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Towards targeting prolactin signaling in human diseases: Stimulate or inhibit?

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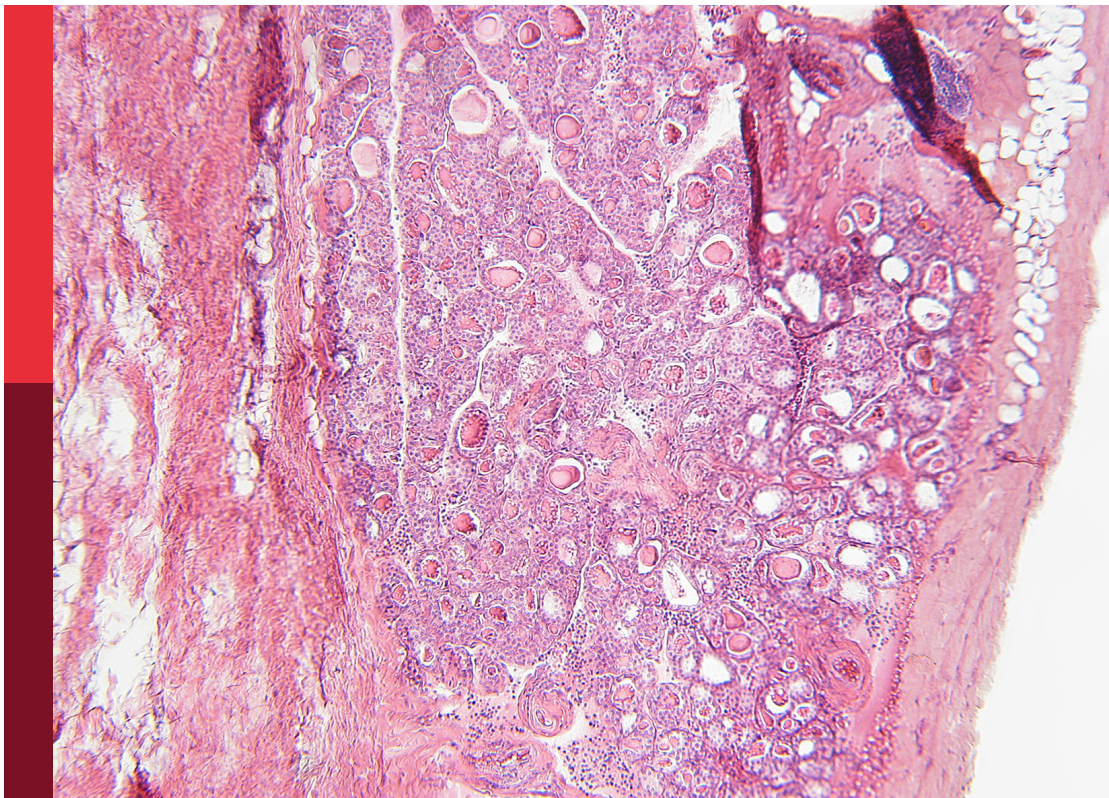

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Towards targeting prolactin signaling in human diseases: Stimulate or inhibit?

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Editorial: Towards targeting prolactin signaling in human diseases: stimulate or inhibit?

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Editorial on the Research Topic

Towards targeting prolactin signaling in human diseases: stimulate or inhibit?

