood vessel growth and function and acquires vascular inhibitory properties after undergoing specific proteolytic cleavage to vasoinhibins by cathepsin D, matrix metalloproteases (MMP), and bone morphogenetic protein-1 (BMP-1)

clarify the advantage of the vasoinhibin nomenclature, and concisely address the generation of vasoinhibins, the effects of PRL and vasoinhibins on blood vessels, and how these vascular actions could affect tissue growth, function, and involution under normal and pathological conditions.

4.2 The Vasoinhibin Term

PRL fragments with inhibitory effects on blood vessels were originally termed "16 kDa PRL." The initial paper by Ferrara et al. [5] used a purified fraction of enzymatically cleaved rat PRL (having 145 amino acids and 16.3 kDa) that showed

an inhibitory effect on endothelial cell proliferation. However, follow-up studies, confirming and extending these vascular actions, used recombinant PRL fragments of different sizes that were still named 16 kDa PRL in an attempt to maintain the connection to the original preparation. Indeed, various laboratories used a recombinant fragment of 14 kDa containing the first 123 amino acids of human PRL [6–8], the same fragment but coupled to a polyhistidine tail [9, 10], a 15.6 kDa fragment containing the first 139 amino acids of human PRL [11–13], or a 17.2 kDa fragment containing the first 150 amino acids of human PRL [14]. The term 16 kDa PRL became even less accurate when studying the endogenous peptides generated by specific proteases. It then became evident that PRL fragments with inhibitory effects on blood vessels are not a single 16 kDa species, but rather a family of peptides with different molecular masses so far ranging from 14 to 18 kDa and all sharing the N-terminal region of PRL.

Different proteases generate the various fragments by cleaving at different sites near or within the long loop connecting the third and fourth α-helices of the PRL molecule (Fig. 4.1). Cathepsin D cleaves rat PRL and mouse PRL into a 16 kDa N-terminal fragment [15, 16], bovine PRL into 14 and 16 kDa N-terminal fragments [17], buffalo PRL into 11, 14, and 18 kDa antiangiogenic fragments [18], and human PRL into 11, 15, 16.5, and 17 kDa N-terminal fragments [19]. Matrix metalloproteases (MMP) predominantly cleave human and rat PRL at amino acids 155 and 153, respectively, to generate 17 kDa N-terminal fragments [20], and bone morphogenetic protein-1 (BMP-1) cleaves after the first 159 amino acids of human and mouse PRL, generating an 18 kDa fragment [21]. Since these peptides share blood vessel inhibitory properties, in 2006 we proposed the term “vasoinhibins” [20, 22] to refer to the whole family of PRL-derived N-terminal fragments that have inhibitory vascular effects. All PRL N-terminal fragments of 14 to 18 kDa that have been tested to date demonstrate blood vessel inhibitory properties, reinforcing the concept that this structure is responsible for the vasoinhibin identity.

4.3 Generation of Endogenous Vasoinhibins

The fact that vasoinhibins are produced by different proteases implies that their generation can occur under different conditions and tissue microenvironments. Cathepsin D is catalytically active at acidic pH (pH < 5.5), and recent findings showed that it is the main vasoinhibin-generating enzyme in anterior pituitary lactotrophs [23]. Cathepsin D is located in PRL secretory granules, which are acidic, and cathepsin-D null mice are devoid of vasoinhibins in the anterior pituitary gland [23]. Accordingly, vasoinhibins can be generated by cathepsin D during the pituitary PRL secretory process and thus, subjected to regulated release. Along this line, estradiol increases the synthesis and activity of cathepsin D [24], and the production of anterior pituitary vasoinhibins is higher in females [23], increases at proestrus with respect to diestrus, and estrogens stimulate their release [25].

Cathepsin D may also be released from secretory granules or lysosomes at anterior pituitary or extrapituitary locations to generate vasoinhibins outside cells. Cathepsin D cleaves PRL in the extracellular milieu of regressing corpus luteum [17] and mammary gland [16], and in cardiomyocytes under oxidative stress [13], conditions in which tissue remodelling and altered metabolic activity can acidify the pericellular pH. Cultured GH4C1 pituitary adenoma cells also secrete cathepsin D, and mimicking the tumor microenvironment by exposure to hypoxia reduces its release [26], suggesting that extracellular production of vasoinhibins could be decreased and favor the proangiogenic condition necessary for prolactinoma growth.

On the other hand, PRL may be physiologically cleaved outside cells by the extracellular matrix-degrading enzymes, MMP and BMP-1, which act at neutral pH and are secreted or anchored to the external cell surface. MMP and BMP-1 released by chondrocytes [20] and embryonic fibroblasts [21], respectively, generate vasoinhibins from PRL, a mechanism that may serve to maintain cartilage avascularity and to limit developmental angiogenesis. MMP and BMP-1 also produce other antiangiogenic factors by proteolytic processing [27, 28]; however, both types of proteases are upregulated in diseased states characterized by blood vessel growth and invasion [29, 30]. As high concentrations of MMP also lead to the degradation of both PRL and vasoinhibin [20], in some cases MMP upregulation may downregulate vasoinhibins to favor pathological angiogenesis.

Consistent with the ubiquitous nature of PRL-cleaving enzymes, endogenous vasoinhibins have been identified in the anterior [23] and posterior pituitary gland [31], hypothalamus [32], cartilage [20], retina [33], cardiomyocytes [13], corpus luteum [17], mammary gland [16], and in biological fluids (serum, amniotic fluid, and urine) [34, 35].

4.4 Vascular Effects of PRL and Vasoinhibins

The effects and signaling mechanisms of PRL and vasoinhibins on blood vessels have been extensively reviewed [4, 22, 36]. Here, we will briefly summarize previous findings with a focus on recent advances. PRL stimulates angiogenesis during development (chick chorioallantoic membrane, CAM) and in adult tissues (corpus luteum, testis, and heart). These observations were recently extended to include the angiogenesis of transplanted pancreatic islets [37] and the neovascularization associated with normal and regenerative liver growth, where inducing hyperprolactinemia increased hepatic endothelial cell proliferation and vascular endothelial growth factor (VEGF) expression [38, 39]. Moreover, in addition to the known effects of PRL on endothelial cell proliferation and VEGF expression, PRL was recently shown to stimulate the migration and tube formation of endothelial cells [40, 41], to reduce vasopermeability by upregulating the expression of tight-junction proteins between endothelial cells [42], and to promote intussusceptive angiogenesis in the CAM [40]. The latter differs from sprouting angiogenesis in that new blood vessels are formed by the splitting of an existing blood vessel in two, which is essentially independent of endothelial cell proliferation and thereby, less energy demanding [43].

PRL can promote angiogenesis by direct actions on endothelial cells. However, the effects of PRL on cultured endothelial cells are modest and not always observed [40, 41, 36]. PRL actions may be limited by underexpressed PRL receptors in endothelial cells. Exposure to ovarian follicular fluid stimulates the expression of the long and short PRL receptor isoforms in bovine umbilical vein endothelial cells (BUVEC), and PRL does not stimulate BUVEC proliferation unless the cells are pretreated with ovarian follicular fluid [44]. In addition, vascular endothelial cells produce and release PRL, so the locally produced hormone may limit the effects of exogenous PRL by occupying its receptors in endothelial cells. Yang and colleagues recently highlighted the role of PRL as an autocrine regulator of angiogenesis. They showed that PRL produced by endothelial cells is a downstream mediator of STAT5-induced endothelial cell migration, invasion, tube formation, and VEGF expression [41]. The fact that STAT5 mediates these angiogenesis events in response to fibroblast growth factor-2 (FGF-2) [45] places PRL in the signaling cascade of potent angiogenesis stimulators. Also, PRL itself activates STAT5 in endothelial cells [40, 41] and stimulates the expression of FGF-2 and VEGF by various nonendothelial cell types [4], suggesting that PRL acts as a positive autocrine and paracrine feedback regulator of angiogenesis.

The complexity of the vascular effects of PRL is further illustrated by conflicting data showing that PRL is unable to stimulate angiogenesis in the mouse cornea, that siRNA-targeting PRL results in angiogenesis in the rat retina, and that disruption of the PRL gene is associated with highly vascularized pituitary tumors in aged mice [4, 36]. Moreover, PRL has opposing effects on vascular resistance, blood volume, and blood flow that depend on the experimental model and conditions [36]. These inconsistencies may involve the proteolytic conversion of PRL to vasoinhibins.

Vasoinhibins inhibit angiogenesis, vasodilation, and vasopermeability, and promote vascular regression in the cornea, retina, heart, and xenografted tumors. They act directly on endothelial cells to inhibit the action of several vasoactive substances including: VEGF, FGF-2, interleukin 1-β, bradykinin, and acetylcholine. Vasoinhibins signal through a still-unidentified receptor distinct from the PRL receptor: (1) to cause cell cycle arrest by blocking activation of the MAPK pathway at the level of Ras, decreasing cyclin D1, and upregulating p21; (2) to inhibit endothelial cell migration by increasing type-1 plasminogen activator inhibitor and thus reducing urokinase activity, and by downregulating the Ras-Tiam1-Rac1-Pak1 pathway; and (3) to induce endothelial cell apoptosis by promoting NFκB-mediated caspase-8 and 9 activation, which in turn stimulate caspase-3 and DNA fragmentation. In addition, vasoinhibins were recently shown to induce the expression of microRNA-146a (miR-146a) in endothelial cells in an NFκB-dependent manner [46]. Silencing miR-146a expression prevented the inhibitory effects of vasoinhibins on endothelial cell proliferation and survival, but not on endothelial cell migration, revealing miR-146a as a mediator of a large fraction of vasoinhibins antiangiogenic effects.

Another key mechanism by which vasoinhibins regulate endothelial cell function, specifically causing vasoconstriction and reduced vasopermeability, is by blocking the activation of endothelial nitric oxide synthase (eNOS). They do so by promoting protein phosphatase 2 A-induced dephosphorylation and inactivation of eNOS, by blocking the activation of phospholipase C and the formation of inositol

1,4,5-triphosphate leading to a reduced release of Ca^{2+} from intracellular stores, and by interfering the expression of transient receptor potential canonical (TRPC) channels [47, 4]. Also, dephosphorylation-mediated inactivation of eNOS can contribute to vasoinhibin inhibition of endothelial cell proliferation and migration. Vasoinhibins block the increase in eNOS activity, migration, and proliferation of endothelial cells overexpressing wild type eNOS but did not affect these responses in cells overexpressing phosphomimetic or nonphosphorylatable eNOS mutants [48].

Besides inhibiting eNOS-mediated vasodilation, vasoinhibins can lower blood flow in developing blood vessels by reducing pericyte coverage of capillaries [49]. Vasoinhibins interfere with pericyte recruitment by disrupting the Notch signaling pathway in endothelial cells, and this action can lead to a dysfunctional vasculature in a murine melanoma tumor model [49]. Finally, vasoinhibins exert proinflammatory actions on blood vessels; they stimulate leukocyte adhesion to endothelial cells and leukocyte infiltration into tumors by activating NFκB and increasing the expression of adhesion molecules in endothelial cells [50]. These actions may also involve vasoinhibin-induced downregulation of eNOS, since VEGF stimulation of eNOS-mediated NO production promotes endothelial cell anergy [51].

In spite of the abundance of data concerning the vascular actions and signaling mechanisms of vasoinhibins, the nature of the vasoinhibin receptor remains unresolved. More than two decades ago, vasoinhibins were shown to bind to a single class of sites on endothelial cell membranes (Kd of 1–10 nM), associating with proteins of 52 and 32 kDa that were distinct from the PRL receptor [52]. Whether these represented receptors or regulatory binding proteins important for vasoinhibin functions is unknown. Difficulties in identifying the vasoinhibin receptor(s) may lie in that they could be forming a complex with other receptors and binding proteins. Similar receptor complexes have been proposed for angiostatin and endostatin, that are also families of antiangiogenic peptides derived by proteolysis from precursor proteins [53, 54].

4.5 Contribution of Blood Vessel Regulation to PRL Biological Effects

The influence of the vascular actions of PRL and vasoinhibins on the regulation of PRL target organs (crop-sac, mammary gland, corpus luteum, retina, cartilage, and heart) has been previously reviewed in a physiopathological [4, 36, 55, 56] and evolutionary [57] context. Here, we extend this discussion by addressing recent findings and promising new avenues.

4.5.1 Mammary Gland

The mammary gland stands as a major PRL target organ. PRL stimulates the growth, differentiation, milk production, and survival of mammary epithelium.

These events are dependent on the expansion and regression of the mammary gland vasculature [58], which may be influenced by PRL and vasoinhibins. PRL promotes the expression of VEGF in mammary epithelial cells [36], and weaning upregulates the expression and activity of the vasoinhibin-generating proteases, MMP, and cathepsin D [59]. Vasoinhibins were recently detected in mouse mammary glands, and their levels increased during involution together with those of the mature cathepsin D isoform [16]. Moreover, PRL stimulates the activation and polarized secretion of cathepsin D by mammary tissue [60]. This mechanism may help attenuate vascular expansion during lactation and promote blood vessel regression during involution since PRL expression and cathepsin D-mediated PRL cleavage increase in the lactating and regressing mammary gland [16, 36].

However, the mechanisms regulating the antiangiogenic effects of vasoinhibins may be altered in the malignant state. Neoplastic breast tissue shows diminished vasoinhibin-generating activity [61] and higher levels of PRL receptors in cells [62], including those of the microvasculature [40]. In contrast to the reduced growth observed in prostate, colon, and melanoma tumors expressing vasoinhibins, tumors derived from breast cancer cells induced to produce vasoinhibins exhibit decreased vascularization but no effect on tumor size [14]. This is surprising as vasoinhibins have the ability to inhibit and promote the growth [14] and apoptosis [16] of breast cancer cells, respectively.

4.5.2 Corpus Luteum

Similar to the mammary gland, the corpus luteum undergoes dramatic expansion and involution at the expense of the vasculature. In rodents, PRL is luteotrophic in pregnancy but luteolytic during nonfertile cycles. These opposing effects may reflect in part the vascular interplay between PRL and vasoinhibins. PRL stimulates the proliferation of endothelial cells in the corpus luteum, and lowering systemic PRL or disrupting the PRL receptor interferes with corpus luteum neovascularization. By using transgenic mice expressing only the long form of the PRL receptor, PRL-induced stimulation of VEGF production and neovascularization of the corpus luteum was specifically linked to the short form of the PRL receptor [63], which is the predominant form found in corpus luteum endothelial cells [64].

4.5.3 Retina

In contrast to reproductive organs, the vasculature is dormant throughout life in most adult tissues and is highly restricted in cases such as the retina. Vasoinhibins help maintain the quiescent state of retinal blood vessels and protect against aberrant vasopermeability and angiogenesis in retinopathy of prematurity and diabetic retinopathy. Retinal vasoinhibins may derive from PRL synthesized in the retina and from systemic PRL accessing the eye via its receptors in the ciliary body [65].

Hyperprolactinemia increases the levels of retinal vasoinhibins, which in turn reduce VEGF and diabetes-induced retinal vasopermeability [65]. Similarly, the transfer to the retina of the vasoinhibin gene via adenoassociated virus type 2 vectors prevents vascular alterations associated with nonproliferative diabetic retinopathy [66].

4.5.4 Heart

Accumulating evidence has linked vasoinhibin overproduction to the pathophysiology of peripartum cardiomyopathy. Increased oxidative stress causes cathepsin D-mediated PRL cleavage to vasoinhibins, which in turn interfere with the growth and function of coronary vasculature required for adequate performance of the maternal heart during pregnancy and lactation. MiR-146a, discovered as a major mediator of vasoinhibin antiangiogenic actions, is also responsible for vasoinhibin effects causing myocardial metabolic dysfunction [46]. Vasoinhibins stimulate the shedding from endothelial cells of exosomes loaded with mirR-146a that, when absorbed by cardiomyocytes, impairs their metabolic activity [46]. Altogether, these concepts have led to the development of promising combination therapies employing bromocriptine and to the evaluation of markers (cathepsin D activity and miR-146a serum levels) for diagnosis and disease monitoring [67].

4.5.5 Other

Other promising research directions relate to the liver, pancreas, and brain. Liver growth is angiogenesis-dependent and coincides with the hyperprolactinemia ocurring during pregnancy and lactation, cirrhosis [68], and after partial hepatectomy [69]. Absence of the PRL receptor confers reduced liver mass, and elevating systemic PRL promotes growth and neovascularization of the normal and regenerating adult liver [38]. During pregnancy, the need for insulin action results in pancreatic islet growth, which is angiogenesis dependent. PRL and placental lactogens stimulate the proliferation, survival, and insulin production by pancreatic β-cells [70], and PRL stimulates vascular density and downregulates the expression of the angiogenesis inhibitor thrombospondin-1 (TSP-1) in transplanted pancreatic islets [37]. Moreover, chronic exposure of isolated human islets to high glucose concentrations impairs angiogenesis, reduces PRL and MMP-9 expression, and increases TSP-1 synthesis [71]. These findings suggest that PRL mediates pancreatic islet neovascularization and growth during pregnancy, and that an altered production of PRL and vasoinhibins may impact abnormal islet angiogenesis in diabetes. PRL acts in the brain to stimulate neurogenesis and neuronal survival [3], which are effects frequently elicited by proangiogenic substances [72]. PRL also reduces the permeability of brain capillary endothelial cells in a NO-independent manner [42], and vasoinhibins inhibit NO-dependent vasopermeability in the retina, thus suggesting that the PRL-vasoinhibin system helps maintain the brain- and retinal-blood

barriers. Finally, exposure to stress reduces the conversion of PRL to vasoinhibins in the hypothalamus, and the intracerebroventricular administration of PRL and vasoinhibins attenuates and enhances stress-related behaviors (anxiety and depression) [32], respectively; these behaviors associate with altered cerebral blood flow and endothelial cell dysfunction [73].

Concluding Remarks

The vascular effects of PRL and vasoinhibins are emerging as novel mechanisms balancing growth, function, and involution. Further research is needed to clarify the regulation of the specific proteases, the receptors, and signaling pathways involved, and how PRL and vasoinhibins interact to affect blood vessel and organ function under health and disease.

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Chapter 5
Tyrosyl Phosphorylated Serine-Threonine Kinase PAK1 is a Novel Regulator of Prolactin-Dependent Breast Cancer Cell Motility and Invasion

Alan Hammer and Maria Diakonova

Abstract Despite efforts to discover the cellular pathways regulating breast cancer metastasis, little is known as to how prolactin (PRL) cooperates with extracellular environment and cytoskeletal proteins to regulate breast cancer cell motility and invasion. We implicated serine-threonine kinase p21-activated kinase 1 (PAK1) as a novel target for PRL-activated Janus-kinase 2 (JAK2). JAK2-dependent PAK1 tyrosyl phosphorylation plays a critical role in regulation of both PAK1 kinase activity and scaffolding properties of PAK1. Tyrosyl phosphorylated PAK1 facilitates PRL-dependent motility via at least two mechanisms: formation of paxillin/GIT1/βPIX/pTyr-PAK1 complexes resulting in increased adhesion turnover and phosphorylation of actin-binding protein filamin A. Increased adhesion turnover is the basis for cell migration and phosphorylated filamin A stimulates the kinase activity of PAK1 and increases actin-regulating activity to facilitate cell motility. Tyrosyl phosphorylated PAK1 also stimulates invasion of breast cancer cells in response to PRL and three-dimensional (3D) collagen IV via transcription and secretion of MMP-1 and MMP-3 in a MAPK-dependent manner. These data illustrate the complex interaction between PRL and the cell microenvironment in breast cancer cells and suggest a pivotal role for PRL/PAK1 signaling in breast cancer metastasis.

5.1 Role of Prolactin in Regulation of Breast Cancer Cell Motility

Prolactin (PRL) is a peptide hormone secreted from the anterior pituitary and was originally discovered in the early twentieth century as a hormone that regulates milk production in mammals [1, 2]. In addition to lactation, PRL was also implicated in mammary gland growth and development [3–6]. Significant progress was made

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in determining PRL-mediated signaling pathways upon the characterization of the prolactin receptor (PRLR) in the 1980s [7]. The PRLR is a transmembrane protein that belongs to the cytokine receptor superfamily and is expressed in variety of tissues, most notably the mammary epithelium [8]. The PRLR has no intrinsic kinase activity and relies on nonreceptor tyrosine kinases to facilitate PRL-mediated downstream signaling pathways. The most well characterized mediator of PRL signaling is the nonreceptor tyrosine kinase Janus-kinase 2 (JAK2) [9–11]. Upon PRL binding to its receptor, PRLRs dimerize, resulting in the activation of JAK2, as characterized by autophosphorylation of Tyr1007/1008, and promoting tyrosyl phosphorylation of the PRLR [12–14]. PRL signaling induces the activation of several signaling cascades, including the signal tranducers and activators of transcription (STATs), mitogen-activated protein kinases (MAPKs), protein kinase C, and phosphatidylinositol 3-kinase (PI3K) [15–21]. Since then, PRL signaling has been shown to regulate a variety of normal and pathological cell processes, one of which is cell motility.

Cell migration is critical for many vital biological functions, including embryonic development, the inflammatory immune response, wound repair, tumor formation and metastasis, and tissue remodeling and growth. The actin cytoskeleton provides both the protrusive and contractile forces required for cell migration via a combination of actin polymerization and depolymerization, actin filament cross-linking, and the interaction of myosin-based motors with actin filaments [22]. The complexity of cell motility and the fact that it is regulated by many hormones, cytokines, and growth factors suggest that multiple signaling mechanisms exist to regulate this process.

Little is known about the mechanisms that underlie the process of PRL-induced cell motility and its putative role in breast cancer metastasis. PRL was previously shown to act as a chemoattractant for human breast carcinoma [23]. Actin-based structures are most commonly controlled by small Rho-GTPases Rac1, Cdc42, and RhoA and these proteins are activated by guanine nucleotide exchange factors (GEFs) and repressed by GTPase-activating proteins (GAPs). PRL can activate Rac1 and several pathways have been implicated in this Rac-dependent regulation [24–26]. The first pathway has been shown to depend on PRL-induced activation of tyrosine kinase Tec which associates with and enhances activity of Vav1, the GEF factor for Rac1 [24]. According to the second proposed mechanism, PRL induces activity of serine-threonine kinase Nek3 (NIMA-related kinase 3) followed by activation of Vav1/Vav2 and subsequent activation of Rac1 [27, 28]. In addition, PRL stimulation also induces an interaction between Nek3 and focal adhesion protein paxillin and significantly increases paxillin serine phosphorylation [28]. In addition to Rac, PRL also activates another small GTPase Cdc42 that plays an important role in development and differentiation of mammary epithelia [25]. We have recently proposed two novel mechanisms to regulate PRL-dependent breast cancer cell motility: (1) through a serine-threonine kinase p21-activated kinase 1 (PAK1) and its substrate, the actin-binding protein filamin A and (2) through regulation of adhesion turnover ([29]; see below).

Cell migration depends on optimal levels of cell adhesion. The mechanisms that regulate focal adhesion assembly, maturation, and turnover are not well understood and have become a critical area of emerging interest. Over 180 proteins are found in adhesions, many of which exhibit multiple protein–protein interactions [30]. Cell adhesion regulated by both PRL- and extracellular matrix (ECM)/integrin-dependent pathways is essential for all aspects of normal mammary gland development and function (reviewed in [31–33]). PRL also regulates activation of numerous proteins participating in breast cancer cell adhesion. Thus, in early studies it has been noticed that PRL dramatically changes adhesiveness of breast cancer cells [34]. PRL activates focal adhesion kinase (FAK) and eventually induces phosphorylation of paxillin, an event that is essential to the rapid turnover of adhesions during cell motility [35]. FAK is a nonreceptor tyrosine kinase that mediates integrin signaling and regulates focal adhesion assembly and maturation during cell spreading and migration through phosphorylation of various adhesion proteins (reviewed in [36]). PRL causes tyrosyl phosphorylation of paxillin in an Src/FAK-dependent manner and serine phosphorylation of paxillin by serine-threonine kinase Nek3 ([28, 37]). In addition, transmembrane glycoprotein signal regulatory protein-α (SIRPα) has been implicated in the PRL- and integrin-activated cross talk in breast cancer cells [38]. We will discuss the role of PRL-activated serine-threonine kinase PAK1 in the regulation of breast cancer cell adhesion (see below). Thus, PRL has evidently been shown to increase cell motility in breast cancer cells.

Epidemiologic studies also linked elevated level of circulating PRL to breast cancer metastases [39–41]. In addition, PRLR expression has been found in patients with colorectal cancer, with high concordance between primary tumors and corresponding metastases [42]. These data, combined with animal studies reporting increased metastases with PRL administration [43], suggest that PRL is involved in the development of metastasis and tumor progression.

We have previously found that the serine-threonine kinase PAK1, a downstream effector for both Cdc42 and Rac1, participates in PRL-dependent signaling. We have shown that PAK1 is a novel substrate of the JAK2 tyrosine kinase and that PRL-activated JAK2 phosphorylates PAK1 in vivo and in vitro. PAK1 tyrosines 153, 201, and 285 were identified as sites of JAK2 tyrosyl phosphorylation by mass spectrometry and two-dimensional (2D) peptide mapping [44].

The aim of this review is to introduce tyrosyl phosphorylated PAK1 as a novel player in the field of PRL signaling and to discuss several mechanisms of pTyr-PAK1-dependent regulation of breast cancer cell motility, adhesion, and invasion.

5.2 p21-Activated Kinase 1 (PAK1)

5.2.1 PAK1 Structure and Activation

The PAKs are an evolutionarily conserved six member family of serine/threonine kinases and can be categorized into two groups based on structure and function:

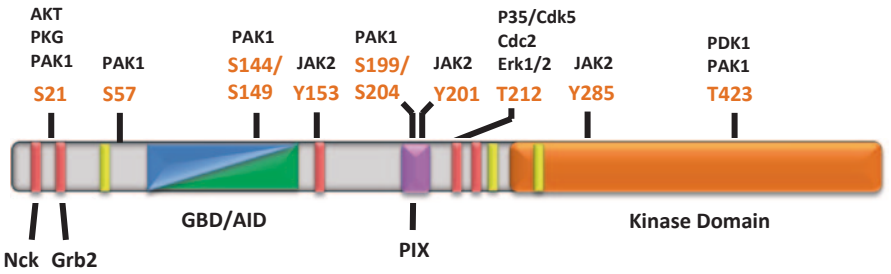

Fig. 5.1 PAK1 domain structure and phosphorylation sites. The N-terminal regulatory region of PAK1 is composed of overlapping GBD/AID domains (aa 70-149, *blue*/*green*), five proline-rich regions (*bright red*), one nonclassical proline-rich region (aa 182–203, *pink*), and three nuclear localization signals (*yellow*). The C-terminal kinase domain (aa 249–545) is represented by the *bright orange* region. The two most N-terminal proline-rich regions (aa 12–18 and aa 40–47) mediate Nck/Grb2 binding, respectively, and subsequent PAK1 membrane localization. The nonclassical proline-rich region regulates PIX/PAK1 binding and subsequent localization of PAK1 to adhesion complexes as well as facilitates PAK1 kinase activity. There are seven PAK1 autophosphorylation sites (S21, S57, S144, S149, S199, S204, and T423) that modulate PAK1 kinase activity, in addition to other sites phosphorylated by protein kinases that mediate PAK1 activity and localization. (Modified from Bokoch 2003)

Group I (PAKs 1–3), which are activated in a GTPase-dependent or independent manner in response to extracellular signals, and Group II (PAKs 4–6), which are generally not regulated by Rho-GTPases but most likely through intramolecular mechanisms (reviewed in [45]). PAK1, a Group I member, is the most well-studied representative of the six PAK family members and is widely expressed in a variety of tissues. PAK1 plays a pivotal role in a range of cellular processes including cell proliferation, survival, motility, and invasion. PAK1 consists of an N-terminal regulatory domain containing a GTPase binding domain (GBD) that is partially overlapped with an autoinhibitory domain (AID). PAK1 enzymatic activity derives from its C-terminal serine/threonine kinase domain. The N-terminal regulatory domain of PAK1 has additional sites of protein–protein interaction that can mediate PAK1 activation and localization, including five classical proline-rich regions (PXXP), two of which facilitate binding to adaptor proteins Nck and Grb2. PAK1 also contains a nonclassical proline-rich region (PXP) that mediates interaction with the p21-interacting exchange factor PIX. In addition, there are three nuclear localization signals (NLS) and multiple phosphorylation sites, seven of which (serines 21, 57, 144, 149, 199, 204, and threonine 423) are sites of PAK1 autophosphorylation. PAK1 activation and localization are dependent on protein–protein interactions and both autophosphorylation and direct phosphorylation of PAK1 by other kinases (Fig. 5.1).

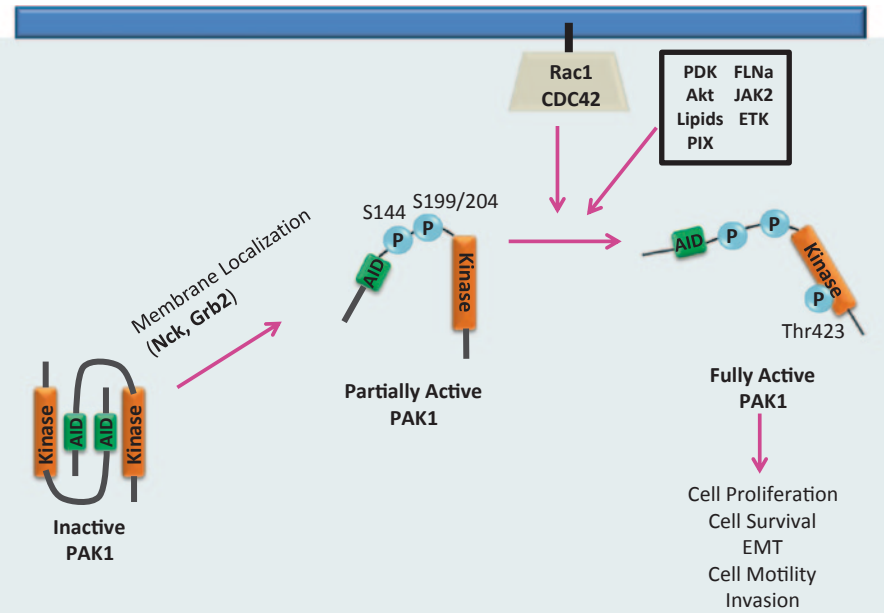

Fig. 5.2 Model for PAK1 activation. Inactive PAK1 is localized to the cytoplasm as a homodimer. Upon binding to Nck or Grb2, PAK1 is localized to the plasma membrane and undergoes a slight conformational change that facilitates partial autophosphorylation. PAK1 is now more susceptible to Rac1/Cdc42 binding, leading to a further conformational change, autophosphorylation at T423, and fully active PAK1 kinase activity. PAK1 can also be activated by interaction with Akt, lipids, PIX, FLNa, ETK, JAK2 or by direct T423 phosphorylation by PDK1. (Modified from Parrini et al. 2009)

PAK1 was initially discovered as an effector protein for two members of the Rho-family of small GTPases, Cdc42, and Rac [46]. These Rho-family GTPases serve as activators of PAK1 kinase activity. Inactive PAK1 resides in the cytoplasm as a homodimer, where the AID of one PAK1 molecule is obstructing the kinase domain of the other and vice versa (Fig. 5.2; [47]). Binding of Cdc42 or Rac1 to PAK1's GBD induces a PAK1 conformational change that allows autoinhibitory relief and autophosphorylation of several sites on PAK1, keeping it in an open and active conformation. Recent studies suggest that membrane localization of inactive PAK1 via the adaptor proteins Nck and Grb2 promotes a semi-open conformation of PAK1 which facilitates autophosphorylation of several serines, including Ser199 and Ser204, and promotes initial kinase activation of PAK1. This semi-open/semi-active PAK1 is more susceptible to interaction with Rac1 or Cdc42. Rho-GTPase/PAK1 binding facilitates a fully open PAK1 conformation and allows autophosphorylation of Ser144 in the GBD and Thr423, the major PAK1 autophosphorylation site mediating PAK1 kinase activity, in the kinase domain [48].

PAK1 activation is not solely dependent on GTPases, since the interaction of PAK1 with a variety of different proteins can regulate PAK1 kinase activity. Membrane localized PAK1 can be activated by direct phosphorylation on the critical

Thr423 by phosphoinositide-dependent kinase 1 (PDK1) [49]. Similarly, certain lipids at the plasma membrane, like phosphatidic acid and sphingosine, can bind to the regulatory domain of PAK1 and induce kinase activation and subsequent autophosphorylation to the same extent as GTPase-activation of PAK1 [50]. Also at the plasma membrane, the actin cross-linking protein filamin A (FlnA) can mediate PAK1 kinase activity in two ways: by binding directly to the PAK1 GBD, therefore stimulating PAK1 activation, and facilitating the interaction of PAK1 with lipids [51, 52]. Akt (protein kinase B) can directly activate PAK1 and phosphorylate Ser21 on PAK1 which negatively mediates Nck/Grb binding and membrane localization [53, 54]. Some proteins, such as the guanine exchange factor (GEF) PIX, can induce PAK1 activation in both a GTPase-dependent and independent manner. The binding of PAK1 to PIX localizes PAK1 to cell–matrix adhesions and can directly mediate PAK1 activation, and since PIX is a Rac1-specific GEF, PIX can indirectly activate PAK1 through activation of Rac1 [55, 56]. Interestingly, PAK1 can also be activated upon tyrosyl phosphorylation. The nonreceptor tyrosine kinase Etk/BMX can tyrosyl phosphorylate PAK1 and induce PAK1 kinase activation; however, the sites for Etk-induced phosphorylation have not been mapped [57]. The nonreceptor tyrosine kinase JAK2 can also tyrosyl phosphorylate and activate PAK1, and we will review this activation and downstream effects on PAK1 signaling in this chapter [44]. Downregulation of PAK1 kinase activity is also important, since hyperactivation of PAK1 can induce mammary gland tumor growth [58]. PAK1 enzymatic activation and localization to focal adhesions can be inhibited when the tumor suppressor protein Merlin is bound to the PAK1 GBD [59]. Likewise, the integrin-binding protein Nischarin can bind to the kinase domain of activated PAK1, greatly reducing PAK1 kinase activity [60]. Cystein-rich protein CRIPak has also been identified as an inhibitor of PAK1 [61]. Human PAK1-interacting-protein 1 (hPIP) binds to first 70 amino acids of PAK1 and blocks kinase activity [62]. P35/Cdk5 phosphorylates PAK1 and inhibits kinase activity while phosphatases POPX1 and POPX2 dephosphorylate threonine 423 of PAK1 and also inhibit it [63, 64]. Protein kinase p110C binds to amino acids 210–332 of PAK1 and inhibits it [65].

The diverse means in which PAK1 is regulated lends PAK1 to participate in a variety of fundamentally different cellular processes (Fig. 5.3).

5.2.2 PAK1 Acts as a Scaffold

While PAK1 kinase activity plays a major role in PAK1 downstream signaling events, PAK1 can also act independent of its kinase activity as a molecular scaffold to facilitate the interaction between different proteins. Thus, PAK1 can regulate the actin cytoskeleton in both kinase-dependent and independent ways. PAK1 mutants with a modified N-terminus have dramatic effects on the actin cytoskeleton regardless of the presence of an active kinase domain [46, 66]. PAK1 overexpression has been shown to increase random cell movement irrespective of its kinase activity [67]. Also, an SH3 domain of PIX protein binds to a noncanonical proline-rich

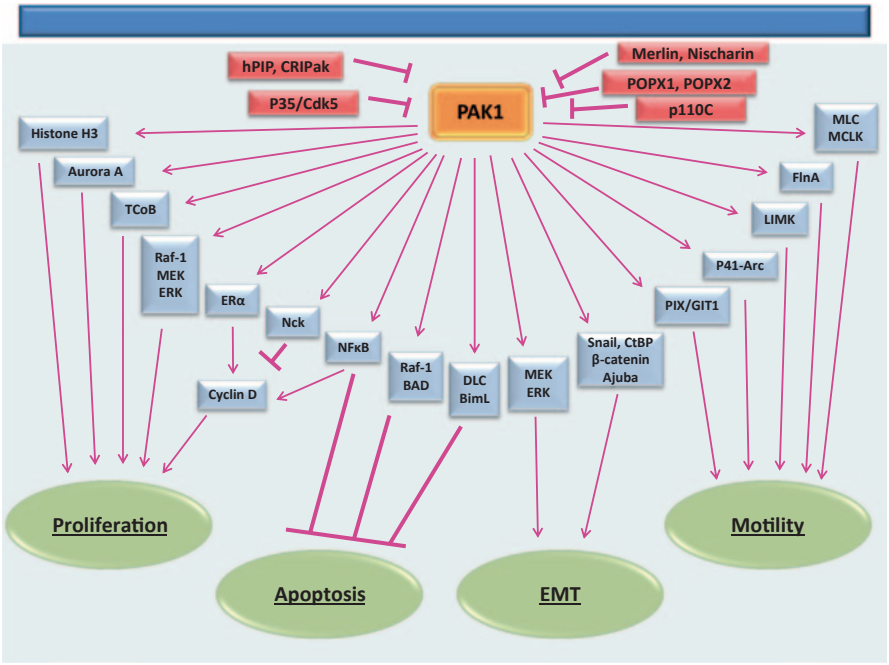

Fig. 5.3 PAK1 regulates different cellular functions, including cell proliferation, survival, cell motility, and EMT. PAK1 kinase activity and/or interaction with various proteins mediate PAK1s variable functions within the cell

region on PAK1 independently of PAK1 kinase activity, and through association with GIT1, localizes PAK1 to the adhesion protein paxillin to regulate cell adhesion [55, 68]. These data, in combination with the fact that overexpression of kinase-dead PAK1 facilitates the formation of focal adhesions and stabilizes stress-fibers [69], suggest a role for the scaffolding abilities of PAK1 in regulation of cytoskeletal and adhesion dynamics.

PAK1 has also been shown to act as a scaffold in coordinating signaling between Raf-1, MEK, and ERK proteins upon cell adhesion to fibronectin or treatment with PDGF [70]. Overexpression of a kinase-dead PAK1 increased phosphorylation of MEK and ERK in kinase-independent way [71]. In addition to the scaffolding function, PAK1 phosphorylates both MEK and Raf-1 to amplify the ERK signaling [72]. The MAPK pathway is not the only pathway that benefits from the nonenzymatic activity of PAK1. Akt, a major regulator of several cell survival pathways, is activated upon phosphorylation by PDK1 and the plasma membrane localization of Akt is important for the activation [73, 74]. Higuchi et al. demonstrated that upon growth factor stimulation Akt binds to the C-terminal domain of PAK1 and is consequently targeted to the plasma membrane. At the plasma membrane, PAK1 can also bind to PDK1, bringing together both Akt and PDK1 thereby facilitating Akt stimulation by PDK1. These findings confirm scaffolding, kinase-independent functions of PAK1

[75]. There is also evidence of kinase-independent roles for PAK1 in regulation of the cell cycle. Overexpression of the kinase inhibitory domain of PAK1 (AID) induces cell cycle arrest and decreases cyclin D1 and D2 expression independently of PAK1 kinase activity [76]. We have previously demonstrated that three tyrosines on PAK1 molecules and PAK1-Nck interaction play a critical role in PAK1-dependent regulation of cyclin D1 promoter activity in response to PRL and proposed that Nck-PAK1 complex (formation of which does not depend on PAK1 kinase activity) can sequester PAK1 in cytoplasm to prevent PAK1 nuclear shuttling thereby inhibiting PAK1-dependent activation of cyclin D1 promoter [77].

The multifunctionality of PAK1 as both a kinase and a scaffolding protein allow PAK1 to modulate a diverse array of cell processes, such as cell proliferation, survival, motility, and invasion.

5.2.3 PAK1 Regulates Cell Proliferation

PAK1 has been shown to stimulate cell proliferation (Fig. 5.3). Thus, highly proliferating human breast cancer cell lines and tumor tissues have been shown to contain hyperactive PAK1 and its upstream regulator Rac3 [78]. Tyrosyl phosphorylation of PAK1 by nonreceptor tyrosine kinase Etk/Bmx leads to increased proliferation of human breast cancer MCF-7 cells [57]. In addition, expression of kinase-active T423E PAK1 mutant in mammary glands induces hyperplasia in the mammary epithelium [79]. One of the first conclusive evidence that PAK1 has a role in cell cycle regulation was the finding that overexpression of activated PAK1 in human breast cancer cells leads to the abnormal accumulation of centrosomes and aberrant mitoses [80]. Furthermore, PAK1 is present at histone complexes, centrosomes, and at mitotic spindles during mitosis [81]. During the early stages of mitosis, DNA must be tightly packed into chromosomes to allow for proper gene segregation through a process called chromosome condensation. This process is highly regulated by various posttranslational modifications to the DNA-bound histone protein complexes. One such event is the phosphorylation of Ser10 on histone H3 that is necessary for the initiation of chromosome condensation [82, 83]. Li et al. reported that active PAK1 can translocate into the nucleus (via the PAK1 NLSs, Fig. 5.1) where it can directly bind to and phosphorylate histone H3 on Ser10, promoting chromosome condensation and aiding in the progression of metaphase to anaphase during mitosis [81]. PAK1 can also mediate proper formation of the mitotic spindle and microtubule dynamics during mitosis. Tight regulation of microtubule dynamics is absolutely required for proper spindle formation and chromosomal segregation. Tubulin cofactor B (TCoB) assists in the assembly of α and β-tubulin and is localized at the centrosomes. PAK1 has been implicated in TCoB activity during mitosis. PAK1 colocalizes with TCoB at the centrosome and phosphorylates two serine residues on TCoB, encouraging the microtubule polymerization activity of TCoB [84]. PAK1 can also regulate the formation of the mitotic spindle at the centrosome through Aurora A. Aurora A is a serine/threonine kinase that is present at the centrosome

throughout mitosis and is responsible for the recruitment of several microtubule-associated proteins required for proper spindle formation. Regulation of Aurora A is important, since knockdown of Aurora A leads to abnormal maturation of the centrosome [85]. Through interaction with PIX/GIT1 complex, PAK1 is localized to the centrosome where it directly induces Aurora A activation by phosphorylating Thr288 and Ser342 [86]. These data combined with the fact that hyperactive PAK1 in cells leads to aneuploidy [80] suggest an important role for PAK1 in chromosome segregation and microtubule regulation during mitosis. PAK1 can also induce expression of cyclin D1, one of the key mediators of cell cycle progression. Overexpression of active PAK1 increases cyclin D1 expression in breast cancer cells while knockdown of PAK1 significantly reduces cyclin D1 expression [87]. Our lab demonstrated that PRL-mediated activation of PAK1 increased nuclear localization of PAK1 and activation of cyclin D1 promoter and that PAK1/Nck binding inhibited PAK1 nuclear localization and cyclin D1 promoter activity [77]. Balasenthil et al. proposed that PAK1 can increase cyclin D1 transcription through two independent pathways—the NFκB pathway and phosphorylation of S305 of estrogen receptor alpha (ERα) [87, 88]. PAK1 has been previously shown to directly phosphorylate ERα at Ser305 and promote its transactivation functions [79]. Interestingly, PAK1 itself is activated by estrogen suggesting a positive feedback loop [89]. Lastly, PAK1 can regulate cell proliferation through activation of the Ras/ERK pathway [90–92]. The regulation of the ERK pathway by PAK1 requires both kinase-dependent and independent functions of PAK1 as stated earlier. Thus, these data describe a multifunctional role for PAK1 in the regulation of cell proliferation and cell cycle progression.

5.2.4 PAK1 Regulates Cell Survival

PAK1 also plays a role in cell survival (Fig. 5.3). PAK1 inhibits the release of pro-apoptotic factors from the mitochondria. BAD is a proapoptotic protein that binds and inhibits the prosurvival proteins Bcl-2 and Bcl-X. Phosphorylation of BAD at Ser112 and Ser136 blocks BAD binding to Bcl-XL and promotes cell survival [93]. PAK1 promotes cell survival by directly phosphorylating these serines on BAD [94]. Also, we had previously mentioned that PAK1 can act as a scaffold for Raf-1, MEK, and ERK, promoting the activation of the MAPK pathway in the regulation of cell proliferation; however, PAK-mediated phosphorylation of Raf-1 can also mediate cell survival. Raf-1 phosphorylation at Ser338 by PAK1 can induce the translocation of Raf-1 to the mitochondria where it binds to Bcl-2 and phosphorylates BAD at Ser112, thus providing an additional mechanism in which PAK1 can regulate BAD activity and prevent apoptosis [95]. PAK1-mediated BAD phosphorylation was described as a critical event in survival signaling induced by the HIV viral Nef protein [96]. In addition to directly regulating BAD activity, PAK1 induces the degradation of proapoptotic proteins such as BimL [97]. Typically, dynein light chain 1 (DLC1) is bound to BimL, preventing BimL from inactivating Bcl-2, thus

promoting cell survival, that is until BimL is released upon proapoptotic signals [98, 99]. PAK1 can phosphorylate both DLC1 and BimL leading to DLC1/BimL degradation and therefore promoting cell survival [97]. Another mechanism in which PAK1 can regulate cell survival is through activation of the NFκB pathway [100–102]. PAK1 mediates NFκB activation by Ras, Raf-1, and Rac1 and expression of active PAK1 can stimulate NFκB on its own without activation of the inhibitor of κB kinases [100]. Friedland et al. discovered that PAK1-induced NFκB activation prevented apoptosis in three-dimensional (3D) cultures of mammary epithelial cells [102]. During *Helicobacter pylori* infection of human epithelial cells, PAK1 activates NFκB via activation of upstream regulatory kinase NIK (NFκB-inducing kinase) [103]. Furthermore, PAK1 also inhibits apoptosis by phosphorylating and inactivating cell survival forkhead transcription factor, FKHR [89]. Hence, PAK1 has both direct and indirect roles to play in the regulation of cell survival.