Prolactin is an anterior pituitary hormone that was originally named for its indispensable role in lactation, but increasingly it is being recognized for pleiotropic roles in metabolism, immune function, pregnancy adaptations and parental behavior. Prolactin secretion is tightly controlled by a short-loop feedback system whereby prolactin stimulates specific neurons in the hypothalamus to release dopamine, which then inhibits prolactin secretion. During pregnancy and lactation, however, this feedback systems adapts to allow prolonged elevations in prolactin secretion, enabling a range of functions specific to these conditions. Prolactin is also released under conditions of stress in both sexes. Prolactin signals exclusively through the prolactin receptor (Prlr), but this is not a simple system. In target cells, prolactin/Prlr engages various signal transduction mechanisms including JAK2/STAT5 (canonical), PI3K/Akt, MAPK and Src family kinases. There is also evidence of local production of prolactin in non-pituitary tissues, leading to autocrine/paracrine receptor triggering independent of circulating hormone. Adding to this complexity, in many species, including humans, there are multiple ligands for the Prlr. These include placental lactogens that supplement prolactin function in pregnancy, and in primates only, pituitary growth hormone. Moreover, specific proteolytic products of these hormones exert important biological actions independent of Prlr. These functions, that are often completely distinct from those of prolactin, have led to the classification of these fragments as a new class of hormones known as vasoinhibins.

Reflecting this molecular and functional complexity, abnormalities in prolactin signaling have been implicated in multiple clinical conditions. There are consequences when circulating prolactin is too high, with hyperprolactinemia causing infertility in both males and females, as well as being associated with a range of metabolic disturbances and mood disorders. But there are also consequences if prolactin is too low, the most obvious

being lactation failure in the absence of prolactin signaling, but many other more subtle deficits are being identified. Changes in autocrine/paracrine prolactin signaling may be extremely important in some conditions, e.g. in modulating inflammation, pain responses, and cancer. The challenge remains when to stimulate and when to inhibit prolactin actions, and this Research Topic dissects different situations that would benefit from either option.

Kavarthapu and Dufau provide an integrated view of the molecular biology of the "target" (Prlr) encompassing its complex transcriptional regulation *via* multiple promoters, the various receptor isoforms resulting from alternative splicing, their specific signaling capacities when homo- or hetero-dimerized, their crosstalk with EGFR/HER2 family members, and how these individual processes can cooperatively promote breast cancer progression. In line with this, Schuler and O'Leary provide a systematic overview of the epidemiological and experimental data documenting the complex actions of prolactin in breast cancer, dichotomizing effects on early lesions *versus* established tumors, and showing how the stromal environment (including matrix stiffness) may alter the responses of target cells to prolactin. This balanced perspective tentatively links to the viewpoint of Ali et al. supporting the beneficial actions of prolactin as a pro-differentiation pathway restricting breast cancer cell plasticity, following emerging evidence that preventing epithelial-to-mesenchymal transition and acquisition of stemness may be a viable approach to temper cancer progression.

This Research Topic also highlights the involvement of prolactin in two diseases besides cancer. Triebel et al. provide an up-to-date discussion of the anti-angiogenic and anti-vasopermeability properties of prolactin and vasoinhibins, which may help restrict the vascularization in the eye of patients with diabetes. The translational potential is advocated by results of a clinical trial in which higher prolactin levels were associated with less diabetic retinopathy.

Prolactinomas are the most frequent functional pituitary tumors causing systemic hyperprolactinemia, with its clinical consequences, and mass effect, locally. The first-line treatment involves dopamine receptor D2 agonists, but a minority of patients with prolactinomas are resistant to this therapy. Ferraris explores impaired autocrine actions of prolactin (a local inhibitor of lactotroph proliferation acting through Prlr), independent of dopamine, in a subtype of medically-resistant prolactinomas. This implies that the pathogenesis of these prolactinomas is not the same as those responding to dopamine therapy, raising Prlr-targeting as a potential therapeutic approach.

Three additional papers emphasize the role of prolactin as a homeostasis hormone:

Macotela et al. discuss evidence for the beneficial actions of functional hyperprolactinemia (moderately-elevated prolactin levels) in metabolic diseases such as obesity and non-alcoholic fatty-liver disease. In clinical practice, beyond the role of prolactin in reproduction, a grey zone of hyperprolactinemia is defined as mild to moderate transient elevations of prolactin levels that are poorly understood. Thus, this review defines 'Homeo Fit-PRL levels' that are required to deal with metabolic challenges. Clapp et al. describe the roles of prolactin in inflammatory responses, and specifically in rheumatoid arthritis where prolactin has both negative and positive outcomes depending on circulating levels. Inflamed tissue is rich in enzymes that cleave prolactin into the bioactive metabolite vasoinhibins. This hormone also influences tissue responses to inflammation, and thus, when prolactin is high, such as during pregnancy and lactation, levels of vasoinhibins are also increased, providing both direct and indirect mechanisms to influence tissue responses. Finally, Garay et al. focus on placental lactogens, which are pregnancy-specific hormones that act through the Prlr. These hormones are critical to adaptive changes in the mother during pregnancy, and low placental lactogen has been associated with impaired pregnancy outcomes. The authors found that maintaining a conscious healthy diet during pregnancy was associated with increased placental lactogen and increased birthweight of babies.

Should Prlr signaling need to be inhibited in cancer or any other disease, Standing et al. provide the most up-to-date toolbox of the various approaches and drug candidates that have been developed so far, including prolactin-based receptor antagonists, receptor neutralizing antibodies and small molecule inhibitors.

In conclusion, this Research Topic aimed to demonstrate how deciphering the complexity of the prolactin/Prlr system in different tissues has clinical relevance in understanding disease. Collectively, these reviews highlight that for normal function, prolactin must be in the "goldilocks zone" – not too high, and not too low – "just right". This evidence constitutes a challenge for any therapeutic intervention aimed at modulating systemic Prlr signaling in disease.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dual Roles of Prolactin and Vasoinhibin in Inflammatory Arthritis

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The term inflammatory arthritis defines a family of diseases, including rheumatoid arthritis (RA), caused by an overactive immune system, and influenced by host aspects including sex, reproductive state, and stress. Prolactin (PRL) is a sexually dimorphic, reproductive, stress-related hormone long-linked to RA under the general assumption that it aggravates the disease. However, this conclusion remains controversial since PRL has both negative and positive outcomes in RA that may depend on the hormone circulating levels, synthesis by joint tissues, and complex interactions at the inflammatory milieu. The inflamed joint is rich in matrix metalloproteases that cleave PRL to vasoinhibin, a PRL fragment with proinflammatory effects and the ability to inhibit the hyperpermeability and growth of blood vessels. This review addresses this field with the idea that explanatory mechanisms lie within the PRL/vasoinhibin axis, an integrative framework influencing not only the levels of systemic and local PRL, but also the proteolytic conversion of PRL to vasoinhibin, as vasoinhibin itself has dual actions on joint inflammation. In this review, we discuss recent findings from mouse models suggesting the upregulation of endogenous vasoinhibin by the pro-inflammatory environment and showing dichotomous actions and signaling mechanisms of PRL and vasoinhibin on joint inflammation that are cell-specific and context-dependent. We hypothesize that these opposing actions work together to balance the inflammatory response and provide new insights for understanding the pathophysiology of RA and the development of new treatments.

Keywords: rheumatoid arthritis, proinflammatory cytokines, joint inflammation, angiogenesis, synovial fibroblasts, endothelial cells, prolactin, vasoinhibin

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INTRODUCTION

Inflammatory arthritis is a collective name for a group of acute and chronic diseases driven by an overactive immune system that causes painful inflammation and stiffness of one or more articular joints. These diseases, broadly classified as non-autoimmune (sepsis arthritis and gout) and autoimmune (rheumatoid arthritis, juvenile idiopathic arthritis, spondyloarthritis, among others) are progressively debilitating if untreated. Rheumatoid arthritis (RA) is the most common chronic inflammatory arthritis affecting around 1% of the global population with a female to male ratio 3 to 1 (1). RA manifests as progressive synovial hyperplasia (pannus formation) and inflammation (synovitis) leading to polyarticular destruction. The etiology of RA is multifactorial, with genetic, environmental, and host-related factors driving early alterations of the innate and adaptive immune

system that result in the recruitment of immune cells into the joints and subsequent chronic inflammation.