5.2.5 PAK1 Regulates the Actin Cytoskeleton

The first described and most well-understood function of PAK1 is the role for PAK1 in the regulation of the actin cytoskeleton and cell motility (Fig. 5.3). Active PAK1 is localized to areas of actin remodeling, such as filopodia and lamellipodia of motile cells, membrane ruffles, and pinocytosis vesicles [46, 104, 105]. Overexpression of kinase-active PAK1 induces the formation of lamellipodia and membrane ruffles [46, 104]. PAK1 phosphorylates a variety of different actin cytoskeleton proteins such as Lim Kinase 1 (LIMK1), p41-Arc, filamin A, myosin light chain (MLC) and myosin light chain kinase (MLCK). LIMK1 is a kinase that upon activation can phosphorylate and inactivate cofilin, an actin-binding protein. Cofilin depolymerizes actin fibers in ruffles and lamellipodia, promoting actin recycling and retrograde flow [106, 107]. PAK1 can directly phosphorylate and activate LIMK1, resulting in downstream inactivation of cofilin and subsequent stabilization of actin filaments [108, 109]. Actin filaments stabilization allows for the efficient formation of protrusive structures, such as lamellipodia and filopodia during cell motility [109]. Proper protrusion formation also relies on the creation of a branched actin network. The Arp2/3 complex is a complex of proteins that facilitates branching actin filaments by binding to existing actin fibers and providing nucleation sites for new actin filaments at a 70° angle from the original filament. The nucleation property of the Arp2/3 complex requires p41-Arc protein, which can be regulated by PAK1. Phosphorylation of p41-Arc on Thr21 by PAK1 induces the localization of p41-Arc to the Arp2/3 complex, facilitating actin nucleation and branching during cell motility, while blocking PAK1-mediated phosphorylation of p41-Arc inhibits cell motility [110]. Similarly, PAK1 can bind to the actin-cross-linking protein filamin A. Serine phosphorylation of FlnA by PAK1 at Ser2152 results in PAK1-dependent membrane ruffling [51]. FlnA in turn activates PAK1, furthering PAK1 downstream actin-modulating signals. Actin stress-fibers are anchored by focal adhesions to provide support and bind to nonmuscle myosins that regulate tension and

contraction. PAK1 can modify actin–myosin binding and focal adhesion assembly by interacting and phosphorylating MLC and MLCK. MLCK typically phosphorylates MLC at Ser19, promoting actin–myosin binding and increased contractility [111]. PAK1, however, can phosphorylate Ser439 and Ser991 of MLCK and inhibit MLCK [112]. Inhibition of MLCK by PAK1 reduces stress fiber formation and leads to the disassembly of focal adhesions, both processes that are necessary to promote cell motility. PAK1 can directly phosphorylate MLC at Ser19, promoting myosin–actin binding which may regulate the contraction of the trailing edge of motile cells [67, 113]. Furthermore, PAK1 facilitates integrin-mediated cell adhesion [114–116], as activation of PAK1 promoted disassembly of actin stress, abolishment of focal adhesion, and reduction of cell attachment, while PAK1 silencing enhanced cell adhesion and/or spreading and led to increased size and number of mature focal adhesion [117–122].

5.2.6 Role of PAK1 in EMT

Epithelial-mesenchymal transition (EMT) is a process where tightly adhered, non-motile epithelial cells lose their epithelial characteristics and display the loose adherence and motile phenotypes of mesenchymal cells. EMT was first described in the context of embryogenesis, where it leads to the generation of mesenchymal cells. Epithelial cells undergoing EMT acquire a morphology that is appropriate for migration through the extracellular environment, and for settlement in areas of new organ formation. In recent years, EMT-like processes have been the focus of active research on their potential role as determinants of cancer cell invasion and metastasis. Certain proteins can be used as markers for pathogenic EMT, including E-cadherin, N-cadherin, and vimentin. EMT is characterized by the reduction of E-cadherin expression and an increase in both N-cadherin and vimentin expression. E-cadherin transcription is controlled by various transcription factors (TFs), one of which is Snail [123, 124]. The Snail superfamily of TFs is composed of two families; the Snail family (Snail, SNAILP, and SLUG), and the Scratch family (SCRATCH1 and SCRATCH2) (reviewed in [125]). Members of the Snail family have been shown to regulate EMT [124, 126].

PAK1 has been implicated in regulation of EMT by findings that E-cadherin expression in MCF-7 cells was downregulated upon transfection of PAK1, and conversely, E-cadherin expression in MDA-MB-435 cells was upregulated through inhibition of PAK1 expression [127]. A similar effect of PAK1 has been also demonstrated in keratinocytes [128]. On the other side, PAK1 was also shown to be required for the stabilization of adherent junctions through a recently discovered target of PAK1 Ajuba, an actin-binding protein that colocalizes with cadherins [129]. The role of PAK1 in the stabilization of E-cadherin cell–cell junction has also been shown during zebrafish epiboly [130].

PAK1 has been shown to regulate E-cadherin expression through Snail [127]. Yang et al. demonstrated that PAK1 phosphorylates Ser246 on Snail. Serine

phosphorylation of Snail facilitates the accumulation of Snail in the nucleus and promotes transcriptional repression of E-cadherin. Knockdown of PAK1, or mutation of serine 246 on Snail to an alanine, leads to increased cytoplasmic Snail and a reduction of Snail repressor activity [127]. In contrast, the same lab had previously demonstrated that PAK1 phosphorylates corepressor CtBP (C-terminal binding protein 1) that leads to translocation of CtBP from the nucleus into the cytoplasm and relieves its corepressor activity toward the E-cadherin promoter. They demonstrated that CtBP-mediated repression of the E-cadherin promoter was relieved by transfection of PAK1 [131]. If so, the effect of PAK1 on E-cadherin expression may be either stimulating through inhibition of CtBP or repressive through activation of Snail.

PAK1 also interacts with β-catenin and promotes β-catenin activation in gastric epithelial cells [132]. Phosphorylation of β-catenin at Ser675 by PAK1 increases the stability and transcriptional activity of β-catenin in colorectal cells [133]. PAK1 knockdown in human colorectal cell lines inhibits β-catenin expression, β-catenin transcriptional activity, and the expression of c-Myc and suppresses the tumor growth and metastasis in mouse model [134].

Recently, PAK1 has been shown to activate β-catenin transcriptional activities and promote EMT in podocytes [135].

In addition, PAK1 can mediate peroxisome proliferator-activated receptor gamma (PPARgamma)-induced EMT of intestinal epithelial cells through activation of the ERK1/2 pathway [136].

5.2.7 Role of PAK1 in Breast Cancer

PAK1 plays an important role in such vital processes like cell proliferation, survival, cell motility and EMT, therefore it is no surprise that misregulation of PAK1 activity is present in many cancers. Altered expression and/or activation of PAK1 is evident in various cancers, including brain, pancreas, colon, bladder, ovarian, hepatocellular, urinary tract, renal cell carcinoma, thyroid, and breast cancers ([137–146], reviewed in [147]). Of these cancers, the role for PAK1 in breast cancer has been studied to the most extent ([58, 80, 87, 148–152], reviewed in [147, 153]). PAK1 is overexpressed or upregulated in some breast cancers. The PAK1 gene is localized within the 11q13 region, and 11q13.5→q14 amplifications involving the PAK1 locus are found in 17% of breast cancer [154, 155]. Overexpression of PAK1 was observed in 34 of 60 breast tumor specimens [87] and expression of PAK1 in human breast tumors correlates with tumor histologic grade [80, 150]. PAK1 expression and activity were higher in human breast tumors as compared to their adjacent controls [156]. Furthermore, expression of PAK1 in human breast tumors correlates with tamoxifen resistance [150]. PAK1 kinase activity can also be increased in human breast tissue by the upregulation of Rac3 activity or Rac-1expression [78, 157]. In a transgenic mouse model, PAK1 hyperactivation (PAK1 T423E mutant) leads to the formation of mammary gland tumors [58]. Of particular

interest, PAK1 plays a critical role in premalignant progression of MCF10 series of human breast epithelial cell lines grown in 3D reconstituted basal membrane overlay cultures [158]. It has been demonstrated that expression of a kinase-dead PAK1 mutant in highly invasive breast cancer cell lines led to reduced invasiveness [69]. Conversely, hyperactivation of the PAK1 pathway in the noninvasive breast cancer cell line MCF-7 promotes cell migration and anchorage-independent growth [80]. As we described above, PAK1 phosphorylates several transcription factors, among them CtBP1 and Snail both of which are important for EMT [127, 131]. Another possible mechanism of PAK1-mediated malignant transformation is the enhancement of PAK1-regulated cell motility because PAK1 kinase activity participates in directional motility and PAK1 directly phosphorylates cytoskeletal proteins as we discussed above. For example, depletion of PAK1 has been shown to contribute to breast cancer cell invasion through cofilin-dependent mechanism [159]. Thus, PAK1 has become one of the focal points in the investigation into the mechanism and onset of human breast cancer. Recently, our lab has demonstrated a role for PRL-mediated tyrosyl phosphorylation of PAK1 in breast cancer cell motility, adhesion and invasion.

5.3 PRL Regulates Breast Cancer Cell Motility Through Tyrosyl Phosphorylated PAK1

5.3.1 JAK2 Tyrosyl Phosphorylates and Activates PAK1 in Response to PRL

In 2007, we demonstrated that PAK1 is a novel substrate of the JAK2 tyrosine kinase and that PRL-activated JAK2 phosphorylates PAK1 in vivo. PAK1 tyrosines 153, 201, and 285 were identified as sites of JAK2 tyrosyl phosphorylation by mass spectrometry and 2D peptide mapping. Our findings indicated that this phosphorylation plays an important role in cell survival and in the regulation of cyclin D1 promoter activity [44, 77].

In an attempt to understand the mechanism of JAK2-dependent activation of PAK1, we first focused on testing PAK1 kinase activity in an in vitro kinase assay with P^{32}-ATP and exogenous H4 histone as a substrate. Indeed, PAK1 kinase activity was increased in the presence of overexpressed activated JAK2 but not kinase dead JAK K882E. Active JAK2 had no effect on the kinase activity of the PAK1 Y3F mutant in which the three JAK2 phosphorylation sites (Tyr(s) 153, 201 and 285) were mutated to phenylalanine [44]. PRL treatment activated both PAK1 WT and PAK1 Y3F (which is catalytically active). However, in the presence of PRL, the kinase activity of PAK1 WT was significantly stronger than PAK1 Y3F in MCF-7, T47D and TMX2–28 breast cancer cell lines (Fig. 5.4; [29, 160]). Heregulin (HRG), a ligand for HER3 (human epidermal growth factor receptor-3) and HER4 (human epidermal growth factor receptor-4), activates both PAK1 WT and

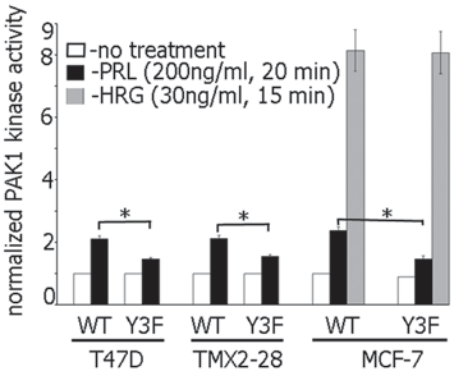

Fig. 5.4 Tyrosyl phosphorylation of Tyr 153, 201, and 285 is required for maximal PAK1 kinase activity in response to PRL but not heregulin. Indicated cell lines stably overexpressing PAK1 WT or PAK1 Y3F were deprived of serum and treated with or without prolactin (*PRL*) or heregulin (*HRG*). Myc-PAK1 was IP'ed and subjected to an in vitro kinase assay with H4 histone as a substrate. Relative PAK1 kinase activity was then normalized by the amount of IP'd PAK1 for each lane and plotted. *Bar* represent mean ± S.E., *, $p < 0.05$ compared with the same cells without treatment ($n = 3$)

PAK1 Y3F to the similar extent confirming that PAK1 Y3F retains its kinase activity. How does PRL activate JAK2-phosphorylation-deficient mutant PAK1 Y3F? Presumably, it works through Rac1. Indeed, both PAK1 WT and PAK1 Y3F were similarly activated by either activated Rac1 V12 or by activated Cdc42 L61 [160]. This Rac1/Cdc42-dependent activation is pTyr-PAK1-independent, therefore PAK1 Y3F mutant exhibits some kinase activity in response to PRL (black bars for PAK1 Y3F in Fig. 5.4).

However, PAK1 is activated by GTPase-independent mechanisms as well. As we discussed above, membrane recruitment of PAK1 by Nck and Grb2 adapter proteins results in the stimulation of PAK1 kinase activity through interaction with lipids such as sphingosine or phosphatidic acid [50]. Membrane-localized PAK1 can also be activated by PDK1 [49]. The nonreceptor tyrosine kinase Etk/BMX can tyrosyl phosphorylate PAK1 and induce PAK1 kinase activation [57]. In addition, PAK1 can be directly activated by Akt [54] and FLNa [51]. Which mechanism acts in response to PRL? It has been demonstrated that PAK1 phosphorylates FLNa on Ser 2152 and FLNa activates PAK1 in a positive feedback loop [51]. We have shown than Ser-phosphorylation of FLNa was increased when FLNa was coexpressed with PAK1 and constitutively active JAK2 V617F as compared to coexpression of FLNa with kinase inactive JAK2 mutant K882E. Furthermore, we have demonstrated that PAK1 phosphorylates Ser 2152 of FLNa to a greater extent when PAK1 is tyrosyl-phosphorylated by JAK2 in response to PRL [29]. Thus, PRL can induce PAK1 kinase activity in two ways: first in a GTPase-dependent manner, activating both PAK1 WT and PAK1 Y3F, but also through pathway(s) that are pTyr-PAK1-dependent, further activating PAK1 WT but not PAK1 Y3F, for example, through filamin A (Fig. 5.5).

Moreover, PAK1 has a dual activity. First, PAK1 is a serine-threonine kinase and this activity depends on activation of the PAK1 kinase domain. Second, as we discussed above, PAK1 acts as a scaffold for many proteins, for example, for Raf-1, MEK and Erk [70] and this PAK1 activity depends on its ability to initiate protein–protein interactions. One might speculate that tyrosyl phosphorylated PAK1 may create additional docking sites to recruit SH2-domain containing proteins to facilitate local activation of recruited proteins and amplify PRL-dependent signaling. In such case, pTyr-PAK1 will be able to recruit additional proteins to function as a scaffold to locally amplify PRL signaling.

Thus, PRL may stimulate both PAK1 activities: kinase activity and scaffolding ability of PAK1 through JAK2-dependent tyrosyl phosphorylation of Tyr(s) 153, 201, and 285.

In our search for scaffolding activities of pTyr-PAK1, we focused on βPIX/GIT1 proteins. A proline-rich motif of PAK1 (residues 182–203) binds directly to the SH3 domain of GEF βPIX [55].The PIX proteins associate with G protein-coupled receptor kinase-interacting target 1 (GIT1), a GTPase activating protein (GAP) for Arf, that targets adhesion complexes by binding to paxillin [68]. βPIX and GIT1 can homodimerize and form large aggregates in the cell [161, 162]. This oligomerization is essential for localization to sites of adhesion since mutations that disrupt either GIT-βPIX association or βPIX homodimerization result in diffuse cytoplasmic localization of both proteins [163, 164]. PAK1 is an important component of this complex and formation of the four-molecule PAK1/βPIX/GIT1/paxillin signaling module transiently targets PAK1 to the sites of adhesion [165–168]. To provide insight into whether tyrosyl phosphorylation of PAK1 increases the ability of PAK1 to bind βPIX and GIT1, we immunoprecipitated PAK1 from the lysates of PAK1 WT and PAK1 Y3F cells treated with PRL over a time course and assessed these immunoprecipitates for endogenous βPIX. The quantification of PAK1 and βPIX bands in the immunoprecipitates showed that PRL increased association of βPIX with PAK1 WT about 8.5-fold. The amount of endogenous βPIX bound to PAK1 Y3F was left unchanged during PRL treatment. Next, we also assessed GIT1 associated with PAK1 WT and PAK1 Y3F upon PRL treatment. We demonstrated that threefold more GIT1 was associated with tyrosyl phosphorylated PAK1 WT than with PAK1 Y3F. These data demonstrate that tyrosyl phosphorylation of PAK1 regulates its binding activity toward βPIX and GIT1. We hypothesize that phosphorylation at position Y285 may affect the interaction with βPIX by inducing a conformational change that makes the proline-rich motif of PAK1 more accessible to βPIX (Hammer et.al. unpublished).

Overall, PAK1 WT and PAK1 Y3F have similar kinase activity in response to active Rac1/Cdc42. PRL stimulates both PAK1 WT and Y3F through Rac1 and additionally activates PAK1 WT (but not PAK1 Y3F) by Rac1-independnet mechanism(s) which depends on tyrosyl phosphorylation (for example, by local activation by filamin A). Furthermore, pTyr-PAK1 is able to recruit additional proteins to function as a scaffold to locally amplify PRL signaling further (for example,

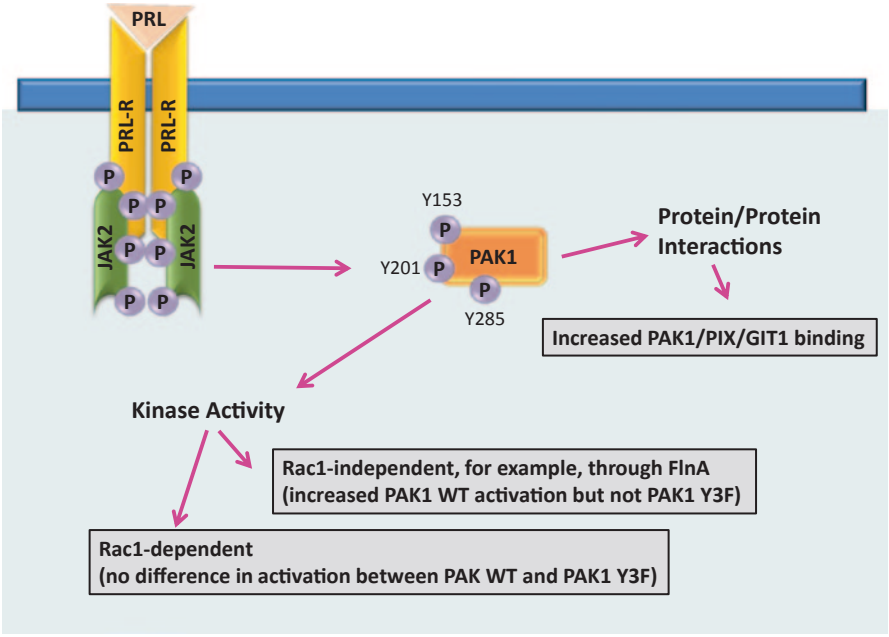

Fig. 5.5 PRL stimulates kinase activity of PAK1 and PAK1 ability to form protein–protein interaction. PRL binding induces dimerization of the PRLR and subsequent activation of JAK2. JAK2 phosphorylates PAK1 on three tyrosines, Tyr 153, 201 and 285. Tyrosyl phosphorylation of PAK1 enhances both a Rac-dependent (Rider et al. 2013) and Rac-independent PAK1 kinase activity (Hammer et al. 2013), and also the ability for PAK1 to act as a molecular scaffold (Hammer et al., unpublished)

via βPIX/GIT1 recruitment). These two activities are interconnected because high local concentration of PAK1 in PAK1/βPIX/GIT1/paxillin complexes permits autophosphorylation, stimulating the kinase activity of PAK1 (Fig. 5.5).

5.3.2 Tyrosyl Phosphorylated PAK1 Regulates the Actin Cytoskeleton and Cell Motility in Response to PRL Through Filamin A

PAK1 is major regulator of actin cytoskeleton dynamics and can bind a variety of different actin-modulating proteins. We have implicated tyrosyl phosphorylation of PAK1 in the regulation of unstimulated phagokinesis, which is a combination of two processes that are dependent upon changes in the actin cytoskeleton: cellular movement and phagocytosis [44]. Later, we have demonstrated that overexpression of WT PAK1 enhanced the ability of PRL to induce cell ruffling. In contrast, overexpression of PAK1 Y3F failed to increase ruffling [169]. Membrane ruffling has been observed in many cell types in response to certain extracellular

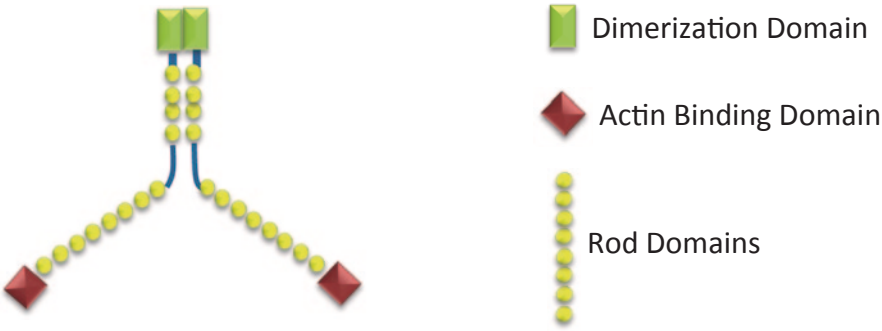

Fig. 5.6 Schematic diagram of FLNa dimer. FLNa consists of an N-terminal actin-binding domain, a *rod* domain containing 24 IgG-like repeats and C-terminal dimerization domain. (modified from Cukier et al. 2007)

factors, and on motile cells where they are believed to be required for directed cell migration. Thus, the formation of membrane ruffles may be considered as a sign of increased response to external stimuli and of elevated cell migration (for review, [170, 171]). We extended our findings and demonstrated that overexpression of PAK1 WT strongly enhances cell migration in response to PRL in both cell wounding and Boyden chamber assays [29]. In an attempt to understand the mechanism of the amplifying effect of tyrosyl phopshorylated PAK1 on cell motility, we focused on filamin A for several reasons. First, the actin-binding protein FLNa is a binding partner of PAK1 [51]. Second, we have previously implicated FLNa in PRL-dependent signaling through adapter protein SH2B1β [169]. Filamin A is a 280 kDa actin cross-linking protein containing an N-terminal actin-binding domain and a rod region containing 24 immunoglobulin-like repeats (Fig. 5.6; reviewed in [172]). The last repeat of the rod region enables the FLNa molecules to dimerize, allowing for a flexible structure mediating the actin gelation activity of filamins. Filamins have >90 interacting partners, including adapter proteins, small GTPases, transmembrane receptors, and membrane channels [173]. FLNa participates in the activation of various kinases as well as being regulated by kinases itself. FLNa binding to PAK1 enhances the kinase activity of PAK1, which subsequently phosphorylates FLNa at Ser 2152, resulting in PAK1-dependent membrane ruffling [51]. FLNa also stimulates PAK1 by interacting with sphingosine kinase 1, which phosphorylates sphingosine, leading to the direct activation of PAK1 [52]. As a potent actin cross-linking protein, FLNa regulates cell migration, although the role of FLNa in this process is controversial. Thus, multiple studies have demonstrated a positive impact of FLNa on the migration of different cell types (for example, [174–177]). One of the first noted defects of FLNa-deficient melanoma cells (M2 cells) was the inability to migrate due to inefficient polarization and continuous blebbing, which was rescued once FLNa was stably reexpressed (A7 cells) [174]. In contrast, FLNa overexpression inhibits neuronal migration [178] and downregulation of FLNa stimulates cancer cell migration, invasion, and metastatic formation [179]. In support of

the latter finding, we demonstrated that the depletion of FLNa increased basal nonstimulated migration of T47D cells. However, PRL-induced cell migration was suppressed by FLNa knock-down. These previously published and our current results suggest that at normal expression levels, FLNa activity should be strongly regulated to coordinate cell migration. In addition, we show that PAK1 phosphorylates Ser 2152 of the actin-binding protein filamin A to a greater extent when PAK1 is tyrosyl-phosphorylated by JAK2 in response to PRL. Downregulation of PAK1 or filamin A abolishes the effect of PRL on cell migration. Thus, these data bring some insight into the mechanism of PRL-stimulated motility of breast cancer cells [29].

We have proposed a model for PRL-dependent regulation of the actin cytoskeleton (Fig. 5.7; [29, 169]). According to this model, upon ligation of PRLR and activation of JAK2, adapter protein SH2B1β translocates to activated PRLR-JAK2 complexes, where it cross-links actin filaments via its two actin-binding domains and binds to FLNa [169, 180]. PRL-activation of JAK2 also leads to tyrosyl phosphorylation of PAK1, thereby increasing PAK1's activities (both the kinase and scaffolding activities) and stimulating phosphorylation of FLNa. FLNa, in turn, activates PAK1, binds to SH2B1β and relocates more SH2B1β to the JAK2/PAK1/FLNa complex. Because SH2B1β enhances the tyrosine kinase activity of JAK2 [181], the formation of this multiprotein complex results in enhancement of JAK2 activation and further activation of the JAK2/PAK1/FLNa-actin complex, leading to actin cytoskeleton reorganization.

5.3.3 PRL-Mediated pTyr-PAK1 Regulation of Adhesion Turnover

Cell adhesion is the basis for cell migration. Dynamic changes in cell–matrix adhesions are necessary for both cell spreading and cell motility. The rapid assembly and disassembly of adhesions during cell migration is called adhesion turnover. Upon contact with the ECM, or in response to external stimuli, there is clustering and activation of the cell-surface proteins integrins, the chief proteins regulating cell–matrix adhesion. Integrin clustering induces autophosphorylation of FAK on Tyr397, enhancing FAK kinase activity and recruits the adhesion scaffolding protein paxillin ([182, 183], reviewed in [184]). Localized FAK and paxillin, along with the actin-binding protein talin, at integrin clusters form small adhesion complexes, called nascent adhesions, at the distal edge of the lamellipodium [185]. Nascent adhesions are unstable and can either immediately disassemble, or mature into larger focal complexes. Nascent adhesion maturation requires FAK-mediated phosphorylation of paxillin on two tyrosines, Tyr31 and Tyr118 [182]. This tyrosyl phosphorylation of paxillin increases the affinity of paxillin for FAK, recruits a variety of kinases, scaffolding proteins, and regulators of GTPase activity, and induces the maturation of the nascent adhesion into a focal complex [185–187]. Focal complexes can then either further mature into

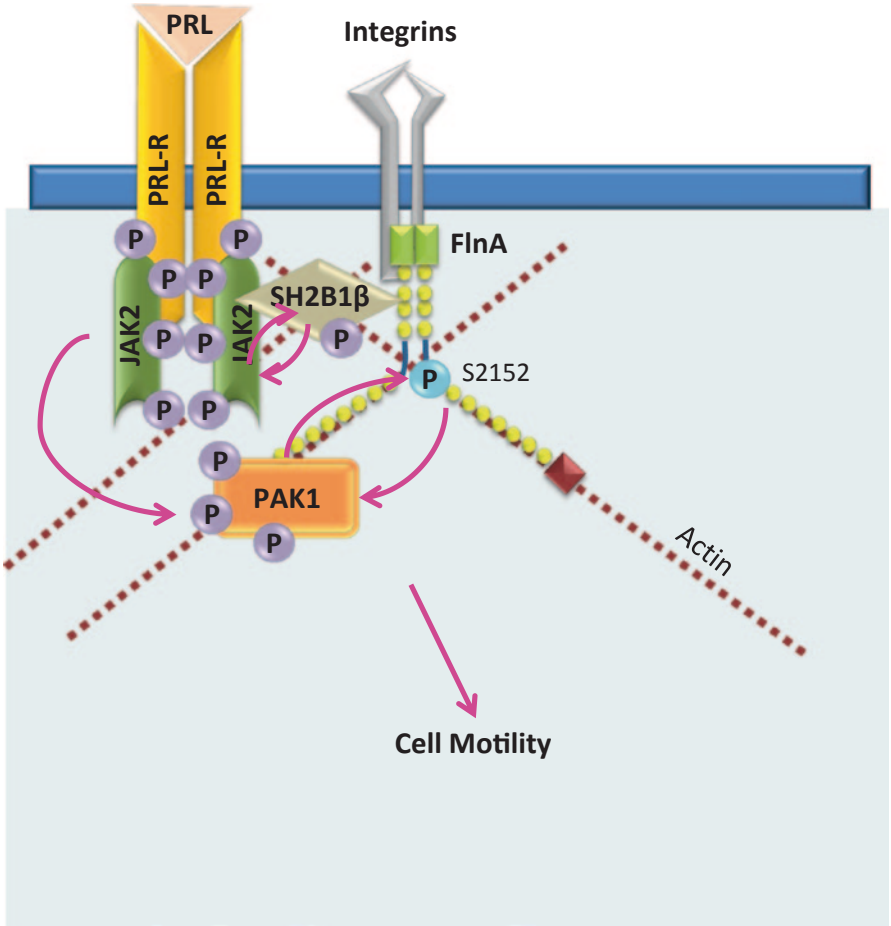

Fig. 5.7 Tyrosyl phosphorylated PAK1 regulates cell motility in response to PRL through filamin A. PRL-activated JAK2 tyrosyl phosphorylates PAK1 increasing PAK1 kinase activity and scaffolding ability of PAK1. pTyr-PAK1 has increased FlnA interaction and phosphorylates FlnA on Ser2152. FlnA activates PAK1 in a positive feedback loop. JAK2 also phosphorylates SH2B1β, which binds to FlnA and cross-links actin filaments. Positive feedback from FlnA to PAK1 and SH2B1β to JAK2 facilitates the formation of more JAK2/SH2B1β/ FlnA/pTyr-PAK1 complexes that regulate actin remodeling during enhanced cell motility in response to PRL

larger focal adhesions, or disassemble (turnover), a process that occurs rapidly during cell motility. Modulation of adhesion dynamics is both tightly regulated and highly complex.

PAK1 activity has been implicated in regulating cell–matrix adhesion dynamics. PAK1 is localized at adhesions, where it regulates both adhesion assembly and disassembly [117, 119, 188, 189]. One of the first observations was that overexpressed PAK1 WT and kinase-dead PAK1 localized to the focal adhesions and caused the accumulation of focal points [117]. These data were conformed later by

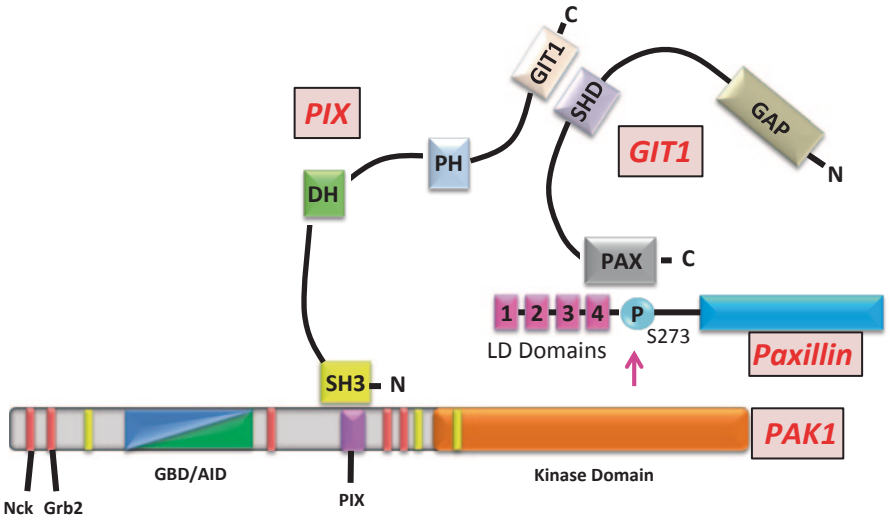

Fig. 5.8 GIT1/βPIX/PAK1/paxillin complex. PAK1 binds to the N-terminal SH3-domain of βPIX. βPIX has a DBL-homology (*DH*) domain and a plextrin homology (*PH*) domain, and binds to GIT1 through a C-terminal GIT1-binding domain. GIT1 has a C-terminal paxillin-binding domain (*PAX*) that binds to the LD4 domain of paxillin and SHD domain that binds to βPIX. PAK1 can phosphorylate Ser273 of paxillin at adhesion complexes

demonstration that overexpression of kinase-dead PAK1 facilitates the formation of focal adhesions [66, 69]. Several years later, PAK1 was shown to be directly involved in mediating adhesion turnover, a process that could be reversed upon expression of the AID domain, suggesting that PAK1 kinase activity was necessary for proper adhesion turnover [119, 189]. It turns out that both PAK1 kinase activity and scaffolding properties are required to modulate adhesion turnover in motile cells. In order for PAK1 to be localized to adhesion complexes, PAK1 must bind to the βPIX protein as well as the GIT1 protein, however this interaction is completely independent of βPIX's GEF activity and GIT1's GAP activity [55, 167]. βPIX binds to noncanonical proline-rich motif on PAK1 (Fig. 5.8) and this interaction is negatively regulated by autophosphorylation of PAK1 at Ser199/Ser204 [55, 165]. As mentioned previously, PAK1/βPIX binding does increase PAK1 activation and PAK1 can subsequently phosphorylate βPIX at Ser340; however, this phosphorylation does not regulate PAK1/βPIX interaction and the physiological relevance of this event has yet to be uncovered [190, 191]. The complex formation of PAK1 and βPIX is not sufficient to locate PAK1 at adhesion complexes. However, βPIX binds to GIT1 and GIT1 binds to paxillin thereby targeting this trimolecular complex to adhesions (Fig. 5.8; [168]). Once the GIT1/βPIX/PAK1 complex arrives at the adhesion complex, PAK1 can phosphorylate Ser273 on paxillin, increasing the affinity of GIT1 for paxillin and recruiting more GIT1/βPIX/PAK1 complexes to the adhesion [189]. At the same time, serine phosphorylation of paxillin reduces the affinity of FAK to paxillin, setting the stage for

adhesion disassembly yet freeing FAK to facilitate the formation of new nascent adhesions [189]. Thus, PAK1 can facilitate adhesion turnover by means of its scaffolding and enzymatic activity.

We have recently shown that PRL-mediated tyrosyl phosphorylation of PAK1 regulates adhesion turnover. When breast cancer cells stably overexpressing either PAK1 WT or PAK1 Y3F were plated on collagen IV in the presence of PRL, PAK1 WT cells displayed a motile phenotype, while PAK1 Y3F cells were more round and well-spread (Hammer et.al., unpublished). Amount of cells adherent to collagen in the presence of PRL was also dependent on tyrosyl phosphorylated PAK1. We have demonstrated that PRL-induced tyrosyl phosphorylation of PAK1 facilitates PAK1/βPIX/GIT1 binding and the localization of PAK1 to small adhesion complexes. These data confirm that tyrosyl phosphorylation of PAK1 increases the ability for PAK1 to create protein–protein interactions. Furthermore, PRL/JAK2 induces kinase activity of pTyr-PAK1, therefore pTyr-PAK1 phosphorylates Ser273 on paxillin in response PRL to a greater extent than PAK1 Y3F mutant. Using phospho-specific antibodies directed to single phosphorylated tyrosines on PAK1, we identified Tyr285 as a site of PRL-dependent phosphorylation of PAK1 by JAK2. Our immunofluorescence analysis revealed that pTyr285-PAK1 localized to small adhesion complexes in the cells treated with PRL. Finally, we have performed time-lapse confocal fluorescence microscopy video recording of the cells treated with PRL. We have shown that PRL-mediated tyrosyl phosphorylation of PAK1 has a direct effect on the rate of adhesion turnover. Tyrosyl phosphorylated PAK1 increased the rates of both adhesion assembly and disassembly in breast cancer cells plated on collagen in response to PRL, while mutation of the single Tyr285 completely abolished the effect of PRL on adhesion turnover (Hammer et. al., unpublished).

We have proposed a model for PRL-dependent regulation of adhesion turnover that integrates our finding with previous studies (Fig. 5.9). PRL-activated JAK2 phosphorylates PAK1 on Tyr285, facilitating the formation of the GIT1/βPIX/pTyr-PAK1 complex and subsequent formation of new paxillin-containing focal complexes. At these complexes, pTyr-PAK1 phosphorylates paxillin at Ser273, recruiting more GIT1/βPIX/pTyr-PAK1 and releasing FAK from the focal complex. The accumulation of GIT1/βPIX/pTyr-PAK1 at the focal complexes facilitates both adhesion assembly and disassembly, thereby regulating cell motility.

5.3.4 Role of PRL-Activated PAK1 in Breast Cancer Cell Invasion

Cells adhere to the ECM throughout most of their lifetime. The molecular composition of the ECM, ECM stiffness, specific association of multiple growth factors/cytokines with the matrix and “dimensionality” play major roles in the response of cells to their local matrix microenvironment [192].

The 3D matrix is a critical component of mammary tissue development not only under physiological but also in pathophysiological conditions. In vivo, women

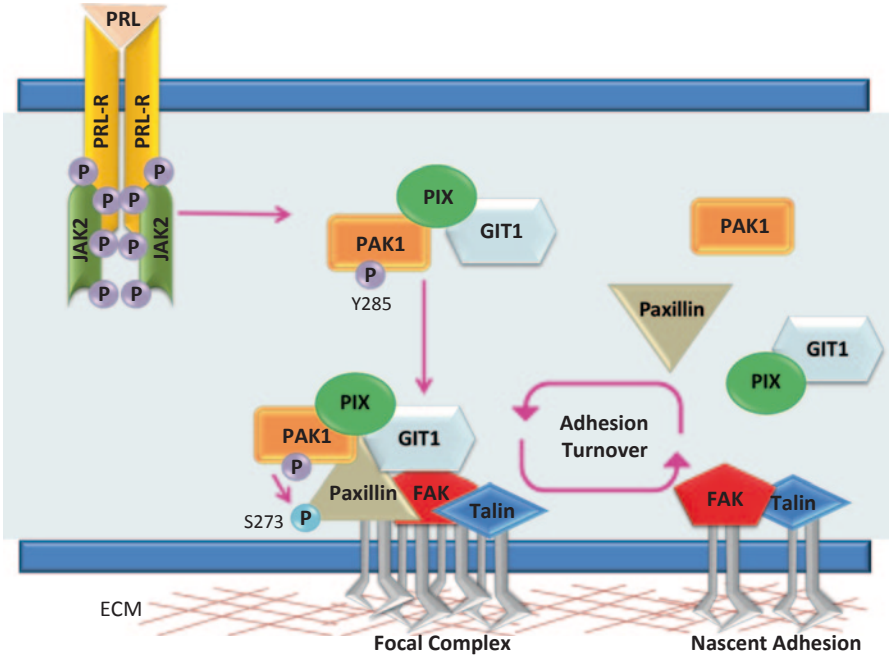

Fig. 5.9 PRL-dependent tyrosyl phosphorylation of PAK1 regulates adhesion turnover. PRL-activated JAK2 phosphorylates PAK1 at Tyr285 and stimulates both PAK1 activities: kinase activity and ability of PAK1 to form the GIT1/βPIX/ pTyr285 PAK1 complex. This complex localizes to small adhesion complexes, the amount of which is increased by PRL treatment. Increased GIT1/βPIX/ pTyr-285PAK1 association leads to enhanced phosphorylation of paxillin on Ser 273 that results in enhanced adhesion turnover and finally to increased cell motility

with dense mammary tissue, which is associated with increasing amount of collagen in the stroma of breast tissue are at 4–6 times greater risk of breast cancer as compared to those with no densities, and have a poor prognosis ([193–196]; reviewed in [197]). In vitro, mammary epithelial cells grown in 3D reconstituted basal membrane overlay cultures form spheroids with lumens (acini) that resemble secretory alveoli of normal mammary tissue. Increasing 3D matrix tension affects mammary cell morphogenesis and physiological functions [198–200]. Furthermore, reciprocal interactions between mammary epithelial cells, ECM and ECM remodeling enzymes are critical for development and differentiation during mammary gland development. Loss of this interaction leads to tumor progression (reviewed in [201]). It is now well documented that the interaction of cells with 2D substrates is significantly different than the more natural 3D environments that cells are embedded within in vivo [202–204]. Furthermore, the molecular composition of the ECM, specific association of multiple growth factors and cytokines with the matrix, along with the aforementioned "dimensionality" play roles in the responses of cells to their local matrix microenvironment [192]. Cells embedded in 3D matrix have higher amounts of ligated matrix-receptors as compared to the cells grown on

the top of a thin film of matrix. Collagen receptors, such as integrins and discoidin domain receptors (DDRs), are signal transducting receptors. Integrin clustering initiates an array of signaling cascades, including activation of the Rho family of small GTPases, MAPKs, and PI3-kinases [205], and any of them can lead to regulation of matrix metalloproteinases (MMP) expression.

MMPs are a family of Zn^{2+}-dependent enzymes composing 23 members. MMP-1 (collagenase 1) is a major proteinase of the MMP family that specifically degrades type I collagen, a major component of the ECM. It also degrades other fibrillar collagens of types II, III, VII, VIII, X, XI as well as gelatins, aggrecan, entactin, tenascin, and perlican [206–208]. As these collagen types are the most abundant proteins in the body, MMP-1 is critical for the modeling and remodeling of the ECM [209]. In clinical studies, increased MMP-1 expression is associated with the incidence or invasiveness of several cancers: colorectal, esophageal, pancreatic, gastric, breast, and malignant melanoma [210–215]. Increased MMP-1 expression is also associated with advanced stages of breast cancer and may be a predictive marker for the development of invasive disease [216]. MMP-3, or stromelysin 1, can degrade a variety of ECM substrates, such as type III, IV, V, VII, and IX–XI collagens, laminins, fibronectin, osteopontin, and proteoglycans. MMP-3 is expressed by stromal cells during normal mammary gland development and is strongly upregulated during postlactational mammary involution when considerable ECM remodeling and alveolar apoptosis occur. MMP-3 is upregulated in many breast tumors and contributes to cancer development. Indeed, mice overexpressing MMP-3 show excessive side branching and eventual tumor formation in the mammary gland ([217–219]; reviewed in [220–222]). MMP-3 induces EMT in mammary cells through cleavage of E-cadherin, expression of Rac1b and transcriptional factor Snail [223]. MMP-2 and MMP-9 are both type IV collagenases that contribute to tumor invasion in vitro because of their ability to break down basement membrane, degrading collagen IV in particular [224, 225]. Elevated circulating MMP-9 levels have been demonstrated in patients with breast cancer and MMP-2 and/or MMP-9 release has been associated with tumor invasion and metastasis ([226, 227]; reviewed in [228–231]). The expression of MMPs is regulated at the transcriptional and posttranscriptional levels (including the stability of mRNA and protein as well as the release and activation of protein) by a number of hormones, growth factors, and cytokines [232]. Despite efforts to discover the cellular pathways regulating MMPs, little is known as to how different cytokines cooperate with cytoskeletal proteins to regulate MMP expression.

PRL regulation of MMP expression and breast cancer cell invasiveness is complex. It has been shown that PRL-induced activation of MAPK/AP-1 pathway is inversely related to PRL-induced STAT5 activation [233–235]. Thus, PRL together with IGF-I promotes MMP-2 expression and cell invasion (AP-1 targets MMP-2 gene) [236]. In contrast, WT STAT5a overexpression inhibits MMP-2 transcription/activity while reduction of STAT5 by siRNA or inhibition of STAT5 activity increases the PRL-dependent transcription/activity of MMP-2 and invasiveness [235, 237]. T47 cells overexpressing degradation-resistant PRLR demonstrated increased proliferation and invasiveness while silencing of PRLR dramatically re-

duced the cell invasion and MMP-9 secretion [238]. In vivo, murine PRL-induced mammary carcinomas with lower level of pSTAT5 demonstrate higher level of MMP-9 expression [234]. Recently, these findings have been linked to the ECM stiffness [233]. Thus, in compliant 3D collagen I, PRL signaling has been shown to be mediated predominantly through the STAT5 pathway. This pathway results in prodifferentiation outcome with no MMP2 expression and absences of invasion. In contrast, in stiff collagen I, PRL signaling is mediated by Src/FAK and pERK 1/2 pathways resulting in MMP2 expression and enhanced invasion. Thus, increased stiffness of the ECM switches the signal in breast cancer cells from differentiation toward enhanced tumorigenic processes [233] PAK1 also plays a pivotal role in the regulation of cell transformation, invasion, and MMP secretion. Role of PAK1 in the EMT and breast and other cancers has been discussed above. Of particular interest, PAK1 plays a critical role in premalignant progression of MCF10 series of human breast epithelial cell lines grown in 3D reconstituted basal membrane overlay cultures [158]. Thus, PAK1 expression and activity increased with premalignant progression from normal mammary cells through hyperplasia, atypical hyperplasia, to ductal carcinoma while dominant-negative PAK1 or knock-down PAK1 reduced cell proliferation, migration, invasion, and pericellular proteolysis of collagen IV in these 3D cultures [158]. PAK1 has been also implicated in MMP regulation. TNFα-induced MMP-9 is mediated through PAK1 and JNK activation [239]. PAK1 regulated IL-1β-induced production and activity of MMP-13 and MMP-14 (MT1-MMP) in synoviocytes during inflammatory joint disease [240] as well as MMP-2 activity in ovarian cancer cells [241]. In addition, inhibition of Rac1 reduces MMP-1 expression [242].

We have recently implicated both PRL and PAK1 together with 3D collagen IV in the regulation of breast cancer invasion. PAK1 stimulates the PRL-dependent invasion of TMX2–28 cells (highly invasive ER-negative clone of MCF-7 cells) through Matrigel. We have shown that TMX2–28 cells stably overexpressing PAK1 WT have upregulated expression and secretion of MMP-1, -2, and -3 when they grow in 3D collagen IV, which makes up 31% of Matrigel protein composition. PRL-induced PAK1 tyrosyl phosphorylation leads to a further increase in MMP-1 and MMP-3 expression and cell invasion in MAPK-dependent manner.

Why have we seen these effects only in 3D collagen IV? Different collagens regulate expression of different MMPs. Collagen I induces expression/secretion of MMP-1 and MMP-9 ([239, 243–247], while collagen IV upregulates expression of MMPs 2 and 9 [248]. MMP-2 is often constitutively expressed and controlled through a unique mechanism of enzyme activation. MT1-MMP (MMP-14)-mediated activation of pro-MMP-2 is upregulated by 3D collagen I in endothelial cells [249–251], fibroblasts [252–254], and cancer cell lines [255–259].

Embedding cells in a 3D matrix can amplify signals from ligated integrins/DDRs and lead to MMP expression. Furthermore, there are numerous reports of “cross talk” and “synergy” between signaling by ECM receptors and by various growth factors and cytokines. Such cross talk involves cooperation in the downstream signal transduction pathways.

Another possible explanation of how 3D collagen results in elevated expression of MMPs as compared to 2D collagen relates to the physical properties of 3D matrixes. ECM physical properties often refer to rigidity, porosity, insolubility, topography, and other characteristics that are essential for its scaffolding role in supporting tissue structure and integrity, and for its role in migration and anchorage of the cell (reviewed in [205, 208]). The aforementioned recent paper from Dr. Schuller's lab has evidently demonstrated the role of 3D collagen I stiffness in the production of MMP-2 [233].

The actin cytoskeleton appears to be the major cellular system for transduction of force generated by the external network. Cytoskeletal stretching correlates with the recruitment of adhesion complex proteins and triggers signals resulting in the induction of a matrix-degrading protease (reviewed in [260, 261]). This may explain our data demonstrated that 3D collagen I induces expression of only MMP-9 while 3D collagen IV upregulates expression of MMP-1, 2, and 3 but not MMP-9 [160]. Collagen I is a fibril-forming collagen while collagen IV is a network forming collagen. We can speculate that cells embedded in the network formed by 3D collagen IV, but not collagen I, can sense geometry and the external force generated by this network. We speculate that, in addition to the ligation of different receptors, physical properties of the 3D collagen IV network activate cytoskeletal-triggered signaling pathways that are distinct from those activated by 3D fibrillar collagen I that results in induction of distinct MMPs.

Another possible explanation of how 3D collagens can induce MMPs expression is the observation that the ECM acts as a "sink" or "reservoir" for growth factors/cytokines. Indeed, the ECM is essential for shaping the concentration gradient for many growth factors, including bone morphogenetic protein, fibroblasts growth factor, Hedgehog, and Wnts [208, 262, 263]. We can speculate that the 3D collagen IV network retains PRL to a better extent than 3D fibrillar collagen I or 2D collagens, therefore leading to amplified PRL signal which leads to MMP-1 and -3 productions.

We have hypothesized that contact with 3D collagen IV may be an important invasive stimulus for breast cancer cells (Fig. 5.10). Mammary cells are normally surrounded by basement membrane, comprised mostly of type IV collagen. In normal cells, signals from collagen IV do not induce MMP expression. In contrast, in breast cancer cells PRL initiates the JAK2-dependent tyrosyl phosphorylation of PAK1, increasing PAK1 signaling. Importantly, PAK1 expression is also elevated in breast cancer [87]. Filamin A can serve as a bridge between activated integrins and pTyr-PAK1 to integrate signals from cytokines (PRL) and the ECM (collagen IV). PAK1 activates Erk 1/2, p38 MAPK, and JNK 1/2, each of which can activate AP-1. Genes encoding MMP-1 and -3 have an AP-1 binding site supporting the transcription of these MMPs after induction by PAK1. MMP-1 degrades type I collagen, which is a major component of the ECM and MMP-3 degrades collagen IV which is a main component of basement membrane. We have also shown that secretion of MMP-1 and -3 is required for PRL-dependent invasion [160]. Given the complexity of these signaling cascades it is likely that additional signaling molecules are also involved in the modulation of MMP expression.

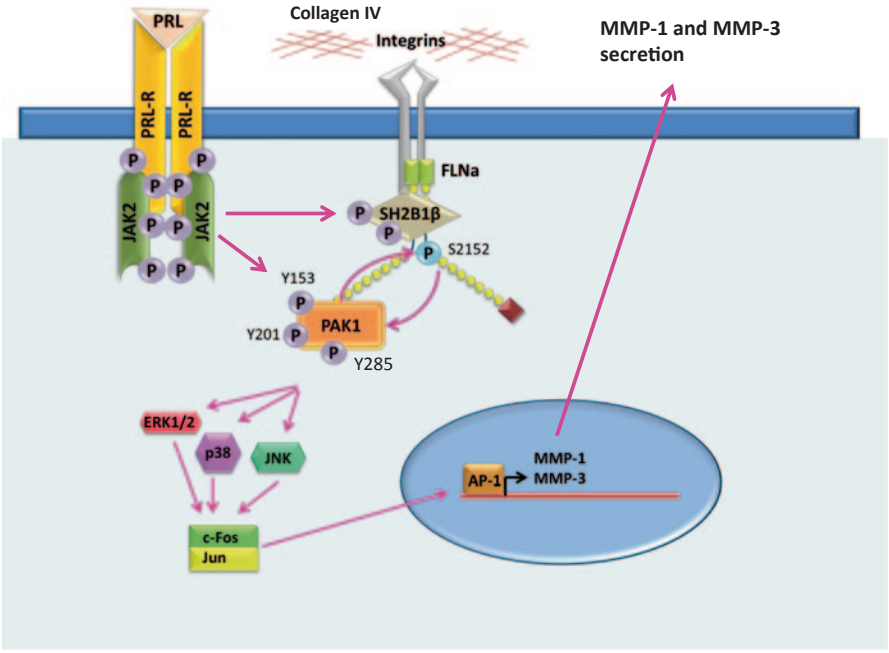

Fig. 5.10 PRL-dependent tyrosyl phopshorylated PAK1 and three-dimensional (3D) collagen IV regulate MMP-1 and MMP-3 production and invasion via MAPK pathways. PRL-activation of JAK2 leads to tyrosyl phosphorylation of PAK1 on tyrosines 153, 201, and 285, thereby increasing PAK1 activities and stimulating phosphorylation of FLNa. Phosphorylated FLNa stimulates the kinase activity of PAK1 in a positive feedback loop. In turn, FLNa binds to β-integrin and transduces signals from surrounding matrix to inside of a cell. 3D collagen IV-induced signals, in combination with pTyr-PAK1, produce intense synergistic increases in MMP-1 and MMP-3 production via MAPK pathways. MMP-1 degrades type I collagen, which is a major component of the ECM and MMP-3 degrades collagen IV which is a main component of basement membrane resulting in increased invasion of breast cancer cells in response to PRL

5.4 Conclusion and Future Directions

PRL binding to the PRLR induces receptor dimerization and activation of the non-receptor tyrosine kinase JAK2. Activated JAK2 phosphorylates the serine/threonine kinase PAK1 on three tyrosines 153, 201, and 285. This tyrosyl phosphorylation of PAK1 enhances such important PAK1 functions as kinase activity and the ability to form protein–protein interactions. Both of these PAK1 activities are important for adhesion, motility, and invasion of breast cancer cells in response to PRL. During cell adhesion, PRL promotes formation of the GIT1/βPIX/pTyr285-PAK1 complex. This complex localizes to small adhesion sites (adhesion complexes), the amount of which is increased by PRL treatment. In these small adhesion complexes at cell periphery PAK1 phosphorylates serine 273 on paxillin that results in enhanced ad-

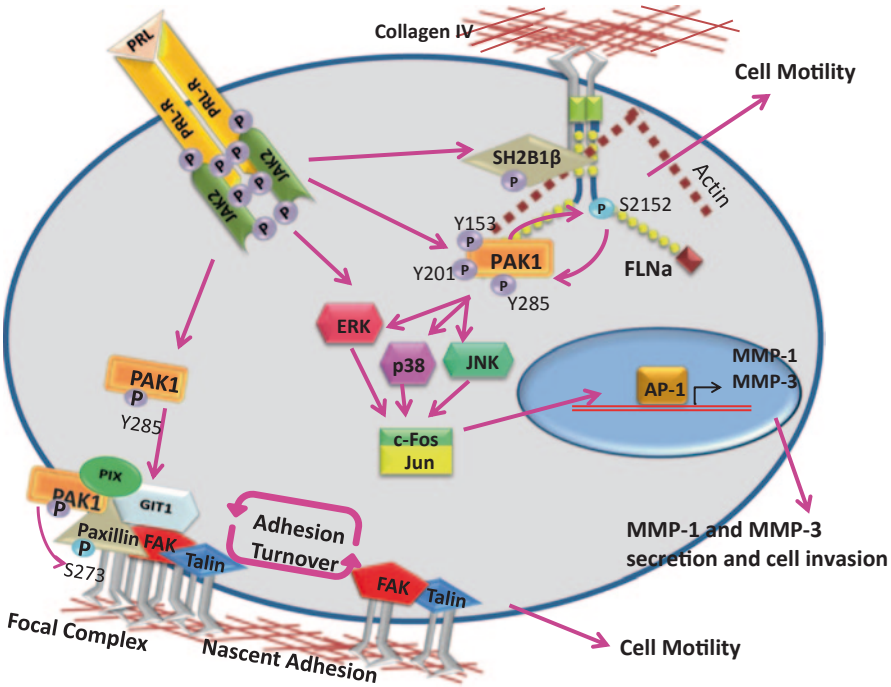

Fig. 5.11 PRL-dependent tyrosyl phosphorylated PAK1 regulates breast cancer cell motility and invasion

hesion turnover. Enhanced adhesion turnover facilitates cell motility and, indeed, PRL stimulates breast cancer cell motility.

In addition to GIT1/βPIX/pTyr285 PAK1-dependent mechanism, we implicated actin-binding protein filamin A in the regulation of cell motility. Tyrosyl phosphorylation of PAK1 in response to PRL increases PAK1/FLNa interaction, and subsequent serine phosphorylation and activation of FLNa. Phosphorylated FLNa stimulates the kinase activity of PAK1 and has increased actin-regulating activity. FLNa directly binds to adapter protein SH2B1β (which is tyrosyl phosphorylated by JAK2 in response to PRL), relocates SH2B1β to the JAK2-PAK1-FLNa complex. Since SH2B1β is the enhancer of the kinase activity of JAK2, the formation of the complex results in enhancement of JAK2 activation and further activation of the JAK2-PAK1-FLNa complex that leads to actin cytoskeleton reorganization via actin-regulating proteins PAK1, FLNa, and SH2B1β, which has two actin-binding sites and cross-links actin filaments [169, 180].

PRL-induced pTyr-PAK1 also activates MAPK pathways, leading to the expression and secretion of MMP-1 and MMP-3 in response to 3D collagen IV microenvironment. MMP-1 degrades type I collagen, which is a major component of the ECM and MMP-3 degrades collagen IV which is a main component of basement membrane resulting in increased invasion of breast cancer cells in response to PRL.

PAK1 is important for a variety of fundamentally different cellular processes therefore it is critical to understand how PAK1 functions are controlled. The role of PAK1 tyrosyl phosphorylation is incompletely understood and there are only a few publications in this field although PAK1 is ubiquitously expressed, subject to growth factors, cytokine and hormone regulation and participates in various cellular functions. Fundamental questions whether PRL-dependent regulation of PAK1 also plays a critical role in normal mammary gland development, growth, and differentiation also remain (Fig. 5.11).

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Chapter 6
Plasticity of the Prolactin (PRL) Axis: Mechanisms Underlying Regulation of Output in Female Mice

P. R. Le Tissier, D. J. Hodson, A. O. Martin, N. Romanò and P. Mollard

Abstract The output of prolactin (PRL) is highly dynamic with dramatic changes in its secretion from the anterior pituitary gland depending on prevailing physiological status. In adult female mice, there are three distinct phases of output and each of these is related to the functions of PRL at specific stages of reproduction. Recent studies of the changes in the regulation of PRL during its period of maximum output, lactation, have shown alterations at both the level of the anterior pituitary and hypothalamus. The PRL-secreting cells of the anterior pituitary are organised into a homotypic network in virgin animals, facilitating coordinated bouts of activity between interconnected PRL cells. During lactation, coordinated activity increases due to the changes in structural connectivity, and this drives large elevations in PRL secretion. Surprisingly, these changes in connectivity are maintained after weaning, despite reversion of PRL output to that of virgin animals, and result in an augmented output of hormone during a second lactation. At the level of the hypothalamus, tuberoinfundibular dopamine (TIDA) neurons, the major inhibitors of PRL secretion, have unexpectedly been shown to remain responsive to PRL during lactation. However, there is an uncoupling between TIDA neuron firing and dopamine secretion, with a potential switch to enkephalin release. Such a process may reinforce hormone secretion through dual disinhibition and stimulation of PRL cell activity. Thus, integration of signalling along the hypothalamo-pituitary axis is responsible for increased secretory output of PRL cells during lactation, as well as allowing the system to anticipate future demands.

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6.1 Introduction

A fundamental aspect of all hormone axes is that they are plastic, responding to physiological need with modification of output, which is then monitored and varied in classical feedback regulatory loops to maintain homeostasis. This is true of systems as diverse as the hypothalamus, pancreas, adrenal gland, testes and, of course, the pituitary gland. As a master regulator of animal physiology, the pituitary gland responds to central and peripheral inputs with highly variable outputs, which both maintain homeostasis and drive physiological processes, such as responses to acute and chronic stress and ovulation.

Prolactin (PRL), the focus of this book, is secreted from specialised cells of the anterior lobe of the pituitary. All of the anterior pituitary hormones have dynamic outputs, which vary to different extents and with a timing which is related to their function. For adrenocorticotrophic hormone, for example, there is both an ultradian and circadian rhythm in unstressed animals [1], whilst for luteinizing hormone in seasonal breeding animals there is also an annual rhythm of output which is dependent on photoperiod [2]. This is also true of PRL, which has a highly variable output dependent on physiological status, although consistent with its physiological functions (see further), the pattern of output depends on the reproductive strategy of the species. For example in both rodents and humans, PRL varies during the reproductive cycle and pregnancy, with a dramatically increased output during lactation, whilst in seasonal breeding animals, such as the sheep, PRL levels reach similar levels in the non-breeding season to those of lactation [3]. The mechanisms underlying the changes from infrequent pulses of PRL output during the estrous cycle and in pregnancy to maintenance of sustained high secretion of PRL in lactation will be reviewed here. We will focus on recent studies in the mouse but extrapolate and contrast with those in other species, in particular rats, where relevant and informative.

6.2 The Pattern of PRL Output in Different Physiological States

Expression of the PRL gene is first detected in the mouse pituitary between embryonic days 15.5 and 17.5 but serum protein is undetectable before birth [4]. There are no studies to our knowledge documenting the changes in PRL secretion that occur before puberty. There is a low secretion of PRL during the estrous cycle of the mouse, with the possible exception of late proestrus or early estrus, where one study has shown a strain-dependent small increase in the concentration of plasma PRL [5] (Fig. 6.1). If mating occurs then vaginal stimulation leads to twice daily surges of PRL, with a peak occurring at the end of the light phase and a smaller nocturnal peak [6]. These continue until mid-pregnancy when placental production of a member of the PRL gene family, placental lactogen, increases and suppresses pituitary PRL [7]. Pituitary PRL remains suppressed until the end of pregnancy, when a nocturnal surge of PRL immediately preceding parturition [8] is followed

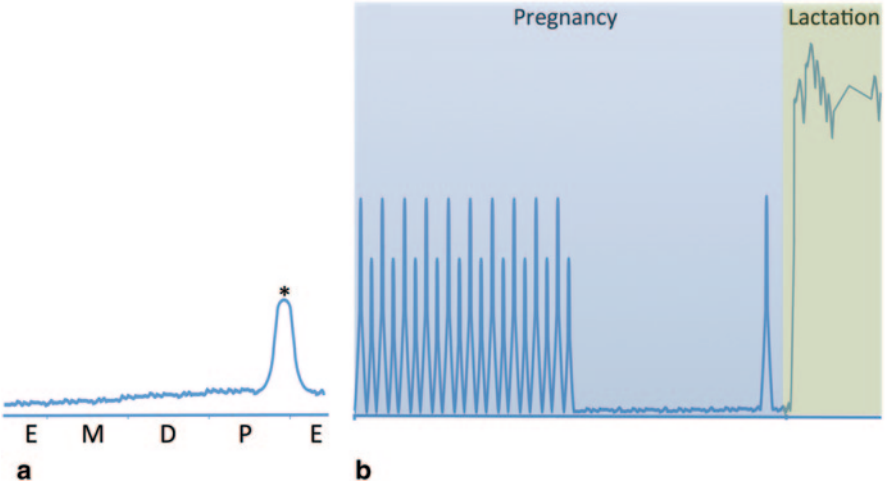

Fig. 6.1 Secretory pattern of prolactin (PRL) at different reproductive stages of the female mouse. **a** In virgin female mice PRL levels are maintained at low levels in estrus (*E*), metestrus (*M*) and diestrus (*D*). *Whilst there is strong evidence for a PRL surge pulse of secretion on the afternoon of proestrus (*P*) in rats, in mice the data is limited and suggests that the level and timing of any increase at proestrus is strain dependent**. b** If mating occurs, vaginal stimulation leads to twice daily pulses of pituitary PRL for the first half of pregnancy, which returns to low levels until a nocturnal surge immediately before parturition and high levels of secretion during lactation

by high-serum concentrations of the hormone during lactation, when PRL secretion largely depends on the strength of the suckling stimulus [9]. At weaning, concomitant with a cessation of the suckling stimulus, the concentration of PRL in the blood rapidly declines [10] and then follows the same pattern as before pregnancy. There is evidence from the rat, however, that reproductive experience results in a lower level of basal secretion compared with virgin animals [11].

6.3 The Functions of PRL and its Regulation

Over 300 functions for PRL have been described [12]. Since there is no obvious phenotype associated with the majority of these in studies of mice with loss of function of either PRL ($Prl^{-/-}$) [13] or its receptor (PRLR, mice with loss of functional receptors are designated $Prlr^{-/-}$) [14], for many of these a non-essential role for the hormone is likely. The most overt phenotype of these knockout mice is in females, with a failure to maintain pregnancy or undergo the mammary expansion required for lactation [13–15], consistent with the main role of PRL in mammals as a hormone which modifies female physiology to maximise reproductive success. Below we will briefly review the reproductive functions described for PRL in female rodents and identify, where possible, the role of each of the distinct secretory patterns.

6.3.1 Physiological Roles of PRL in Female Rodents

Maintenance of Pregnancy PRL is required for post-mating rescue of the corpus luteum (CL) in mice, which maintains progesterone secretion early in pregnancy. The vaginal stimulation of mating induces the two daily peaks of PRL secretion associated with the first half of pregnancy and these are essential for maintenance of the CL, acting to both increase progesterone synthesis [16] and reduce its degradation [17]. Consistent with this is the reproductive failure of *Prl*$^{-/-}$ [13] and *Prlr*$^{-/-}$ [14] mice.

Adult Mammary Development and Maintenance of Lactation A role for PRL in adult mammary gland development is demonstrated in *Prlr*$^{-/-}$ mice. Although prepubertal development of the mammary gland in this mouse model is normal, the increase in ductal side-branch and alveolar bud density which occurs in wild-type animals with each estrous cycle fails to occur [15]. In pregnancy, the mammary glands of normal mice develop further, with alveolar buds developing into lobuloalveoli, a process with an absolute requirement for PRL signalling [15]. In addition, there is a clear and unequivocal requirement for high levels of pituitary PRL for maintenance of lactation (reviewed in [18]).

Suppression of Fertility Hyperprolactinaemia leads to a loss of fertility in female rodents (reviewed in [19]), suppressing pulsatile luteinizing hormone (LH) at both the level of the hypothalamus and pituitary [20, 21]. This suggests an important role for PRL in timing of fertility to prevent a conflict between the metabolic requirements of pregnancy and lactation. Whilst rodents have suppressed fertility during lactation, they have evolved a window of opportunity for mating with a postpartum ovulation immediately after birth, although there are mechanisms which delay implantation so that the subsequent litter is not born until the current pups are weaned [22]. Recent studies in rats have suggested that the pathway leading to this suppression of fertility is via kisspeptin neuron inhibition by the high PRL levels during lactation [23], together with direct effects upon LH release from pituitary gonadotrophs [20, 24].

Maternal Behaviour During parturition the behaviour of rodent mothers changes to promote care of offspring, including nursing and licking pups and their retrieval to the nest site [25]. Many studies have shown that PRL has a role in this change of behavior (reviewed in [26]), and that this is mediated by the central nervous system [27], in part by a stimulation of neurogenesis in the subventricular zone of the forebrain [28, 29]. In elegant studies, reducing the magnitude of the twice-daily surges of PRL in early pregnancy, Larsen and Grattan [6] demonstrated that these are required for induction of full maternal behavior. Interestingly, although both *Prl*$^{-/-}$ and *Prlr*$^{-/-}$ mice have deficits in aspects of maternal behavior, this is less severe in *Prl*$^{-/-}$ females [14, 30]. Thus, exposure of female pups to their mother's PRL in early lactation may also modify their subsequent maternal behavior, invoking an indirect role for the high-PRL levels of lactation, since the hormone has been shown to be present in milk [31] and to be absorbed intact from the neonate intestine in rodents [32, 33].

Altered Stress Response There is marked reduction in anxiety and the response of the hypothalamo-pituitary-adrenal to stress in pregnant and lactating rodents (reviewed in [34]), in part to protect the conceptus from the damaging effects of glucocorticoids during development. High levels of PRL have been shown to attenuate maternal anxiety and stress responses [35, 36], and the PRL surges of early pregnancy have again been implicated by studies of the effect of their suppression [6].

Regulation of Oxytocin Neurons Oxytocin is a critical factor in the processes of parturition and milk ejection (reviewed in [37]), as well as a modifier of maternal behavior (reviewed in [38]). This neuropeptide may also act as a physiological regulator of PRL release itself [39–41]. Pertinently, infusion of a PRL antagonist into the brain of pregnant rats disrupts parturition [42], whilst in lactating animals PRL stimulates both oxytocin gene expression [43] and secretion [44], suggesting a role for PRL in regulating oxytocin, and possibly its own secretion, in both of these processes.

Alteration of Metabolism Pregnancy and lactation are associated with a dramatic alteration of maternal metabolic demand to support the growth of the fetus, parturition and lactation [45]. PRL and placental lactogen have been shown to alter a number of processes leading to a myriad of adaptations that are required to alter nutrient supply in pregnancy and lactation, for example by inducing leptin resistance to promote hyperphagia [46].

Other Reproductive Functions As well as the maintenance of CL function, there is evidence from rats that PRL may have a role in luteolysis [47]. In addition, PRL may alter female receptivity to mating [48] and modify the uterine environment, as demonstrated by the failure of embryo implantation in *Prlr*$^{-/-}$ mice [49].

Fetal and Early Post-Natal Development A role for maternal PRL in fetal development is suggested by the expression of PRLR in pre-implantation embryos [50]. Although widespread expression has been described later in embryonic development, at this time pituitary PRL secretion is low, so it is unlikely to play a role. The expression in the olfactory system in the rat in late-gestation and in the neonate [51], however, suggests that there may be role for the maternal PRL present in milk (see above).

6.3.2 Roles for PRL at Different Stages of Reproduction and Lactation

Ascribing specific roles for the different levels and patterns of PRL secretion during the estrous cycle, pregnancy and lactation is complicated by the interaction between these processes—without a normal cycle pregnancy does not occur and without a pregnancy there is no lactation. As described previously, however, a large number of physiological, pharmacological and genetic manipulations have demonstrated roles at different stages of reproduction and lactation. Based on these, a role for the different stages of PRL secretion is suggested (Table 6.1).

Table 6.1 Roles of prolactin at different stages of its patterned secretion in adult female rodents

Stage of increased prolactin secretion	Modifying role in female physiology
Proestrous surge	Luteolysis Receptivity to male Embryo implantation Adult mammary development
Surges of early pregnancy	Maintenance of corpus luteum Adult mammary development Maternal behaviour Programming of offspring's maternal behaviour Reduced stress and anxiety Programming of offspring's stress axis
Late pregnancy surge	Induction of parturition Initiation of lactation
Lactation	Maintenance of lactation Metabolism Suppression of fertility Maternal behaviour Programming of offspring's maternal behaviour Reduced stress and anxiety Milk ejection Neonatal development/bonding

6.3.3 Normal Regulation of PRL Cell Number, Gene Expression and Secretion

Cell Number The anterior lobe of the pituitary gland is the principal source of circulating PRL in rodents and the hormone is secreted from a specialised group of cells, lactotrophs. The gland develops from oral ectoderm with proliferation and commitment to the hormone-secreting cell types of the anterior pituitary controlled by a cascade of transcription factors [52] after exit from the cell cycle [53]. The transcription factor repertoire defining lactotrophs has not been fully defined but a majority derive from a subset of cells expressing the transcription factor *Pou1f* [52]. At birth only a few cells expressing PRL can be detected but there is a rapid post-natal expansion, increasing in mice exponentially from birth to 5 weeks, before stabilising around 8 weeks of age [54]. The post-natal expansion of lactotrophs is regulated by a number of peripheral, hypothalamic and intrapituitary factors, including insulin-like growth factor 1, epidermal growth factor and estradiol [55]. In the adult, it is clear that dopamine (DA) [25], acting through the dopamine 2 receptor (D2R) is a major suppressor of lactotroph proliferation [56, 57], but the increased severity of lactotroph hyperplasia found in $Prl^{-/-}$ or $Prlr^{-/-}$ mice compared with those with a loss of functional DA signalling demonstrates that other factors also regulate lactotroph proliferation [58]. Estradiol [59], along with a range of paracrine factors [60], control lactotroph proliferation in the adult gland but in combination with DA the steroid also regulates lactotroph apoptosis [61]. Trans-differentiation of other pituitary cell types to lactotrophs has also been described as a mechanism

regulating their number [62], although recent studies suggest this is unlikely to have an important role in mice [63].

Gene Expression A range of factors have been described which affect PRL gene expression in rats (reviewed in [64]), but it is the regulation by DA and estrogen, which is the most clearly defined in rodents. The *Pou1f1* transcription factor, required for adult PRL gene expression, mediates both the positive and negative regulation of PRL gene transcription by estrogen [65] and hypothalamic DA [66], respectively.

Secretion The regulation of PRL secretion from lactotrophs is unusual because it is principally under the inhibitory control of hypothalamic DA (reviewed in [67]). This is mediated predominantly by the D2R, as demonstrated by the very high concentrations of serum PRL in mice deleted for the D2R, or treated with D2R-antagonists [56, 57]. The source of inhibitory DA is from three populations of hypothalamic neurons: the tuberoinfundibular dopaminergic (TIDA) neurons, which originate in the dorsomedial arcuate nucleus and project to and secrete DA into the median eminence (ME) [68]; the tuberhypophyseal dopaminergic (THDA) neurons with cell bodies in the rostral arcuate nucleus which project to the neural and intermediate lobes of the pituitary [69]; and the periventricular hypophyseal (PHDA) dopaminergic neurons which arise in the periventricular nucleus and project to the intermediate lobe of the pituitary [70]. DA is transported to the anterior pituitary from the ME by the portal circulation and from the intermediate and neural lobes via short-portal vessels [71]. A large number of factors which stimulate PRL secretion [67] have also been described, including estrogen, vasointestinal peptide, oxytocin, thyrotrophin-releasing hormone and galanin, but there is no clear evidence for a physiological role for these.

6.3.4 Interaction Between PRL and Hypothalamic DA Neurons

As may be expected from the diverse range of physiological changes associated with pregnancy and lactation, there are a number of neuronal populations in the forebrain which are responsive to PRL [72]. Amongst these, TIDA neurons are of special interest because of their known involvement in the regulation of PRL secretion. Experiments using both intraperitoneal and intra-cerebroventricular injections of increasing doses of PRL allow comparison of the relative sensitivity of the different neuronal populations [73]. These data show that TIDA neurons respond to much lower doses of the hormone than other neuronal populations, possibly resulting from their localisation in the arcuate nucleus which has an increased access to circulating molecules [74] due to lack of a complete blood–brain barrier [75]. As well as having an augmented sensitivity to PRL in adult animals, the development of TIDA neurons in the neonate is dependent on PRL [76], further demonstrating the strength of their interaction.

6.3.5 *Potential Mechanisms Leading to Altered Output*

Clearly, there are a number of mechanisms that may underlie the altered output of PRL in different physiological states which support murine reproduction and lactation:

Altered Exposure to Inhibitory or Stimulatory Factors The acute changes in PRL output, the pro-estrous surges and the twice-daily pulses of release in early pregnancy, are most likely to be a result of altered inhibition by hypothalamic DA, since it is tonic inhibition by this factor which primarily regulates PRL secretion. It is also possible that a PRL-stimulating peptide may mediate these pulses and oxytocin is a candidate for this, although it may have a complex interaction with DA [77]. Of course, alterations in exposure of the pituitary gland to these regulatory factors could also have a role in the long-term alteration in PRL secretion that accompanies lactation.

Altered Number of Lactotrophs Whilst the number of lactotrophs will not vary at a sufficient rate to account for the acute changes in PRL, a variation in their number has been suggested as a mechanism to account for the ability of the gland to secrete the large amounts of PRL required for lactation [62]. Recent studies, however, have shown that a similar increase in the number of lactotrophs does not occur in mice [63, 78], discounting this as one of the mechanisms underlying the increased PRL secretory capacity during lactation. The pituitary gland of mice is enlarged during lactation but this primarily results from a doubling in lactotroph volume, with only a modest and non-cell-type specific increase in mitosis [79].

Altered Gene Expression Increase in PRL gene expression could account for some aspects of the surges of PRL secretion at pro-estrous and early pregnancy if combined with altered inhibition or stimulation of the cells. Indeed, the stimulation of PRL secretion by estrogen can be accounted for by its effect on gene expression [80]. It is apparent that the large amounts of PRL secreted in lactation could only be maintained by an increased production of the hormone by lactotrophs, although simply increasing the amount of hormone per cell will again not account for the altered output of the gland per se.

Altered Cell Function in Response to Stimulation or Inhibition There are several potential mechanisms where an alteration of lactotroph cell function could underlie changes in their secretory activity. The most obvious of these would be an altered expression of receptors for factors inhibiting or stimulating secretion, for example a downregulation of D2R. Gene expression profiling of lactotrophs in virgin and lactating animals, however, failed to show dramatic changes in D2R or any of the other known cellular factors which could account for the altered secretory activity (Le Tisser, Mollard, Coutry, Baudry and Drouin unpublished). An alternative mechanism would be a change in the cellular response to the factors regulating secretion, either by altered regulation of cell signalling pathways, ion channel activity or cell–cell interactions.

Of course, it is possible that a combination of these mechanisms could account for the dramatic changes in PRL secretion that occur in female mice and which are required to support successful reproduction. Further we will describe our recent studies at two levels of regulation of the stage where the most dramatic alteration of PRL secretion occurs, during lactation.

6.4 Plasticity at the Level of the Pituitary

The three-dimensional arrangement of endocrine cells within the mammalian pituitary influences intercellular communications by supporting the display of complex dynamics at the population level. Regulation of hormone secretion by the tissue context has been demonstrated in studies showing that cells plated as a monolayer fail to properly organise their electrical and exocytotic activities in response to secretagogue [81–83]. Indeed, the pituitary is a highly compartmentalised tissue, with the majority of embedded cell populations structurally and functionally organised as networks [84–87], which may account for plasticity in physiological output [88].

6.4.1 The Network Organisation of Lactotrophs

Notably, lactotrophs form a homotypically-interconnected network that occupies the ventral and lateral portion of the pituitary gland in female PRL-DsRed animals [89]. When viewed at higher magnifications, the PRL network possesses a distinctive honeycomb appearance with recurrent rosette motifs, an arrangement that likely arises due to the close juxtaposition of lactotrophs with the capillary meshwork that pervades the gland parenchyma [87, 89]. Of functional relevance, anatomical interactions between the PRL network and the vasculature may be essential to both dynamically compensate the energy requirements of highly-metabolising lactotrophs, as well as facilitate the extrusion of secreted hormone from the gland [90, 91]. Perhaps as a consequence of sharing a common progenitor, the PRL and growth hormone (GH) networks are tightly intermingled, and as for the LH and proopiomelanocortin networks [92], this may have repercussions both for development (e.g. mutual cell network scaffolding) and for regulation of hormone secretion (e.g. through enhanced paracrine communications). When viewed at the nanoscale level using electron microscopy, a key observation is that lactotrophs are heterotypically connected to folliculostellate (FS) cells via gap junctions (GJs) more often than would be expected due to chance. Since FS cells are non-endocrine and form an electrically-coupled network that allows the long-distance propagation of signals across the entire pituitary [93, 94], they may facilitate interactions between lactotrophs by functionally connecting and entraining activity between distant regions of the PRL cell network via GJ exchanges.

6.4.2 PRL-Network Plasticity During Lactation

During lactation, the PRL cell network must increase its secretory capacity to maintain high levels of serum PRL. The increase in lactotroph cell volume associated with lactation results in alterations to the structural connectivity of the PRL network due to an increased frequency and density of homotypic cell–cell contacts. A tantalizing prospect is that such changes in connectivity may also be associated with migration of lactotrophs, similar to that recently described for somatotrophs in the GH network during the pubertal spike in GH secretion [95]. This re-organisation of the PRL network is directly linked to the strength of the suckling stimulus, since dams nursing a reduced number of pups display impaired levels of cell–cell connectivity [89].

6.4.3 Changes in Cell Activity Resulting from Altered Organisation

To determine the contribution of network structure to the population dynamics that orchestrate hormone release, granule exocytosis must be simultaneously monitored in hundreds of cells residing within the intact tissue. However, this is largely constrained by the poor signal-to-noise ratio of currently available tools, meaning that intracellular calcium (Ca^{2+}) rise must instead be measured as a surrogate for Ca^{2+} -dependent exocytosis [96, 97]. Assuming that collective activity contributes to the same output, Ca^{2+} -imaging can be combined with correlation analyses to map episodes of coordinated cell-pair behaviour, allowing the visualisation of the functional connectivity that drives PRL secretion. Using these methods, it can be shown that, in pituitary slices from PRL-DsRed virgin females, basal hormone release is supported by a sparsely-connected network due to the infrequent display of coordinated cell–cell behaviour [89]. During lactation, the appearance of highly-connected node cells results in dramatic increases in correlated lactotroph population Ca^{2+} -spiking activity, driving five–tenfold more PRL than under basal conditions. Using two-photon excitation to capture a deep snapshot of network architecture immediately preceding Ca^{2+} -imaging, this could be directly linked to an increase in the underlying structural connectivity. It should be noted that the changes documented during lactation likely stem from network-endogenous processes encoded in vivo, rather than removal of DA tone (see below), since heightened functional connectivity was not detected in pituitary slices from dams treated with a D2R antagonist.

Mechanistically, the increases in coordinated cell–cell activity observed during lactation are primarily due to improvements in GJ-signaling. These intercellular channels are composed of connexin protein and allow the passage of ions, nucleotides and second messengers between neighbouring cells in a highly charge- and size-selective manner [98]. Importantly, cell–cell communications are highly up-regulated during lactation, as evidenced by both an increase in the number of GJ-coupled cells, as well as the extent of cell–cell dye transfer [89]. The promotion of coordinated PRL network activity via GJ exchanges is suggested by the observation that current injection into identified lactotrophs results in similar patterns of cell–cell

entrainment to those seen during Ca^{2+} –imaging experiments. GJ-coupling is also detected between lactotroph, GH and FS-cells, and as alluded to above, heterotypic intercellular communications with the latter may provide an important route for the long-distance propagation of information. Moreover, autocrine (e.g. PRL), juxtacrine (e.g. annexin) and paracrine (e.g. ATP) signalling are likely to contribute to lactotroph–lactotroph communications, although this is difficult to examine due to the myriad of potential candidates (see references [97, 99, 100] for detailed reviews).

6.4.4 *Interaction of PRL-Network with Regulation of Gene Transcription*

As well as the electrical activity underlying hormone secretion, the PRL-network also integrates and supports other critical processes including transcription. Indeed, PRL gene transcription is coordinated at the population level, a trait which is lost when cells are dispersed and cultured in two-dimensions [101, 102]. Likewise, lactotroph transcription is pulsatile during embryogenesis before stabilising in the postnatal period [103]; it is tempting to speculate that this transition may coincide with the final specification, maturation and placement of the PRL-network. However, whether cycles in lactotroph transcription are the cause or consequence of PRL-network development remains unknown.

6.4.5 *Long-Lasting Post-Weaning Changes at the Pituitary Level*

Following weaning, the increase in PRL secretion and pituitary weight detected during lactation are thought to subside due to decreases in cell size. However, when pituitary glands from weaned dams were subjected to Ca^{2+} -imaging, a lactating-like distribution of cell correlations could still be detected, albeit with less-densely connected nodes. Remarkably, such wiring patterns were still retained 3 months post-weaning, suggesting that the PRL-network possesses the inherent capability to maintain a long-term ‘memory’ of prior demand [89]. Strikingly, repeat stimulation of the PRL network by a second lactation was associated with amplified PRL secretion due to the display of evolved population behaviour in the form of highly synchronous Ca^{2+} rises (see Fig. 6.2 for a schematic). This ‘endocrine memory’ appears to be encoded by suckling-evoked changes to the strength and extent of structural connectivity. Indeed, the augmented PRL secretion observed in multiparous dams during lactation could be prevented by reducing the number of suckling pups to alter maternal demand and disrupt the onset of highly synchronous Ca^{2+} rises.

6.4.6 *Network Memory and Functional Adaptation*

The possession of endocrine or network memory by the lactotroph population may have wide-ranging repercussions for homeostasis. As described previously, in

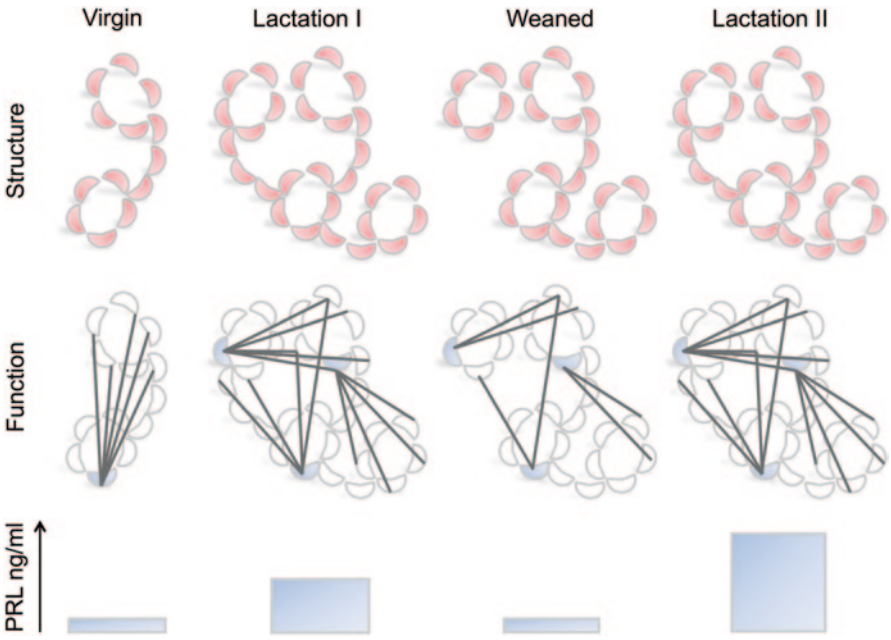

Fig. 6.2 Schematic depicting structural and functional plasticity of the PRL-network during physiological demand. In virgin animals, poor structural and functional connectivity are associated with basal PRL release. During lactation, an increase in cell–cell contacts drives increases in functional connectivity that support elevated PRL levels. Following weaning, plasma PRL concentrations fall, but a lactating-like wiring pattern persists due to residual structural connectivity. During a second lactation, the PRL network displays emergent behaviour, boosting PRL secretion beyond that measured during the first lactation

addition to ensuring nourishment of rapidly growing offspring, PRL also regulates a range of neural outcomes in the mother including appetite, stress and reproduction. Indeed, mothers who have experienced one or more lactations display incrementally better performance at spatial memory tasks, a phenomenon which could feasibly be mediated by the effects of PRL upon oxytocin neurons [104, 105]. Thus, experience-dependent responses are an emergent feature of the pituitary gland, and may underlie functional adaptation of hormone release in the face of changing demands, altering maternal physiology accordingly. Importantly, this may offer advantages for the development of offspring by improving nursing, milk output and growth.

6.5 Plasticity at the Level of the Hypothalamus

In classical feedback loop conditions, PRL alters DA secretion in a composite manner over a range of time scales. For example, PRL can increase the rate of DA synthesis, which can be divided into protein-synthesis-independent (within 4 h) and

-dependent components (> 12 h) [106], as well as acting more rapidly (within minutes) by direct activation of TIDA neuronal electrical activity [107, 108].

6.5.1 PRL Feedback on DA Synthesis

All TIDA neurons show transcriptional responses to PRL, including an increase in the expression of mRNA encoding tyrosine-hydroxylase (TH), a rate-limiting enzyme in DA synthesis, leading to increased DA production [109]. PRL also increases the phosphorylation of TH though a pathway involving intracellular protein kinases [110], enhancing activity of the enzyme and increasing DA synthesis. TH phosphorylation at several serine residues is the primary mechanism responsible for regulation of DA production, with phosphorylation at Ser^{40} being the most important for activation of the enzyme [111, 112]. The phosphorylation state of TH in the stalk-ME is modulated by PRL, and reflects the TH activity in TIDA neurons and their potential to produce DA. Modulation of TH phosphorylation has been demonstrated in different physiological situations, including cycling female rats [113] and during suckling-induced PRL surges [114]. Using mice with deficiencies in specific isoforms of the signal transducer and activator of transcription (STAT) protein, STAT5, it has been shown that STAT5b is the principal mediator of the action of PRL in the hypothalamus of the mouse [115], although its direct involvement in the induction of either TH phosphorylation or its gene expression has not yet been demonstrated.

6.5.2 TIDA Neuron Electrical Activity and its Modulation by PRL

TIDA neurons in brain slices display spontaneous firing, including bursting, the most efficient way to secrete neurotransmitters in various neuronal populations [116, 117]. In male rats, bursting and GJ-mediated synchronous membrane potential oscillations have been described in TIDA neuron subpopulations [118]. In mice, TIDA neurons constitute a more heterogeneous group of neurons if classified according to their firing properties (regular, bursting or irregular) [108], with ~20 % of the cells displaying bursting firing. TIDA neurons, like numerous other neuroendocrine systems [119], do not display a strong coordinated bursting electrical activity.

In mice, TIDA neurons display long-term oscillations in their firing rate, with alternating periods of elevated and decreased firing. The anatomy of TIDA neurons is such that their cell bodies in the arcuate nucleus can be studied in parallel to their terminals at the ME in the same coronal brain slice, allowing simultaneously monitoring of electrical activity and DA secretion. Amperometric recordings at the ME demonstrated that these oscillations are likely the basis of a larger functional network, as they are harmonised between neurons and are directly involved in the generation of DA secretion from virgin female slices [108]. Compared with a classical

neuron–neuron connection, the neuroendocrine neurohemal synapse has the peculiarity of secreting large amounts of material on a timescale of tens of minutes, or hours. Secretion of sufficient DA at the appropriate time by such a small neuronal population necessitates coordination of secretory events at the level of multiple neurons. In the TIDA system this happens through long-term modulation of firing rates rather than synchronisation of single action potentials [120], with TIDA neurons acting as integrators. Integrators can summate temporally dispersed (asynchronous) inputs before transforming them into a common physiological output, i.e. DA secretion in the case of TIDA neurons. This provides a mechanism whereby firing rate co-modulation between cells can provide a robust harmonisation of the functional activity within the neuronal population, whilst maintaining plasticity. Such activity patterns enable adaption to very different physiological demands which would not be possible in the case of strictly coordinated/synchronised cells.

Several studies have demonstrated that PRL in rodents stimulates TIDA neuron electrical activity [107, 108, 121], to drive increased DA release [108]. In pre-pubertal male rats, this is due to activation of a mixed cation current with TRPC-like pharmacological characteristics and BK-type channels [107]. Notably, the action potential width increases significantly in the presence of PRL, which may potentiate the release of DA. However, the acute application of PRL (10–20 min) has no long-lasting effects on either the patterning or the harmonisation of the electrical activity after wash out of the PRL.

6.5.3 Plasticity of TIDA Neuron Function During Lactation

To maintain the long-term hyperprolactinemia required during lactation, the regulatory feedback of DA secretion needs to be suppressed, even in the presence of an ongoing PRL stimulus [8, 106, 122, 123]. The basis of this escape from the TIDA neuronal tone is decreased secretion of DA in the portal blood. TIDA neurons are described as ‘inactive’ during this period, with reduced activity, as typified by decreases in TH mRNA and protein expression in the arcuate [124], DA turnover at the ME [125] and secretion of DA from terminals [126]. During lactation in mice, the Ser^{40} phosphorylation activating TH enzyme activity is strongly decreased, particularly in the external zone of the ME [108], with no change in the total TH content. Therefore, reduced phosphorylation of TH, possibly alongside a decrease in *TH* mRNA levels [124], could explain the absence of secretable DA. This decreased phosphorylation may be a result of the uncoupling of the PRLR from its classical JAK/STAT intracellular signaling pathway [127]. Indeed, although there is no evidence of a downregulation of the PRL receptor in TIDA neurons during lactation [128], PRL-induced activation of STAT5 is markedly reduced [127, 129]. This change effectively renders the neurons unresponsive to PRL via STAT5b-mediated activation of DA release, and may be mediated by a family of endogenous inhibitors, the suppressors of cytokine signalling (SOCS) proteins, several of which are increased in the arcuate nucleus during late pregnancy and lactation [130].

6.5.4 Conservation of TIDA Neuron Electrical Activity in Lactation

The observation that DA output from TIDA neurons is markedly reduced during lactation, despite elevated PRL levels, has led to the assumption that TIDA neurons become inactive and unresponsive to PRL [106, 122, 125, 126]. In mice, the reduced DA release from TIDA neurons during lactation could be confirmed using amperometry but, contrary to expectations, the electrical properties of these neurons, at the single-cell and network level, are maintained. Surprisingly, they continued to respond electrically to PRL at the cell-body level [108] and, as a consequence, lactation can be considered as a state of uncoupling between firing and DA secretion, rather than a period of TIDA neuron electrical silencing. During lactation, the intracellular pathway(s) induced by PRL is therefore profoundly different from that of a virgin animal, a discrepancy not reflected at the level of the electrical activity. The exact link between PRLR and the electrical activity detected during lactation still requires further study.

6.5.5 The Opioid Switch?

The finding that TIDA neurons still electrically integrate PRL signals during lactation suggests that the neurons may still have a physiological role, albeit not that of inhibiting PRL secretion. Previous studies have reported a change in the characteristics of these neurons in lactating rodents, with detection of increased opioidergic immunoreactivity in the arcuate nucleus [131, 132] and specific expression of enkephalin (Enk) in TIDA neurons. Positive effects of endogenous opioids on PRL secretion have been described [133], which could result from inhibition of TIDA neurons themselves via suppression of DA turnover [134] or TH activity [135]. Thus, a role for Enk may be to reinforce the suppression of DA secretion from TIDA neurons, although as there are numerous other targets for opioids within the hypothalamus, as well as other regions of the brain [136], it is tempting to speculate that Enk may instead modify secretion of potential PRL-releasing factors. Indeed, it is possible that the PRL-releasing factor is Enk itself, with its secretion from the ME resulting from adaptations to TIDA neuron secretory apparatus having a direct effect on the pituitary. An opioid-dependent modification of the lactotroph secretory response to thyrotropin-releasing hormone has been described in rats [137]. Undergoing a phenotypical switch may therefore allow the TIDA population to conserve its basic physiological properties, such as electrical activity, whilst drastically changing its output function (i.e. switching from DA to Enk secretion) so that it may provoke rather than inhibit PRL secretion. This could be advantageous in terms of cellular economy, as a stimulus from the same hormone (PRL) can be used to determine different physiological effects, and can be rapidly reverted in response to altered physiological status (i.e. weaning when TIDA neurons are expected to resume normal DA-releasing activity).

6.5.6 *A Memory in the TIDA System? Reproductive Experience*

Basal serum concentrations of PRL are significantly decreased in both reproductively experienced animals [127, 138] and women [139]. The basis of the reduced PRL secretion is not clear and may involve both pituitary and hypothalamic levels. If the TIDA system itself is capable of a memory of reproductive experience, one potential mechanism could involve an increased sensitivity to PRL feedback by both the TIDA neurons or by afferent cells such as kisspeptin and opioid neurons, both of which have been shown to contribute to this system [134, 140].

Conclusions

Historically, PRL secretion has been considered to be primarily under the control of brain orders with suppression of hypothalamic TIDA activity driving large increases in release of the peptide during lactation. However, over the past decade, pioneering studies on transgenic mouse models have significantly changed our view of the ultra-short feedback regulation of pituitary lactotroph function. Indeed, the hypothalamo-lactotroph axis can now be best described by a dual-integrator model where inputs are decoded at the level of both the 3D lactotroph network and hypothalamic TIDA neurons to deliver PRL pulses into the bloodstream. Importantly, it is the remarkable plasticity of both these sites that allows PRL secretion to be dynamically adapted to physiological demands. Using lactation to selectively and repeatedly stimulate PRL release, it can be shown that the lactotroph population undergoes experience-dependent responses to heighten PRL release during subsequent lactations, and this may be important for maternal behaviour and pup growth. In addition, TIDA neurons are phenotypically switched during pregnancy such that their output is no longer inhibitory, and could potentially be stimulatory; this may act to further reinforce PRL secretion while allowing the neuronal population to maintain their electrical properties (i.e. PRL-responsiveness; see Fig. 6.3 for a revised overview of PRL regulation during lactation).