The close association between RA, sex, reproductive state, and stress have long-linked the sexually dimorphic, reproductive, stress-related hormone prolactin (PRL) to disease progression (2). However, the role of PRL in RA is more complex than anticipated. Clinical and pre-clinical studies have shown that PRL can be both pro-inflammatory and anti-inflammatory in a context-dependent manner. The detailed essentials of the association of PRL and RA are beyond the scope of this article and can be found in several reviews (2–5). Here, we briefly summarize the bases of the association between PRL and RA and focus on recent findings in arthritic rodents showing direct effects of PRL on joint tissues and the influence of the proteolytic conversion of PRL to vasoinhibin, a PRL fragment with dual actions on vascular and non-vascular cells of joint tissues that affect inflammatory reactions. Finally, we discuss how this information may be translated into novel therapeutic interventions.

PROLACTIN AND RA

The fact that RA is more frequent in women and disparity is greater at younger ages (6) encouraged investigating the female reproductive history. Early studies showed a higher risk of RA in nulliparous than parous women and a higher risk and worsening of RA in the postpartum period in association with breastfeeding (7). Because both risk factors (reduced fecundity and breastfeeding) associate with hyperprolactinemia, PRL was suggested as a biological explanation (8). Furthermore, the frequently observed adverse relationship between stressful events and RA (9) pointed to the upregulation of PRL in response to stress (10) as a contributing factor. However, other evidence suggested the opposite. A large population cohort, controlled for breastfeeding among parous women, did not support nulliparity as risk factor and revealed that breastfeeding for >12 months was inversely related to the development of RA (11). Also, RA improves or goes into remission during pregnancy (12) when the circulating levels of PRL and placental lactogen are high. Moreover, stress worsens but also attenuates RA dependent on the duration and type of stressors and in association with stress hormones, including PRL (9). For example, acute exposure to hyperprolactinemia enhances inflammation during stress, whereas chronic hyperprolactinemia is immunosuppressive (13). Finally, controversies were found when measuring PRL levels in the circulation of patients with RA (reviewed by Clapp et al., 2016 (2)). Higher, similar, and even lower PRL levels, within the normal range (≤ 20 μg/L), occurred with no clear association to disease severity. Lowering or increasing circulating PRL levels with dopamine D2 receptor agonists or antagonists, respectively, were both effective and ineffective against RA. Altogether the contrasting findings have indicated dual outcomes of PRL in RA and encouraged the search for clarifying mechanisms.

Opposite effects of PRL on the immune response have been known for more than three decades and are essentially associated to PRL concentration (14), with lower levels (≤25 μg/L) being immunostimulatory and higher levels (≤100 μg/L) immunosuppressive (15). It is possible that systemic levels of PRL in RA are confounded by PRL produced and metabolized at the inflamed joint. Infiltrated leukocytes and fibroblasts of the RA synovium produce PRL (16) and matrix metalloproteases (MMPs) upregulated in the joints of patients with RA (17) cleave this hormone to vasoinhibin (18), a PRL fragment with potent anti-angiogenic and pro-inflammatory properties (19). Following is a summary of the PRL/vasoinhibin axis, an integrative framework able to alter joint inflammation by influencing the levels of systemic and local PRL and vasoinhibin.