However, many questions remain unanswered regarding the role of hypothalamic and pituitary oscillators in PRL secretion. For example, the focus has been on lactation, but what happens to oscillatory function during other periods of demand (i.e. proestrous PRL surge, pregnancy)? What are the mechanisms that underlie PRL secretion in non-rodent species including seasonal breeders and humans? What is the nature of the upstream arcuate nucleus neural circuits which impinge upon TIDA neuron function? How do other important homeostatic processes, for example food intake and energy balance, interact with PRL secretion at the hypothalamic and pituitary levels? It is expected that, with the development of mouse genetics and imaging techniques, such questions should be addressable both in vitro and in vivo within the next decade.

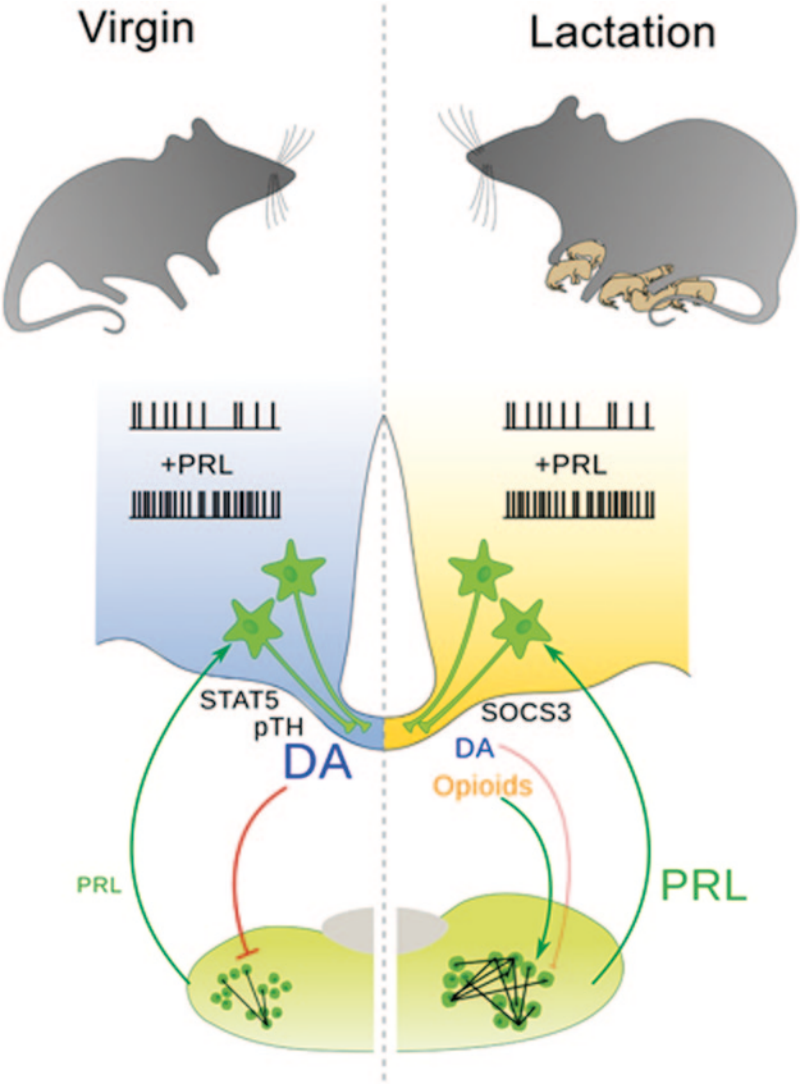

Fig. 6.3 Schematic of the changes which occur in the activity of hypothalamic dopamine (DA) neurons and lactotrophs in virgin and lactating mice. Prolactin secretion in virgin animals feeds back at the level of DA neurons to activate *STAT5*, which leads to increased phosphorylation of tyrosine hydroxylase (TH) and DA release. This leads to basal PRL secretion from the poorly coordinated PRL network (correlated lactotrophs indicated by *black lines*). During lactation, there is no change in the electrical activity of DA neurons but a reduced synthesis and secretion of DA, possibly through a SOCS-induced reduction in STAT5 signalling. This may also lead to a switch from DA to opioid secretion, which results in both a disinhibition and stimulation of PRL secretion. In parallel, PRL network structural connectivity is heightened due to increases in lactotroph volume, and this facilitates more coordinated activity between cells (N.B. the 3-dimensional network organization is not apparent in this 2-dimensional representation). The net effect is a large increase in PRL output required to support mammary gland development and offspring nourishment during lactation

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Chapter 7
Role of Src Family Kinases in Prolactin Signaling

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Abstract Prolactin (PRL) is a polypeptide hormone/cytokine mainly synthesized by the lactotrophic cells of the adenohypophysis. In addition to the best-known role in mammary gland development and the functional differentiation of its epithelium, PRL is involved in regulation of multiple physiological processes in higher organisms contributing to their homeostasis. PRL has been also associated with pathology, including breast cancer. Therefore, it is relevant to determine the molecular mechanisms by which PRL controls cellular functions. Here, we analyze the role of Src family kinases (SFKs) in the intracellular signaling pathways controlled by PRL in several model systems. The data show that SFKs are essential components in transmitting signals upon PRL receptor stimulation, as they control activation of Jak2/Stat5 and other routes that regulate PRL cellular responses.

Abbreviations

BaF-3 cells	Mouse pro-B lymphocytes
BrdU	5-bromo-2'-deoxyuridine
CEF	Chick embryo fibroblasts
CSK	Carboxyl-terminus Src kinase
EGF	Epidermal growth factor
EGFR, ErbB1	Epidermal growth factor receptor
Erk1/2	Extracellular signal-regulated kinase 1/2
Fak	Focal adhesion kinase
FERM domain	F for 4.1 protein, E for erzin, R for radixin, and M for moesin
FGF	Fibroblast growth factor

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FKHRL1, FOXO 3	Forkhead box O3 transcription factor
GH	Growth hormone
Gab2	Grb2-associated-binding protein 2
Grb2	Growth factor receptor-bound protein 2
GSK3ß	Glycogen synthase kinase 3beta
IEG	Immediate early genes
IL-3	Interleukin 3
IGF-IR	Insulin-like growth factor I receptor
IP	Immunoprecipitation
Jak	Janus family kinases
JH domain	Jak homology domain
Mek1/2	Mitogen-activated protein kinase 1/2
NDF	Neuregulin differentiation factor
PDGF	Platelet-derived growth factor
PRL	Prolactin
PRLR	Prolactin receptor
PI3K	Phosphoinositide 3-kinase
RCAS A or B	Replication-competent avian retroviral vectors derived from RSV
RSV	Rous Sarcoma Virus
SFKs	Src family kinases
SH3 domain	Src homology domain 3
SH2 domain	Src homology domain 2
Shp2	Tyrosine phosphatase containing two SH2 domains
Stat	Signal transducers and activators of transcription
WB	Western blot
W53 cells	BaF-3 cells expressing the long form of the PRLR from rat ovary

7.1 Introduction

The landmark in prolactin (PRL) molecular signaling was the cloning and characterization of prolactin receptors (PRLR) in the late 1980s [51]. The PRLR is a member of the type I cytokine receptor family, which also includes receptors for growth hormone, interleukins-2–7, erythropoietin, granulocyte colony stimulating factor, granulocyte–macrophage colony stimulating factor, etc. [9, 40, 96]. Although several isoforms of PRLR have been described, the short, intermediate, and long forms are the most common in biological systems. The short and the long forms, generated by alternative RNA splicing, differ in length and sequence of the cytoplasmic domain. The intermediate form of the receptor, found in the rat thymoma cell line Nb2, is generated from the long form through a partial in-frame deletion of 198 amino acids of the cytoplasmic domain [15].

PRLR isoforms do not exhibit intrinsic enzymatic activity. However, in NB2-11C cells it was promptly shown that PRL induced tyrosine phosphorylation by association and activation of a 120 kDa protein kinase [81], subsequently identified as Jak2 in Nb2, in mouse mammary explants [17] and in BaF-3 cells (mouse pro-B lymphocytes) expressing the long form of PRLR [24]. Soon after, it was found that the mammary gland transcription factor MGF/Stat5, a central component of the lactogenic hormone-signaling pathway, was phosphorylated and activated by Jak2 upon PRL stimulation [34]. In this context, Jak2 conditional knockout mice showed the essential role of this kinase for proliferation, differentiation of alveolar cells, and maintenance of lactation [93]. All these data doubtlessly established the role of Jak2 for PRL signaling.

In the early 1990s, collaborating with my colleague J.P. García-Ruiz, who found that a 60 kDa protein had been phosphorylated upon PRL stimulation of hepatocytes from lactating rats, we established association and activation of the proto-oncogenic c-Src (pp60-c-Src) to PRLR [11]. Similarly, PRL caused activation of Fyn, another member of the Src family kinases (SFKs), in Nb2 cells [20].

Since these first observations several groups have worked to learn about the role of Src kinases in PRL signaling. Here we revise these data, but first, we describe some information about structure and function of Jak and Src family of tyrosine kinases.

7.2 The Janus Protein Tyrosine Kinase Family

The Jak family ("Janus kinase" or "Just another kinase", Jak) [98, 99] contains four members: Jak1, Jak2, Jak3, and Tyk2 that exhibit a high degree of homology. Mammalian expression of Jak1, Jak2, and Tyk2 is ubiquitous whereas Jak3 expression is predominantly hematopoietic [44]. Jak family proteins are located at the plasma membrane where they interact with the cytokine receptor [39].

The Jak family has a size between 120 and 140 kDa corresponding to about 1100 amino acids. Its members have a conserved structure in insects, birds, and mammals and display seven Jak homology domains (JH) (Fig. 7.1). The main characteristic of this family is the presence, at the extreme C-terminus, of a tyrosine kinase catalytic domain (JH1) followed by an inactive pseudo-kinase domain (JH2), which is involved in the regulation of enzyme activity. Indeed, it suppresses basal kinase activity and allows stimulation upon binding of the ligand to the receptor [83]. At the N-terminus, the JH3, JH4, and JH5 domains possess homology with SH2 domains

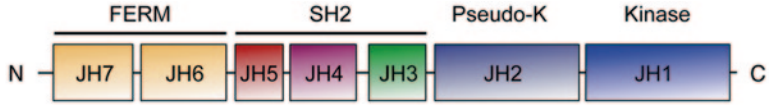


Fig. 7.1 The Janus protein tyrosine kinase family (Jak): structural domains.

(SH2-like) and through them may interact with proteins containing phosphorylated tyrosine residues. The JH6 and JH7 domains are homologous to the FERM domain, found in molecules as Band 4.1, radixin, myosin, etc. The FERM domain has 300 amino acids in length. In addition regulating the kinase activity by interacting with the JH1 domain [41, 107], FERM is responsible for the association of Jak2 with Box I of PRLR [41, 56]. This interaction is not dependent on ligand binding to the receptor [6].

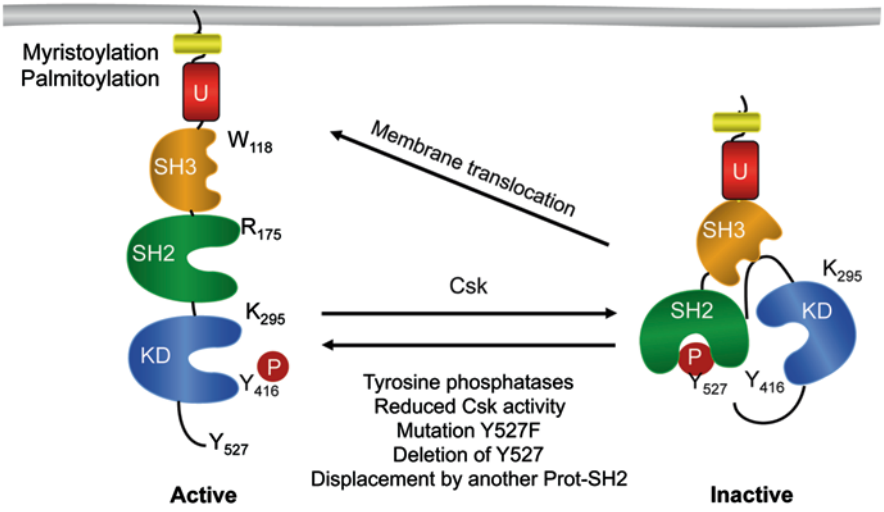
Jak2 is associated and activated by a large number of cytokine receptors [17, 43, 88], and it is considered the major effector for PRLR signaling [76, 81, 82, 86, 100]. Therefore, it is not surprising that conditional knockout mice for Jak2 and PRLR show similarities in their mammary gland phenotypes [92].

Activation of Jak2 by PRLR (Fig. 7.4) occurs by dimerization and the conformational change of the receptor after the ligand binding. This causes the juxtaposition of two Jak2 molecules and their autophosphorylation/transphosphorylation on tyrosine residues Y1007 and Y1008, which are located in the activation loop of the kinase domain [28, 61]. Besides the Y1007 and Y1008, there are other autophosphorylation residues that regulate Jak2 catalytic activity, among them, Y813, between JH1 and JH2 domains, which allows binding of the SH2-Bß adapter molecule. In addition, Y221 in the FERM domain positively modulates Jak2, while Y570 [7, 27, 53], and S523 phosphorylation, between JH3 and JH2 domains, negatively regulate Jak2 enzymatic activity [46, 64].

7.3 The Src Family of Tyrosine Kinases

Identification and characterization of Src have been associated with several milestones on molecular and cellular biology. It started more than a century ago when Peyton Rous described “a transmissible avian neoplasm” [79] and is still continuing today. The efforts of many scientists to decipher the structure and function of the Rous Sarcoma Virus (RSV) have given rise to multiple biological concepts, including retrovirus, oncogene, proto-oncogene, Src homology domains SH2 and SH3, later found in many other proteins involved in cellular signaling [74, 91], tyrosine protein kinases, protein tyrosine phosphorylation/dephosphorylation, etc. that have changed our understanding of cell biology as well as oncology [62].

Src is the prototype member of the SFKs, a family of non-receptor tyrosine kinases consisting of nine members (Src, Yes, Fyn, Fgr, Lyn, Hck, Lck, Yrk, and Blk), four of them Src, Yes, Yrk, and Fyn are ubiquitously expressed [91]. The structure of SFKs is modular (Fig. 7.2). At the amino-terminus it contains signals for interactions with fatty acid (myristic and palmitic acids), which allow them to associate with the cellular membrane. The first 40–60 amino acids constitute the unique domain (U, also named as SH4), which is the most dissimilar region among family members, therefore providing specificity. The Src homology domain 3 (SH3) allows intra/intermolecular interactions with proline-rich sequences. The Src



c-Src mutants	Kinase activity	SH3	SH2
SrcK$^-$: K295M (ATP_{BS})	Inactive	Inactive/Active	Inactive/active
SrcDN : K295M/Y527F	Inactive	active	active
SrcΔK : Δ A259-L533	Inactive	active	active
Src-SH3$^-$: W118A	Active	Inactive	Active
Src-SH2$^-$: R175L	Active	Active	Inactive

Fig. 7.2 Structure and functionality of the SFKs. The SFKs have a modular structure; the nine members have at amino-terminus sequence an acylation signal, allowing their association with plasma membrane, which is followed by the unique domain (U or SH4), the SH3, SH2, the linker or bridge, the kinase domain (KD, SH1), and a regulatory sequence. The indicated residues, which positions referred to chicken nomenclature, are essential to maintain functionality of the Src family members. The K295 is required for binding to ATP (ATP_{BS}) and auto-phosphorylation of Y416 induces maximal enzymatic activity. Phosphorylation of Y527 by CSK facilitates its intramolecular interaction with the SH2 domain. This inactive conformation, stabilized by the contact of the proline residue of the linker/bridge, prevents functionality of the SH2, SH3, and KD. This restrained structure can be released by several mechanisms causing Y527 to be unphosphorylated: displacement by another SH2-containing protein with higher affinity for the p-Y527, mutation of Y57F or deletion of this carboxyl-terminus tail, as occurs in the oncogenic form of SFKs. Furthermore, mutations of W118A and R175L cause functional inactivation of SH3 and SH2 domains, respectively, provoking constitutive activation of KD.

homology domain 2 (SH2) binds to tyrosine phosphorylated proteins. The linker, a connecting sequence between the SH2 and the catalytic domain, contains a proline residue. The catalytic domain (KD or SH1) includes the amino-terminus lobe, which contains the K295 (chicken nomenclature) residue required for ATP-binding and the carboxyl-terminus activation loop. In contrast to the viral proteins (v-Src), the proto-oncogenic (c-Src) has a carboxyl-terminus regulatory tail containing a tyrosine residue (Fig. 7.2) [74, 91].

This conserved modular structure among family members suggests a common regulatory mechanism. Interestingly, tyrosine phosphorylation/dephosphorylation was found to control conversion between inactive and active conformation of SFKs. In this context, Src was identified as a phosphorylated protein of 60 kDa (pp60-c-Src) with tyrosine kinase activity [91]. Two tyrosine residues Y416 and 527 (chicken nomenclature, used from now on) were identified. The Y416 resides in the activation loop of the kinase domain and its autophosphorylation fully activates enzymatic activity. The Y527 is at the carboxyl-terminus regulatory tail that when phosphorylated by the CSK (carboxyl-terminus Src kinase) interacts intramolecularly with the SH2 domain. At the same time, the SH3 domain binds the linker containing a proline residue. Together, these intramolecular interactions induce a closed conformation of the SFKs and restrain their catalytic activity. Under this condition not only the kinase activity is blocked but also the adapter SH2 and SH3 domains are occupied and, therefore, cannot interact with other cellular proteins to form signaling complexes [91] (Fig. 7.2).

The SFKs members can be activated through the interaction of a variety of receptors for growth factors, cytokines, steroid hormones, etc. [16, 26, 31, 65, 68, 74, 87, 91] and transmit intracellular signals upon receptor activations. Also, integrins activate the Fak (focal adhesion kinase)/Src complex, which in turn regulates adhesion, migration, invasion, etc. [66]. Therefore, we can conclude that the SFKs are implicated in the regulation of many cellular signal transduction pathways that control division, motility, adhesion, migration, angiogenesis, survival, differentiation, etc. [29, 35, 91, 102]. It is then not surprising that deregulation of their expression and/or activity is associated with a variety of tumors [35, 90, 97, 104]. However, there is no evidence for Src kinase mutations associated with overexpression and/or hyperactivation of these proto-oncogenes in cancer. It is the action of CSK by phosphorylating the carboxyl-terminus tyrosine residue Y527 and inactivating SFKs [71], or the counteracting protein tyrosine phosphatases de-phosphorylating Y527 and activating SFKs [13, 70], the major players of this game.

7.4 Interaction of Prolactin Receptor and SFKs

The first published report about activation of SFKs by PRL described the interaction of PRLR with Fyn in Nb2 cells. In these cells, which express the three major isoforms of the PRLR (short, intermediate, and long forms), Fyn is constitutively associated with all isoforms of PRLR. PRL stimulation of Nb2 causes activation of Fyn. Furthermore, PRLR dimerization appears to be a requisite for Fyn activation [20]. In early 1990s, my colleague J.P. García-Ruiz (Centro de Biología Molecular Severo Ochoa (CBMSO), Madrid) observed a protein of 60 kDa tyrosine-phosphorylated in PRL-stimulated hepatocytes from lactating rat, and in collaboration with her we identified this protein as c-Src (pp60-c-Src). In this biological model, PRL increases association of c-Src to PRLR and its activation [11]. The short form of PRLR is predominant in rat liver [67].

As shown that PRLR interacts with Src and Jak kinases, a question was raised as to what was the interrelationship between these tyrosine kinases and PRLR. Using chick embryo fibroblasts (CEF) that do not express PRLR and the retroviral expression vectors RCAS with different envelope proteins (RCAS-A and RCAS-B) [42], PRLR long isoform from rat ovary [72], Jak2, and Src mutants were expressed in these cells [30].

As a common feature of the cytokine receptor superfamily, the PRLR isoforms contain a proline-rich sequence (PPVPGP) within the cytoplasmic juxtamembrane region named Box I, which is required for Jak2 association and activation by PRL. Therefore, mutations or deletions of these proline residues within Box I prevent binding of Jak2 and receptor functionality [22, 24, 56, 75]. In this context, while PRL stimulation of the wild-type receptor in CEF induces phosphorylation of the receptor, Jak2, and Src, expression of a PRLR mutant with all proline residues of Box I substituted by alanine residues (AAVAGA, $PRLR_{4P\text{-}A}$) inhibits tyrosine phosphorylation of the PRLR and association/activation of Jak2, but does not block Src stimulation. However, expression of Jak2 with the kinase-domain deleted (Jak2ΔK) together with the wild-type PRLR prevents receptor phosphorylation but not SFKs activation, indicating that Jak2 phosphorylates PRLR. Furthermore, a c-Src kinase-defective mutant ($SrcK^{-}$), containing the K295M point mutation, which avoids interaction of SFKs catalytic domain with ATP, does not alter PRLR tyrosine phosphorylation upon PRL stimulation [30].

To further determine the SFKs requirement for interaction with the PRLR, CEF were infected with RCAS-A comprising the wild-type long isoform of the receptor, and RCAS-B, containing different c-Src mutants with deletion of either myristoylation signal (precluding association to the plasma membrane), SH3 (c-SrcΔSH3), SH2 (c-SrcΔSH2), or the kinase domain (c-SrcΔK). The results show that the SH2 or the SH3 domains of Src are not required for interaction with the PRLR. However, the non-myristoylation mutant abrogates PRLR/c-Src interaction, indicating that location of c-Src at the plasma membrane is essential (Fig. 7.3)

These data indicate that the long form of PRLR interacts with both c-Src and Jak2. Then, PRL activates both kinases, but it is Jak2 that phosphorylates the receptor [30] (Fig. 7.4). Depending on the biological system and the physiological conditions, the consequences of these activations could induce proliferation, survival, or differentiation responses. Other receptors also activate SFKs and Jak2, among them receptors for GH, erythropoietin, thrombopoietin, interleukins, etc. [45, 48, 54, 80, 92, 101].

We can conclude that the three major isoforms of PRLR are able to interact and activate SFKs members in different cellular models [1, 2, 11, 18, 20, 30, 31, 33, 87, 77]. However, the short form of PRLR is not able to induce gene transcription of milk proteins [12, 21, 57, 85].

Phosphorylation of PRLR by Jak2 has multiple biological consequences. Thus, phosphorylated tyrosine residues at the carboxyl-terminus region serve as docking sites for Stat5 association and subsequent phosphorylation by Jak2. Indeed, substitution of the last tyrosine residue for phenylalanine (Y/F) results in abrogation of PRL induction of Jak2/Stat5/ß-casein [3, 55, 76].

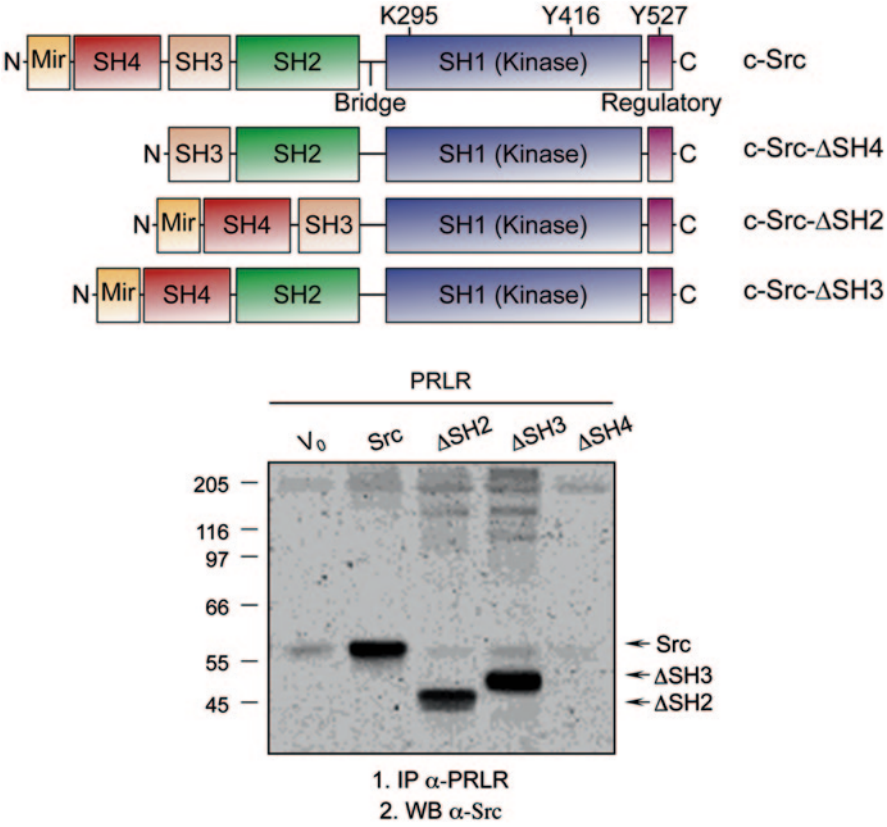


Fig. 7.3 Interaction between PRLR and SFKs. Chicken embryo fibroblasts (*CEF*) were retrovirally infected to express the long isoform of the PRLR from rat ovary and different forms of c-Src. The association of PRLR and c-Src forms was determined by immunoprecipitation (*IP*) of PRLR followed by western blot (*WB*) detection of Src.

7.5 Regulation of Jak2 by SFKs

The association of Src kinases and Jak2 with PRLR and their activation by PRL-induced receptor dimerization brings about the question of whether or not there is a relationship between these two tyrosine kinases.

Interestingly, PP1, a SFKs catalytic activity inhibitor, does not alter PRL-induced PRLR tyrosine phosphorylation and Jak2/Stat5 activation in W53 cells, indicating that SFKs enzymatic activity is not involved in PRL stimulation of the PRLR/Jak2/Stat5 pathway [31]. Similarly, PP1 does not inhibit Jak2 autophosphorylation in T47D [1]. In this context, another SFKs inhibitor, SU6656, also fails to inhibit PRL activation of Jak2 in T47D [2]. As shown in CEF [30], in W53 cells tetracycline-conditional (Tet-On system) expression of $SrcK^{-}$ does not block PRL activation of the Jak2/Stat5 pathway. However, the dominant negative c-Src mutant (SrcDN,

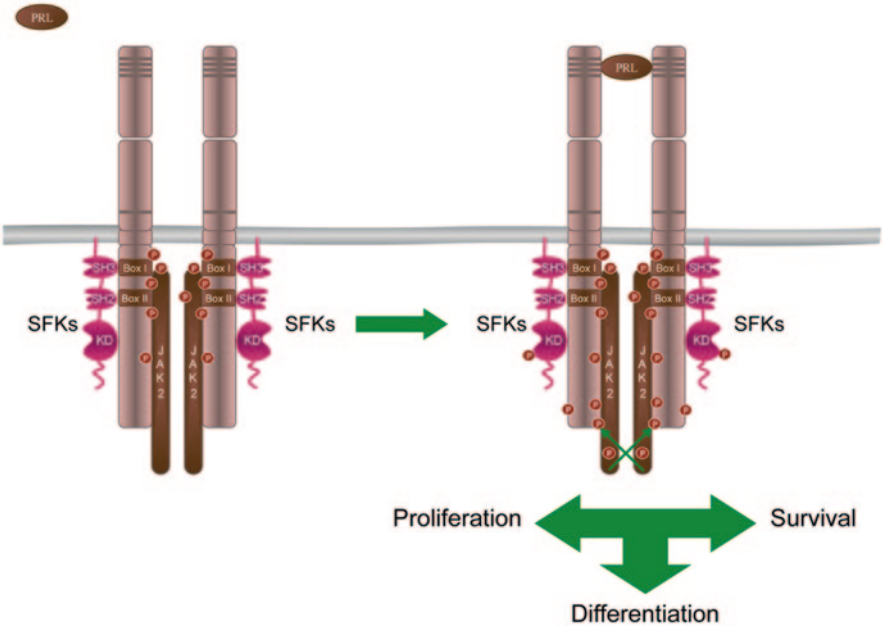


Fig. 7.4 PRLR dimerization and Jak2 and SFKs activation. Src kinases and Jak2 are associated with PRLR. Upon cellular stimulation with PRL, receptor dimerization takes place and Src kinases and Jak2 activate. Jak2 phosphorylates PRLR under the control of Src kinases. Subsequently, several signaling pathways induce cellular responses that, depending on physiological conditions, could be proliferation, differentiation, survival, etc.

c-Src-K295M/Y527F), devoid of enzymatic activity but with functional SH2 and SH3 domains, inhibits PRL activation of Jak2/Stat5, suggesting that these docking regions of SFKs may control this event. Consistently, expression of SrcΔK, which only contains functional SH2 and SH3 domains, blocks PRL stimulation of the Jak2/Stat5 pathway. However, conditional expression of Jak2ΔK in W53 cells does alter PRL activation of SFKs [33]. Furthermore, in MCF7 cells, conditional expression of SrcDN also abrogates Jak2/Stat5 activation. Moreover, functional inactivation of either the SH2 domain (c-Src-SH2^{-}, c-Src-R175L) or the SH3 domain (c-Src-SH3^{-}, c-Src-W118A), which consequently have constitutive kinase activity, inhibits Jak2/Stat5 pathway. Together, these results support the conclusion that the function of the SH2 and SH3 adapter domains, independently of the catalytic activity of SFKs controls the Jak2/Stat5 pathway. Besides, constitutive suppression of c-Src in MCF7 cells by means of a specific shRNA significantly represses PRL activation of Jak2/Stat5 pathway. Also, *src*$^{-/-}$ mice appear to have normal mammary gland development during pregnancy but fail the secretory activation. These animals show normal levels of PRL but reduced expression of PRLR at postpartum with diminished activation of Stat5 [95]. In extracts from the mammary gland isolated from *src*$^{-/-}$ mice at lactation, Jak2/Stat5 is impaired as compared to controls from *src*$^{+/+}$ mice [33]. In this context, in mammary epithelial cells isolated from mid-pregnant *Jak2*$^{-/-}$ and

Cdkn2a$^{-/-}$ mice PRL induces Src/Fak/Erk1-2 activation [84]. Moreover, stimulation of NE1 and A431 cells by EGF or NDF activates the Jak2/Stat5 pathway, which is blocked by selective inhibitors of SFKs catalytic activity PP1 and CGP77675, as well as by expression of SrcDN. These authors propose that EGF binds EGFR (ErbB1) inducing receptor dimerization and autophosphorylation, which in turn recruits Src that phosphorylates and activates the receptor preassociated Jak2 and Stat5 [73]. CGP77675 directly inhibits Jak2 activity in in vitro kinase assays (unpublished data).

7.6 Role of Shp2 in SFKs Regulation of Jak2 Activation

We have been unable to detect direct interaction between SFKs and Jak2 by co-immunoprecipitation assays, which suggests the existence of another interconnecting molecule. In this context, the tyrosine phosphatase Shp2 has been implicated in PDGF-stimulated SFKs activation in fibroblasts [94, 104] and in Jak2 activation by PRL and IL-3 [10, 103]. Shp2 contains two amino-terminus SH2 domains followed by the catalytic domain, and carboxyl-terminuses Y542 and Y580, separated by a proline-rich sequence [69] (Fig. 7.5a). Crystallographic studies of C-terminus-truncated Shp2 demonstrated that its basal catalytic activity is inhibited because of interaction between N-SH2 and phosphatase domains [38]. A role of Y542 and Y580 phosphorylation in the regulation of the phosphatase activity has been suggested. Thus, substitution of these residues by non-hydrolysable phosphonyl amino acids stimulates phosphatase activity, which in combination with mutations of the SH2 domains indicates that the interaction between phosphorylated Y542 and N-SH2 domain releases basal inhibition [59]. Moreover, phosphatase activity is required for all actions of Shp2, including signaling cascades stimulated by growth factors, cell adhesion molecules, and cytokines [3, 69, 78]. However, it remains to be determined if Jak2 and SFKs can regulate Shp2 activity through Y542 and Y580 phosphorylation. This event occurs in sequential fashion [5] inducing conformational changes that promote Shp2 functions [59, 60, 69]. Indeed, conditional expression of Shp2-Y542F-Y580 in W53 cells prevents PRL-induction of Shp2-Y580 phosphorylation (Fig. 7.5b). SFKs functionalities play a role in PRL stimulation of Shp2 phosphorylation. While conditional expression of SrcK^{-}, SrcDN, or SrcΔK mutant in W53 cells shows Shp2 colocalized with all c-Src interfering mutants at the plasma membrane (Garcia-Martinez, unpublished data), its phosphorylation is blocked by SrcDN and SrcΔK but not by SrcK^{-} (Fig. 7.6a). Interestingly, the endogenously expressed Src family members Fyn and Lyn, which are the only ones activated by PRL in W53 cells [31], interact with unphosphorylated Shp2 (Garcia-Martinez, unpublished data). Nonetheless, the Fyn-Shp2 and Lyn-Shp2 complexes are reduced upon induction of SrcDN and SrcΔK, presumably because overexpression of Src docking domains facilitates SFKs-Shp2 association (Fig. 7.7a). In this context, interaction of c-Src-SH3 domain and Shp2-proline-rich sequence, demonstrated by pull-down experiments [94], is responsible for stabilization of the active form of

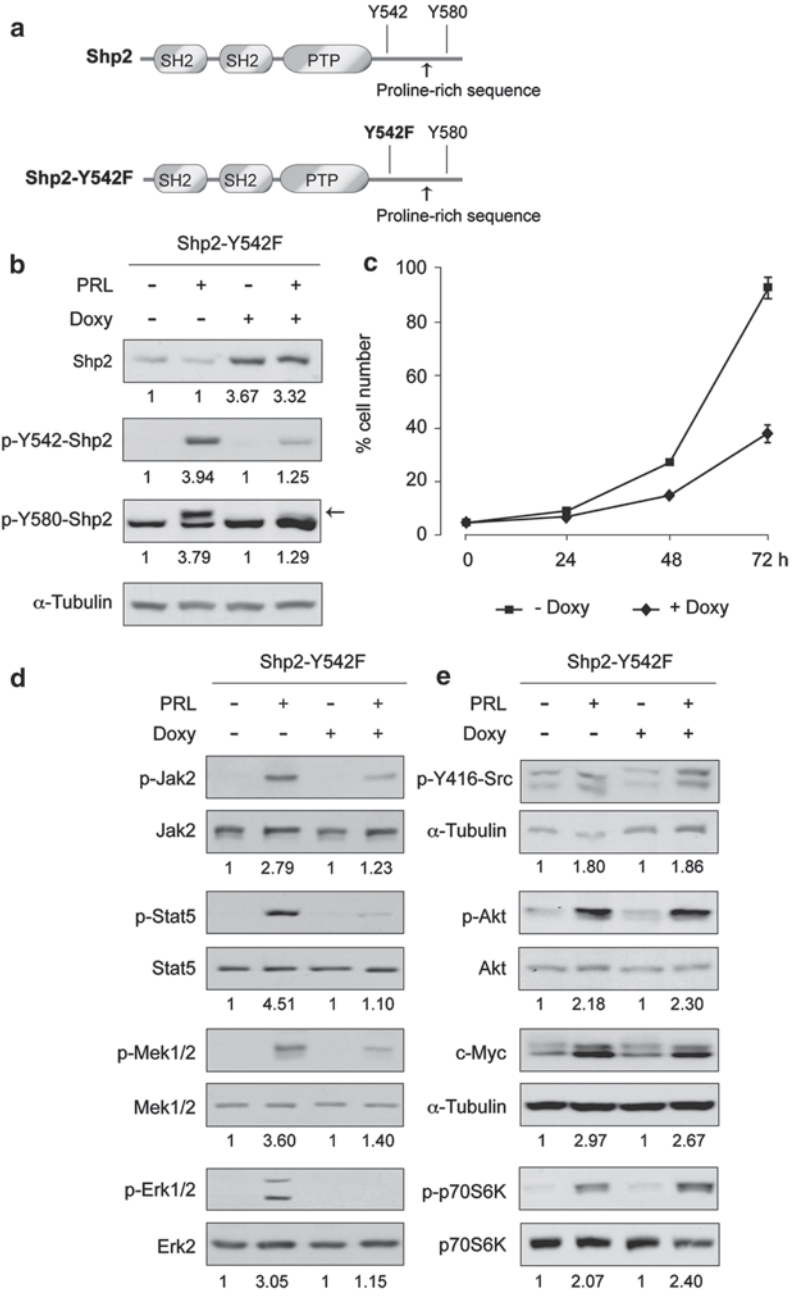


Fig. 7.5 Role of Y542-Shp2 phosphorylation on PRL stimulation of cell proliferation and signaling. **a** Schematic structure of Shp2 and Shp2-Y542F-Y580 mutant. **b** Exponentially growing cultures of W53 (5×10^5 cells/ml) with conditional expression (Tet-On system) of Shp2-Y542F-Y580 were transferred overnight to PRL-depleted media in the absence or presence of Doxy (2 μg/ml).

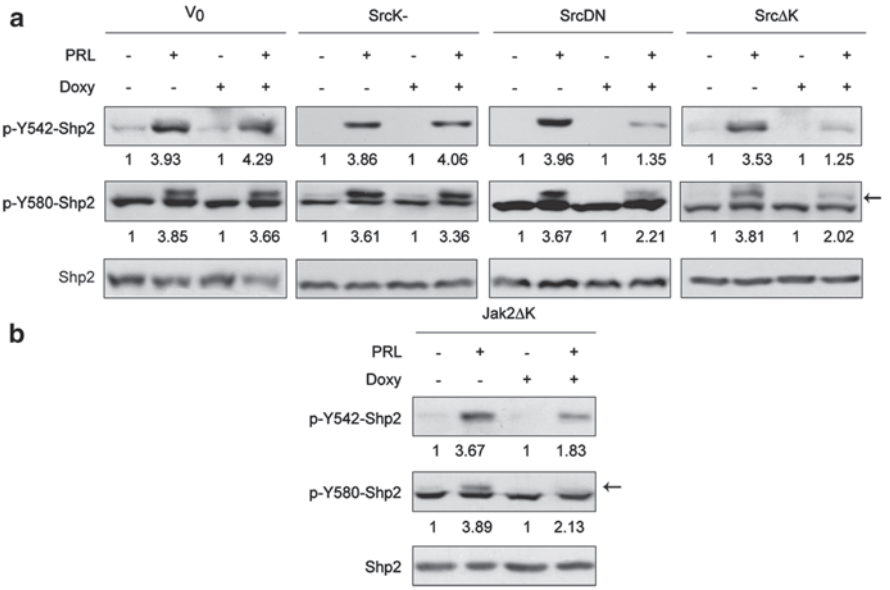


Fig. 7.6 Role of SFKs and Jak2 kinases on PRL stimulation of Shp2 phosphorylation. **a** Exponentially growing cultures of W53 (5×10^5 cells/ml) with conditional expression (Tet-On system) of SrcK$^-$, SrcDN and SrcΔK or empty vector V_0 were treated as in Fig. 7.5b. Protein extracts (20 μg) were used to determine p-Y542- and p-Y580-Shp2 by WB with specific phospho-antibodies. Membranes were then reblotted with anti-Shp2 as a loading control. **b** Extracts (20 μg) from exponentially growing cultures (5×10^5 cells/ml) of cells with conditional expression of Jak2ΔK were treated as in (**a**) and used to determine the specific phosphorylation sites of Shp2. The results represent one of three independent experiments.

SFKs [37]. Furthermore, SrcDN, SrcΔK, as well as Jak2ΔK and Shp2-Y542F-Y580 reduce Shp2-PRLR complex (Fig. 7.7b). This finding is likely due to inhibition of tyrosine phosphorylation of Shp2 by these interfering mutants (Figs. 7.5b and 7.6), which induce a closed conformation with inaccessible SH2 domains, as previously shown [59, 60, 69]. In addition, these mutants inhibit phosphorylation of PRLR, to which the carboxyl-terminus Shp2 binds [3]. In turn, Shp2 appears to be

Cells were then stimulated with PRL (100 ng/ml, 15 min). Protein extracts (20 μg) were used to determine Shp2, p-Y542-, and p-Y580-Shp2 by WB. The arrow indicates the p-Y580-Shp2 band. The lower band is, according to the company New England Biolabs, nonspecific. **c** To determine proliferation, cells were plated at 3 × 104 cells/ml in complete medium containing 5 ng/ml of PRL, and cultured in the absence or presence of Doxy (2 μg/ml) for 24, 48, and 72 h. Cells were collected by centrifugation, incubated with trypan blue, and viable cells were counted. The percentage of cell growth was calculated considering the number of control cells at 72 h as 100 %. The results shown represent the average ± SD of three independent experiments carried out in triplicate**. d** Detection of p-Jak2, p-Stat5, p-Mek1/2, and p-Erk1/2 by WB was carried out as above. **e** WB detection of p-Y416-c-Src, p-Akt, Myc, and p-p70S6K was carried out as in panel (**b**). The quantifications were normalized by the values of their loading controls and referred to conditions-PRL, considered as 1. The results represent one of three independent experiments.

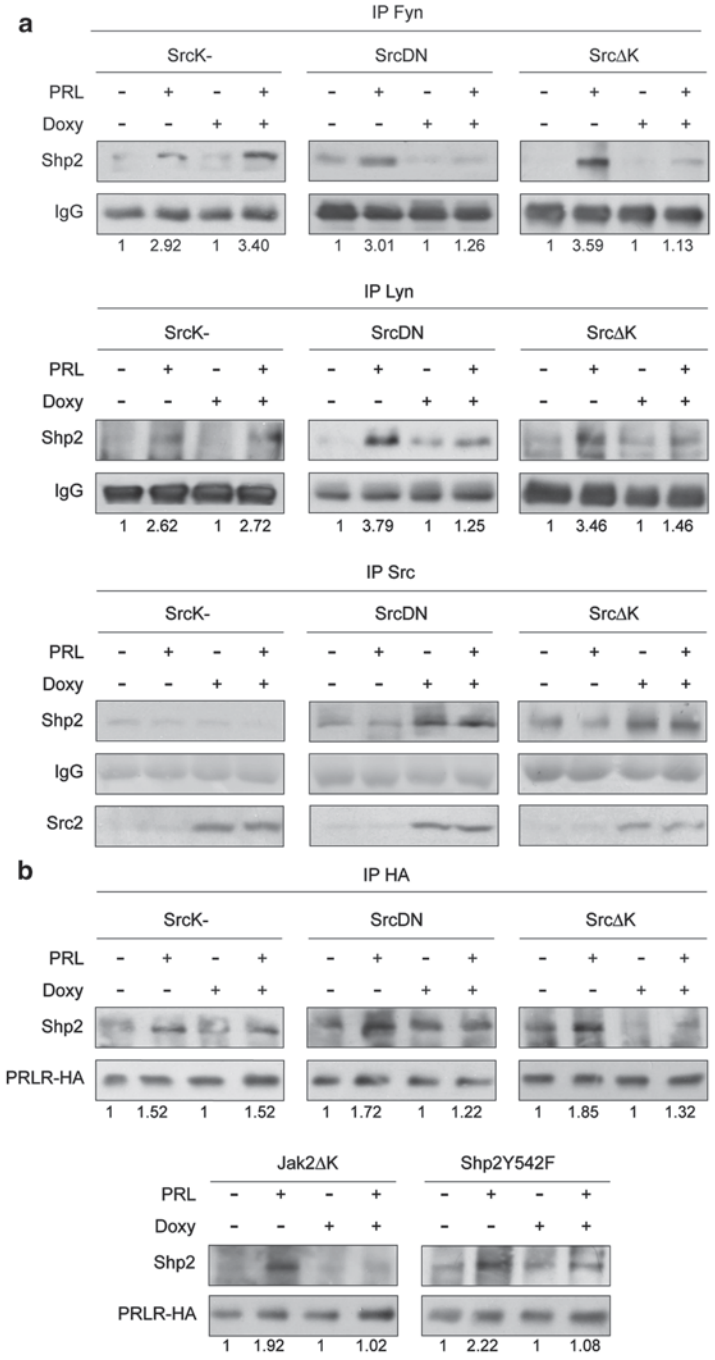


Fig. 7.7 Interaction of Shp2 with SFKs and PRLR. Exponentially growing cultures of W53 (5×10^5 cells/ml) were treated as in Fig. 7.5b. **a**, **b** Clear cell lysates were used to immunoprecipitate Fyn, Lyn, or c-Src mutants (**a**) or the PRLR HA-tagged (**b**). Immune-complexes were used to

involved in Jak2 activation, as Shp2-Y542F-Y580 blocks PRL activation of Jak2/Stat5 (Fig. 7.5d). In this context, Shp2 by dephosphorylating Jak2 Y1007 impedes SOCS-1 association and ubiquitination, which ultimately leads to Jak2 stabilization [4]. This reciprocal modulation between Shp2 and Jak2/Stat5 is also observed in mice with selective suppression of Shp2 in the mammary gland, where PRL stimulation of PRLR-Jak2/Stat5 complex and the subsequent activation of Jak2/Stat5 are impaired [50]. Similarly, Shp2 is required for physical association/activation of Stat5a and milk protein gene transcription in PRL-stimulated HC11, a nontumorigenic mouse mammary epithelial cell line [8], and in GH-treated T47D [19]. In addition, in MCF7 cells, expression of SrcDN or c-Src depletion inhibits PRL-induced Jak2 and Shp2 phosphorylation (Fig. 7.8) [33]. Interestingly, in this breast cancer cell line SFKs catalytic activity appears to be involved in PRL regulation of Shp2 association and dephosphorylation of IGF-IR, which blocks its internalization maintaining its activation [18]. In contrast, suppression of Shp2 in T47D does not prevent PRL activation of Jak2/Stat5 [49]. Furthermore, we cannot exclude the possibility that Shp2 may have different functions while interacting with either SFKs or Jak2, since the proline-rich sequence and the tyrosine-phosphorylated residues could also serve as docking as shown for Grb2 [5] and Gab2 [3].

Together, these findings indicate that SFK-scaffold functions regulate Shp2 activation in PRL-stimulated W53 and MCF7 cells. Considering these results and those published by others we propose a model in which PRL causes receptor dimerization and activation of Jak2, Fyn, and Lyn. These activated SFKs bind to unphosphorylated Shp2. At the same time, Jak2 phosphorylates PRLR, which favors Shp2–SFKs complex dissociation due to the interaction of SH2 domains of Shp2 with the phosphorylated PRLR. In turn, Jak2 sequentially phosphorylates Shp2 at Y542 and Y580, as proposed [58, 63]. Alternatively, another kinase may be involved in Shp2 phosphorylation. The phosphorylated/activated Shp2 then controls Jak2 activation and also serves as docking site for Grb2 and Gab2 [3, 5] (Fig. 7.9).

7.7 Role of SFKs in PRL Induction of Cell Proliferation

PRL can induce survival, proliferation, or differentiation depending on the cell type, the physiological condition, and the stimuli. Cell survival as a result of PRL-induced SFKs activation has been observed in a variety of cell systems, including W53, MCF7, T47D, etc.[1, 2, 23, 36].

Several pieces of evidence support the role of SFKs in mammary gland development and lactogenesis. In HC11, expression of SrcK$^-$ inhibits ß-casein production

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detect Shp2, c-Src mutants, or PRLR by WB. Detection of immunoglobulins and anti-HA from immunoprecipitation served as loading controls. Values were normalized as in Fig. 7.5. The results represent one of three independent experiments.

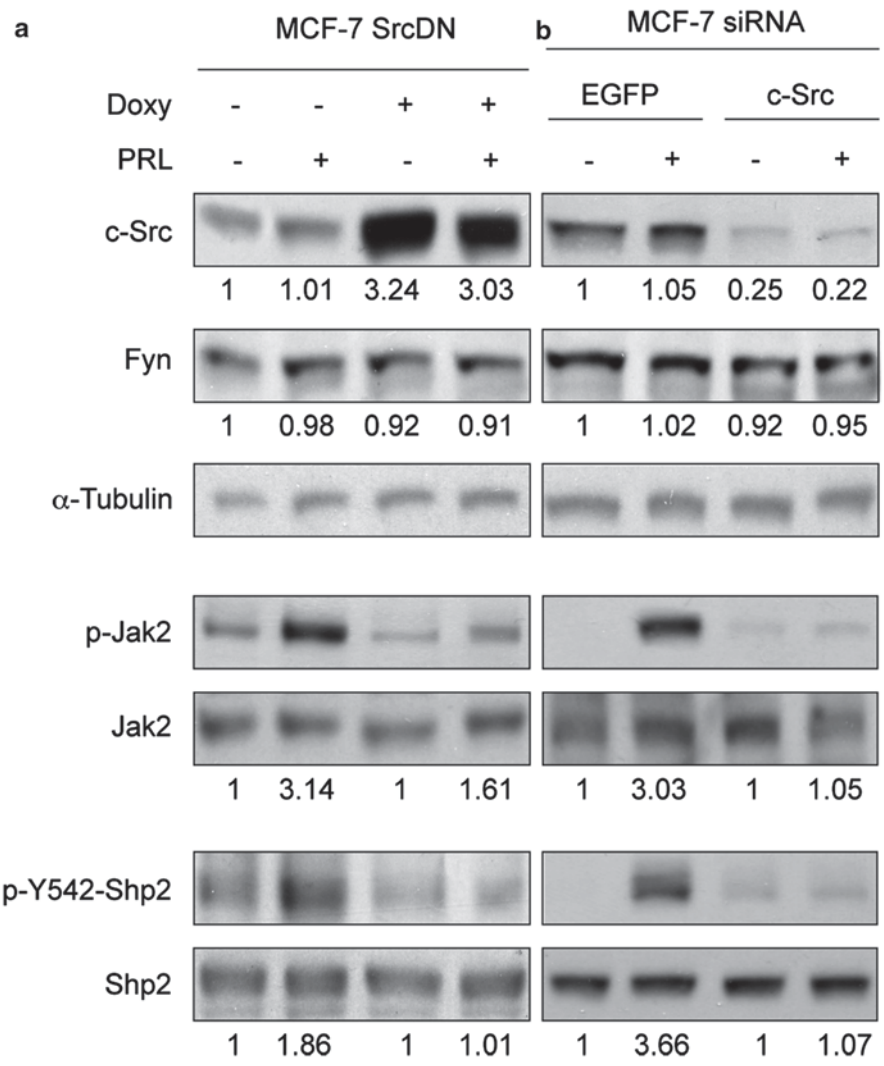

Fig. 7.8 Role of c-Src in PRL stimulation of Jak2 and Shp2-Y542 phosphorylation in MCF7. **a**, **b** Exponentially growing cell cultures of MCF7-Tet-On-SrcDN (**a**) and of c-Src-siRNA-MCF7 and EGFP-siRNA-MCF7, as a control (**b**), were transferred for 48 h to serum-free media for starvation. The MCF7-Tet-On-SrcDN cultures were maintained during this time in absence or presence of Doxy (2 μg/ml). Cell cultures were then stimulated with PRL (100 ng/ml, 15 min). Protein extracts (20 μg) were used to determine the levels of c-Src, Fyn, and Jak2 and Shp2-Y542 phosphorylation by WB. Membranes were reblotted with α-tubulin, anti-Jak2, and anti-Shp2 for loading controls. The quantified values were normalized to their loading controls and referred to condition-PRL, considered as 1.

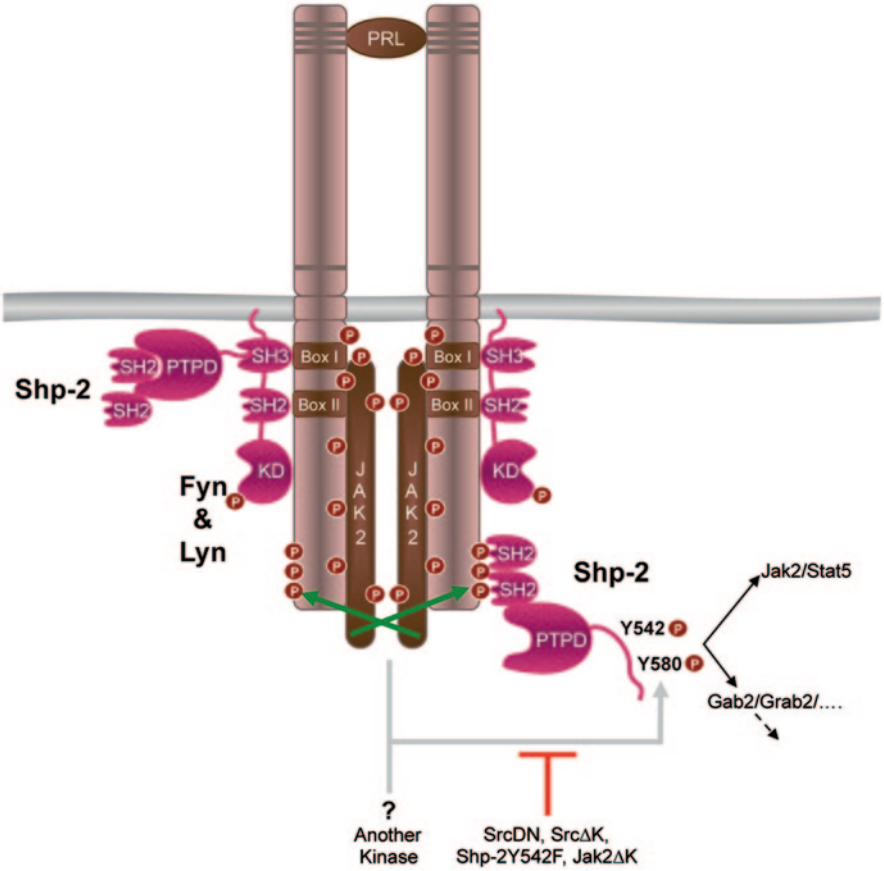

Fig. 7.9 Complex interaction between SFKs, Jak2, and Shp2. The interaction PRL-PRLR causes activation of Fyn, Lyn and Jak2 and phosphorylation of PRLR. Shp2 associates through its proline-rich region with the SH3-SFKs. Upon tyrosine phosphorylation of PRLR, Shp2 forms a complex with the receptor via its carboxyl-terminus SH2, facilitating Y542 and Y580 phosphorylation by Jak2 or another kinase. These phosphorylations in Shp2 eliminate its restricted conformation. Shp2 then positively regulates Jak2/Stat5 and serves as docking molecule to form complexes with Gab2, Grab2, etc., diversifying signaling pathways.

induced by the lactogenic complex (PRL, insulin, hydrocortisone) [89]; we have also observed this effect by conditional expression of SrcDN (unpublished results). However, overexpression of viral Src blocks mammary gland epithelial cell differentiation, inhibiting ß-casein gene expression [47]. Deletion of c-Src in mice causes defects in development and lactation [32, 52, 95] and prevents PRL activation of Jak2/Stat5 in mammary gland explants [33]. In pancreatic ß-cells, inhibition of SFKs catalytic activity by PP2 completely abolishes the increase of intracellular [Ca^{2+}] and insulin secretion induced by ovine PRL [106].

SFKs are also required for PRL stimulation of cell proliferation. Multiple pieces of evidence support this premise. In hepatocytes from lactating rats, PRL activates c-Src

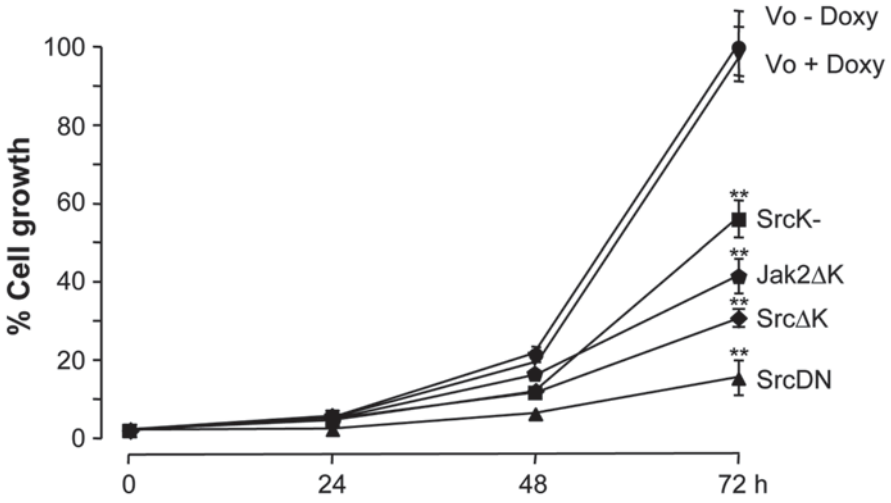

Fig. 7.10. Role of Src kinases and Jak2 in PRL stimulation of cell proliferation. W53 cells conditionally expressing c-Src mutants or Jak2ΔK were plated in complete medium containing 5 ng/ml of PRL, and cultured with or without Doxy (2 μg/ml) for 24, 48, and 72 h. Cells were collected, incubated with trypan blue and viable cells were counted. The percentage of cell growth was calculated considering the number of control cells (Vo, empty vector) at 72 h as 100 %. The results represent the average ± SD of three independent experiments carried out in triplicate. $^{**}p \leq 0.01$ ($n=3$). [33].

and induces expression of c-*fos* and c-*jun* [11]; in Nb2 cells PRL stimulation of Fyn matches proliferation [20]. Different and complementary approaches, such us the use of inhibitors of the SFKs catalytic activity or conditional expression of c-Src mutants, later confirmed the involvement of SFKs in cell proliferation control by PRL.

The inhibitors of SFKs catalytic activity PP1 and herbimycin A abrogate [^{3}H]-thymidine incorporation in PRL-stimulated W53. Furthermore, BrdU pulse-label experiments show that in these cells PP1 blocks cell cycle in G1, indicating that the tyrosine kinase activity of SFKs is required for the G1/S transition [30]. PP1 and PP2 also reduce cell proliferation of MCF7 and T47D induced by PRL [1, 23], while SU6656, another SFKs inhibitor, blocks PRL signaling pathways required for MCF7 and T47D proliferation [2, 36].

Furthermore, transient expression of PRLR together with Csk or with c-SrcK$^-$ in BaF-3 cells partially prevents PRL-induced [^{3}H]-thymidine incorporation. SrcDN and Jak2ΔK inhibit more effectively PRL-induced cell proliferation than Csk or c-SrcK$^-$ [31]. Supporting these observations, conditional expression of c-SrcK$^-$, c-SrcDN, c-SrcΔK, or Jak2ΔK in W53 cells causes analogous results [33] (Fig. 7.10). The strongest inhibition of cell proliferation observed with SrcDN could be explained by the fact that, in addition to being a kinase-dead mutant, it also has an open conformation that exposes its SH2 and SH3 domains. Indeed, SrcΔK that only expresses fully active SH2 and SH3 causes similar effect. These findings suggest the involvement of SH2 and/or SH3 domains of SFKs in the control of PRL-induced cell proliferation. In addition, these results also indirectly support the dependency

of Jak2 activation on the SFKs, as the inhibitory effect of Jak2ΔK is similar to that obtained by SrcDN or SrcΔK. Moreover, SrcDN blocks PRL activation of Jak2 in W53 and MCF7 cells [33].

7.8 Role of SFKs-depending Signaling Pathways in the Regulation of IEG Expression

Cell cycle progression through the G1 phase is a prerequisite for DNA replication (S phase) and cell division. The immediate early genes (IEG) expression is required for G1/S transition. Since inhibition of SFKs blocks W53 cell cycle at G1 [31], it is important to study the signaling pathways controlling IEG expression to understand the involvement of SFKs in the control of cell proliferation by PRL.

In hepatocytes from lactating rats, PRL activates c-Src and induces expression of c-*fos* and c-*jun* [11]. PRL stimulation of W53, MCF7, and T47D proliferation induces expression of IEG c-*fos*, c-*jun*, and cyclin D1, which in turn controls c-Myc expression. The cellular levels of these essential factors are controlled, at least in part, by catalytic activity of SFKs, as their expression is reduced by PP1/PP2/SU6656 [1, 2, 23, 31].

Also, conditional expression of SrcK^{-}, SrcDN, and Src-ΔK in W53 cells significantly reduces c-Myc expression [33]. Induction of c-*myc* expression by PRL depends on the SFKs/PI3K/Akt pathway. Upon phosphorylation/activation, Akt translocates to the nucleus where it phosphorylates FKHRL1 and induces nuclear export of this transcription factor that represses c-Myc transcription. Akt also phosphorylates and inactivates GSK3ß, which in turn abrogates Myc phosphorylation and its subsequent degradation. Consequently, these mechanisms stabilize c-Myc expression. Interestingly, while in W53 cells one can delineate a SFKs/Akt/FKHRL1 and GSK3ß pathway for the control of Myc expression, which is not altered by conditional expression of Jak2ΔK [23, 33], studies in mammary epithelial cells isolated from conditional Jak2$^{-/-}$ mice indicate that Jak2 controls Akt activation and cyclin D1 expression [84].

Expression of c-*fos* in W53 cells does not require the SFKs/PI3K dependent pathway, as it is not affected by LY294002. Instead, it is regulated by Mek1/2-Erk1/2 pathway as PRL induction of c-*fos* is abrogated by PD184352, an inhibitor of Mek1/2 activity [23]. Since Jak2ΔK prevents Mek1/2-Erk1/2 activation and SFKs regulates Jak2, the SFKs/Jak2/Mek1/2-Ekr1/2 pathway appears to control c-*fos* expression [33]. Furthermore, Shp2 association with SFKs, tyrosine phosphorylated PRLR, and Jak2 ends in Shp2 phosphorylation at Y542 and Y580. Phosphorylated Y542-Shp2 in response to FGF or PDGF serves as docking site for Grb2 that is required for Ekr1/2 activation [5]. Then Shp2 may be mediated through its interaction with Grb2 in PRL induction of c-*fos*.

Analyses of the signaling cascades that control IEG expression below SFKs show that PRL induction of cell proliferation involves different mechanisms depending

on the cell type. Thus, in MCF7 and T47D induction of c-Myc and cyclin D1 by PRL requires Src activation of the PI3K and Mek1/2-Erk1/2 pathways, as shown by inhibition of these pathways by PP1, LY294002, and PD184352, respectively. The control of PI3K and the Mek1/2-Erk1/2 pathways by SFKs appears to be independent one from each other, as inhibition of PI3K by LY294002 does not alter activation of the Mek1/2-Erk1/2 and vice versa [1]. Also, in MCF7, PRL induction of AP1 is controlled by c-Src, Jak2, PI3K, and PKC through the control of Erk1/2 activation. However, in this study there is no hierarchical analysis among these pathways for Erk1/2-AP1 activation, with Jak2 seeming to be the major mediator [36]. Moreover, inhibition of SFKs enzymatic activity by SU6656 blocks PRL stimulation of Fak-Y925, PI3K/Akt, Mek1/2-Erk1/2 in MCF7 and T47D without affecting Jak2 phosphorylation [2], which is consistent with previous results obtained with PP1/PP2 [1].

7.9 Concluding Remarks

The data obtained in several biological models undoubtedly demonstrate the fundamental role of the SFKs and Jak2 for PRL signaling. These tyrosine kinases are preassociated with the three major isoforms of the PRLR and are activated upon receptor dimerization induced by ligand binding. Within the receptor juxtamembrane region there is a proline-rich sequence named Box I, which is present in most of the cytokine receptor superfamily. Multiple and undisputable evidence show that this is the domain where Jak2 binds to the receptor. It is also clear that Jak2 is mainly responsible for the tyrosine phosphorylation of the PRLR. As to where and how the receptor interacts with the SFKs there are no complete answers. Mutations at the proline residues of Box I of PRLR, which prevent binding and activation of Jak2, and consequently receptor phosphorylation, do not affect PRLR binding and activation of SFKs. Moreover, catalytic inactivating mutations of Jak2 reproduce the same effect. These data imply that the SFKs SH2 or SH3 domains may not be required for interaction with the PRLR. Indeed, deletion of c-Src SH2 or SH3 domains does not preclude binding to PRLR. Furthermore, the catalytic activity and the kinase domain are also not needed. However, it requires membrane location of c-Src. In this context, it will be important to precisely determine the structural requirements of the PRLR and the SFKs to interact with each other.

While SFKs and Jak2 appear to be needed for many of the biological actions of PRL, several pieces of evidence support the fact that inhibition of the enzymatic activity of one of them does not affect the other in PRL signaling. However, suppression of c-Src alters PRL activation of Jak2. Furthermore, the adapter domains SH2 and/or SH3 seem to be necessary for Jak2 activation. While both SFKs and Jak2 bind and are activated by PRL, there is no clear evidence for direct interaction between them. Considering the results obtained by several groups and our own data, we propose that the tyrosine phosphatase Shp2 is the nexus between both enzymes,

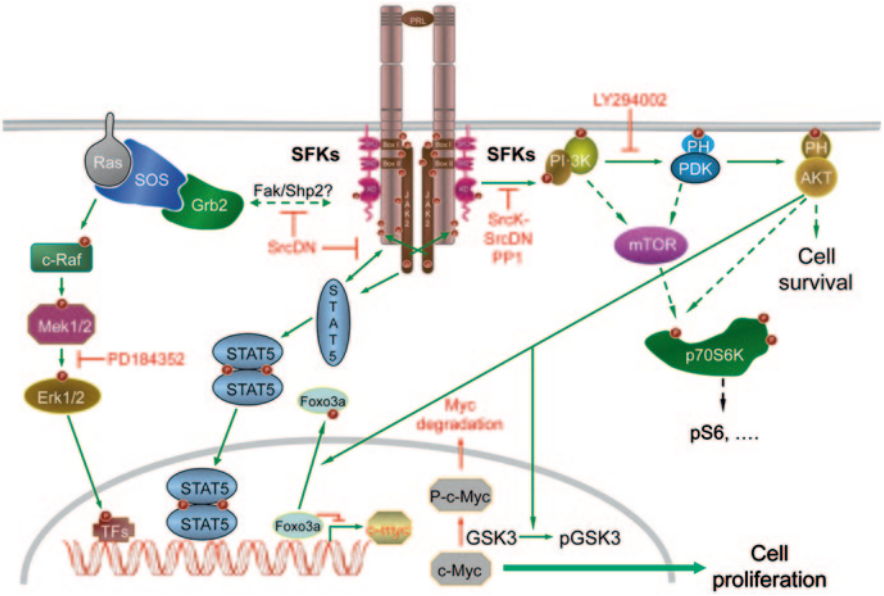

Fig. 7.11 Role of SFKs in PRL cellular signaling. PRL-induced PRLR dimerization causes activation of SFKs and Jak2, which in turns phosphorylates PRLR, in addition to the regulation of Shp2, which is involved in the control of Jak2 activation by SFKs (see Fig. 7.9). SFKs, through its tyrosine kinase activity and the adapter domains SH2 and SH3, regulate several signaling pathways, as deciphered by conditional expression of c-Src, Fak, Jak2, Shp2, Akt, and FKHRL1, as well as by the use of selective inhibitors of SFKs, IP3K, Mek1/2, and GSK3ß. The results obtained in several cell types [1, 11, 23, 30, 31, 33] allows us to delineate this incomplete schema, which describes some of the signaling cascades that control PRL signaling through the SFKs.

playing a role in the regulation of Jak2 by SFKs and subsequent regulation of some signaling pathways.

Integration of the results obtained in our multiple experimental systems led us to propose this complex signaling model for PRL (Fig. 7.11).

A variety of cytokines and growth factors through activation of their membrane receptors activate the SFKs, which in turn act as connecting networks controlling multiple signaling pathways eliciting different cellular responses depending on the biological model and the physiological conditions.

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Chapter 8
Prolactin-Induced Protein in Breast Cancer

Ali Naderi

Abstract Prolactin-induced protein (PIP) is a 17-kDa single polypeptide chain that is secreted by a number of normal apocrine cells, such as milk, saliva, and seminal fluid. PIP is widely expressed in breast cancer and is commonly used as a diagnostic biomarker for the histopathological diagnosis of this disease. Expression of PIP in breast cancer is regulated by androgen and prolactin through a number of transcription factors and signaling cross-talks, including STAT5, Runx2, and CREB1. Notably, PIP is induced by a positive feedback loop between androgen receptor (AR) and extracellular signal-regulated kinase (ERK). The available data indicate that PIP has an aspartyl protease activity that can degrade fibronectin. Importantly, PIP is necessary for outside-in activation of integrin-β1 signaling pathway and regulation of key downstream signaling targets of this pathway such as interaction of integrin-β1 with integrin-linked kinase 1 (ILK1) and ErbB2. Furthermore, the importance of PIP in cell proliferation has been demonstrated by the fact that purified PIP promotes growth of breast cancer cells and PIP expression is necessary for the proliferation of T-47D and MDA-MB-453 cell lines. In addition to cell proliferation, PIP mediates invasion of breast cancer cells in a process that partially depends on the degradation of fibronectin by this protein. Therefore, PIP is a breast cancer-related protein that is expressed in a majority of breast tumors and has a significant function in the biology of this disease.

8.1 Introduction

Prolactin-induced protein (PIP) is a 17-kDa glycoprotein that was originally identified as gross cystic disease fluid protein 15 (GCDFP-15) and a major component of breast cyst fluid, human milk, and saliva [21, 22]. Subsequently, a similar actin-binding protein "secretory actin binding protein" was discovered in seminal fluid [1]. PIP gene is located on chromosome 7q32–36 and is composed of four exons and only one 900 base mRNA transcript has been identified [32, 33]. Translated PIP is a

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146-amino acid long polypeptide that is found in salivary and lacrimal glands, prostate, mammary gland, and other organs [29].

PIP is shown to exhibit structural properties related to the aspartyl proteinase superfamily and site-specific mutagenesis of Asp22 has confirmed that this protein is an aspartic-type protease [8]. In this respect, the only known substrate for PIP protease activity is fibronectin [8]. This protease activity raises the possibility of a role for PIP as a secreted protein to mediate extracellular matrix degradation. The other established binding partner of PIP is CD4 and it is known that PIP binds to this receptor and blocks CD4-mediated T cell programmed death [19]. Therefore, the abundance of PIP in mucosa, saliva, tears, submucosal glands of the bronchi, and apocrine glands of the skin, suggests that PIP may play a role in mucosal immunity [24].

Other proposed physiological functions of PIP include inhibition of bacterial growth and a role in fertilization [24]. The salivary form of PIP binds to the surface of bacterial strains that are colonized in the mouth, ear canal, and skin resulting in the inhibition of bacterial growth [31]. PIP has the ability to bind to several bacterial strains and may inhibit their growth [31, 39]. Moreover, secreted PIP in the seminal fluid is localized on the postacrosomal region of ejaculated spermatozoa and may play a role in fertilization through fibronectin cleavage during liquefaction of seminal fluid [4, 24].

8.2 PIP Expression in Breast Cancer

PIP is expressed in a majority of breast tumors and has been used as a biomarker for the diagnosis of this disease [13, 23]. Genomic studies have revealed that PIP is overexpressed in luminal A and molecular apocrine subtypes of breast cancer [2, 15, 42]. Molecular apocrine is a subtype of estrogen receptor (ER)-negative breast cancer characterized by a steroid-response gene signature that includes androgen receptor (AR), FOXA1, TFF3, and a high frequency of ErbB2 overexpression [15, 17, 42]. AR expression is present in 40–50 % of ER-negative breast tumors and a majority of these cases also have ErbB2 overexpression [20, 34, 37].

We have studied PIP as one of the top ranking genes in molecular apocrine signature. This was established based on the fold-change for gene expression that was extracted from a meta-analysis microarray study performed by our group on 186 ER-negative breast tumors and an expression microarray study of ER-negative cell lines by Doane et al. [15, 42]. The combination of top 8 genes in Teschendorff et al.'s study and top 6 genes in Doane et al.'s study resulted in 12 unique molecular apocrine genes (Table 8.1), [35]. Importantly, PIP is present among the top ranking molecular apocrine genes in both ER-negative breast tumors and cell lines (Table 8.1).

We also investigated PIP protein expression using immunohistochemistry (IHC) in a cohort of 24 ER-negative breast tumors [35]. ER-negative breast tumors were classified into AR+ (molecular apocrine) and AR− subgroups. Importantly,

Table 8.1 Fold changes of top ranking molecular apocrine-signature genes in two studies. (This table is adapted from the study by Naderi et al. [35])

Gene	Fold change (Teschendorff et al.)	Fold change (Doane et al.)
FOXA1	2.55	7.8
TFAP2	2	44.7
BANP	1.9	N/A
S100A8	1.82	N/A
PER2	1.8	N/A
ErbB2	1.72	N/A
SOX11	1.71	N/A
DUSP6	1.66	N/A
AR	1.66	4.95
AZGP1	1.5	13.8
PIP	1.4	17.8
TFF3	1.11	6

N/A not applicable

AR+ breast tumors showed a markedly higher PIP expression (57 % ± 6) compared to AR− tumors (16 % ± 4) suggesting that molecular apocrine tumors have a higher level of PIP expression among ER-negative breast tumors (Fig. 8.1), [35]. In agreement with our findings, a recent study has shown that PIP is one of the best biomarkers for the immunohistochemical identification of molecular apocrine breast tumors [28].

8.3 Secreted PIP and PIP Antibodies in Blood

PIP is secreted in both benign and malignant conditions and blood levels of PIP are measurable in the low ng/ml range in normal individuals. A proportion of patients with metastatic breast cancer have high circulating blood levels of PIP up to 70,000 ng/ml. Early studies have revealed that PIP is a circulating glycoprotein tumor marker in approximately 40 % of patients with advanced breast cancer [14, 30]. In addition, in patients with advanced breast cancer androgen therapy could cause significant increases in plasma levels in the absence of disease progression [14]. Furthermore, urinary excretion of PIP usually parallels the increase in plasma levels of this glycoprotein [14]. Despite these early observations a potential role for PIP as a circulating biomarker for breast cancer has not been fully explored.

In a separate study, antibodies against PIP were measured in the sera from patient with benign and malignant breast lesions. Authors observed that 2.6 % of mammary disease patients were affected by benign conditions and 5.5 % of patients carrying malignant mammary gland tumors expressed statistically significant amounts of antibodies against PIP [38]. The highest circulating PIP antibody levels occurred in

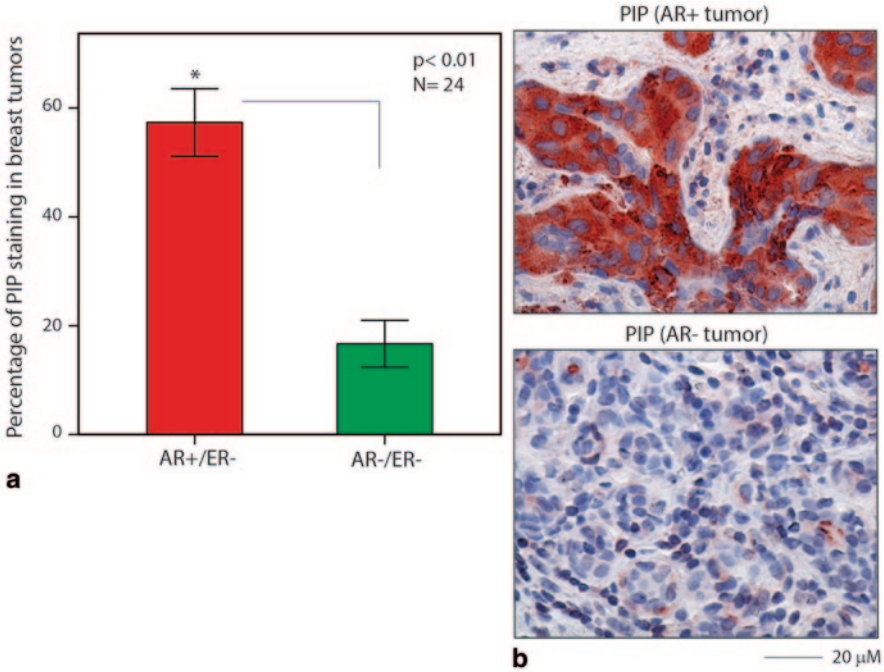

Fig. 8.1 PIP protein expression in primary breast tumors. **a** Immunohistochemistry (IHC) staining for PIP in ER-negative (ER−) breast tumors. Percentage of cells with positive staining are demonstrated for each group. *$p < 0.01$ is for AR+ vs. AR−. Error Bars: ±2SEM. **b** IHC staining for PIP in AR+ and AR− breast tumors. Magnification is at 60×. This figure is adapted from the study by Naderi et al. [35]

patients with highly malignant ductal or lobular carcinoma of breast and in patients with dysplasia [38]. A bimodal correlation with the percent of invaded lymph nodes was also observed. In addition, IgM and IgG isotypes were detected among the circulating PIP antibodies, suggesting the involvement of a T-cell-mediated immune response [38]. Authors suggested a possible role for PIP as an antigen for antitumor vaccination in breast cancer.

8.4 Transcriptional Regulation of PIP

PIP expression is induced by both prolactin (PRL) and androgen hormone (dihydrotestosterone, DHT) [32]. In addition, some of the cytokines, including IL-1α, IL-4, and IL-13, upregulate PIP expression and 17β-estradiol downregulates the expression of this gene [6, 7, 40]. Importantly, there is evidence of cooperation between several transcription factors in the regulation of PIP expression.

One such cooperation is between PRL-induced signal transducer and activator of transcription 5 (STAT5) and AR [9]. Carsol et al. have shown that DHT or PRL

alone only slightly modulates PIP-promoter luciferase activity. In contrast, a maximal increase in luciferase activity is observed using a reporter gene construct after exposure to both DHT and PRL [9]. In addition, a half-site androgen responsive element and two consensus STAT5-binding elements are required for this transcriptional response [9]. Furthermore, the functional cooperation between STAT5 and AR in the transcriptional regulation of PIP depends on the PRL-induced phosphorylation of Tyr694 in STAT5A and Tyr699 in STAT5B as well as the activities of transactivation, DNA-binding, and ligand-binding domains of AR [9].

Another established cooperation in regulation of PIP transcription involves AR and Runx2 [2]. Baniwal et al. found that Runx2 expression synergizes with AR to promote PIP expression, whereas its knockdown in T47D cells abrogates basal as well as hormone-stimulated PIP expression [2]. In addition, Runx2 and AR co-occupy an enhancer element located 11 kb upstream of the PIP open reading frame and Runx2 facilitates AR recruitment to this enhancer [2]. Moreover, PIP knockdown in T47D cells compromises DHT-stimulated expression of multiple AR target genes, including PSA, FKBP5, FASN, and SGK1 that is attributable at least in part to abrogation of AR nuclear translocation [2]. The authors concluded that Runx2 controls a positive feedback loop between androgen signaling and PIP [2].

Moreover, we have identified a positive feedback loop between the AR and ERK signaling pathways in molecular apocrine subtype of breast cancer [12]. In this feedback loop, AR regulates ERK phosphorylation through the mediation of ErbB2 and, in turn, ERK-CREB1 signaling regulates the transcription of AR in molecular apocrine cells [12]. We have recently demonstrated that this AR-ERK feedback loop regulates the transcription of some of the top ranking genes in molecular apocrine signature [35]. Among these genes, we observed that PIP, DUSP6, S100A8, and FOXA1 expression were consistently reduced following the inhibition of AR-ERK signaling [35]. Notably, we found that PIP is the most regulated molecular apocrine gene by the AR-ERK feedback loop and is also regulated by AR-ERK signaling in xenograft models [35]. These observations are explained by the fact that PIP is a target gene of ERK-CREB1 pathway and is induced by AR activation [35].

Therefore, the available data suggest that PIP transcription in breast cancer is regulated by cooperation and cross-talks between AR and other transcription factors, including STAT5, Runx2, and CREB1 (Fig. 8.2).

8.5 PIP and Proliferation in Breast Cancer

Several studies have investigated the role of PIP in proliferation of breast cancer cells. Cassoni et al. examined the effect of purified PIP on proliferation of four human breast cancer cell lines (MCF7, BT474, MDA-MB231, and T47D) and a "normal" human immortal breast-cell line (MCF10A) [10]. These breast cell lines showed a mitogenic response to PIP at 10 μg/ml and PIP-enhanced cell growth of the MCF10A, MCF7, BT474, and MDA-MB231 cell lines at both 48 and 96 h of

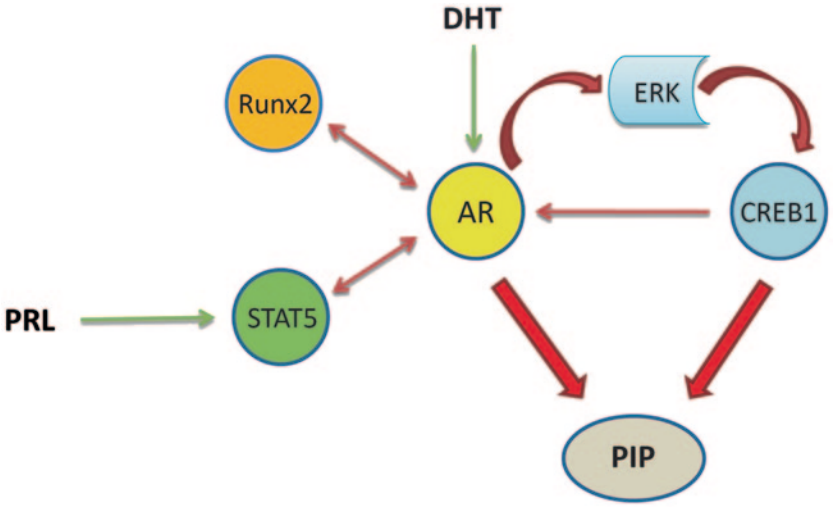

Fig. 8.2 Cooperation between transcription factors in the regulation of PIP expression. Schematic diagram depicts a transcriptional network that regulates PIP expression in breast cancer. *DHT* dihydrotestosterone, *PRL* prolactin. *Arrows* denote stimulatory effects

exposure [10]. The authors noted that the mitogenic effect of PIP was observed in both "normal" and malignant breast epithelial cells [10].

Moreover, other studies have revealed that PIP expression is necessary for the proliferation of breast cancer cell lines. In a study by Baniwal et al. the authors observed that PIP knockdown suppressed the proliferation of ER-positive cell line T47D driven by either serum growth factors or DHT as well as the proliferation of estrogen dependent and tamoxifen-resistant T47D cells[2, 3]. In addition, our group has demonstrated that PIP expression is necessary for the proliferation of ER-negative "molecular apocrine" cell line MDA-MB-453 [35]. Collectively, these findings suggest that PIP promotes cell proliferation in both ER-positive and ER-negative breast cancer cells and argue in favor of targeting PIP as a treatment strategy in the management of breast cancer.

8.6 PIP and Cell Invasion in Breast Cancer

Since PIP is a secreted protein with protease activity in degradation of fibronectin and the established role of fibronectin in extracellular matrix function, we have examined whether PIP expression is required for the invasion of breast cancer cells [35]. Cell invasion was assessed in vitro using a basement membrane and a fluorometric cell invasion assay. Notably, we observed a marked reduction in cell invasion by approximately threefold following PIP knockdown in MDA-MB-453 cell line compared to the control group [35]. This finding suggests that PIP expression is necessary for cell invasion in breast cancer.

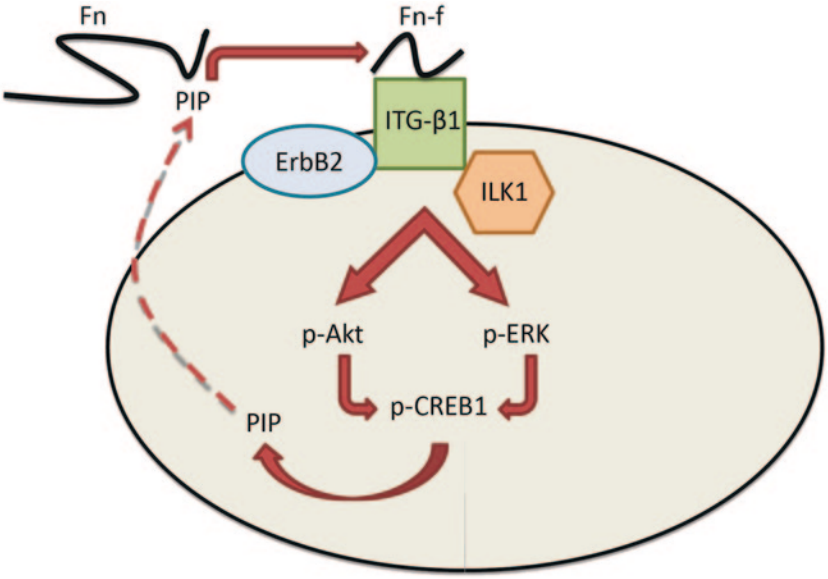

Fig. 8.3 Schematic diagram of the PIP-integrin β1 signaling in breast cancer. *Red arrow* denotes a stimulatory effect. *ITG-β1* integrin-β1, *Fn* fibronectin, *Fn-f* fibronectin fragment. This figure is adapted from the study by Naderi et al. [35]

8.7 PIP-Modulated Signaling Pathways

8.7.1 PIP Regulation of ERK and Akt Phosphorylation

Some of the signaling pathways regulated by PIP in breast cancer have been recently investigated. In this respect, we have studied the effect of PIP expression on the phosphorylation of ERK and Akt, since these phosphorylations are key signaling events in cell proliferation [11]. Notably, we observed a marked reduction in phospho-ERK/total-ERK ratio between 0.2 and 0.5-fold following PIP knockdown in MDA-MB-453 cell line [35]. Similarly, PIP knockdown resulted in a reduction of phospho-Akt/total-Akt ratio between 0.4 and 0.7-fold [35]. We next assessed the effect of PIP knockdown on the phosphorylation of CREB1 since it represents a critical downstream mediator of the EGFR-ErbB2 pathway, which is activated by both Akt and ERK signaling [29]. Consistent with phospho-ERK and phospho-Akt data, we observed a marked reduction in phospho-CREB1/total-CREB1 ratio between 0.2 and 0.4-fold following PIP knockdown in MDA-MB-453 cells [29]. These findings suggest that PIP expression is necessary to maintain the phosphorylation of ERK, Akt, and their downstream target CREB1 in breast cancer cells (Fig. 8.3).

8.7.2 PIP Regulation of Outside-in Integrin-β1 Activation

Integrin-β1 receptor is activated by fibronectin fragments (Fn–fs) following the enzymatic degradation of intact fibronectin (Fn), [25, 43]. It is known that the activation of integrin-β1 promotes cell adhesion and invasion and induces some of the key signaling pathways such as MAPK/ERK and PI3K/Akt that are involved in cell proliferation [5, 18, 42]. Therefore, we have investigated a function role for PIP in the regulation of integrin-β1 in breast cancer cells [35].

Integrin-β1 activation by Fn-fs leads to the binding of this receptor to its binding partners. One of the integrin-β1 key binding partners is integrin-linked kinase 1 (ILK1), which binds to the activated integrin-β1 and mediates downstream signaling effects such as the activation of Akt [27, 44]. In view of these facts, we examined the effect of PIP knockdown on the binding between integrin-β1 and ILK1 using immunoprecipitation assays and observed a marked reduction of integrin-β1 binding to ILK1 by 0.1 to 0.3-fold following PIP knockdown in MDA-MB-453 cells compared to the control [35].

It has previously been reported that integrin-β1 binds to ErbB2 in human carcinoma cell lines [16]. Since ErbB2 overexpression is present in the majority of molecular apocrine subtypes, we also examined the association between integrin-β1 and ErbB2 in MDA-MB-453 cells and evaluated a possible role for PIP expression in this process. We observed that integrin-β1 binds to ErbB2 in the control experiment and this binding is markedly decreased to 0.1-fold following PIP knockdown compared to the control [35].

In agreement with our findings, a study by Baniwal et al. have reported that PIP-silencing significantly reduces FAK phosphorylation in T47D cells at the Tyr397 position, which is a key downstream target of integrin-β1 activation, but it did not significantly affect the total FAK protein level in this cell line [3, 36].

Moreover, we have studied whether the effects of PIP knockdown in the reduction of integrin-β1 binding to ILK1 and ErbB2 can be reversed by Fn–fs. This was assessed by the addition of α-chymotryptic Fn fragment 120 K 24 h after transfection of MDA-MB-453 cells with PIP-siRNA. Transfection with non-targeting siRNA and treatment with vehicle only was used as a control. Importantly, we observed a nearly complete reversal of integrin-β1 binding to ILK1 and ErbB2 in PIP-knockdown experiments following the addition of Fn–fs to levels similar to that of control [35]. All together, these findings suggest that PIP expression is necessary for outside-in activation of integrin-β1 as measured by the binding of this protein to ILK1 and ErbB2 in a process that is partially mediated through the fragmentation of Fn (Fig. 8.3).

8.7.3 PIP Signaling Feedback Loop

Our data suggest that there is a positive feedback loop between PIP and ERK-Akt signaling in molecular apocrine cells (Fig. 8.3). Following the induction of PIP expression by CREB1, the secreted PIP mediates protease degradation of Fn to Fn–fs,

which results in the activation of integrin-β1 signaling (Fig. 8.3). Importantly, in the absence of PIP there is a marked reduction of integrin-β1 binding to its binding partners ILK1 and ErbB2 that can be reversed by the addition of Fn–fs [35]. ILK1 is a key binding partner of activated integrin-β1 receptor that mediates the induction of Akt and ERK signaling pathways [27, 41, 44]. In addition, integrin-β1 is associated with the EGFR-ErbB2 receptor family and mediates an EGF-independent activation of the EGFR-ErbB2 signaling pathway, which in turn results in the induction of MAPK/ERK signaling and cell proliferation [26, 45]. In fact, we observed a marked reduction in the phosphorylation levels of ERK, Akt, and their downstream target CREB1 following PIP knockdown in molecular apocrine cells [35]. Since PIP is a CREB1 target gene, this regulation of CREB1 phosphorylation by PIP provides a positive feedback loop mechanism between PIP and CREB1 mediated through the integrin-ERK and integrin-Akt signaling pathways [35], (Fig. 8.3).

8.8 Summary

PIP is a widely expressed protein in breast cancer that is necessary for the proliferation and invasion of breast cancer cells. PIP is transcriptionally regulated by cooperation between a number of transcription factors, including AR, STAT3, Runx2, and CREB1. Available data suggest that PIP regulates outside-in activation of integrin-β1 and its downstream signaling pathways in molecular apocrine cells. Future studies are required to understand the molecular mechanisms of PIP action and translational implications of this protein in breast cancer.

Competing Interests Author has no competing interests to disclose.

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Chapter 9
Modeling Prolactin Actions in Breast Cancer In Vivo: Insights from the NRL-PRL Mouse

Kathleen A. O'Leary, Michael P. Shea and Linda A. Schuler

Abstract Elevated exposure to prolactin (PRL) is epidemiologically associated with an increased risk of aggressive ER+ breast cancer. To understand the underlying mechanisms and crosstalk with other oncogenic factors, we developed the NRL-PRL mouse. In this model, mammary expression of a rat PRL transgene raises local exposure to PRL without altering estrous cycling. Nulliparous females develop metastatic, histotypically diverse mammary carcinomas independent from ovarian steroids, and most are ER+. These characteristics resemble the human clinical disease, facilitating study of tumorigenesis, and identification of novel preventive and therapeutic approaches.

9.1 Prolactin (PRL) and Breast Cancer

PRL plays critical roles in the proliferation and differentiation of lobuloalveoli during pregnancy (for review, [1]). Its ability to drive growth and differentiation in concert with ovarian hormones and other established players in breast cancer suggests that PRL, too, contributes to this disease. However, this idea remained controversial for some time, in part because of conflicting data from small epidemiologic studies, failure to appreciate the multiple agonists for the PRL receptor (PRLR) and the extensive PRL expression in extrapituitary sites in humans compared to experimental models. However, a myriad of studies over the last decade now support the importance of PRL in breast cancer.

Two lines of evidence link PRL action to the development of breast cancer, which are discussed in recent excellent reviews [2–4]. First, in a large prospective epidemiologic study nested within the nurse's health study, elevated circulating PRL levels were found to significantly raise the risk of cancers that express estrogen receptor alpha (ERα), independent of circulating estrogen levels [2]. A recent follow-up study found that elevated circulating PRL 10 years prior to diagnosis was associated with the risk for metastatic ER+ disease [5]. Second, recent studies

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have identified polymorphisms in the *PRLR* and *PRL* genes associated with breast disease [6–8].

A link between PRL action (elevated circulating PRL or high expression of PRLR in tumors) and tumor progression, metastasis and/or therapeutic resistance has also been observed, although the supporting epidemiologic evidence is more sparse than that correlating PRL and tumorigenesis (for review, [2]). PRLR is expressed in the vast majority of breast cancers, including both ERα+ and ERα− tumors [9], and PRLR mRNA is higher in tumors than adjacent normal tissue [10]. In seeming contrast to those reports, however, activation of STAT5A, the best understood mediator of PRL signals in normal mammary physiology, is associated with a better prognosis [11, 12]. However, PRL initiates activation of multiple other signaling cascades (Fig. 9.1; for reviews, [13–15]), which may in part account for the apparent disparity in these observations (see below). Data from many in vitro studies also demonstrate that PRL can drive processes underlying tumor progression. PRL increases proliferation and survival (for review, [15]), and increases chemotherapeutic resistance of breast cancer cells in culture [16, 17]. However, similar to the clinical findings, PRL-activated STAT5A promotes differentiation in breast cancer cells in vitro [18,19]. Interestingly, PRL signals to STAT5 are inversely related to its ability activate an AP-1 enhancer in a panel of breast cancer cell lines [20], and the stiffness of the matrix in 3D cultures inversely modulates PRL-activated STAT5 and ERK1/2 [21]. Together, these observations suggest that features of both the cancer cell itself and the surrounding microenvironment can alter the signaling repertoire of PRL, which may have profound effects on the biological outcome.

The growing strength of the epidemiologic data suggests preventive and therapeutic opportunities directed at the actions of PRL in breast cancer. However, in order to exploit the clues from the clinical data and studies in individual breast cancer cell lines, we need to understand the signals and gene targets of PRL in diverse mammary cell contexts, and the interplay of its actions and the microenvironment in breast pathology over time. Genetically modified mouse models enable examination of the dynamic processes of tumor development and progression and interactions among oncogenic players. They permit investigation of the underlying mechanisms which can reveal potential targeted strategies, and evaluation of the likelihood of their success. Here, we describe the mammary pathology that develops in the NRL-PRL transgenic mouse. This model recapitulates many features of clinical breast cancer, especially aggressive ERα+/PR− cancers, which account for the majority of breast cancer deaths [22].