THE PRL/VASOINHIBIN AXIS

The PRL/vasoinhibin axis is a newly described endocrine axis where the proteolytic cleavage of PRL to vasoinhibin is regulated at the hypothalamus, the pituitary, and the target tissue levels (20). Disruption of this axis contributes to the pathogenesis and progression of diabetic retinopathy (21), retinopathy of prematurity (22), peripartum cardiomyopathy (23), pre-eclampsia (24), and inflammatory arthritis (25, 26). Vasoinhibin comprises a family of PRL fragments that range from 5.6 to 18 kDa that correspond to the first 48 to 159 amino acid residues of PRL depending on the cleavage site of proteases that include MMPs (18), cathepsin D (27), bone morphogenetic protein 1 (28), thrombin (29), and plasmin (30). Vasoinhibin signals through receptor/binding protein complexes distinct from the PRL receptor (31) to exert effects frequently opposite to those of the full-length hormone. PRL stimulates angiogenesis, whereas vasoinhibin inhibits angiogenesis, vasodilation, and vasopermeability (19). Vasoinhibin acts as proinflammatory cytokine upregulating inducible nitric oxide synthase (iNOS) in lung tissues (fibroblasts and type II epithelial cells) (32), whereas PRL attenuates proinflammatory cytokine-induced iNOS expression in these cells (33). PRL inhibits and vasoinhibin stimulates anxiety- and depression-related behaviors (34) as well as neuronal apoptosis (35), respectively. However, both PRL and vasoinhibin stimulate the release of vasopressin by the hypothalamo-neurohypophyseal system (36). Because opposing actions reside within the PRL molecule, proteolytic cleavage represents an efficient mechanism for balancing functions. Recent work showed that a short linear motif of just three residues (His46-Gly47-Arg48) is the functional antiangiogenic determinant of vasoinhibin and that such motif is concealed in PRL by salt-bridges between Arg48 and Glu161 and 162 located in PRL fourth alfa-helix, a part of PRL removed during vasoinhibin generation (37).

The influence of the PRL/vasoinhibin axis in arthritis is suggested by the presence of PRL in the synovial fluid and of PRL, vasoinhibin, and PRL-cleaving MMPs in joint tissues including chondrocytes, vascular endothelial cells, synoviocytes, fibroblasts, and immune cells (reviewed by Clapp et al., 2016 (2)). While RA remains a uniquely human disease, animal models with induced synovial inflammation are an essential component of drug development (38) that have helped investigate the influence of the PRL/vasoinhibin axis in RA.

PRL, VASOINHIBIN, AND INDUCED ARTHRITIS IN RODENTS

Murine adjuvant arthritis (AA) and antigen-induced arthritis (AIA) are models of inflammatory arthritis, including RA, where disease is mediated by antigen-specific immune responses by T and B lymphocytes (39). The AA model is commonly induced by a single intradermal injection of complete Freund's adjuvant in rats and mice and is characterized by a reliable, rapid onset and progression of robust and easily measurable polyarticular inflammation, cartilage degradation, and bone loss. AIA is usually induced in mice immunized by intradermal and subsequent intra-articular injection of antigen (methylated bovine serum albumin) that causes acute monoarticular inflammation and eventual joint destruction.

A first study, carried out 40 years ago, suggested a detrimental effect of PRL by showing that hypophysectomized rats do not develop AA unless treated with this hormone (40). However, adrenocortical deficiency due to hypophysectomy confounded PRL action. In the absence of hypophysectomy, rats made hyperprolactinemic by placing anterior pituitary grafts under the kidney capsule showed less severe AA and higher corticosterone circulating levels (41). The PRL beneficial action was recently confirmed and extended by showing that hyperprolactinemia induced by anterior pituitary grafts, osmotic minipumps delivering PRL, or treatment with the dopamine D2 receptor blocker, haloperidol, reduced joint inflammation and pain, cartilage loss, and bone erosion in AA rats (42, 43). Reduced inflammation involved systemic (lower levels of circulating C-reactive protein and TNFα) and local mechanisms (43). The long isoform of the PRL receptor was upregulated in arthritic joints where hyperprolactinemia inhibited enhanced expression of proinflammatory cytokines (TNFα, IL-1β, IL-6, INFγ), elevated chondrocyte apoptosis, and increased osteoclast differentiation (42, 43). Furthermore, PRL-receptor null mice (*Prlr-/-)* exhibited a more severe AA (43), which was consistent with previous reports showing that targeted disruption of the PRL receptor (44) or PRL (45) enhances immune responses and mortality under stress-related conditions.