9.2 Mammary PRL Synthesis

Expression of the PRL gene in pituitary lactotrophs is driven from a proximal promoter that is conserved across mammals (for review, [23]). In humans, transcription of the PRL gene can also be driven from a second upstream promoter that drives expression in many tissues apart from the pituitary, including the mammary gland

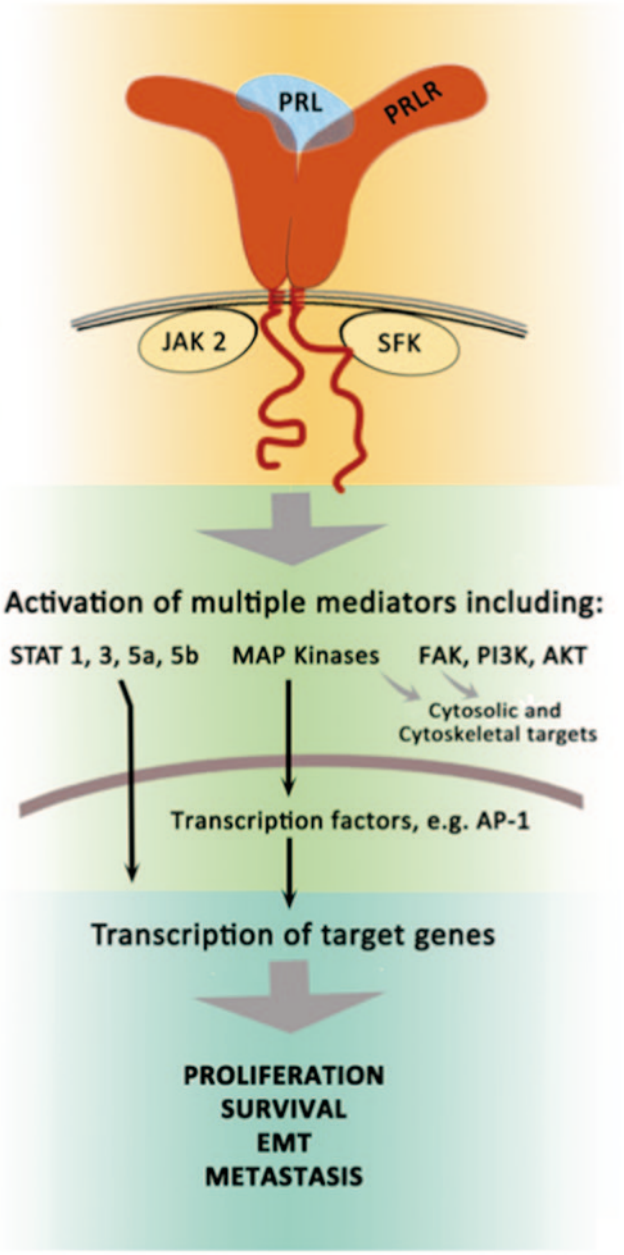

Fig. 9.1 PRL actions on mammary epithelia may contribute to breast cancer by multiple pathways. PRL binds to its receptor (PRLR), stabilizing a homodimer, and altering receptor conformation to activate associated kinases (JAK2 and Src Family Kinases (SFKs)). Subsequent phosphorylation cascades activate multiple signaling mediators, which may vary in strength depending on features of the tumor cell (e.g., levels of mediators, p53 status), as well as features of the microenvironment (e.g., ECM composition and stiffness, growth factors)

[24–27]. Local PRL expression is elevated in many breast cancers [28, 29]. Although incompletely understood, the potential for different regulation conferred by usage of the distal promoter [25, 30] would explain the independence of expression in nonpituitary tissues from dopamine, and subsequent insensitivity of these breast cancers to treatment with dopamine agonists [31].

Despite the restrictions imposed by a single promoter, rodents also express detectable PRL in their mammary glands, particularly during late pregnancy, as well as in mammary tumors [32–34]. Indeed, local PRL is necessary for epithelial differentiation and milk synthesis in mice [34, 35]. This requirement for endogenous PRL at the end of pregnancy (also discussed in [36]) suggests the intriguing possibility that mammary PRL initiates distinct signals. Interestingly, MCF7 breast cancer cells also responded differently to inducibly overexpressed PRL than to exogenous PRL [37]. Whether these observations simply reflect locally elevated ligand concentrations, or preferential signals initiated by autocrine action, remains to be determined.

Regardless, locally produced PRL also would add to the mammary milieu. In combination with the other lactogenic hormones (placental lactogens, primate growth hormone) [38, 39], it is clear that the examination of circulating PRL alone underestimates the exposure of mammary tissue to PRLR agonists.

9.3 Mouse Models of Elevated PRL

Rodents have been used extensively to model PRL actions in breast cancer. Pregnancy and pseudopregnancy elevate PRL activity in concert with the other hormonal changes of pregnancy [38]. A rich literature describes the use of pituitaries transplanted under the kidney capsule to raise circulating PRL for prolonged periods by protection from inhibitory hypothalamic dopamine (for reviews, [14, 40–42]). Like the hormonally complex physiologic states, the latter technique also raises ovarian steroids, particularly progesterone [41, 42], via effects of the high circulating PRL on corpus lutea [43]. While PRL and progesterone physiologically cooperate in mammary function [1, 44] and may also crosstalk in breast cancer [45], manipulations that increase circulating PRL levels preclude study of actions of the individual hormones.

More recently, transgenic PRL has been employed to augment PRL exposure, as we have previously reviewed [46]. In addition to widespread epithelial PRL expression driven by the metallothionein promoter [47], local mammary PRL concentrations have been raised by mammary selective promoters. These include the whey acidic protein promoter [48] and the rat neu-related lipocalin (NRL) promoter ([49], described in detail below). These transgenic models overexpress the secreted PRL ligand, revealing the outcome of direct and indirect actions of PRL on the many different mammary cell types that may contribute to breast disease, including not only epithelial subpopulations, but also immune cells, endothelial cells and fibroblasts, and other stromal cells.

Table 9.1 Differences in the NRL-PRL and *Stat1*$^{-/-}$ models of mammary cancer

	NRL-PRL	*Stat1*$^{-/-}$
Model		
Mouse strain	FVB/N	129S6/SvEV, Balb/c
PRL specificity	Yes	Multiple cytokines
Parity	Nulliparous	Multiparous
Tumor phenotype		
Tumor incidence/mean latency	70%/17 months	65%/23 months
Spectrum of tumor histotypes	Broad	Narrow
Steroid receptor expression	ERα+/PR−; ERα−/PR−	ERα+/PR+
Metastasis	Yes	No
Estrogen sensitive growth	No	Initially, then lost
Dependence on Jak2	Little	Much
Cooperation with other oncogenes	Yes NRL-TGFα, p53$^{-/-}$	Yes MMTV-neu, p53$^{-/-}$

Although both NRL-PRL and *Stat1*$^{-/-}$ females exhibit similar early mammary pathology and develop ERα+ tumors, the established tumors display marked differences in appearance and behavior. *Stat1*$^{-/-}$ mammary phenotype from [50, 51, 88]

Interestingly, genetic ablation of Stat1 also results in ERα+ tumors in multiparous 129S6/SvEV and Balb/c mice [50, 51]. Like the secreted PRL transgene in the NRL-PRL model, germline silencing of Stat1 reveals the outcome of potential effects on multiple cell types. Recent mechanistic studies have demonstrated that failure to downregulate PRLR in mammary epithelial cells (MECs) via Socs1 is a contributor to the mammary pathology [51]. Stat1 and Socs1 modulate signaling of multiple cytokines in addition to PRL, which are produced by the mammary stroma as well as MECs (for reviews, [50, 52]). Stat1 can act as a tumor suppressor by promoting cytotoxic T lymphocyte activity [50], and SOCS1 modulates inflammation, thereby altering tumorigenesis and therapeutic responses (for review, [53]). Nonetheless, the well-differentiated nonmetastatic ER+/PR+ mammary tumors that develop in *Stat1*$^{-/-}$ 129S6/SvEV multiparous females provide an interesting contrast to the aggressive ER+/PR− carcinomas that develop in NRL-PRL females (Table 9.1 and discussed further below).

9.4 NRL-PRL Model

9.4.1 Model

In the NRL-PRL model, transgene expression is driven by a 3015 bp fragment of the rat NRL proximal promoter that confers selective expression in mammary epithelia beginning at a young age [49, 54]. Transplanted transgenic MECs to a cycling syngeneic female develop adenocarcinomas [46], confirming that PRL expression

by these cells is sufficient to drive mammary tumorigenesis. The identity of the MEC subpopulation targeted by the NRL promoter is not well defined. However, as noted above, the secreted transgenic PRL exposes multiple mammary cell types, of which many may contribute to tumorigenesis. This promoter fragment is not sensitive to estrogen or PRL [49], but unlike the endogenous gene [55], it is highly responsive to progesterone [54].

Two independently derived NRL-PRL lines in the FVB/N strain background have been extensively characterized [TgN(Nrl-Prl)23EPS, NRL-PRL 1655-8 and TgN(Nrl-Prl)24EPS, NRL-PRL 1647-13]. The NRL-PRL 1655-8 line exhibits modestly elevated circulating lactogenic activity, while the NRL-PRL 1647-13 line has normal levels [49]. Females of both lines cycle similarly to nontransgenic controls, indicating that effects on the corpus luteum are not a component of the subsequent pathology. Although some differences in early pathology were observed [49], both lines develop similar histologic spectra of carcinomas with comparable latency. To reduce the likelihood that transgenic PRL influences the mammary gland via secondary effects on another tissue, many of our studies have focused on NRL-PRL 1647-13.

9.4.2 Early Pathology

Ductal and alveolar epithelia of nulliparous NRL-PRL females exhibit increased proliferation and slightly increased apoptosis [49]. The increased turnover of morphologically normal mammary epithelium in vivo contrasts with PRL-augmented survival of human breast cancer cell lines in vitro (reviewed in [15]). Mammary pathology is detectable at 12 weeks of age. We observe focal dilated ducts with irregular epithelium, and later mammary intraepithelial neoplasms (MINs), resembling ductal carcinoma in situ (DCIS). Diffuse alveolar development is evident early in some females, and with age, all nulliparous females show extensive epithelial hyperplasias [49]. In aged females, the myoepithelial layer, associated with tumor suppressive properties, is markedly reduced in the presence of transgenic PRL [56]. Generally about 70 % of transgenic females develop single carcinomas after a long latency (mean, about 17 months) [49, 57].

An essential role for PRL signals mediated by Jak2 in tumorigenesis was demonstrated by MMTV-Cre-mediated conditional deletion of the *Jak2* gene in the NRL-PRL model [58]. However, the scope of the downstream effectors is unclear. Stat5 isoforms are likely to be one set of mediators, consistent with the importance of the Jak2-Stat5a pathway in the physiologic actions of PRL (for reviews, [1, 59]), and the modest but significant increase in the number of cells with nuclear pStat5 in epithelial hyperplasias of aged NRL-PRL females [56]. Interestingly, however, mammary glands of 12 week old NRL-PRL females displayed dramatic increases in Thr202/Tyr204-pErk1/2 and Ser473-pAkt, compared to age-matched nontransgenic controls [56], suggesting that other signaling cascades contribute to PRL-induced oncogenesis. PRL can also drive signaling pathways via SFK, [60, 61], which may be important in the observed pathology.

Jak2 is also critical for mammary cancer in *Stat1*$^{-/-}$ mice [51]. With the caveat that this model may reflect the actions of multiple cytokines in addition to PRL, these data support a role for Jak2/Stat3/ Stat5 in ER+ carcinogenesis.

The cell cycle regulator, cyclin D1, is the major D cyclin in the mammary gland and is overexpressed in many breast cancers (for review, [62]). This D cyclin is a mediator of the PRL-driven alveolar proliferation that occurs during pregnancy [63]. In order to understand its role in the pathogenesis induced by PRL, we compared nulliparous NRL-PRL females with wild type *Ccdn1* to those with genetic ablation of this gene [64]. Although the absence of cyclin D1 dramatically reduced PRL-stimulated epithelial proliferation and the incidence of PRL-induced carcinomas, PRL nonetheless was able to augment epithelial proliferation to levels in females with wild-type cyclin D1 and induce epithelial hyperplasias and MINs. A partial compensatory mechanism was suggested by the increased nuclear cyclin D3 in luminal epithelia in NRL-PRL/D1$^{-/-}$ glands in the absence of effects on mRNA levels, indicating that PRL may stabilize cyclin D3 post-translationally by mechanisms similar to those described for cyclin D1 [64, 65]. Cyclin D3 is high in some clinical cancers, frequently associated with elevated cyclin D1 [66–68]. Our data suggest that PRL is able to employ both D cyclins to augment MEC proliferation, in concert with its effects on other cell cycle regulators [69].

The MEC subpopulation(s) that gives rise to PRL-induced tumors and the target cells of PRL that contribute to this outcome are not known. Despite the clear role for PRL in physiologic alveologenesis but not ductal development, transgenic PRL increases ductal as well as alveolar proliferation and results in preneoplastic lesions attributable to both populations [49]. Transgenic PRL in the presence of ovarian steroids does not alter the ratio of luminal to basal MECs (O'Leary, unpublished observations). However, although PRLR mRNA is restricted to luminal MECs, transgenic PRL alters gene expression in both subpopulations, indicating paracrine effectors. Ongoing studies will dissect the crosstalk of PRL with ovarian steroids on progenitor activity, permitted by the NRL-PRL model. Progesterone is of particular interest, since it augments stem cell activity [70, 71]. These studies will illuminate the direct and indirect actions of PRL that contribute to the development of ERα+ breast cancer, and also the ERα− carcinomas that develop in the NRL-PRL model [49, 57, 72]. In addition, they will shed light on the roles of established lineage regulators in PRL activity, such as Stat5a, Gata3, and Elf5 (for review, [73]). In light of the emerging significance of Elf5 as a suppressor of ER expression and antiestrogen sensitivity in luminal cancers [74] and suppressor of the epithelial–mesenchymal transition [75], understanding these networks is particularly important.

9.4.3 Carcinomas

Nulliparous females of both NRL-PRL transgenic lines develop aggressive, histologically diverse carcinomas with a range of latency from 14 to 21 months. These carcinomas aggressively invade nearby tissue, and metastasize to local lymph nodes and the lungs. They demonstrate wide histological diversity, including

adenocarcinomas of several subtypes, adenosquamous carcinomas, and spindle-cell carcinomas. The majority are ERα+ (defined as more than 10 % positive cells). However, negligible progesterone receptor (PR) expression is detectable in most tumors. In contrast to the responsiveness of morphologically normal MECs in NRL-PRL females to estrogen [76] and the ability of supplemental 17β-estradiol to reduce tumor latency [56], growth of established carcinomas is not sensitive to estrogen despite continued ERα expression [57]. Although tumor pStat5 levels were not correlated with ER expression [57], and ablating Jak2 did not reduce ER in established tumors [58], reduction of Stat5 expression in cell lines established from PRL-induced ER+ tumors lowers ERα expression (Brockman and Schuler, unpublished observations), suggesting that PRL is one contributor to ER expression. The aggression and lack of estrogen responsiveness of the ER+ carcinomas in NRL-PRL mice resembles clinical luminal B breast cancers [77–79]. Since few mouse models develop ERα+ tumors (for reviews, [80–82]), the characteristics of the NRL-PRL tumors suggest that they are useful models of aggressive ERα+ clinical disease.

Consistent with their histotypic diversity, PRL-induced carcinomas display disparate patterns of phosphorylated signaling mediators [57]. Well-differentiated adenocarcinomas exhibited highest levels of pStat5, a key physiological mediator of PRL, and also lower MMP-9 mRNA. This models the correlation between high STAT5 activation and better clinical outcomes [11, 83]. Many adenocarcinomas also exhibit high levels of pStat3 ([58], Arendt and Schuler, unpublished observations). Despite the requirement for Jak2 in tumor development, tumor transplantation was not dependent on Jak2, and although activation of Stat5 was dependent on Jak2, Stat3 was constitutively phosphorylated [58]. PRL also activates many MAP kinases, which may alter cell behavior [84]. The distribution of pErk1/2 differed with histotype. Stromal cells within adenocarcinomas displayed high levels of pErk1/2, whereas spindle-cell carcinomas exhibited nuclear and cytoplasmic staining of tumor epithelium [57]. One potential downstream target of Erk1/2 is the transcriptional enhancer, AP-1. AP-1 proteins, including c-Fos and c-Jun, are expressed in PRL-induced tumors at highest levels in tumors with lowest pStat5, consistent with the inverse activity of these pathways observed in breast cancer cells in vitro [20]. Ongoing studies are exploring the role of other upstream kinases, including SFK, in PRL-driven tumor progression. All NRL-PRL tumor histotypes displayed varying levels of pAkt within the tumor epithelium; the phosphatidylinositol 3-kinase pathway is dysregulated in many clinical cancers [85].

PRL-induced ERα+ adenocarcinomas differ from many commonly used mouse models of breast cancer in that they are composed of heterogeneous populations of epithelial cells. These tumors display both luminal and basal epithelial subpopulations when analyzed by flow cytometry using antibodies to EPCAM and CD49f (Shea and Schuler, ms in prep). In contrast, luminal but ER- mammary carcinomas from MMTV-Her2/neu mice consist of a single population of luminal cells [86], and claudin-low carcinomas that develop from *p53*$^{-/-}$ mammary cells are composed of a single basal cellular subpopulation [87]. Interestingly, both subpopulations within NRL-PRL carcinomas are capable of regenerating heterogeneous tumors of the same histotype and ERα expression following transplantation, indicating tumor

initiating cells (TICs) in both subpopulations. The biologic and therapeutic consequences of this heterogeneity and characteristics of the TICs are currently under investigation. Since some PRL-induced carcinomas are ER−, similar studies will provide insight into the origin and progression of these tumors, and differences from the ERα+ adenocarcinomas.

Increased PRL signals also contribute to the mammary pathology induced by genetic ablation of Stat1, yet the appearance and behavior of these tumors is substantially more benign than the carcinomas of the NRL-PRL mouse [51, 88] (Table 9.1). In contrast to NRL-PRL tumors, these adenocarcinomas are generally ER+/PR+, and metastases have not been reported. Moreover, the majority is initially dependent on ovarian hormones, although this is later lost in a subset of tumors [51]. Interestingly, in contrast to NRL-PRL ERα+ adenocarcinomas, tumors that develop in *Stat1*$^{-/-}$ mice exhibit a single luminal MEC population as determined by flow cytometry. Unlike carcinomas in NRL-PRL animals, tumors in *Stat1*$^{-/-}$ females are highly dependent on Jak2, and display high levels of Jak2-dependent pStat5 and pStat3 [51]. Comparison of the regulation of tumor behavior in the *Stat1*$^{-/-}$ and NRL-PRL models using similar perturbations and endpoints will illuminate the underlying differences, and may reveal key regulators of the more malignant processes in NRL-PRL tumors. Such findings likely would important implications for aggressive ERα+ clinical breast cancers.

9.4.4 Crosstalk with Estrogen, ERa

Similar to the clinical epidemiologic studies [2], both the incidence and latency of PRL-induced tumors are independent of postpubertal ovarian steroids [56]. However, supplemental 17β-estradiol accelerates tumor formation in ovariectomized animals. This cooperation between transgenic PRL and estrogen is demonstrated in the proliferation of morphologically normal mammary epithelium [76], and resembles the observed interaction of these hormones in breast cancer cells in vitro [89–93].

Multiple mechanisms of estrogen/PRL crosstalk have been shown in studies of breast cancer cell lines in vitro. PRL and estrogen can increase expression of the other's receptor [37, 94]. PRL and estrogen can also cooperatively activate ERα-mediated transcriptional signals, such as activation of AP-1 enhancers [89]. Further, STAT5, one mediator of PRL actions, has been observed to positively or negatively impact estrogen-induced signals, depending on the experimental system [95–98]. Recent reports have begun to elucidate the outcomes of PRL-estrogen crosstalk on target genes in various breast cancer cell lines [91, 92].

PRL also activates ERα in the absence of estrogen ligand in the mammary glands of the NRL-PRL mouse, demonstrating that this mechanism, which is believed to contribute to antiestrogen resistant breast cancer [99], is active in vivo [100]. Although PRL initiates phosphorylation of ERα in the AF-1 domain (Ser118, Ser167) [101] and induces recruitment of ERα to target genes [102] in breast cancer cells, the ability of this mechanism to exert physiologically significant effects in vivo has not been clear. In order to examine this directly, we utilized the ERα(G525L)

mouse, which expresses a mutant ERα that is refractory to 17β-estradiol, but can be activated by kinases downstream of growth factors and cytokines [103]. NRL-PRL females with the ERα(G525L) mutation displayed ER-dependent ductal elongation and gene expression at puberty [100], confirming the ability of PRL to act via this mechanism. Moreover, ligand independent activation of ERα also mediates a significant portion of the crosstalk between PRL and the EGFR ligand, TGFα ([100], see Sect. 9.4.5 below).

Together, these studies underscore the multiple mechanisms of cooperation between PRL and estrogen in carcinogenesis. Interestingly, however, established carcinomas in NRL-PRL females do not respond to estrogen [57], resembling antiestrogen resistant, luminal B clinical tumors, as discussed further below.

9.4.5 Crosstalk with Growth Factors

Advancing lesions can coopt other agents, such as local growth factors, to promote progression. Indeed, PRL-induced tumors express varying levels of erbB family members, including erbB2 [58]. In breast cancer cells in vitro, PRL strongly cooperates with EGF, TGFα, and IGF-1, prolonging phosphorylation of ERK1/2 and AKT and altering ligand-induced EGFR and IGF-1R trafficking [104], suggesting positive interactions in vivo.

In order to examine the effect of crosstalk between mammary PRL and EGFR ligands in the dynamic in vivo environment, we employed NRL-TGFα animals [54], which overexpress the EGFR ligand, TGFα, similar to many breast cancers [105–107]. Compared to single NRL-TGFα females, mammary tumor development was dramatically accelerated in bitransgenic NRL-PRL/TGFα females [108], and pErk1/2 and pAkt were further elevated in mammary tissue, as predicted by the cooperative interaction in in vitro studies [76, 108]. Interestingly, the stroma surrounding PRL/TGFα-induced tumors exhibited significantly increased cell and collagen density, compared to that surrounding tumors induced by TGFα alone [108]. In light of the increasing recognition of the importance of the stroma on tumor progression [109–111], our data suggest that PRL actions on the microenvironment may be significant contributors to PRL-induced mammary pathology (see Sect. 9.4.7 below).

Elevated growth factor activity is linked to resistance of ERα+ cancers to antiestrogen therapies [112–115]. These resistant clinical cancers also display the lack of responsiveness to estrogens [77–79]. Similarly, we found that the proliferation of mammary epithelia in NRL-PRL/TGFα females was strikingly insensitive to ovarian steroids or supplemental 17β-estradiol despite high levels of ERα expression, in contrast to the strong response of MECs in nontransgenic and single transgenic NRL-PRL and NRL-TGFα mice [76]. Moreover, 17β-estradiol also failed to induce PR expression. Consistently, tumor incidence and latency in these animals were not altered by ovariectomy or additional estrogen. These findings suggest that tumors that express high levels of PRLR and erbB family members are likely to be less responsive to aromatase inhibitors, but that therapies directed at the PRLR in combination with growth factors may impact therapeutic outcome.

Although MEC proliferation and tumor latency in PRL/TGFα females were independent of estrogen, the strong crosstalk between PRL and TGFα to kinase cascades that phosphorylate the AF-1 domain of ERα suggests that ERα may nonetheless play a role in the tumorigenesis induced by these oncogenes in combination. In order to investigate the role of ligand-independent activation of ERα in vivo, we employed the ERα(G525L) mouse, which expresses an ERα that can be activated by phosphorylation, but is refractory to 17β-estradiol [103]. As predicted, this model revealed that ligand-independent activation of ERα accounted for a significant portion of PRL/TGFα-stimulated MEC proliferation [100]. Interestingly, ER-dependent signals were not necessary for development of PRL/TGFα-induced ERα+ tumors. ER-dependent signals did alter gene expression; however, indicating that even when ER is not a strong mediator of mitogenic signals, it may alter tumor phenotype and behavior.

Notably, the potent crosstalk of local transgenic PRL and TGFα also resulted in mammary tumors in male mice, which did not occur in the single transgenic animals [101]. Although breast cancer is relatively rare in men compared to women, the incidence is rising and the disease is less responsive to treatment [116–118]. Interestingly, hyperprolactinemia secondary to medications or prolactinomas is a significant risk factor [119–122], suggesting that the NRL-PRL mouse may be a useful tool for male breast cancer.

9.4.6 Crosstalk with p53

TP53 is mutated in many breast cancers across subtypes, and is associated with poor outcomes [85]. In order to examine the effect of PRL exposure on the cancers that develop in this context, we transplanted NRL-PRL x $p53^{-/-}$ mammary cells to mammary glands of syngeneic wild-type mice [72]. PRL decreased the latency of the resulting carcinomas compared to $p53^{-/-}$ cells alone, and increased their proliferation and invasiveness. Strikingly, PRL also increased the proportion of triple negative claudin-low carcinomas. PRL/$p53^{-/-}$ tumors displayed elevated levels of AP-1 components associated with aggression of clinical tumors [123, 124], compared to $p53^{-/-}$ tumors, and altered levels of transcripts associated with cell cycle progression, invasion and stromal reactivity. The latter included those for Col1a1 and Vegfa, supporting a role for PRL in modulation of the tumor microenvironment. Interestingly, imaging of collagen surrounding PRL/$p53^{-/-}$ tumors using polarized microscopy of picrosirius red-stained sections demonstrated that many carcinomas displayed straight collagen fibers oriented perpendicularly to the tumor edge, which is associated with more aggressive behavior clinically [125], compared to $p53^{-/-}$ tumors (see Sect. 9.4.7 below). Together, these studies suggest that PRL can promote the development of more breast cancer subtypes than just luminal tumors, which may have been overlooked in epidemiologic studies that pooled all ER− cancers, and that actions on nontumor cells in the microenvironment may mediate some of its oncogenic effects.

9.4.7 Interaction with the Extracellular Matrix (ECM)

Accumulating evidence indicates that the development and progression of breast cancer can be strongly influenced by the density and composition of the ECM (for reviews, [126, 127]). Hormones, including PRL, are associated with elevated mammographic density (increased stromal cells and/or ECM) in some clinical studies [128–130], which is also linked to increased risk of breast cancer [131, 132]. In established carcinomas, the density of the surrounding ECM is frequently elevated (desmoplasia) [109, 133]. Moreover, aligned collagen fibers that are oriented perpendicularly to the tumor predict a threefold relative increased risk for relapse, particularly in patients with ER+ cancers [125]. These epidemiologic observations are supported by experimental evidence that increased mechanical stiffness of the ECM promotes tumorigenesis [134, 135].

PRL increases synthesis of mammary ECM components in mouse models. Mammary glands of *Prlr*$^{-/-}$ females contain reduced transcripts for many ECM proteins [136]. Consistently, mammary glands of 3 month old ovariectomized NRL-PRL females contain increased transcripts for collagens 1a and 5 (Rugowski and Schuler, unpublished data). Furthermore, mammary glands of aged NRL-PRL females also exhibit increased tenascin C mRNA compared to age-matched nontransgenic females [56], which is also associated with progression of clinical disease [137, 138]. These observations indicate that PRL potentially can augment ECM density and alter its composition, with consequences for breast disease.

The NRL-PRL model in the context of a second oncogene has permitted us to examine the effect of PRL on the ECM surrounding tumors of similar histotypes. Mammary tumors in NRL-PRL/TGFα females displayed increased surrounding stromal density, including increased cellularity, compared to those in NRL-TGFα females tumors [108]. PRL action in the absence of p53 revealed the ability of PRL to modify the alignment of collagen fibers in addition to increase collagen synthesis [72], associated with increased metastases and poor outcomes [125].

Conversely, the ECM also modulates PRL actions, providing insight into apparent disparity between the association of PRL exposure with poor outcomes in breast cancer and positive prognosis associated with activated STAT5a [2, 11]. In ER+ breast cancer cells in vitro, we have observed that matrix stiffness modulates the spectrum of PRL-induced signals and subsequent cell behavior [21]: stiff collagen matrices prolong PRL signals via SFK-FAK-ERK1/2 and reduce those via STAT5 compared to compliant matrices, associated with increased MMP-2 synthesis and activity and MMP-dependent invasion. Moreover, PRL aligns stiff matrices, increasing radially oriented collagen fibers [21], consistent with invasive carcinomas [125]. Matrix stiffness also increases protumorigenic outcomes of crosstalk between PRL and 17β-estradiol (Barcus and Schuler, ms submitted). We are extending these in vitro studies to the more complex in vivo environment, taking advantage of a mouse model with mutated collagen 1a (*col1a1*^{tmJae}), which is resistant to degradation [139]. In combination with the NRL-PRL mouse, these studies will shed light on the interplay of PRL and estrogen signals with stiffness of the ECM in breast cancer development, progression and therapeutic responsiveness.

9.5 Summary and Future Directions

The NRL-PRL model recapitulates the epidemiologic link between PRL exposure and aggressive ER+ breast cancer in women, and has confirmed the potency of PRL cooperation with multiple other factors implicated disease progression. Indeed, PRL in this model cooperates with all of the oncogenes that we have examined (TGFα, absence of p53, estrogen, as well as elevated β-catenin ($APC^{min+/-}$) ([56, 72, 76, 101, 108], O'Leary and Schuler, ms in prep).

As described herein, NRL-PRL mice enable discovery of PRL actions in mammary tumorigenesis, and dissection of crosstalk with ovarian steroids, apart from the complex hormonal milieu of pregnancy. Our studies to date provide the foundation to identify the role(s) of PRL on the dynamics of MEC subpopulations, and illuminate the position of PRL in paracrine networks. These studies will point to origin of ER+ tumors, and the contributions of PRL to other breast cancer subtypes, which may not have been distinguished in the epidemiologic studies.

The estrogen-insensitive metastatic carcinomas that develop in this model provide the tools to investigate the progression, metastasis and therapeutic responsiveness of ER+ cancers, and hormonal contributions to these processes. These studies are likely to reveal significant influences of PRL on the tumor microenvironment, including the ECM, and other mammary cell types. For example, PRL promotes endothelial migration and VegfA transcription [140] (Friedl, this book), and increases GMCSF expression [141], indicating potential roles in angiogenesis and immune responses. They will also elucidate the importance and determination of the components of the repertoire of PRL-initiated signals in oncogenesis and tumor progression, including the roles of Stat5 isoforms.

The ability of the mammary phenotype of the NRL-PRL mouse to mimic clinical breast cancer underscores the value of characterized mouse models to investigate this disease. Building on our extensive knowledge of murine mammary biology, the immunocompetent NRL-PRL model will illuminate hormone actions in the dynamic processes of breast cancer development and progression.

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Chapter 10
Prolactin-Induced Prostate Tumorigenesis

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Abstract The physiological role of prolactin (PRL) in the prostate gland is not clearly understood. Genetically-modified mouse models that have invalidated actors of the PRL signaling axis failed to identify an essential regulatory function on this tissue. However, a large body of evidence suggests an important role for PRL in prostate tumorigenesis. Mainly through the activation of its downstream target STAT5, PRL can induce growth and survival of prostate cancer cells and tissues in several experimental settings. In the clinic, PRL expression and STAT5 activation in human prostate tumors correlate with disease severity. Available data point to a role of local (autocrine/paracrine) rather than circulating (endocrine) PRL in the induction of disease progression. In mice, transgenic expression of PRL in the prostate leads to enhanced epithelial hyperplasia and dysplasia, with amplification of basal/stem cells which have been recently identified as prostate cancer-initiating cells. Thus, targeting PRL receptor (PRLR)/STAT5 signaling may provide an alternative therapy for the treatment of prostate cancer. Corresponding targeted therapies currently in preclinical development include antagonists or blocking antibodies for the PRLR and small molecule inhibitors directed against the tyrosine kinase JAK2 upstream of STAT5. Present efforts are aimed at validating these therapies for the treatment of prostate cancer, while understanding the mechanisms of disease progression induced by PRL/STAT5.

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10.1 Introduction: Prolactin (PRL) in Prostate Physiology

The role of prolactin (PRL) in male physiology is not completely understood. Many organs have been shown to express the PRL receptor (PRLR) and among the ~300 actions of PRL that have been described [9], only a few relate to male reproduction. In the testis PRL regulates the levels of leutinizing hormone (LH) and follicle stimulating hormone (FSH) receptors, and in the epididymis and seminal vesicles; effects of PRL on energy metabolism and fluid contents have been described (for a review, see [9]). In the human prostate, the presence of local PRL expression and functioning PRLR [43, 55] suggest a role for autocrine/paracrine PRL signaling (in addition to circulating PRL). However, not much is known about the physiological role of PRL in the prostate. Reported actions of PRL in the rodent prostate gland include increased epithelial secretions and energy metabolism, with enhanced production of citric acid, increased expression of IGF-1 and IGF-1 receptor, and of other prostate proteins [9, 21, 47].

In rodents, Prl injection results in generalized epithelial phosphorylation of signal transducer and activator of transcription 5 (Stat5). Other signaling pathways activated by Prl in other organs, such as Stat3, Stat1, PI3K/Akt or MAPK, do not seem to be activated by constitutive expression of PRL in the rodent prostate in vivo [63] or in organ cultures [2]. PRL stimulation also resulted in decreased apoptosis when prostate organ cultures were deprived of androgen stimulation [1]. Some of the effects of PRL stimulation seem to be stronger in certain regions of the rodent prostate as compared to others. In contrast to the human prostate, the rodent prostate is organized in lobes: anterior (also called coagulating gland), ventral, lateral, and dorsal lobes are disposed around the urethra in symmetrical pairs (left and right). The dorsal and lateral lobes seem to be the most responsive to Prl action in terms of Stat5 phosphorylation, induction of epithelial hyperplasia and survival upon androgen deprivation [2, 67] (Fig. 10.1.).

The activation of PRLR signaling appears not to be essential for prostate development or function. This is regardless of the proliferative and antiapoptotic action observed in organ cultures. Rodents with no Prl (*Prl*-knockout mice) displayed significantly reduced ventral prostate lobes and normal anterior lobes. Unfortunately, no data are available on their prostatic histological features or the weights of lateral and dorsal lobes [72]. *Prlr*-knockout mice, in turn, displayed minor changes in prostate morphogenesis and function, with slightly heavier ventral and dorsal lobes than wild-type prostates and unaffected anterior lobes. In terms of histological features, *Prlr*-knockout mice displayed a small decrease in epithelial content of the dorsal lobe, with unchanged ventral lobe histology [62]. In addition, the dorsal lobe epithelium displayed more marked regression in $Prlr^{-/-}$ than wild-type animals upon castration, while ventral lobes showed no difference from controls [62]. Mice lacking Stat5a (one of the two closely-related Stat5 molecules) showed relatively

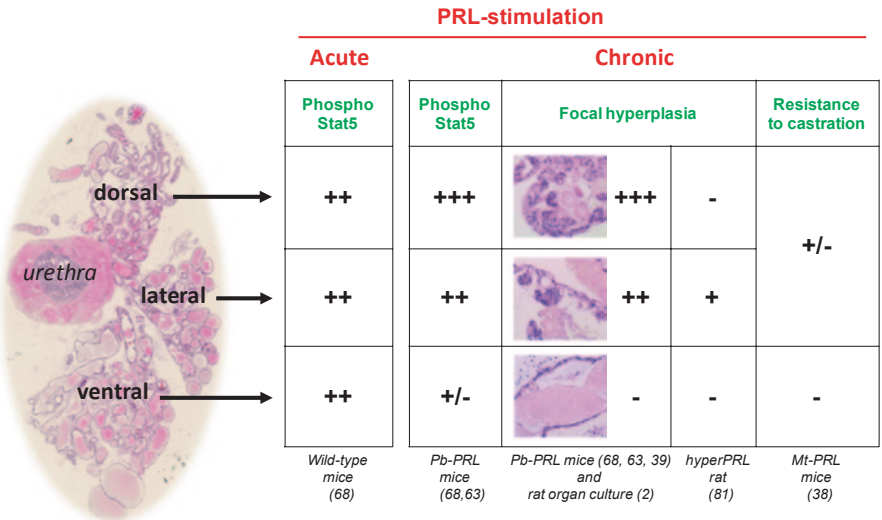

Fig. 10.1 Lobe-specific responses of rodent prostate to acute or chronic Prl stimulation. The picture on the *left* shows the left dorsal, lateral, and ventral lobes of a *Pb-Prl* mouse prostate (hematoxylin/eosin staining), and the *right* part of the figure shows the activation of Stat5, the presence of focal epithelial hyperplasia and the resistance to castration as determined using the various models indicated *below* the table.

more marked histological defects, including cyst formation with shedding and desquamation of epithelial cells in the ventral prostate lobe [56]. On the other hand, the dorsal and lateral lobes seemed unaffected, and regarding sizes, no differences were reported in any lobes when compared to those of wild-type mice. Overall, the available data suggest a minor physiologic role of PRL in prostate development and homeostasis. Unfortunately, no isolated PRL defects have been reported in men to offer clinical insights on the effects of PRL deficiency in the human prostate. A loss-of-function mutation of the PRLR (H188G) was recently reported, with different reproductive phenotypes in three sisters. However, the men in the family carrying the same mutation displayed no clinical symptoms, notably their father who was tested at the age of 69 years [57].

A role of PRL in benign prostate hyperplasia (BPH) has been proposed, given the phenotypes of *Prl*-transgenic mice (see below) and that primary cell cultures from BPH specimens displayed higher proliferation when stimulated with PRL [73]. However, in humans there is no obvious epidemiological link between circulating PRL levels and development of BPH although the data are scarce [61, 13]. No data concerning local PRL expression being available, a role for local PRL, rather than its circulating form, can be excluded. For a more thorough review, see [25].

In summary, the data currently available in the field suggest that PRL is not mandatory for prostate development, function, or homeostasis.

10.2 PRL and Prostate Cancer

10.2.1 Clinical Evidence (Observational)

10.2.1.1 Epidemiology

As opposed to testosterone or growth hormone (GH) levels which decrease in old age, circulating PRL levels stay constant or may even increase with age [4, 82]. Thus, upon the physiological decrease of other hormonal regulators, PRL might become a key player in the pathophysiology of the aging prostate. As tempting as it may seem to speculate about the correlation of this putative increase of PRL influence in the prostate and the well-established increasing risk of prostate cancer with advancing age, data to support this hypothesis are lacking. Initial studies demonstrated that prostate cancer patients displayed higher circulating PRL levels [34, 68], but currently available epidemiological evidence indicates that circulating PRL levels are not linked to prostate cancer risk in humans [19, 22, 71]. A recent study even suggested that hyperprolactinemic patients might be protected from prostate cancer possibly due to the establishment of hypoandrogenism as a result of PRL interfering with pulsatile GnRH release [6]. In addition, there is no clear evidence to suggest that potential PRLR activation by increased GH levels in acromegalics (human GH can bind the human PRLR) might increase prostate cancer risk, since acromegalic patients do not present enhanced rates of this disease [84]. In brief, there is currently no strong evidence to support the involvement of endocrine PRL in prostate tumorigenesis.

10.2.1.2 PRLR Levels

With respect to the PRLR, we are aware of two studies that have addressed whether the PRLR could be a biomarker of prostate cancer. The first one investigated PRLR expression in healthy/pathological prostates and concluded that PRLR levels were increased in preneoplastic stages (dysplasia/prostate intraepithelial neoplasia (PIN)) [43]. However, this study faced some limitations as it involved a relatively small series of samples (18 carcinomas, 20 dysplasias, 5 BPH, and 10 “lesion-free” tissues from radical prostatectomy) in which high Gleason grades were poorly/not represented. Importantly, PRLR protein expression was evaluated using the so-called immunoglobulin M (IgM) monoclonal antibody B6 (mAb B6) that was later commercialized as B6.2. The latter was recently proposed to recognize a PRLR-associated protein [23]. The lack of specificity of commercial antibodies is an issue that is not restricted to this mAb, therefore the conclusions of any study investigating PRLR expression levels based on immunostaining should be considered with caution. Fortunately, this study also analyzed the mRNA expression of the long PRLR isoform using in situ hybridization. By this approach the authors showed that poorly differentiated high-grade carcinomas displayed lower levels of PRLR mRNA and with a more heterogeneous distribution than low-grade tumors, where PRLR mRNA expression was similar to normal tissue. An increase in PRLR mRNA

levels also was reported for PIN lesions as observed by immunological staining [43]. The second study we are aware of in the field suggested that the PRLR was overexpressed in ductal adenocarcinoma, which is a less frequent form of the disease than acinar carcinomas [67]. This study presented similar limitations to the previous one, as it also involved a small number of cases (11 acinar, 5 ductal, and 20 mixed ductal-acinar carcinomas) and used a PRLR mAb (SPM2123) that is not commonly used in the field. However, the authors reported that PRLR transcripts (estimated by microarray analysis of microdissected samples) were approximately seven times higher in ductal adenocarcinoma than acinar adenocarcinoma. Their finding of increased transcription (by 17-fold) of lipocalin 2, a known target gene of the PRLR, was also in line with the increase in PRLR signaling that could be expected due to higher PRLR levels.

These observations raised the hypothesis that amplification of the *PRLR* gene/locus may be a mechanism by which PRLR signaling could be increased in some prostate cancers. Interestingly, recent integrative genomic profiling of human prostate cancer revealed that the most frequently observed genetic anomalies in prostate cancer involved chromosomal rearrangements (copy number alteration, translocations) much more than somatic point mutations [75]. Expectedly, tumors bearing the highest copy number alterations were highly enriched in metastatic cancers and displayed unfavorable prognosis. In this respect, the *PRLR* gene (chromosome 5p13.2) is located in a region that was found to show high genomic instability in prostate tumor tissues: amplification of the 5p13.1-p13.3 locus was indeed significantly associated with a negative outcome [75]. This is in agreement with a former study that identified a prostate cancer susceptibility locus at 5p13-q11 by linkage analysis [70]. Strikingly, the 5p13.1–p13.3 locus contains several genes that are relevant to prostate cancer, including the prostate cancer biomarker alpha-methylacyl-CoA-racemase (*AMACR*) [10], mTOR signaling (*RICTOR*), S-phase Kinase-associated Protein 2 (*SKP2*) and two genes whose products trigger the STAT5 pathway: interleukin-7 receptor (*IL7R*) and the *PRLR*. Clearly, such molecular analyses cannot identify which of these genes is/are relevant to disease etiology/progression, but open the door to a possible role for *PRLR* gene amplification in prostate cancer subgroups.

10.2.1.3 The Autocrine/Paracrine PRL/STAT5 Activation Loop

Studies on prostate cancer cohorts support a role in disease progression for paracrine/autocrine PRL/STAT5 signaling. As opposed to circulating PRL levels, local expression of PRL and activation of STAT5 in prostate tumors correlated to disease progression and recurrence. Overall, analyses of prostate tumors have found that PRL was expressed in more than 50% of prostate tumors (including local, locally advanced, and hormone refractory) and over 60% metastases (67% to regional lymph nodes and 60% to other organs) [14, 44]. In addition, PRL expression was associated with high disease severity (high Gleason score) and activation of its signaling molecule STAT5 [44].

Other studies have shown that STAT5 activation correlates with disease severity (Gleason score) and predicts recurrence after androgen-ablation therapy [44, 45]. STAT5 activation was associated with a lower progression-free 15-year survival

rate (44% compared to 65% in patients with negative STAT5 activation) and was proposed as an additional criterion for treatment decision in patients with intermediate Gleason score or PSA levels [45]. The same study showed that patients exhibiting tumors of intermediate Gleason grade 3–4 (cohort of 325 tissue microarray samples) had a 1.7-fold higher risk of experiencing disease progression if their tumors exhibited STAT5 activation compared to those whose tumors were negative for active STAT5 [45]. These findings were recently confirmed by the same group on two different patient cohorts, showing that STAT5 activation predicted recurrence after prostatectomy and cancer-specific death both after prostatectomy and deferred palliative therapy [50].

The importance of STAT5 implication in prostate cancer progression was highlighted by the finding of active STAT5 in over 70% of primary tumors treated by androgen ablation and in 95% of castration-resistant prostate cancers (CRPC), which is the fatal stage of the disease [74]. Interestingly, STAT5 was almost three times more likely to be activated in tumors under androgen deprivation than those that did not undergo such treatment [74]. This notion underscores the importance of STAT5 as a survival factor of androgen-independent tumors. Activation of STAT5 was found in 81% of regional lymph node metastases and 33% bone metastases, with activation observed in 46% of metastases to other organs [29]. In addition, a recent study showed that the *STAT5A/B* locus was amplified in a significant number of prostate cancers, with a pattern supporting a role for enhanced STAT5 signaling in prostate cancer progression: this amplification was observed in 3% of Gleason 6 versus 40% of Gleason 8–9 tumors, and in 16% of organ-confined tumors versus 29% of distant metastases [33]. It remains to be established whether amplified STAT5 molecules can signal as transcription factors independently of their upstream activation (phosphorylation) via PRLR signaling. For instance, the activation of STAT5 in DU145 prostate cancer cells was sensitive to inhibition of SRC kinase rather than JAK2 [29]. Interestingly, the activating mutation of JAK2 (V617F), described in myeloproliferative diseases, has not been detected so far in locally-confined prostate cancer or CRPC [30].

In summary, endocrine PRL seems harmless whereas paracrine/autocrine PRL expression appears more relevant to prostate cancer, giving rise to STAT5 activation and disease progression. It remains to be established whether PRLR expression may be used as a biomarker of bad prognosis in the clinic.

10.2.2 Experimental Evidence (Mechanistic)

10.2.2.1 In Vitro Studies

PRL/STAT5 Signaling

Human prostate cancer cell lines and human/rodent organ cultures have been used in the search for mechanistic explanations regarding the role of PRL/STAT5 signaling in prostate cancer progression. Most of the classical cell lines were shown

to express PRLR mRNA at low but detectable levels [60]. Studies in the 1990s and early 2000s reported that PRL-stimulation led to enhanced proliferation and survival of both normal and malignant prostate epithelial cells [1, 36, 49, 53, 55]. Using long-term organ cultures of normal rat prostate tissue, Ahonen and colleagues demonstrated that PRL induced the survival of androgen-deprived epithelial cells in the lateral and dorsal lobes. Ventral lobes, however, seemed not to be affected by PRL stimulation [1]. Using the same experimental setting, this group later showed that PRL induced hyperplasia in epithelia of lateral and dorsal but not ventral prostate lobes (Fig. 10.1.). Activation of Stat5 was observed in all lobes of rat prostate in organ cultures [2]. In fact, Stat5 was the only activated signaling pathway downstream of the Prlr in explant cultures of normal rat prostate [1]. Similar results were obtained in prostate cancer cell lines [3]. To our knowledge, only one report concerning PC-3 cells has proposed a survival role for PRL upon TRAIL-induced apoptosis on these cells via the activation of Akt [64]. Organ cultures of human prostate cancer samples also displayed STAT5 activation upon PRL stimulation [44].

Importantly, the PRL/STAT5 cascade was reported to be critical for the survival of the androgen-independent CWR22Rv cell line [14]. Consistently, STAT5 was found to play an antiapoptotic role in CWR22Rv and androgen-dependent LNCaP cells, whereas androgen-independent PC-3 cells, which did not express active STAT5, were not affected by a dominant negative form of this signaling molecule [3]. Similarly, two noncancerous human prostate epithelial cell lines RC165N and RC170N were not affected by the expression of a dominant-negative STAT5 mutant [28]. Inhibition of STAT5 by genetic means also induced the death of androgen-dependent LNCaP and androgen-independent DU145 and CWR22Rv cells [15]. Similar inhibition of cell growth and invasive potential in vitro was reported for cell lines derived from the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model when stably transfected with a dominant-negative STAT5B mutant (C-terminal truncated form lacking the transactivation domain, STAT5deltaB) [37]. The effects of STAT5 on prostate cancer growth and survival were proposed to be mediated by BCL2-like 1 protein and cyclin D1, whereas BCL2 and STAT3 were unaffected by STAT5 inhibition [15]. Of the two STAT5 homologs, STAT5B seemed to play a more important role than STAT5A in viability and survival of human prostate cancer cells, as shown for DU145 cells [28]. Finally, a constitutively active STAT5 variant engineered by mutating the C-terminal serine residue (STAT5a S710F) led to increased motility of three prostate cancer cell lines (DU145; PC-3 and LNCaP), with decreased cell-surface E-cadherin expression in LNCaP cells and increased heterotypic adhesion of DU145 and PC-3 epithelial cancer cells to human endothelial cells. Respectively, these alterations are reminiscent of the epithelial to mesenchymal transition and of the attachment needed for extravasation; two processes leading to metastasis [29].

Thus, in line with what was observed in human prostate tumors, STAT5 signaling appears to play a key role downstream of the PRLR in most experimental models investigated so far.

Autocrine PRL Expression

The evaluation of *PRL* expression in human prostate cancer specimens and prostate cancer cell lines showed that they were positive for the hormone's mRNA. Both androgen receptor (AR)-positive (LNCaP, CWR22Pc, and CWR22Rv) and AR-negative (DU145 and PC-3) cell lines were tested as well as xenografts issued from these cells (see below) [14, 88]. These observations argued for the existence of an autocrine/paracrine loop in at least some prostate cancer cell lines as observed in primary human prostate cell cultures. With respect to the PRL protein, conditioned medium from DU145 cell cultures was shown to induce mild proliferation of PRL-responsive Nb2 cells—Nb2 is the rat pro-B lymphoma cell line used as the classical in vitro proliferation assay for lactogens [88]. However, this type of bioassay does not firmly identify the growth factor involved. In this respect, we are not aware of studies that have convincingly identified the PRL protein produced or secreted by these cell lines using classical biochemical approaches. Attempts to do so from cultured cells (using immunoblot) or cell line xenografts (using immunoblot and immunohistochemistry) were so far unsuccessful in our laboratory, suggesting that the amount of PRL produced is probably very low. In fact, the functional relevance of the autocrine/paracrine loop in prostate cancer cell lines is indirectly supported by functional evidence using PRL-core based analogs. We demonstrated that the pure PRLR-antagonist Del1-9-G129R-hPRL, a hPRL mutant that competes with PRL for receptor binding, was able to reduce constitutive STAT5 phosphorylation and survival of androgen-independent CWR22Rv cells [14]. Using S179D-hPRL, a molecular mimetic of serine-phosphorylated hPRL, Xu and colleagues reported that this analog was able to inhibit the growth of various androgen-independent prostate cancer cell lines in vitro, and both the take rate and growth of DU145 cell xenografts in vivo [88]. However, a subsequent study from the same group suggested that the beneficial effect of S179D-hPRL treatment may essentially result from increased expression of the SF1b PRLR isoform more than inhibition of the autocrine/paracrine PRL loop. This is in good agreement with earlier findings indicating that (i) S179D-hPRL is in fact an alternative PRLR agonist and not a competitive antagonist [7] and (ii) this analog activates a specific PRLR signaling pattern resulting among others in the upregulation of short PRLR isoforms which can act as dominant negative of long PRLR isoforms [35, 87].

The transcriptional regulation of the *PRL* gene in prostate tissue remains poorly understood (for a review, see [48]). Interestingly, *PRL* transcription in prostate cancer specimens and cell lines was driven by the proximal (pituitary) and distal promoters [14], somehow against the conventional picture suggesting that extra-pituitary PRL gene expression is regulated by the distal promoter [48]. Androgen levels have been shown to regulate PRL expression in the rat prostate epithelium, supporting the concept that the autocrine/paracrine PRL loop could mediate some of the actions of androgens and potentially take over androgen regulation of prostatic cancer cells during the development of androgen-independent growth [54].

STAT5/AR Crosstalk

Already in 2002, Ahonen and colleagues reported an increase in Prl-induced phosphorylation of Stat5 in rat prostate organ cultures when testosterone was present in the culture medium [2]. Even though the evaluation of a direct interaction between Stat5 and the AR in rat prostate organ cultures gave negative results [2], later studies in human cancer cell lines reported otherwise. Coimmunoprecipitation and functional in vitro studies by the same group revealed synergistic activity for STAT5 and AR, such that the activation of STAT5 favored AR action independently of androgen stimulation and, vice versa, presence of ligand-bound AR enhanced STAT5 activity regardless of phosphorylation [74]. Recently, testosterone and PRL were shown to act synergistically to increase nitric oxide production via upregulation of carboxypeptidase-D, resulting in increased survival of prostate cancer cells in culture [76]. Interestingly, these effects were abolished only when antiandrogens (flutamide) and Del1-9-G129R-hPRL were used together [77].

This synergy or crosstalk between AR and STAT5 might be critical for prostate tumor recurrence after androgen-ablation therapy, where despite the lack of androgen stimulation, AR expression persists. Of note, chronic hyperprolactinemia was shown to induce hyperplasia of dorsolateral prostate in castrated mice, further supporting that PRL can intrinsically exert tumorigenic actions in the absence of androgens [38]. Taken together, these data suggest that the combination of antiandrogen and anti-PRLR signaling targeted therapies may be relevant for the treatment of prostate cancer.

10.2.2.2 In Vivo Studies

Although in vitro models have been extremely useful to decipher the molecular events following PRL stimulation in prostate cancer cells, evidence for the protumorigenic actions of PRL have been provided by in vivo studies. Various preclinical in vivo models have been used to that end. Cell line xenografts present the advantage that they involve human cells and address the effect of PRLR signaling on the progression of established tumors, which is a suitable preclinical set up to test drug candidates that could be used in patients with prostate cancer. However, these models have shown their restricted reliability to predict the actual effects of anticancer drugs in part because cell lines are over-simplified models that do not take into account the whole complexity of human tumors. In this respect, it is noteworthy that recent studies from Rui's group showed that bovine PRL, which is present in the fetal calf serum added to most culture media, is in fact a poor agonist of the human PRLR [79]. This suggests that the immortalized human prostate cancer cell lines that have been passaged for decades in such culture conditions have become adapted to growth in the nearly absence of functional hPRLR agonists. Thus, these cell lines may have lost their sensitivity or dependence on PRL for growth in vitro

as well as in vivo. This further strengthens the importance of assessing the presence of the autocrine PRL loop in cell lines that are used as preclinical models for PRLR signaling-related issues. Another important limitation of cell line xenografts is that they do not mimic the architecture of endogenous prostate tissue, including stromal-epithelial interactions which are known to play a key role in the regulation of prostate cancer progression [5]. In this respect, genetically-modified mouse models such as *Prl*-transgenic mice offer the opportunity to study the pathogenesis induced by Prl in a healthy prostate that harbors physiological and anatomical characteristics (histology, architecture). These mouse models allow the elucidation of the molecular and cellular events that may be triggered by PRLR signaling in the early phases of prostate tumorigenesis, although we acknowledge that evidence is currently lacking to support a role of PRL in human prostate cancer initiation.

Xenograft Studies

Proof-of-concept for STAT5 stimulatory effects on survival and growth in prostate cancer was obtained by the group of M. Navalainen through the use of human prostate cancer cell line xenografts [15]. The constitutively active STAT5 mutant (STAT5a S710F) enhanced the growth in soft agar and the formation of lung metastases when expressed in DU145 cells injected into nude mice [29]. In addition, adenoviral infection of CWR22v cells with a construct coding for a dominant-negative STAT5A/B protein led to significantly lower incidence and growth of tumor xenografts in nude mice compared to constructs encoding WT-STAT5B or LacZ [15]. These adenoviral vectors were tested on local injections of established CWR22Rv xenograft tumors measuring 8 mm of diameter. A significant decrease in tumor growth was obtained through injection of an adenoviral vector coding for dominant-negative STAT5A/B compared to WT-STAT5B [15]. Similar results have been reported by another group using the tumorigenic and metastatic C2H cell line derived from the TRAMP mouse model. A stable C2H cell line expressing a dominant-negative STAT5B mutant (STAT5deltaB) could not form tumors in nude mice, as opposed to cells stably expressing WT-STAT5B or control vector [37].

Unfortunately, human prostate cancer cell xenograft models have suffered from the low affinity of endogenous (circulating) mouse Prl for the human PRLR [78, 79]. This species-specificity problem has so far prevented the accurate study of the role of PRL on STAT5 activation and growth of xenografted human tumors. The recent generation of mice expressing human PRL represents a promising solution for the advancement of the field [12, 65].

Genetically Modified Mouse Models

As discussed at the beginning of this chapter, genetic inactivation of any component of the Prlr/Stat5a signaling pathway has moderate consequences on mouse prostate physiology. In contrast, upregulation of Prlr signaling as observed in *Prl*-transgenic

mice was shown to have more dramatic effects. In the first model that was developed, the so-called *Mt-Prl* transgenic mouse, the ubiquitous metallothionein promoter ensured *Prl* overexpression in most if not all tissues from neonatal stages. Besides the occurrence of mammary gland adenocarcinomas, that was the scrutinized phenotype of this model [85], enhanced Prlr signaling was also able to promote prostate hyperplastic growth. Indeed, after a few weeks of *Prl* overexpression, *Mt-Prl* mouse prostates displayed classical features of BPH, including stromal hyperplasia and focal areas of epithelial dysplasia [86]. As systemic hyperprolactinemia was accompanied by elevated testosterone levels in *Mt-Prl* mice, a subsequent study from the same group showed that normalization of serum testosterone levels by castration and implantation of testosterone pellets in *Mt-Prl* males did not impact Prl-induced prostate hyperplasia. Furthermore, supraphysiological testosterone levels also failed to affect prostate hyperplasia in *Mt-Prl* mice, clearly indicating that Prl effects were independent of elevated androgen levels [38]. Nevertheless, these authors concluded that androgens seemed to be required for maintaining the BPH phenotype induced by Prl since castration of 12-week old mice without testosterone substitution led to similar rates of prostate weight regression in *Mt-Prl* compared to WT animals. It is interesting to note that while involution of the prostate epithelium after androgen-deprivation normally occurs rapidly after castration, the weight of dorsolateral lobes of *Mt-Prl* mice at 8 weeks after castration remained significantly higher than that of control mice. This again suggests that enhanced Prl signaling may in some instances counteract the effects of androgen deprivation.

As mentioned above, autocrine/paracrine PRL rather than circulating PRL likely plays an important role in human prostate tumorigenesis as increased PRL expression and STAT5 activation are characteristic of the majority of high grade human prostate tumors, especially in the recurrent phase. Interestingly, the two *Mt-Prl* transgenic strains that were analyzed by Wennbo and colleagues exhibited similar prostate phenotypes despite of very different Prl circulating levels (~15 ng/ml vs. ~250 ng/ml), further suggesting that prostate enlargement resulted more from *Prl*-transgene expression in prostate tissue itself than from the action of the circulating hormone [86]. *Probasin (Pb)-Prl* mice were developed in the early 2000s to address this hypothesis by generating a model of enhanced Prl expression specifically in the prostate [39]. The *Pb* gene promoter is one of the most popular promoters for directing prostate-specific expression of transgenes in prostate tissue [80]. The small *Pb* promoter (−426 to +28 bp) used in *Pb-Prl* mice is expressed in all prostate lobes, albeit with different efficacy [39]. As observed in *Mt-Prl* mice, *Pb-Prl* mice harbor hyperplastic prostates. In terms of histology, *Pb-Prl* prostates display distended glands full of secretions and increased density of the stroma [39]. Infiltrating inflammatory cells are also commonly observed, although the actual nature of these cells remains to be established. At the molecular level, we showed that Stat5 was widely activated in the prostate epithelium [63]. No other canonical signaling pathways appear to be activated by local Prl in the *Pb-Prl* prostate, such as Stat3, PI3K/Akt, or MAPK pathways. Thus, *Pb-Prl* mice can be viewed as a model that recapitulates the elevated activation of the PRL/STAT5 pathway observed in human prostate cancer.

As has been reported for rat organ culture assays (see above), mouse prostate lobes can respond differently to Prl stimulation (Fig. 10.1.). In the case of *Pb-Prl* mice, we detect markedly higher activation of Stat5 in dorsal than ventral lobes, with intermediate-high activation of Stat5 in lateral lobes. A high scale expression study performed by Dillner and colleagues [18] has reported the genes that are affected in the *Pb-Prl* model, looking at differences between the ventral and dorsolateral lobes (dorsal and lateral lobes pooled together). This study adds molecular evidence to the lobe-specific differences in *Pb-Prl* mice mentioned above. Interestingly, lobe-specific differences in Stat5 activation are not observed when wild-type mice are acutely injected with PRL and analyzed shortly after ~1 h, suggesting that, at least in *Pb-Prl* mice, the lobe-specific variations in Stat5 activation must rely on regulatory mechanisms resulting from long-term exposure to Prl. Prostate lobe differences in response to chronic hyperprolactinemia have also been described in rat models [81] with no real understanding of the underlying mechanisms.

The prostate epithelium of *Pb-Prl* mice displays hyperplastic regions (consisting of multiple layers of epithelium) and which sometimes present nuclear atypias, representing preneoplastic intraepithelial (PIN) lesions [39]. We have noted that the frequency of development of these regions displaying hyperplasia and PIN lesions is higher in the dorsal than in the ventral lobe (Fig. 10.1). Therefore, the development of abnormal epithelial lesions seems to follow the same lobe-specific pattern of Stat5 activation in *Pb-Prl* mice [68]. These characteristics seem to indicate that Stat5 activation is directly responsible for the hyperplasia and PIN lesions observed in prostates of *Pb-Prl* mice.

The Stem Cell Hypothesis

Many efforts have been directed at determining what kind of cell was responsible for the development of cancer in the prostate epithelium. Given the loss of basal markers and the luminal characteristics prevailing in prostate tumors, a transformed luminal cell was traditionally thought to be responsible for tumor development. Recently, more attention has been attributed to basal cells, given their capacity to differentiate into luminal cells. Stem cells in the prostate are believed to be localized in the basal compartment, sharing morphological features with other nonstem basal cells. In terms of epithelial lineage, stem cells in the basal layer are proposed to give rise to all three lineages, namely basal (called “basal/stem”), luminal and neuroendocrine cells [27]. Castration studies in rodents have shown that only luminal cells are dependent on androgen stimulation for survival [87]. This is the rationale for treating prostate tumors (which display a luminal phenotype) with androgen-ablation therapies. In contrast, basal/stem cells do not need androgens to survive and can resist castration, giving rise to gland regeneration once androgen stimulation is reinstated [87]. This androgen-independent phenotype has led to the proposal that basal/stem cells in the prostate epithelium are responsible for the regeneration of castration-resistant tumors after androgen-ablation treatment [27].

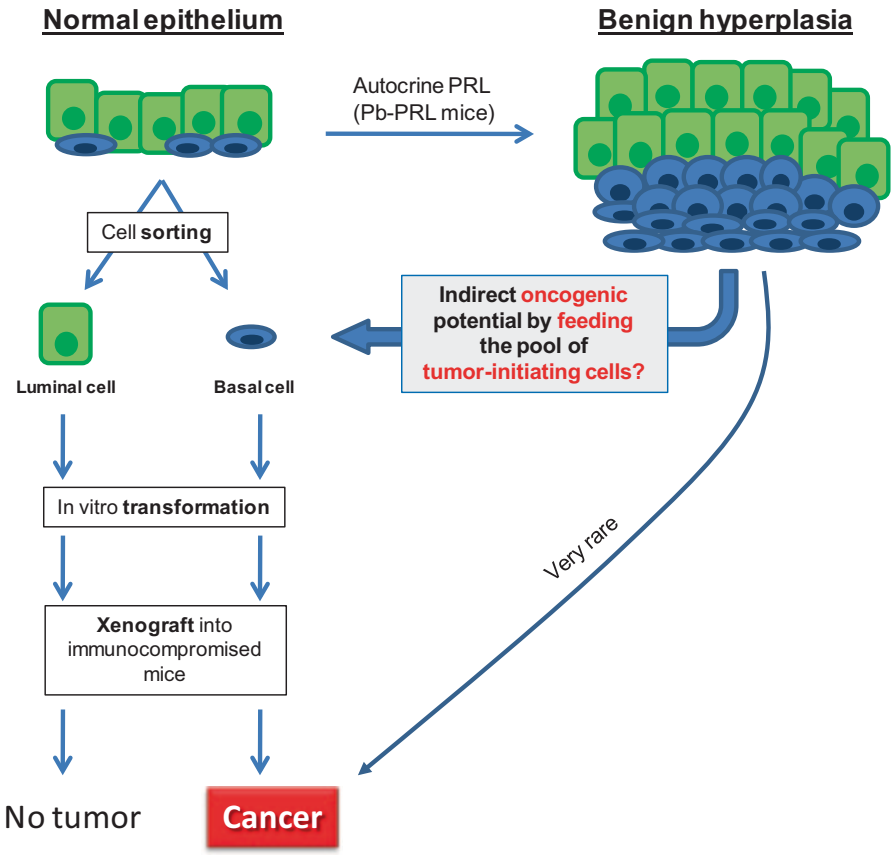

Fig. 10.2 The stem cell hypothesis. Recent studies have shown that the basal/stem cell compartment, in contrast to luminal cells, had tumor-initiating properties when expressing oncogenes. Although the basal/stem cell compartment is markedly amplified in the *Pb-Prl* prostate, the occurrence of carcinomas is very rare. One mechanism by which Prlr/Stat5 signaling may contribute to prostate tumor progression is by feeding the pool of tumor-initiating cells, thereby facilitating cell transformation by oncogenic hits

A breakthrough in the field was reported in recent years by the group of O. Witte, showing that basal/stem cells can be cancer-initiating cells in human and mouse prostate (Fig. 10.2). Using sorted basal/stem cells that were transformed with oncogenes frequently found in human prostate tumors (*AR*, *AKT*, and *ERG*), they showed that these cells gave rise to tumors displaying luminal-like characteristics when grafted in immunodeficient mice [26, 42]. In contrast, transformed luminal cells were not able to generate tumors in these conditions (Fig. 10.2). Subsequent studies have shown that the differentiation of adult basal/stem cells into luminal cells normally may be a slow process [12], but that environmental factors such as

inflammation may speed basal-to-luminal differentiation, possibly favoring cancer development [41].

Based on studies performed on the *Pb-Prl* mouse model, we have recently reported that Prl can induce the amplification of prostate basal/stem cells. Our data show a frank increase in the number of basal/stem cells as detected by immunostaining of basal cell markers (p63; cytokeratin 5) in *Pb-Prl* compared to wild-type epithelium [63, 68]. To further characterize this amplification, we have analyzed amplified basal cells and their surrounding luminal cells in terms of their differentiation status, Stat5 activation and proliferation. We have found that Stat5 is activated in luminal cells but not basal cells of *Pb-Prl* prostates [63]. Interestingly, the frequency of basal cell clusters (consisting of amplified basal/stem cells) was higher in the dorsal than the ventral lobe, showing a tight correlation with the levels of activated Stat5 in each lobe, as mentioned above [68]. It is tempting to speculate that Stat5 activation is directly responsible for the amplification of basal/stem cells, which in turn, via differentiation into luminal cells, might lead to the observed hyperplasia and PIN lesions in the *Pb-Prl* prostate epithelium. Of note, given the lack of Stat5 activation observed in basal/stem cells, an indirect (paracrine) mechanism might be responsible for basal cell amplification, rather than a direct effect of Prl on basal/stem cells [68].

Although locally-produced Prl induced expansion of prostate tumor-initiating cells, the occurrence of adenocarcinoma in *Pb-Prl* mice is very rare, indicating that enhanced luminal Stat5 signaling and basal/stem cell amplification cannot on their own lead to prostate cell transformation. Based on this evidence, one might speculate that a possible mechanism by which PRL could contribute to prostate cancer progression is to feed the pool of prostate tumor-initiating cells that could subsequently increase the probability that oncogenic events lead to cell transformation (Fig. 10.2).

10.3 Therapeutics

Systemic therapies of prostate cancer patients are primarily directed to the advanced stages of the disease since local treatments (surgery, radiotherapy) of early-stage tumors are fairly successful. Medical therapies of advanced prostate cancers mainly involve hormone therapy (androgen deprivation) and chemotherapy (taxanes). Despite recent improvements in patient survival in metastatic CRPC, prognosis remains highly variable and can still be pretty poor in this lethal form of the disease [89]. Novel compounds such as androgen synthesis inhibitors (abiraterone acetate) [66], new generation AR antagonists (Enzalutamide®) [69], or radioisotope drugs (radium-223) [59] are associated with increased overall survival compared to placebo in both pre- and postchemotherapy settings, however they remain insufficient due to the appearance of mechanisms of drug resistance. Hence, the challenge of personalized medicine is to treat patients individually by targeting the pathways that

are activated in their specific tumor. Several signaling cascades have emerged as new candidates for targeted therapy. These include PI3K/Akt/mTOR, IGFR signaling, SRC, c-Met, c-Kit, CXCR-4/2, and FGFR2 to only cite a few. It is unlikely that targeting any single pathway will definitely cure recurrent tumors as resistance to treatment appears to be a general rule; multiple weapons are obviously required. In this context, targeting PRLR signaling is emerging as a relevant perspective.

The classical clinical treatment for downregulating PRL signaling in hyperprolactinemic patients consists in the administration of dopamine agonists that block PRL production from the pituitary gland lactotrophs [51]. However, this kind of therapy decreases circulating PRL levels with no expected effect on PRL production in extrapituitary tissues. Given the evidence discussed above, targeting PRL levels in prostate cancer concerns mainly its autocrine production as opposed to circulating PRL. Regulation of *PRL* gene expression in the prostate is still poorly understood [48] and accordingly there is no known drug able to abolish prostate *PRL* gene expression. Of note, although androgens were suggested to increase prostate PRL expression in experimental models (see above), this regulation may be altered in a cancer context since autocrine PRL appears to parallel cancer progression. For all these reasons, targeted blockade of PRLR signaling in the target tissue (i.e., the prostate) seems a more appropriate strategy than targeting the expression of the PRL hormone.

The inhibition of PRLR signaling for prostate cancer may be performed at various levels, including the ligand-receptor interaction and the activation of JAK2 tyrosine kinase and STAT5 transcription factor whose role in promoting prostate cancer has now been clearly established. While inhibition of the former involves the development of either competitive PRLR antagonists based on the PRL-core or of neutralizing anti-PRLR antibodies, inhibition of JAK2 involves small molecule inhibitors. Of note, direct targeting of STAT5 has been achieved so far using genetic approaches (adenoviral delivery of dominant-negative STAT5A/B, antisense oligonucleotides, or STAT5A/B siRNA); pharmacological approaches, e.g., phosphotyrosyl peptides or peptidomimetics which bind the SH2 domain and prevent recruitment to the receptor, still require further development before considering clinical application [46]. These various strategies and lead compounds have been reviewed recently [16, 23, 25, 40] therefore the section below is aimed at briefly summarizing and updating these reviews.

The pure antagonist developed by our group (Del1-9-G129R-hPRL) [7] was shown to inhibit autocrine PRL-driven effects in various target tissues (for a review, see [23]). With respect to the prostate, it was able to inhibit the proliferation/survival of the human androgen-independent CWR22Rv prostate cancer cell line in vitro [14], and when expressed chronically in transgenic mice, it inhibited autocrine Prl-driven prostate tissue hyperplasia and normalized its major hallmarks including Stat5 activation and basal/stem cell amplification [63]. Its effects on human prostate cancer xenografts are yet to be tested.

The proof-of-concept data supporting the in vivo antitumor growth properties of the PRLR neutralizing antibody developed by Novartis (LFA102) rely on its ability

to inhibit the growth of Nb2-11C lymphoma cell xenografts and of DMBA-induced endogenous mammary tumors in rats [17]. No evidence for its ability to block human prostate cancer cell xenografts were published so far, which did not prevent a phase I clinical trial to be initiated (http://www.clinicaltrials.gov).

Finally, the JAK2 inhibitor AZD1480, which also abolishes downstream activation of STAT5, was shown to inhibit primary androgen-dependent prostate cancer growth as well as castrate-resistant growth of recurrent prostate cancer which occurs after regression of the original tumors in mice subjected to androgen deprivation [31]. In addition, this compound was recently reported to inhibit metastatic dissemination of prostate cancer in nude mice [32].

Based on the absence of damaging phenotypes in *Prlr*$^{-/-}$ males, inhibiting PRLR signaling is not expected to have deleterious/strong side effects or toxicity [24]. In contrast, systemic inhibition of JAK2 or STAT5 may have much more deleterious impact in terms of toxicity (undesirable side effects) due to the large distribution of these signaling molecules in many cell types. Strikingly, many cytokines regulating the immune system signal through STAT5 [58]. As an illustration of the potential deleterious effects of STAT5 inhibition, inactivating mutations of the *STAT5B* gene have been shown to lead to many immune disorders in addition to dwarfism due to impaired GH signaling [52]. A recent clinical trial using a JAK1 and 2 inhibitor (ruxolitinib) identified anemia and thrombocytopenia as the most common adverse events, and occasionally transformation to acute myeloid leukemia in some patients [83]. Therefore, although small molecule inhibitors may show better efficacy than current biomolecules (antagonists, antibodies) the inhibition of JAK2 or STAT5 may have unsuitable collateral effects in the human prostate cancer context.

10.4 Conclusions and Perspectives

There is currently a large body of clinical and experimental evidence supporting the involvement of PRL/STAT5 signaling in prostate tumorigenesis and cancer progression. The fact that autocrine rather than endocrine PRL is involved adds a level of complexity to the relevance of this pathway. The possibility that PRL/STAT5 signaling, in addition to promote survival of luminal (cancer) cells, could also target tumor-initiating cells is an attractive hypothesis that awaits the identification of the downstream mediator(s) that promote basal/stem cell amplification. Immediate perspectives are the validation of efficient therapeutic compounds that target this pathway, and the identification of relevant biomarker(s) to stratify patients eligible for such therapies. Since circulating levels of PRL cannot be used for that purpose, tissue biomarkers such as phosphorylated STAT5, PRL, or PRLR expression levels are probably the best candidates. Finally, the emerging evidence that STAT5 and AR signaling may cooperate suggests the relevance of combining anti-AR and anti-PRLR targeted therapies to prevent prostate cancer progression.

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Chapter 11
Prolactin in Inflammatory Response

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Abstract Prolactin (PRL) is a peptide hormone produced by the pituitary gland and diverse extrapituitary sites, which triggers activation of various signaling pathways after binding to its receptor (PRLr) resulting in the activation of specific genes associated with the pleiotropic activities of PLR. To date, various PRLr isoforms have been described, generated by post-transcriptional or post-translational processes. PRL has been associated with the modulation of a variety of actions in the immune response and inflammatory processes in several physiologic and pathologic conditions. However, PRL can have opposite effects, which might be regulated by interaction with the various isoforms of PRLR and PRL variants, as well as the cellular and molecular microenvironment influence.

11.1 Introduction

Prolactin (PRL) is defined as a pituitary-secreted polypeptide hormone and is a member of the PRL/growth hormone/placental lactogen family. It was initially known as a hormone synthesized in the pituitary gland, which develops the mammary gland and promotes lactogenesis, function for which it is named. PRL was discovered 84 years ago. The first findings about a pituitary factor capable of inducing milk secretion in rabbits date back to the late 1920s and early 1930s' French researchers [107, 121]

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PRL has more actions than all other pituitary hormones combined. The initial step in the action of PRL, like all other hormones, is binding to a specific membrane receptor, the PRL receptor (PRLr). At least 300 physiological functions such as immune modulation, osmoregulation, metabolism, maternal behaviour and non-lactational aspects of reproduction have been discovered over time [39], but the question remains open as to which of them are really relevant in humans. The different functions are imputed in part to extrapituitary sites of PRL production [8] and expression of different PRLr isoforms [70]. PRL has been implicated in alterations of cellular and humoral arms of the immune system [104]. Also, PRL can stimulate and inhibit immune responses regarding to different concentrations of the hormone [41]. PRL concentration is an important modulating factor of the inflammatory response leading to opposing effects, but the mechanisms responsible for these regulatory processes remain undefined. Decidua, brain, endometrium, as well as cells and tissues of immune system are some of the most established extrapituitary sites of PRL production. Particularly, in immune system, PRL acts as a cytokine and plays an important role in human immune responses, including autoimmune and chronic diseases [27, 29, 70]. Likewise, the expression of an autocrine loop of PRL in a lymphocyte [101, 138] implies that PRL and its receptor (PRLr) must be synthesized as well as its ligand is secreted for the same cell; and have an autocrine bioactivity (e.g. proliferative responses). Although the PRL expression in peripheral blood mononuclear cells (PBMC) has been noted [31, 82], there is no complete evidence of an autocrine loop expression with the participation of PRL in monocytes. Nonetheless, given the versatility and adaptive nature of PRL and selective extrapolation from different in vitro animal and human models should be done judiciously. In this chapter, we discuss the expression of an autocrine loop of PRL during the inflammatory response in monocytes and the relationship between the eventual synthesis of PRL and PRLr isoforms with the inflammatory response elicited by lipopolysaccharide (LPS), and culture filtrate proteins (*CFP*) *of M. bovis* in monocytes, also a differential expression of PRLr isoforms in macrophages (MØ) after *M. bovis* exposure.

11.2 Neuroendocrine Immunoregulation

The relationship between neuroendocrine and the immune system has been analyzed since the 1980s, spotlighting to new knowledge in the neuroendocrine-immune field [135]. The nervous and the immune systems share functional responses towards danger signals. Although considered to be spatially separated there is a certain degree of anatomical connection because sites for immune control exist in the nervous system (NS) [7, 118]. The neuroendocrine performance is based on interactions between the nervous and the endocrine systems. The neuroendocrine network can both directly and indirectly impact on the developmental and functional activities of the immune system. In turn, the immune system can collaborate in endocrine activity regulation [61]. All these mechanisms of bidirectional interactions, in order to main-

tain homeostasis and health, are mediated by the complex network designates as neuroendocrine-immune system. This regulation is accomplished by hormones such as those from the hypothalamic–pituitary–adrenal and gonadal (HPA-G) axis [90]. The neuroendocrine-immune system works in harmony with all other physiological systems at the level of the whole organism. These two systems reciprocally regulate each other, and share common ligands and receptors. The immune system regulates the central nervous systems through immune mediators and cytokines that can cross the blood–brain barrier, or signal indirectly through the vagus nerve or second messengers. Furthermore, an entire assortment of neurotransmitters and neuroendocrine hormones are endogenously synthesized by the immune system components, while the hypothalamus and pituitary gland are capable to produce different cytokines [30] (Fig. 11.1). In addition, immune, endocrine and neural cells express receptors for hormones, cytokines, neurotransmitters and neuropeptides. Hence, these products act in an autocrine, paracrine and endocrine manner thereby supporting the postulated bidirectional interactions of the neuroendocrine-immune system [55]. Endocrine glands are not the only source of hormones and neuropeptides production; they are also secreted by many extra gland sites including the immune cells, and these molecules are capable of stimulating or suppressing the immune cells activity by binding to its receptors [12]. The inflammatory cytokines interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and leukemia inhibitory factor (LIF) produced by innate immune cells in the periphery in response to danger signals cross the blood–brain barrier to activate neurons in the anterior hypothalamus, in order to induce the febrile response [4, 97, 108, 119]. Conversely, preventing overshooting inflammation and associated damage also underlies neuronal regulation through the so-called inflammatory reflex [130]. In some autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis, high levels of hormones such as estrogens and PRL, and low levels of anti-inflammatory hormones such as glucocorticoids have been described in the active phase. High levels of estrogens and PRL can increase interferon-gamma (IFN-γ) and interleukin-2 (IL-2) by Th1 cells activation, but also autoantibody production through Th2 lymphocytes activation. Alterations in hormonal levels might be coordinated by bidirectional communications between neuroendocrine and the immune systems [29, 67]. Therefore, hormones, neuropeptides and neurotransmitters participate in innate and adaptive immune response (Fig. 11.1).

11.3 Prolactin

PRL belongs to a large family of proteins, which also includes growth hormone (GH), placental lactogens (PL), PRL-like proteins (PLPs) and PRL-related proteins (PRPs). All these members share structural homology and biological characteristics. These proteins are expressed in pituitary and no pituitary sites. A single PRL gene is expressed on human chromosome 6 [8]. In rodents, many PRL-related genes clustered on chromosome 13 and 17 in mice and rats, respectively, are expressed. PRL

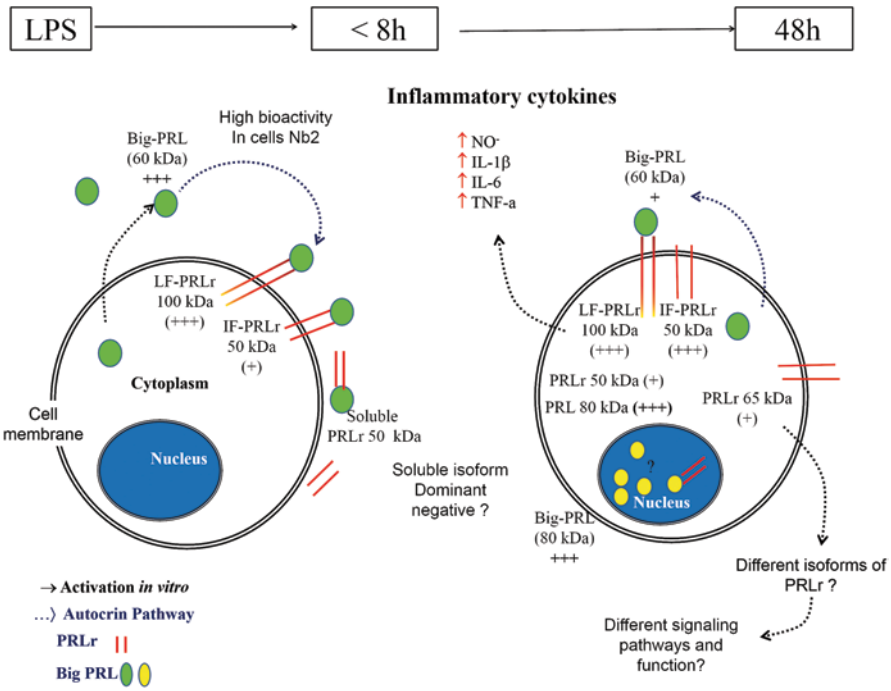

Fig. 11.1 Schematic representation of autocrine loop of PRL in activated monocytes by LPS (Lipopolysaccharides from enteric Salmonella serotype typhimurium) elicits differential expression of PRLr isoforms and a big PRL in time-depending manner. After 1 h and before 8 h, monocytes synthesize PRLr isoforms of 100 and 50 kDa as well as big PRL of 60 kDa; and later after 48 h also express PRLr of 65 kDa and big PRL of 80 kDa instead 60 kDa. PRLr 50 kDa is a product of alternative splicing and/or hydrolysis of the long isoform of 100 kDa. This short PRLr of 50 kDa could exert dominant negative function and/or activate alternative signaling pathways. Big PRL of 60 kDa displays proliferative bioactivity lactogen-dependant-Nb2 cells. This big PRL is a PRL storage and source of functional peptides hydrolysis derived. Interaction of big PRL with long form of PRLr might activate JAK2/STAT1 and the proinflammatory cytokine release. Interaction of pituitary PRL with intermediate form of 65 kDA might activate PI3k/Akt and IL-10 release. Interaction of big PRL complex with chaperon with short PRL in the nucleus affects transcription rates of some target genes

gene is composed of five exons and four introns with an overall length of 10 kb. The mature hPRL contains 199 aa, corresponding to 23 kDa of molecular weight. All PRLs identified so far are 197–199 aa and contain six cysteines forming three intramolecular disulfide bonds (Cys 4–11, 58–174 and 191–199 in hPRL). Primary structure of PRL is highly conserved among diverse species, e.g bovine and human PRLs share 74% aa identity [84, 106, 115]. Secondary structure studies have shown that PRL is an all-α-helix protein and contains almost 50% of α-helices, while protein remainder appears to fold into no organized loop structures. Mature PRL can be modified by post-translational changes, including glycosylation, phosphorylation and proteolytic cleavage. [115, 134]. Glycosylated PRL has a lower PRLR binding affinity and promotes a decrease on its actions at target cells and

tissues (e.g. mitogenic activity). Also, glycosylation may alter proteolytic cleavage, distribution control or clearance process of PRL [9, 115]. In addition to synthesis and secretion by lactotrophic cells of the anterior pituitary gland, PRL is also produced by numerous other cells and tissues. PRL gene expression has been found in various regions of the brain, decidua, myometrium, thymus, spleen skin fibroblasts, mammary epithelial cells and tumors, lacrimal and sweat glands, as well as circulating and bone marrow lymphoid cells [8]. Extrapituitary PRL is regulated at the transcription level due to presence of various enhancer and silencer domains, and also because of the formation of chromatin loops with the consequent transcription mechanisms [35]. In addition to serum, PRL can thus be found in several fluid compartments, such as cerebrospinal, amniotic and follicular fluids, as well as tears, milk and sweat; suggesting an important role of extrapituitary PRL for compensate pituitary PRL actions under some specific conditions [92]. Also, extrapituitary PRL can act directly, i.e. as a growth factor, neurotransmitter or immunomodulator, in an autocrine or paracrine way. Thus, locally synthesized PRL can act on adjacent cells (paracrine) or on the PRL-secreting cells itself (autocrine). In addition to the 23 kDa PRL, other isoforms can be found in human serum as big PRL with a molecular weight between 40–60 kDa; and macroprolactin (also known as big-big PRL of 100 kDa and more). Also, proteolytic cleavage of full-length 23-kDa PRL originates to N-terminal fragments [22]. PRL proteolysis induced by cathepsin D generates a single 16-kDa vasoinhibin in rat [3], whereas in human, the same PRL proteolysis mechanism produces casoinhibin of 15 kDa, 16.8 kDa and 17.2 kDa, corresponding to amino acids 1–132, 1–147 and 1–150 respectively [102]. These peptides act on endothelial cells to inhibit their proliferation [21, 123] and migration [68], reduce vasodilation [48] and vasopermeability [42] and promote apoptosis-mediated vascular regression [32, 77, 122]. The PRL variants of prolactin could alter their biological activity.

11.4 Receptor of Prolactin

PRL is a hormone, whose functions initiate with PRLr binding, followed by activation of signaling pathways leading to physiological actions via paracrine, autocrine and endocrine well confirmed in vitro and in vivo [8, 46, 128]. The gene encoding human PRLr is localized on chromosome 5p13-p14 [14]. The PRLr is a member of the cytokine type I receptor superfamily composed of three major domains; extracellular domain (ECD), transmembranal region (TM) and intracellular domain (ICD). Members of type I cytokine receptors family share sequences for the TM and ECD but differ in the ICD. On the other hand, most of the sequence similarities between cytokine receptors are found within their ECD. The ECD cytokine receptor is formed of 200 aa, known as cytokine receptor homology (CRH) region [136]. PRLr is also a single-pass transmembrane chain as all cytokine receptors. The TM is 24 aa of length. The cytoplasmic domain (ICD) of cytokine receptors has more restricted sequence similarity compared with the ECD. Two relatively conserved regions, called box 1 and box 2 belong to the ICD [60]. Box 1 is an 8 aa membrane-proximal

region highly enriched in prolines and hydrophobic residues. Box 2 is a consensus region much less conserved than box 1 and consists in the succession of hydrophobic, negatively and then positively charged residues (aa 288–298). While box 1 is conserved in all membrane PRLr isoforms, box 2 is not found in short isoforms [46, 60]. Structural alterations are observed in the ECD or ICD, while in the TM variation have not been reported and its structure is conserved in all PRLr isoforms. Expression of PRLr variety of isoforms are probably products of distinct genes [28], differential transcript mechanism including alternative splicing within exons, intron retention, alternative transcription start and termination sites, deletion of partial exon, an alternative promoter [13, 23, 64, 101, 133] and/or post-translational modifications, like cleavage process [13, 53, 62, 64, 125, 133]. These PRLr variants were defined as long form (LF) [16], intermediate form (IF) [63], ΔS1 and the other seven short forms on the basis of their molecular weights and structures. The short forms include S1a, S1b, Δ4-SF1b, Δ7/11, Δ4-Δ7/11 and two short soluble forms (SS1 and SS2). Also, a PRL-binding protein (PRLBP) of 32 kDa which was identified in human serum and milk [23]. Initially the description of the PRLr isoforms have been designated based upon their intracellular domains length [95], but the ΔS1 and S1a are longer than the IF PRLr [16, 53, 63, 64, 133] The mature LF is the hPRLr largest, constituted by 598 residues distributed as a 210-residue EC domain, a 24-residue TM domain and a 364-residue ICD. Changes of hPRL cytoplasmatic domains could modulate the IC signaling systems in distinct pathways.

Therefore, it is likely that the structural heterogeneity of PRLr is associated with pleiotropy. PRLr activation is regulated by a sequence of processes, which initiated with increased concentration of PRL, which in turn promotes binding to monomeric or dimeric forms of the PRLr and also induce structural change in its ECD , to form the ligand/receptor complex; in the next step, the ligand-induced structural changes in the ECD and also induce structural changes to the ICD. These changes in the structure of the ICD promote several processes that allow PRLr activation. The activated ICD docks signaling pathway molecules that modulate cellular processes, all of these reactions are modulated and eventually finished [19]. PRLr expression can be regulated by various stimuli such as inflammatory mediators. A differential expression of mRNA PRLr was observed in an acute inflammation murine model [25]. While in fibroblasts, proinflammatory cytokines induce LF-PRLr [24], and in PBMC from breast cancer patients, the IL-10 was associated with depletion of LF and increased expression of SF-PRLr [99]. Differential expression of PRLr could be a mechanism for orchestrating anti-inflammatory responses on chronic inflammation process.

11.5 Prolactin in the Immune System

Innate immune recognition is based on the detection of constitutive and conserved microbial products known as pathogen-associated molecular patterns (PAMPs), which include LPS and lipotheicoic acid (LTA), that are recognized through pattern

recognition receptors (PRRs), including toll-like receptors (TLRs). The recognition of a variety of bacterial components by individual TLRs in MØ and dendritic cells (DCs) induces inflammatory cytokines, chemokines and triggers functional maturation of DCs by upregulating the receptors CD80 and CD86 [65] and leads to antigen-specific adaptive immune responses initiation and the functional differentiation of T cells. Innate immunity therefore acts as link to acquired immunity control. The HPA axis stimulates natural immunity and suppressor/regulatory T cells, which downregulate the adaptive immune system [10]. Taken together these evidences, PRL might be a cornerstone in the immunoneuroendocrinology network [105]. During immune response, PRL stimulates T, B, and NK lymphocytes, MØ, neutrophils, CD34 hematopoietic cells, as well as DCs [45, 47, 83]. All these effects can be achieved by pituitary and extrapituitary PRL. The extrapituitary sites of PRL production including tissues and cells of the human immune system; PRL mRNA expression in normal and abnormal human lymphoid tissues was observed in thymus, spleen, tonsil, lymph node, and lymphoid tumors, as well as in lymphocytes, epithelial and vascular endothelial cells. A PRL-like molecule is secreted by PBMCs, and PRLr can be found in many cell types, including monocytes and lymphocytes. Therefore, PRL and PRLr expression in immune cells suggest that the hormone may act in an auto- or paracrine way [54, 70, 71, 86].

The immune and neuroendocrine systems are intimately linked and involved in bidirectional communications. Innate and adaptive immunocompetences are maintained by hormones of the HPA axis, like PRL, vasopressin (VP), cytokines and catecholamines [10].

PRL can mediate or alter the cellular and humoral arms of the immune system. It can stimulate and inhibit immune responses, in dose-dependent manner [41, 104]. Also, innate responses modulations as activation of MØ [11] and superoxide anion production responsible for killing pathogenic organisms are mediated by the PRLr [33, 40]. The PRLr is distributed throughout the immune system and belongs to a superfamily, which includes PRL, GH, leptin, IL-2, IL-3, IL-4, IL-6, IL-7, erythropoietin and leukemia-inhibiting factor. After the attachment of PRL/PRLr, several signaling pathways are activated, which include the Janus kinase-signal transducer and activator of transcription (JAK/STAT), the mitogen-activated protein kinases (MAPK) and the phosphoinositide 3 kinase (PI3K). Activation of these cascades results in endpoints such as differentiation, proliferation, survival and secretion [9].

PRL expression in T lymphocytes is regulated by cytokines, both IL-2 and IL-4 reduced PRL mRNA levels in these cells, but PRL has been described as an important mediator for maintaining the function of the thymus and T-cell [29, 43]. Also, PRL stimulates inducible nitric oxide syntheses (iNOS) expression, Ig's and cytokine release in human leukocytes [66, 78, 81], significantly enhances the expression of CD69, CD25 and CD154 and cytokines secretion, as well as modulates B cells development [26, 88, 124]. In the same context, enhanced B-cell activity is mediated by high-dose of PRL as well as the suppression of natural killer cell-mediated cytotoxic function in vivo [104]. PRL (100 ng/mL) is capable of inducing the interferon regulatory factor (IRF-1) expression, a key transcrip-

tion factor driving the Th1 phenotype in T cells and may promote the development and function of this cellular subset [74, 120]. In the same context, later findings have shown that PRL acts as a T-cell mitogen through the PRLr/JAK/STAT/IRF-1 signaling pathway and NF-kB signals [139]. The divergent immune effects of low and high-dose PRL may involve modulation of T-bet, a key transcription factor directing T helper type 1 inflammatory responses. T-bet is also modulated in a $CD4^+$ T-cell line by PRL exposure [129]. Also, the expression of PRL and its receptor in Treg and effector T (Teff) cells has been reported. PRL treatment in cultures, favored a Th1 cytokine profile, with increased production of TNF-α, IFN-γand IL-2 in Teff cell, but inhibited the suppressive function of Treg cells, apparently through the induced secretion of Th1 cytokines [29, 69]. In autoimmune diseases, non-organ-specific such as systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, and psoriasis arthritis, as well as in organ-specific autoimmune diseases such as celiac disease, type 1 diabetes mellitus, Addison's disease, and autoimmune thyroid diseases hyperprolactinemia condition has been described [56]. In autoimmune diseases are involving alterations in the balance of proinflammatory and antiinflammatory responses, as well as in increasing circulating PRL level that might be related in their pathogenesis. The immunomodulatory activities of PRL may arise from increasing nuclear transcription factors such as IRF-1 and NFkB, which play a pivotal role in many immune functions and pathophysiological processes in physiological hyperprolactinemic states (e.g. pregnancy) [17]. However, PRL is known to have other contradictory actions on the immune system that depend upon the concentration (e.g. it can inhibit lymphocyte proliferation at high concentrations, while it enhances proliferation at lower concentrations [79, 80, 87].

The effects of PRL on immune responses are stimulatory and enhance production of cytokines, such as IFN-γ, IL-12 and IL-10, as well as T-cell proliferation. PRL also alters the functions and selection of B cells, resulting in the breaking of tolerance of autoreactive B cells [78]. Consistent with this, bromocriptine administration abrogates the estradiol-induced breakdown of B cell tolerance [100]. However, effects of PRL on the immune system are complex. The removal of pituitary gland weakens thymus growth [91] and immune reaction to immunogenic factors in rats. On the other hand, hypoprolactinemia, in mice injected with *Listeria monocytogenes* or *M. tuberculosis*, increases mortality associated with impaired lymphocyte proliferation and decreased MØ-activating factor production (IFN-γ) by T lymphocytes [11]. Also PRL has been described as a potent positive modulator of immunity to some protozoan parasites that stimulates IFN-γ and many other Th1-type cytokines production during *Toxoplasma gondii*, *Leishmania sp.* and *Acanthamoeba castellanii* infections. PRL has been proposed as a regulator of antiparasitic activity against *Plasmodium falciparum*. On the other hand, hyperprolactinemia-associated to pregnancy may have a relevant contribution to reactivation of latent infections caused by many helminthic parasites, like *Ancylostoma* sp. or *Necator* sp. It is possibly connected with the process of transmammary transmission of hookworm infection to breast-fed newborns [37, 103]. The large number of data described above support the role of the PRL in immune system.

11.6 Prolactin in Inflammatory Response in Myeloid Cells

Injury or pathogens agents induce alarm signals that lead to development of the acute inflammatory response in order to lead to containment and elimination of microbial invaders. This process is a complex organized sequence of events that includes activation of endothelial cells, adhesive interactions between leukocytes and the vascular endothelium, recruitment of leukocytes, activation of tissue MØ, activation of platelets as well as their aggregation, activation of the complement, clotting and fibrinolytic process, and release of proteases and oxidants from phagocytic cells. All these mechanisms contribute to re-establish homeostasis after the state of injury. Uncontrolled inflammation becomes excessive or prolonged, results in serious damage of tissues and organs and leads to the development of the physiopathologic basis to the wide range of inflammatory diseases. Resolution phase of the inflammatory response is orchestrated by mechanisms including leukocytes removing through lymphatics or by apoptosis ways, to give an end to the ongoing inflammatory response. This response is subject to very tight regulation to contain the cascades before they lead to damage to tissues and organs. There are numerous anti-inflammatory factors naturally occurring: inducible cytokines such as IL-4, IL-10 as well as IL-12 in very low concentrations that participate in acute inflammatory response and in containment processes by stabilizing IκBα and the blockade of NF-κB activation. Also there are several transcriptional regulatory factors such as suppressor of cytokine signaling 3 (SOCS3) and STAT3 that block the proinflammatory genes activation, resulting in a decrease of proinflammatory factors concentration.

The inflammation events result in an increase of polymorphonuclear leukocytes (PMNs) in the inflamed area at the onset of the lesion, which are later gradually replaced by mononuclear cells, mainly monocytes, which then differentiate into MØ [114]. Myeloid cells display extensive plasticity of their phenotype in response to various stimuli. This characteristic directly impacts polarization and activity of lymphocytes, also is controlled by changes at both transcriptional and translational level. The surface receptors of the MØ and closely related myeloid cells regulate a range of functions, including differentiation, growth and survival, adhesion, migration, phagocytosis, activation, and cytotoxicity [127]. MØ surface-expressed molecules have capacity to recognize a diversity of endogenous and exogenous ligands, and implement an appropriate response, which has a pivotal role in MØ functions like homeostasis, host defense in innate and acquired immunity, autoimmunity, inflammation and immunopathology [49, 57]. Diverse process as an enhanced phagocytosis have been recently investigated, the results point to classic opsonins (antibody and complement), also into sensing mechanisms of a range of microbial ligands by toll-like receptors (TLR) and families of cytosolic proteins (e.g., NODs, NALPs) [59, 96, 65].

After recognizing their respective pathogen-associated molecular patterns (PAMPs), TLRs initiate signaling pathways activation that results in specific immunological responses adapted to the PAMPs expressed by pathogens. The TLRs specific response is dependent of the recruitment of, TIR domain-containing adaptor

protein (e.g., MyD88, TIRAP, TRIF or TRAM) [58. 59]. MyD88 transmits signals culminating in NF-kB and MAPK activation followed by the induction of inflammatory cytokines and is utilized by all TLRs as well as members of IL-1 receptor family [59].

During inflammatory process, the host responds with a defensive reaction, the acute phase response mechanisms, subsequently injury is matching with alterations in immune, metabolic, neuroendocrine and behavioral functions [6], cells enhance cytokine production and orchestrate diverse important immunomodulatory roles associated with this response. MØ can be either classically or alternatively activated depending on the cytokine profile of the surrounding inflammatory environment [114]. These changes can lead to an imbalance in immune responsiveness and susceptibility to infections. The immune cells provide a very important feedback component to the brain. In addition to acute inflammation, there is a range of other clinical conditions in which peripheral cytokine signals might modulate brain function [72].

Monocytes (Mo) originate in the bone marrow from a common myeloid progenitor shared with neutrophils. They are then released into the peripheral blood, where they circulate for several days before entering tissues to restock the tissue MØ populations. Circulating Mo constitute lower than 10% of blood immune cells, nonetheless serve a critical role as primary responders to infection [44, 89]. Circulating blood Mo give rise to a variety of tissue resident MØ throughout the body, as well as to specialized cells such as dendritic cells (DCs) and Langerhans cells in the skin. Pro-inflammatory stimuli elicit increased recruitment of Mo to peripheral sites, where differentiation into tissue MØ and DCs occurs. However, this differentiation pathway is still poorly understood in vivo. Several studies have provided evidence that there is substantial heterogeneity in the phenotype of Mo. This diversity may reflect the specialization of individual tissue MØ populations within their environments [50, 76]. Given that MØ play pivotal roles during tissue repair, the questions arise, which signals and mechanisms control the accumulation of MØ at the wound site, and whether one of the Mo subsets may be preferentially recruited. The “inflammatory” subset expresses high levels of the chemokine receptor CCR2 and low levels of the fractalkine receptor CX3CR1 [1], whereas the “non-inflammatory” subtype is characterized by low expression of CCR2 and high expression of CX3CR1 [2]. MØ are generally classified as either classically (M1) or alternatively (M2) activated [50]. While this nomenclature is based on the phenotype MØ acquire in response to defined stimuli in vitro, inflammatory characteristics of MØ at sites of inflammation in vivo are less well-studied. In particular, the phenotype of MØ found during resolving inflammation is little unknown. The signals that control their post-inflammation repopulation and the subtypes that confer protection and signal homeostasis are unknown at this stage. Thus, MØ are important for resolution and restoration of homeostasis after inflammation with the phenotype (M1, M2, or rM) dictating whether inflammation abates or progresses to wound healing. This will depend on the degree of inflammation and associated tissue injury signals in situ.

In mononuclear phagocytes, reprogramming is a regulatory process useful during inflammatory response, driven by several cytokines and some hormones [38,

71, 75]. Although the role of PRL in different cells during inflammation has been investigated, the results might be considered controversial due to masked effects of other molecules released by differentiated inflammatory cells into the culture medium. Inflammatory response after a brain injury, such as proliferation and activation of glia is enhanced by PRL [85]. The production and secretion of PRL by activated human MØ was previously reported [110]. Secretion of PRL by activated Mo as early as 6 h following complete Freund's adjuvant injection could support a role for local PRL in contributing to the early inflammatory response. Therefore, increased PRL at this early time point may be important in the initiation of a cascade of immune responses [111, 112]. In MØ, PRL stimulation significantly enhanced IL-6 production in response to TNF-α or CD40L, first evidence that PRL is produced locally in the synovium of patients with inflammatory arthritis, and contributes to the activation of MØ in the presence of other inflammatory stimuli was given by Tang et al. [126]. Others studies show that rodent and human MØ synthesize PRL in response to inflammation and high glucose concentrations [15]. PRL is also known to enhance immune functions in fish as in mammals. The phagocytic activity of fish leukocytes is stimulated by administration of PRL [5, 51]. The signaling pathway involved in the activation of fish MØ by PRL and, in particular, its cross-talk between JAK/ STAT and NF-kB signaling pathways, a mechanism which promotes MØ polarization in fish to a proinflammatory M1/classically activated phenotype is characterized by the production of reactive oxygen species (ROS) and proinflammatory cytokines [94, 109]. In vitro and in vivo MØ treated with PRL induced an enhanced superoxide anion production, elevated phagocytic index and increased phagocytic activity [98].

The precise molecular mechanisms by PRL modulating the inflammatory response is controversial, various data show the action dichotomy of this hormone. PRL modulating role of inflammatory response in myeloid cells has been described previously. Peritoneal MØ respond to PRL significantly enhanced NO production through protein tyrosine kinases, MAPK and Ca^{++} channeling pathways activation [131]. Also in the same experimental model, PRL significantly enhances production of IL-1β, IL-12p40 and IFN-γ [116] through JAK/STAT1 and JNK MAPK [132] also Ca^{++} and p42/44 MAPK pathways activation [117]. However, higher doses of the PRL (1000 ng/ml) induced IL-10 synthesis with significant abrogation in proinflammatory cytokines production in the same cells production correlated with pSTAT3 expression [116].

However, the activation of molecules associated to PRL/PRLR signaling pathways could open new fields for understanding the effects of PRL in inflammatory processes in myeloid cells and in other cell types and tissues. In this context, the role of STAT3 in different inflammatory processes has been described, e.g. in tumoral processes STAT3 promotes inflammation thought activation of NFκB and IL-6 production [140], on the other hand, displays anti-inflammatory activity, and it can suppress both IL-6 and TNF-α synthesis in LPS-stimulated MØ [137]; may be mediated by IL-10 increase. Also, in Crohn's disease constitutive activation of STAT3 has been observed mainly in intestinal T cells from biopsies of patients [73]. Furthermore, in synovial fibroblasts, activated STAT3 was not able to suppress IL-6

synthesis, therefore suggesting that the cellular environment plays an important role to lead to a pro- or anti-inflammatory response by STAT3 [137]. Also, MAPK pathways can be activated by PRL. The ERK 1/2 pathway is activated by mitogenic stimuli-like growth factors, while the p38/MAPK pathway is stimulated by stress and environmental inflammatory cytokines profiles [20]. Therefore, activation of MAPK pathways is responsible for phosphorylating and activating several transcription factors that in turn stimulate inflammatory cytokines synthesis [18, 34].

Little is known regarding the expression of extrapituitary PRL and PRLr isoforms in myeloid cells [31, 82]. Moreover, the precise role and mechanism of action of PRL in mononuclear phagocytes still remains elusive. Further, PRL causes a significant increase in the phosphorylation level of p38 MAPK in mononuclear cells [52]. Among other genes induced by PRL are several members of SOCS family and iNOS [93, 113].

We demonstrated the expression of a full-autocrine loop of PRL enhances the inflammatory response in activated Mo. PRLr mRNA and PRL mRNA RT-PCR assays were performed to determine if THP-1 Mo treated with LPS were able to synthesize. The results showed that the expression of total PRLr mRNA increased over 300-fold from 1 h to 72 h after LPS treatment, and the expression of PRL mRNA increased 80-fold after 1 and 2 h of LPS stimulation. In addition, two PRLr isoforms of 100 and 50 kDa and two PRL variants (big PRL 60 kDa and bigger PRL 80 kDa) were identified in THP-1 Mo. Mo expressed these same isoforms from healthy subjects after stimulation with LPS. PRL and PRLr synthesized by these cells were related with nitrites and proinflammatory cytokines (IL-β, TNF-α and IL-6). This response mediated by big PRL may contribute to the eradication of potential pathogens during innate immune response in Mo but may also contribute to inflammatory disorders (Fig. 11.2**)** [71].

Our working group also observed that the expression of autocrine PRL and overexpression of short isoforms PRLr in Mo is stimulated with *CFP-Mycobacterium bovis (M. bovis)*. Our results suggest that *CFP-M. bovis* induces overexpression of short and intermediate isoforms and autocrine synthesis of Big-PRL and bigger-PRL in myeloid cells. Both molecules were associated with the induction of apoptosis because inhibiting the big PRL and PRLr at 48 h induced the decrease of apoptotic cells stimulated with *CFP-M. bovis* (Fig. 11.3)**.** This autocrine mechanism might play an important role during the inflammatory response in Mo. Therefore, the pleiotropic functions of PRL might be mediated by different isoforms of its receptor (PRLr) (López-Rincon et al. in preparation).

The PRL role in modulating responses against pathogens as mycobacteria, as well as other immune processes is controversial. PRL promotes intracellular multiplication of *Mycobacterium avium* subspecies *paratuberculosis* in bovine peripheral blood Mo, which in turn may contribute to the progression of the infectious state, but on the other hand, no significant change in the phagocytic function of the cells was observed [36]. Recently, we confirmed PRLr mRNA synthesis in MØ after *M. bovis* exposure and proposed that molecular pathogen patterns of *M. bovis* might modulate inflammation during bovine tuberculosis (bTB) through expression of the PRLr isoform in MØ. PRLr isoforms expressed in Mo and MØ were observed in infected cattle. Induction of specific isoforms of PRLr by *M. bovis* in cattle was

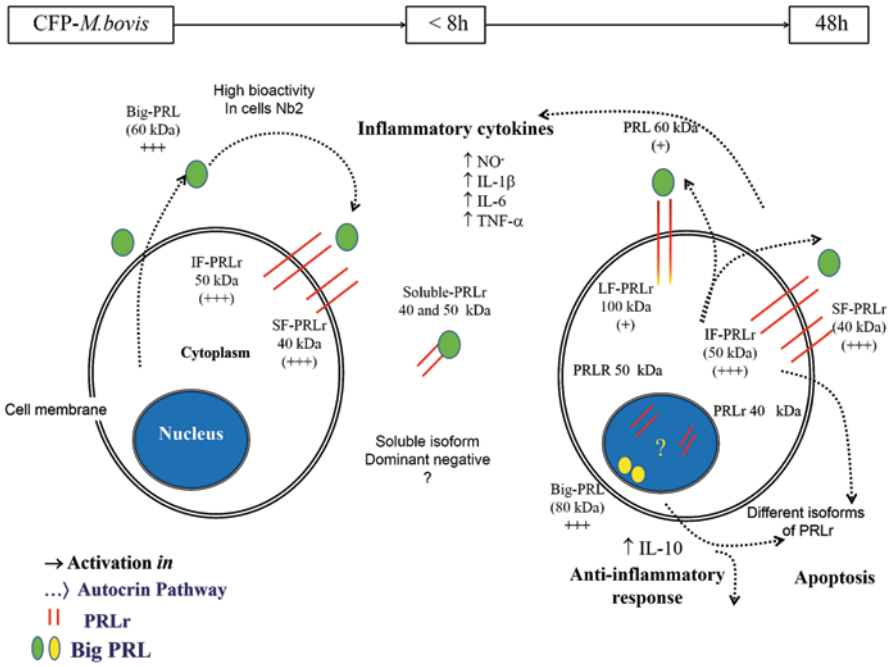

Fig. 11.2 Schematic representation of autocrine loop of PRL in activated monocytes by culture filtrate proteins (CFP) of *Mycobacterium bovis* elicit differential expression of PRLr isoforms (intermediate and short) and a big PRL in time-depending manner. After 1 h and before 8 h, monocytes synthesize PRLr isoforms of 40 and 50 kDa as well as big PRL of 60 kDa; and later after 48 h also express PRLr of 40, 50, 65 kDa and big PRL of 80 kDa instead 60 kDa. The overexpression of PRLr isoforms (intermediate and short) was observed. These isoforms could exert dominant negative function and/or activates alternative signaling pathways (apoptosis). Big PRL of 60 kDa displays proliferative bioactivity lactogen dependant-Nb2 cells. This big PRL is a PRL storage and source of functional peptides hydrolysis derived. Interaction of big PRL with long form of PRLr might activate JAK2/STAT1 and the proinflammatory cytokines release. Interaction of pituitary PRL with intermediate form of 40, 50 and 65 kDA might activate PI3k/Akt and IL-10 release. Interaction of big PRL complex with chaperon with short PRL in the nucleus affects transcription rates of some target genes

confirmed in peripheral blood Mo and derived MØ. We propose that molecular patterns of *M. bovis* might modulate chronic inflammation during tuberculosis; modulating expression of PRLr isoforms in MØ. Further analyses are necessary to elucidate the role of PRLr in the antimycobacterial defense or inmmunopathogenesis of bTB [70]. Mammary gland (MG) displays variants of PRL-PRLr depending on the physiological state, milking or involute. In lactating cows, MØ present in MG express short PRLr isoforms (65 and 40 kDa) depending on the *M. bovis* infection (Pereira-Suárez et al. in preparation).

Based on the diverse data of the PRL/PLRr system, it suggests a novel purpose in the biology of this regulatory mechanism. In the classical view, pituitary PRL (23 kDa) acts in diverse homeostasis process through mainly LF, but also IF PRLr (100, 75-80 kDa).

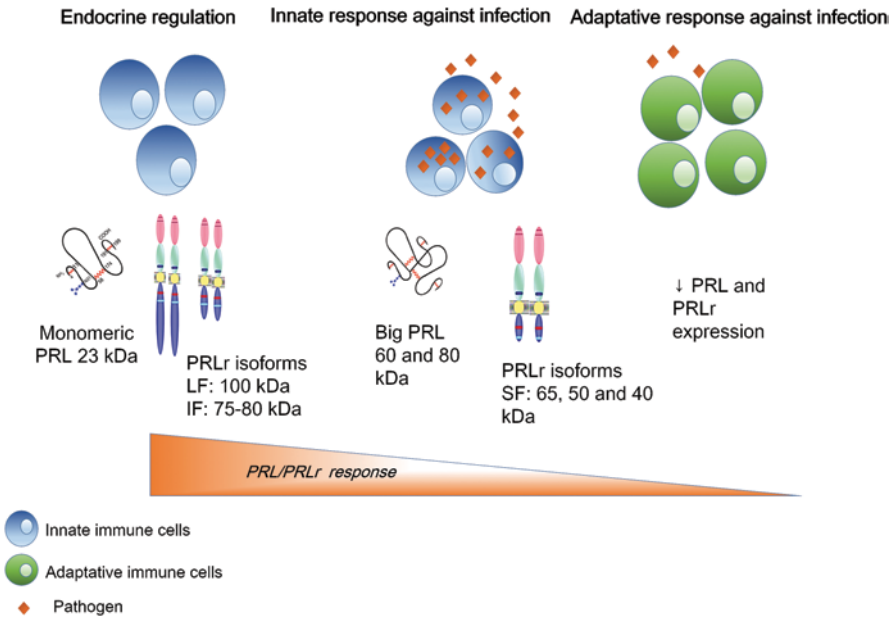

Fig.11.3 Novel views in PRL biology. The classical view is represented on the *left*, while novel concepts in immune cells and infection are shown in the *middle* and *right side*. Endocrine PRL acts in monocytes and macrophages through LF PRLr. Extrapituitary synthesis of PLR stimulates inflammatory response induced in myeloid cells. The system is regulated by the diverse PRLr isoforms

In infectious states, variants of autocrine big PLR have been described (60 and 80 kDa) and also a predominant expression of SF PLRr (65, 50 and 40 kDa) in innate immune cells. Big PRL is associated with an increase in inflammatory responses, but the SF of PRLr may participate as a negative regulator of the LF isoform, by inmunocompetence or ligand-increased affinity. The PRL/PRLr system actions are downregulated in adaptative immune response against infection (Fig. 11.3), suggesting a possible pivotal role the modulation of inflammation process in infectious states by the diversity in PRL/PRLr complex conformation.

Conclusions

In summary, the neuroendocrine and immune systems communicate bidirectionally via shared receptors and messenger molecules, such as hormones, neurotransmitters and cytokines. The immune capacities of PRL are related, among others, to co-mitogenic activity, prevention of immune cell apoptosis, interleukins stimulation and antibodies production. The role of PRL during pathogenic inflammatory conditions such as autoimmune diseases has been strongly studied and documented. To date, it is well recognized that PRL enhances the progression of immune process in autoimmune diseases.

Changes in endocrine responses in chronic infections such as tuberculosis, has been poorly characterized. The cellular immune response with a bias towards a Th1 cytokine pattern in the early stages of infection is the most efficient way to solve the disease because it promotes hypersensitivity reactions and activation of MØ. Endocrine responses act parallel to the immune response to infectious agent and influence the course of infection. The cytokines effects on the hypothalamus–pituitary–thyroid–adrenal–gonadal axis mediate some of the defence mechanisms.

The immune-modulatory activities of PRL may arise from increasing nuclear transcription factors such as IRF-1 and NF-kB, which play a pivotal role in many immune functions. Proinflammatory mediators such as production of cytokines, chemokines or nitric oxide (NO) release, have been associated to PRL. However, its role during microbial pathogen infection and its association in modulating the expression of different PRL and PRLr isoforms needs to be further studied, in order to understand the diversity of mechanism PRL-regulated in different cell types in physiological as well as pathological conditions.

Finally, the autocrine synthesis of PRL and PRLr expression in myeloid cells in infectious inflammatory processes and/or chronic responses could be heading in the knowledge and application of the regulatory network that could elucidate a key role of PRL in immune-inflammatory response as well as resolution processes by different mechanisms.

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Chapter 12
A Positive Feedback Loop Between Prolactin and STAT5 Promotes Angiogenesis

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Abstract The signal transduction events that orchestrate cellular activities required for angiogenesis remain incompletely understood. We and others recently described that proangiogenic mediators such as fibroblast growth factors can activate members of the signal transducers and activators of transcription (STAT) family. STAT5 activation is necessary and sufficient to induce migration, invasion and tube formation of endothelial cells. STAT5 effects on endothelial cells require the secretion of the prolactin (PRL) family member proliferin-1 (PLF1) in mice and PRL in humans. In human endothelial cells, PRL activates the PRL receptor (PRLR) resulting in MAPK and STAT5 activation, thus closing a positive feedback loop. In vivo, endothelial cell-derived PRL is expected to combine with PRL of tumor cell and pituitary origin to raise the concentration of this polypeptide hormone in the tumor microenvironment. Thus, PRL may stimulate tumor angiogenesis via autocrine, paracrine, and endocrine pathways. The disruption of tumor angiogenesis by interfering with PRL signaling may offer an attractive target for therapeutic intervention.

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12.1 Introduction

Angiogenesis, the formation of a new microvasculature, is a critical process during normal organ development as well as tumor initiation, progression, and metastasis [1–3]. Angiogenesis requires the orchestration of a sequence of events at a spatial and temporal level, which include the degradation of the vascular basement membrane, migration, and invasion of endothelial cells (ECs) into the perivascular space of the stroma surrounding a preexisting vessel, proliferation along the migration path, differentiation into a new vessel tube, and vessel maturation [4, 5].

Malignant neoplasms depend on the induction of new blood vessels (angiogenesis) for continued growth and metastatic spread [4–6]. Angiogenesis combined with aberrant endothelial cell proliferation is evident in many types of malignant neoplasms, particularly in high-grade gliomas [7, 8]. Due to the apparent reliance of lethal tumors such as gliomas on angiogenesis and the lack of effective targeted treatments, angiogenesis inhibition carries promise as an alternative therapy. The rational design of such treatment strategies, however, requires a more complete understanding of the biology of angiogenesis regulation.

12.2 Proangiogenic Factors Induce STAT Activation in Endothelial Cells

A myriad of secreted growth factors and peptide hormones are capable of inducing the generation of new blood vessels [3, 9, 10]. However, the autocrine and paracrine signaling networks and the intracellular signal transduction events that orchestrate the cellular activities required for angiogenesis are still incompletely understood. Some of the most potent proangiogenic factors such as FGF family members and VEGF primarily signal via the ras-raf-MAPK and PI3K-Akt signaling pathways. However, reports also implicate STATs in FGF signaling and consequently, these transcription factors may play a role in angiogenesis [11]. Furthermore, phosphorylated STAT3 can be detected in activated endothelial cells of high-grade gliomas, suggesting a role for STAT family members in angiogenesis of this tumor type [12].

12.2.1 FGF-Induced STAT Activation

We recently identified the activation of STAT, especially STAT5, as an important event in FGF-induced angiogenesis. Interestingly, the patterns of STAT activation are likely organsite-specific. In mouse brain microvascular endothelial cells, both FGF2 and FGF8b activate STAT5 and to a lesser extent STAT1 in a time-dependent fashion, whereas STAT3 phosphorylation remains unchanged. Conversely, in microvascular endothelial cells from bone, both FGFs trigger robust but transient phosphorylation of STAT3 and STAT5 but not STAT1. The different STAT family members activated in endothelial cells from different organ sites may be related to

differential expression of FGFR isoforms in these cells. In order to function as transcription factors, STATs must enter the nucleus after tyrosine phosphorylation [13] and, as expected, both STAT5 and STAT1 proteins translocate to the nucleus upon stimulation of mouse brain endothelial cells with FGF. Importantly, FGF-induced STAT5 activation is also observed during angiogenesis in vivo, using the subcutaneous matrigel plug assay; and in human glioma samples [14].

12.2.2 STAT5 Is Not Involved in FGF-Induced Mitogenesis but Is Required for Endothelial Cell Migration, Invasion and Tube Formation

These observations demonstrate STAT activation in response to FGF but do not necessarily indicate a direct role of STAT activity in angiogenesis. As a first step to explore a potential requirement of STATs in angiogenesis, we examined whether STAT5 is involved in regulating endothelial cell mitogenesis. The expression of a constitutively active STAT5A mutant (CA-STAT5A) failed to induce endothelial cell proliferation, suggesting that FGF-induced mitogenesis in brain endothelial cells is independent of STAT5 activation. In contrast, CA-STAT5A stimulated endothelial cell migration, invasion, and endothelial tube formation in matrigel or collagen, arguably the most relevant in vitro angiogenesis assays [2]. Conversely, the introduction of a dominant-negative STAT5 abolished FGF2- and FGF8b-induced in vitro angiogenesis. These observations strongly suggest that STAT5 mediates specific FGF-induced angiogenic events.

This raises the question whether STAT activation represents a general signaling pathway for other proangiogenic growth factors. Indeed, the potent angiogenic stimulator VEGF also induces tyrosine phosphorylation of several STAT family members, including STAT1, 3, 5, and 6 [14–18], and at least VEGF-mediated STAT3 activation is essential for migration and tube formation in human dermal microvascular endothelial cells [17]. The angiopoietin receptor Tie-2 has also been shown to activate STAT1, 3, and 5 [19]. Interestingly, activated STAT3 was detected in tumor vessel endothelial cells of gliomas [12], further suggesting that STATs play roles in tumor angiogenesis.

12.3 Activation of STAT5 in Endothelial Cells Induces the Expression of Prolactin Family Members

12.3.1 STAT5 Induces Proliferin 1 (Plf1) Expression in Mouse Endothelial Cells

Activation of STAT5 is proangiogenic by inducing endothelial cell migration, invasion, and tube formation, all of which are critical components of the angiogenic cascade. However, the mechanism by which STAT5 mediates angiogenesis remains

unclear. In an attempt to explore the signaling pathway downstream of STAT5, we discovered that conditioned media produced by endothelial cells transduced with CA-STAT5A exhibit a significant proangiogenic activity, suggesting that STAT5 activation in endothelial cells leads to the expression and secretion of a single or multiple soluble factors that specifically promote angiogenesis via an autocrine mechanism. Thus, we compared global gene expression signatures between endothelial cells transduced with CA-STAT5A, dominant negative (DN)-STAT5A, and control virus. With this method, we identified 31 transcripts corresponding to 20 unique genes that were overexpressed at least fivefold in CA-STAT5-transduced cells compared to the other cells. Out of these 20 genes, we selected Prl2c2 for further study because it encodes proliferin-1 (PLF1), a secreted protein which has been reported to stimulate endothelial cell migration in vitro and neo-vascularization in vivo [20, 21]. Indeed, subsequent experiments confirmed that PLF1 secretion is responsible for all STAT5-induced proangiogenic endothelial cell responses in vitro. As expected, the proangiogenic growth factors FGF2, FGF8b, and VEGF induce the secretion of PLF1 by endothelial cells. [14]. CA-STAT5 increases PLF1 transcript levels and binds to the PFL1 promoter region as determined by chromatin immunoprecipitation (ChIP) assay, suggesting that STAT5 participates directly in the transcriptional regulation of PFL1 [22].

12.3.2 STAT5 Induces Prolactin (PRL) Expression in Human Endothelial Cells

Conditioned medium from human CA-STAT5-transduced endothelial cells induces angiogenesis in vitro, similar to conditioned medium from mouse endothelial cells. In the mouse, PLF1 is a member of an extensive family of paralogous genes that originated through gene expansion. In humans, PRL appears to be the sole member of this family unless growth hormone and placental lactogen are included as members of a wider group of genes [20, 23–25]. Therefore, we investigated whether active STAT5 induces PRL secretion in human endothelial cells and whether PRL induces angiogenesis in vitro. Indeed, after STAT5 activation in human brain endothelial cells, elevated PRL protein secretion into the conditioned medium is detected, and PRL mRNA levels are increased, while growth hormone (GH) and placental lactogen (PL) remain unchanged [26]. The induction of PRL by CA-STAT5 could be the result of direct transcriptional regulation or it could be mediated by an indirect regulatory pathway. A NCBI Entrez Gene database analysis revealed multiple potential STAT5 binding sites within the PRL gene promoter region and ChIP confirmed STAT5 binding to the PRL promoter region in an activation-dependent manner [26]. Together, these observations in mice and humans point to an evolutionarily conserved mechanism where active STAT5 induces the secretion of a prolactin family member to mediate or synchronize proangiogenic events.

12.3.3 STAT5-Induced PLF and PRL Promote Endothelial Cell Migration, Invasion and Tube Formation but Not Mitogenesis

PRL and PLF1 act as downstream effectors of STAT5, however, their precise functions on endothelial cells had to be further defined. The conditioned medium from CA-STAT5A but not from DN-STAT5A-expressing endothelial cells induces the phosphorylation of the PRLR and of ERK1/2. When PRL expression is silenced by RNAi, the CA-STAT5A conditioned medium failed to induce PRLR or ERK1/2 phosphorylation, demonstrating that PRL is bioactive and responsible for activation of the PRLR signaling pathway [26]. As expected from the mouse experiments, STAT5-induced PRL failed to stimulate human endothelial cell mitogenesis. Similarly, recombinant PRL used at a concentration of up to 1 μg/ml failed to induce a significant mitogenic effect. The mitogenic effect of PRL on endothelial cells is controversial. While some reports describe the induction of endothelial cell proliferation by PRL [27], we and other investigators have failed to observe this activity [26, 28]. This apparent discrepancy may be due to different endothelial cells types studied or different functional states of the target cells. However, silencing of PRL expression in the producer cells or neutralization of PRL activity in the conditioned medium consistently abolishes CA-STAT5A-induced endothelial cell migration, invasion, and tube formation. Moreover, recombinant PRL can rescue endothelial cell tube formation in conditioned medium produced by cells in which PRL expression had been knocked down, implicating PRL as the active proangiogenic constituent. The proangiogenic effect of STAT5-induced PRL is not limited to human brain endothelial cells but is also seen in human umbilical cord endothelial cells (HUVEC) [26] (Yang, Friedl, unpublished data).

Coexpression of PRL and STAT5 was detected in endothelial cells of some but not all human glioma tissue samples. STAT5 is found primarily in the endothelial cell nucleus, suggesting activation of this transcription factor in this cell type [26]. STAT5 is also seen in scattered glioma cells, while PRL is expressed in the majority of glioma cells. It should be pointed out that PRL also localizes to the nucleus of endothelial cells. This subcellular location is somewhat unexpected for a secreted hormone, however, it is consistent with prior reports of PRL localization to the nucleus of lymphocytes and breast carcinoma cells and has been shown to be functionally relevant [29–31].

12.4 PRL and PLF as Proangiogenic Factors

12.4.1 Autocrine and Paracrine PRL

Prolactin (PRL) is a secreted pituitary hormone and, along with growth hormone (GH), placental lactogen (PL), prolactin-like proteins (PLPs), and prolactin-related

proteins (PRP), forms the greater PRL family of hormones. The PRL family shares structural similarities and probably resulted from the duplication of a sole ancestral gene [32, 33]. For example, there is approximately 85% sequence similarity between the peptide sequence of human GH and PL, while human PRL shares about 25% identity with GH or PL [34]. Apart from peptide sequence homology, the biological activities, particularly proangiogenic roles, also appear to be conserved across species with PLF fulfilling this role in mice [20].

While lactotrope cells in the anterior pituitary gland represent the major site of PRL production [32, 33], extrapituitary cells such as decidual cells, adipocytes, T lymphocytes, macrophages, and epithelial cells can also secrete PRL, suggesting that this hormone can act locally as an autocrine or paracrine factor [33, 35]. PRL is also secreted by various endothelial cells, a cell type that also receives PRL signals due to the expression of PRLR [26, 28, 36–40]. The PRL produced by different cell types can act locally as growth factor, neurotransmitter, or immunomodulator in an autocrine or paracrine manner. The synthesis and release of prolactin by the lactotrope cells in the pituitary is tightly regulated by multiple factors including dopaminergic neurons which exert an inhibitory effect [41, 42]. Little is known about the regulatory mechanisms that control expression of prolactin at extrapituitary sites but extrapituitary prolactin synthesis does not appear to be sensitive to dopamine [43].

12.4.2 Proangiogenic Activities of PLF and PRL

PLF1 was originally described as a protein secreted by embryonic fibroblasts in response to serum growth factors and was identified in a mouse placenta expression library [44, 45]. As a nonclassical family member of PRL/GH/PL family of polypeptide hormones, PLF1 is primarily produced by deeply invasive trophoblast giant cells in the pituitary gland and the placenta during midpregnancy in most species but not human [24, 46]. Evidence for a proangiogenic activity of PLF1 came from the work of Jackson and coworkers, who demonstrated that the protein promotes angiogenesis in vitro and in vivo [20]. Further indication for a role in angiogenesis came from the observation of PLF1 binding to endothelial cells with high affinity [47]. A role for PLF1 in tumor angiogenesis was first evaluated by Toft et al., who found that fibrosarcoma cells secrete increasing amounts of proangiogenic PLF1 while progressing to a more aggressive phenotype [21]. Interestingly, proliferin-related protein (PRP) suppresses angiogenesis, possibly by antagonizing PLF1 activity [20].

The mechanism underlying PLF1-induced angiogenesis remains unclear. PLF1 binds to the insulin-like growth factor 2/mannose 6-phosphate receptor, which is required for PLF1 activity, but how this receptor induces downstream angiogenic events is unresolved [48]. The 16 kDa PRL fragment competes with PLF1 for high-affinity endothelial cell binding, suggesting that the PRL receptor may also be involved [39, 49]. While the precise mechanism of receptor activation remains obscure, it appears to involve a G-protein-coupled pathway and mitogen-activated phosphokinase [50].

Compelling evidence indicates that both PRL and PLF regulate angiogenesis in vitro and in vivo [20–22, 26, 27, 34, 39, 40, 51–56]. As circulating hormone and as paracrine/autocrine factor, PRL can either stimulate or inhibit various stages of angiogenesis, including migration, proteolytic matrix remodeling, apoptosis, and possibly endothelial cell proliferation [27, 28, 34, 36, 51, 57]. PRL binds to endothelial cells and affects endothelial cell adhesion [40], stimulates endothelial cell proliferation [27, 53] and improves engraftment and function of transplanted pancreatic islets [58]. PRL also promotes angiogenesis in vivo [51, 54]. For example, invasive prolactinomas, primary, pituitary, PRL-secreting tumors, are highly vascular [52, 55, 59] and the injection of a PRL-encoding construct increased angiogenesis in the testis [54].

The intact 199 amino acid, 23 kD human PRL molecule is proangiogenic [51, 60–66]. However, shorter 16 kD fragments of PRL family members potently inhibit angiogenesis and are therefore termed angioinhibins [49, 51, 60, 66, 67]. The 16 kD fragments are generated by proteolytic cleavage of full length PRL, GH, and PL at the N-terminus, catalyzed by several enzymes including cathepsin D, matrix metalloproteases (MMPs) and bone morphogenic protein (BMP) [66, 68, 69]. The dual function of full length and shorter fragments of PRL as stimulators and inhibitors of angiogenesis constitutes an efficient and unique regulatory mechanism and might involve the utilization of different signaling receptors [49, 51]. While full-length PRL utilizes the PRLR, proteolytic fragments of PRL appear to signal via distinct receptors which have so far eluded characterization [39, 70].

12.5 PRL Activates STAT Through the PRLR

12.5.1 PRLR Signal Transduction

The pleiotropic activities of PRL are mediated through binding to the PRLR cell surface receptor, a member of the cytokine class-I receptor superfamily, which is expressed in a wide variety of cells and tissues including endothelial cells [71–75]. A number of receptor isoforms are generated through differential splicing and post-translational processing [71, 76–78]. The human long form of PRLR, consists of 590 amino acids and is capable of mediating all of the PRL signaling events. The PRLR consists of an extracellular ligand binding domain, a single transmembrane region, and an intracellular domain [79–81]. The PRLR contains no intrinsic cytoplasmic tyrosine kinase domain but instead recruits cytoplasmic tyrosine kinases of the JAK2 and Src families [79–84]. The shorter PRLR isoforms distinguish themselves from the long form by an altered ligand binding domain or an impaired ability to engage intracellular signaling partners [85]. Several signaling cascades are activated upon ligand binding and receptor dimerization, including the JAK2/Src/STAT5, Ras/Raf/MAPK, Tek/Vav/Rac1 and PI3K/Akt pathways [72, 74, 78, 86, 87]. The concerted action of JAK2/STAT5 is especially important for angiogenic and survival phenotypes propagated by PRL [72, 74, 78, 86, 87], and thus will be discussed more extensively below.

12.5.2 JAK2-STAT

The JAK family consists of the four members JAK1, JAK2, JAK3, and Tyk2 [88–90]. Among these, JAK2 is constitutively associated with the PRLR [91], and is considered the major PRLR-associated Janus kinase, although JAK1 is also possibly involved in some situations [92].

JAK2 is activated quickly following PRLR stimulation, suggesting that JAK2 fulfills a critical role within the PRLR signaling pathway [93]. Consistent with this notion, PRLR mutants with defects in their ability to interact with or activate JAK2 fail to signal [81, 91, 94, 95]. Upon activation, JAK2 phosphorylates tyrosine on different substrates including the PRLR and STATs. PRL-mediated activation of JAK-STAT signaling results in transcriptional induction of a variety of genes primarily important in lactation, cell cycle regulation and survival [25, 96, 97]. Apart from JAK2, downregulation of JAK1 also interferes with PRL-induced activation of STAT3 and activation of JAK2, suggesting a role for JAK1 as an activator of STAT3 and also as an enhancer of the JAK2–STAT5 pathway [92].

STATs form a family of latent cytoplasmic proteins involved in cytokine receptor signaling [13, 89, 98–100]. Seven STAT family members have been identified in humans to date: STAT1 (α and β), STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (or IL-4 STAT). STATs contain a DNA-binding domain, SH3-like and SH2 domains and a C-terminal transactivating domain. All isoforms carry the functionally essential tyrosine 694, which is phosphorylated by JAK2 [101]. To date, three members of the STAT family have been identified as signaling molecules within the PRLR–JAK2 pathway: STAT1, STAT3 and, mainly, STAT5 [14, 26, 76, 87].

Apart from tyrosine phosphorylation, serine/threonine phosphorylation events are believed to be also important for full STAT activation. The ERK2/MAP kinase pathway, PAK and PKCα have been proposed as possible candidates for serine phosphorylation of STAT5 or STAT3 despite the fact that the putative STAT target sequence does not perfectly match the MAP kinase consensus site [102–107]. While the functional difference between STAT5A and STAT5B has not been resolved entirely, it might be related to suspected serine/threonine phosphorylation sites.

12.6 PRL and STAT5 Participate in a Positive Feedback Loop

As described above, STAT proteins are well characterized as critical transcription factors downstream of the PRLR that mediate many PRL activities, including angiogenesis [14, 22, 25, 108–110]. In endothelial cells, PRL family members rapidly activate PRLR/JAK2 and consequently STAT family members, especially

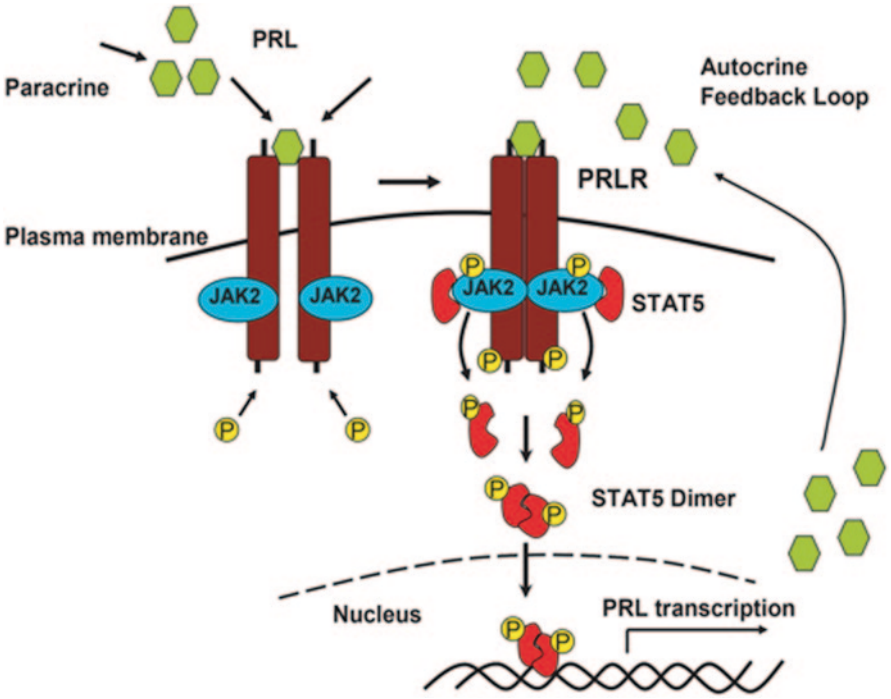

Fig. 12.1 Schematic representation of autocrine feedback loop involving *PRL* and *STAT5*. *PRL* derived from endocrine, paracrine or autocrine sources binds to the *PRLR*, leading to *JAK2* and *STAT5* activation. *STAT5* promotes *PRL* production completing the *autocrine feedback loop*

STAT5 [14, 22, 72, 86, 87]. However, in addition to this linear signaling cascade, we find that STAT5 activation in human brain endothelial cells significantly elevates PRL (PLF1 in mouse endothelial cells) mRNA, protein production and secretion [22, 26]. STAT5-induced PRL is bioactive since it activates PRLR and stimulates the phosphorylation of STAT5, STAT1 and to a much lesser extent STAT3. Since active STAT5 induces expression and secretion of PRL, these observations point toward a positive feedback loop that would drive enhanced and sustained STAT5 activation via autocrine PRL (Fig. 12.1). As many growth hormones and cytokines exhibit biological activity through activation of STAT proteins, the potential involvement of STAT5 upstream and downstream of PRL (PLF1) would set the stage for amplified and sustained proangiogenic signals through this positive feedback loop. Importantly, the feedback loop between PRL and STATs might also explain why a single hormone might exert such a range of diverse endocrine functions [90, 111].

12.7 Role of PRL-STAT Signaling in Tumor Angiogenesis

PRL has been associated with certain tumor types and may be directly or indirectly involved in tumor growth. Compelling data demonstrate that physiologically relevant concentrations of prolactin can induce the proliferation of primary human breast tumor cells [64, 108, 112–120]. Active STATs, including STAT5, are found in carcinomas of breast and prostate and in gliomas [121–127] suggesting that tumor cells might also be a source for PRL, given that STAT5 stimulates PRL production. Indeed, tumor cells, such as MCF-7 and T47D breast carcinoma cells and U251 malignant glioma cells produce bioactive PRL, implying an autocrine, growth promoting feedback loop [128, 129](Yang, Friedl, unpublished observations). Tumor cell-derived, secreted PRL would also be expected to raise PRL levels locally, adding to PRL of pituitary origin and PRL derived from endothelial cells. Cumulative PRL in the tumor microenvironment would be available to stimulate and sustain angiogenesis in concert with other proangiogenic factors. The contribution of PRL relative to other proangiogenic factors to tumor angiogenesis in vivo is currently unknown and the subject of further study.

The central position of PRL and STAT family members in proangiogenic signaling pathways renders them potentially attractive targets for anti-angiogenic therapeutic approaches. PRL receptor antagonists have been described [130–132], which could be used alone or in combination with STAT5 blockers [133, 134] to disrupt the positive feedback loop. The viability of such strategies for the antiangiogenic therapy of tumors remains to be tested in preclinical models

12.8 Summary

STAT transcription factors are critical downstream mediators of PRL signaling via the PRLR. Recent work has shown that not only does PRL activate STATs but active STATs induce the production and release of PRL, thus triggering a positive, autocrine (and possibly paracrine) proangiogenic feedback loop. Since other proangiogenic growth factors and cytokines activate STATs and thereby feed into this pathway, the STAT-PRL signaling loop may have an important role in synchronizing, amplifying, and sustaining proangiogenic stimuli.

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Erratum

Recent Advances in Prolactin Research
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The Publisher regrets to inform that the addendum of the chapter 4 is currently missing and should read as given below:
During the review process of the manuscript a paper was published, characterizing plasminogen activator inhibitor-1 (PAI-1) as a mediator of the antiangiogenic effects of 16K PRL (1). In this paper, it is reported that PAI-1 is a binding partner of 16K PRL and that the antiangiogenic activity of 16K PRL is lost in PAI-1 KO mice and restored upon re-expression of PAI-1. In addition, 16K PRL demonstrated profibrinolytic effects by reducing the antifibrinolytic effects of PAI-1 in vitro and was demonstrated to exert a thrombolytic effect in vivo. These novel insights raise important functional implications not only for 16K PRL, but also for the other members of the vasoinhibin family, such as smaller (14K PRL) and larger (18K PRL) PRL fragments, and similar molecules derived from growth hormone and placental lactogen (2). It is possible that the mediation of antiangiogenic effects after binding to PAI-1 is not restricted to 16K PRL, but applies for the whole vasoinhibin family of antiangiogenic peptides.

1. Bajou K, Herkenne S, Thijssen VL, D'Amico S, Nguyen NQ, Bouche A, Tabruyn S, Srahna M, Carabin JY, Nivelles O, Paques C, Cornelissen I, Lion M, Noel A, Gils A, Vinckier S, Declerck PJ, Griffioen AW, Dewerchin M, Martial JA, Carmeliet P, Struman I (2014) PAI-1 mediates the antiangiogenic and profibrinolytic effects of 16K prolactin Nature Medicine. 20 (7):741-747.

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2. Clapp C, Thebault S, Jeziorski MC, Martinez De La Escalera G (2009) Peptide hormone regulation of angiogenesis. Physiological reviews 89 (4): 1177-1215.

Also, in the last paragraph, the grant number “179496” should be substituted for “179506”.

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Index

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