The positive role of PRL in murine arthritis contrasts with its controversial action in RA. Because high PRL levels are immunosuppressive (15), the magnitude of the induced hyperprolactinemia (>60 μg/L) (42, 43) could be an explanatory mechanism. Another contributing factor may be the cleavage of PRL to vasoinhibin. Hyperprolactinemia promotes the conversion of PRL to vasoinhibin by providing more substrate to cleaving proteases. Hyperprolactinemic mice overexpressing PRL in the liver have enhanced levels of circulating vasoinhibin (46), and pharmacologically induced hyperprolactinemia results in higher levels of vasoinhibin in ocular tissues and fluids of rats (47) and humans (48). In agreement, the activity of major PRL-cleaving proteases, MMPs and cathepsin D, is upregulated in the joints from AIA mice (26), vasoinhibin increases in the circulation of *Prlr-/-* mice when subjected to AIA (26), and *Prlr-/-* mice are hyperprolactinemic (49).

Upregulation of vasoinhibin can contribute to the beneficial outcome of PRL in arthritis by means of its inhibitory effects on blood vessels. Exacerbated vasopermeability and angiogenesis promote synovial inflammation and inhibition of angiogenesis is a promising therapy in RA (50). Hypervasopermeability results in edema formation and joint swelling, and pannus formation requires new blood vessels to cope with the increased requirement of oxygen and nutrients and the delivery of inflammatory cells and molecules. Consistent with this notion, the intra-articular delivery of the vasoinhibin gene *via* a recombinant adeno-associated type 2 vector (AAV2-Vi) reduced pannus vasopermeability and angiogenesis, joint inflammation, and bone loss in mice under severe AIA (25).

Nevertheless, the role of vasoinhibin in arthritis is not a simple matter, as vasoinhibin is also proinflammatory. Higher circulating vasoinhibin levels coincide with exacerbated arthritis in *Prlr-/-* mice (26, 43) and vasoinhibin has proinflammatory effects in lung tissues (32). A recent study showed that vasoinhibin indirectly inhibits and directly stimulates joint inflammation depending on vasoinhibin concentration and the severity of the disease in which it acts (26). While the AAV2-Vi vector indirectly (via an antiangiogenic mechanism) ameliorated severe joint inflammation (25), it enhanced arthritis under mild inflammatory conditions (26). Vasoinhibin gene delivery in mice subjected to mild AIA enhanced joint swelling, synovial leukocyte infiltration, and expression of proinflammatory mediators (*Il1b, Il6, Inos, Mmp3, Icam1, Cxcl1, Cxcl2, Cxcl3, and Ccl2*) by a direct action on synovial fibroblasts (26). The magnitude of vasoinhibin transgene expression was higher under mild vs. severe AIA suggesting that, depending on the inflammatory context, higher levels of vasoinhibin are needed to promote inflammation but not anti-inflammation in arthritis.

Altogether evidence shows that dual actions of PRL extend to vasoinhibin and are dependent on the local concentration of each hormone, the level of inflammation, the activity of local proteases, and the activation of specific cells and signaling pathways.

TARGETED CELLS AND SIGNALING PATHWAYS

PRL signals directly on chondrocytes and synovial fibroblasts to inhibit cartilage degradation, synovial inflammation, and osteoclastogenesis in arthritis (42, 43). Articular chondrocytes express the long form of the PRL receptor (51) and PRL inhibits the apoptosis of cultured chondrocytes in response to proinflammatory cytokines (Cyt: IL-1β, TNFα and INFγ) by preventing the induction of p53 and decreasing the BAX/BCL-2 ratio through a NO-independent, JAK2-STAT3 dependent pathway (42) (**Figure 1**). Furthermore, the Cyt upregulate the long PRL receptor in synovial fibroblasts (43) which are key cells in the initiation and perpetuation of joint inflammation and destruction (52). PRL induces the phosphorylation/activation of STAT3 in cultured in synovial fibroblasts to inhibit Cyt-induced expression of IL-1β, IL-6, and receptor activator of nuclear factor

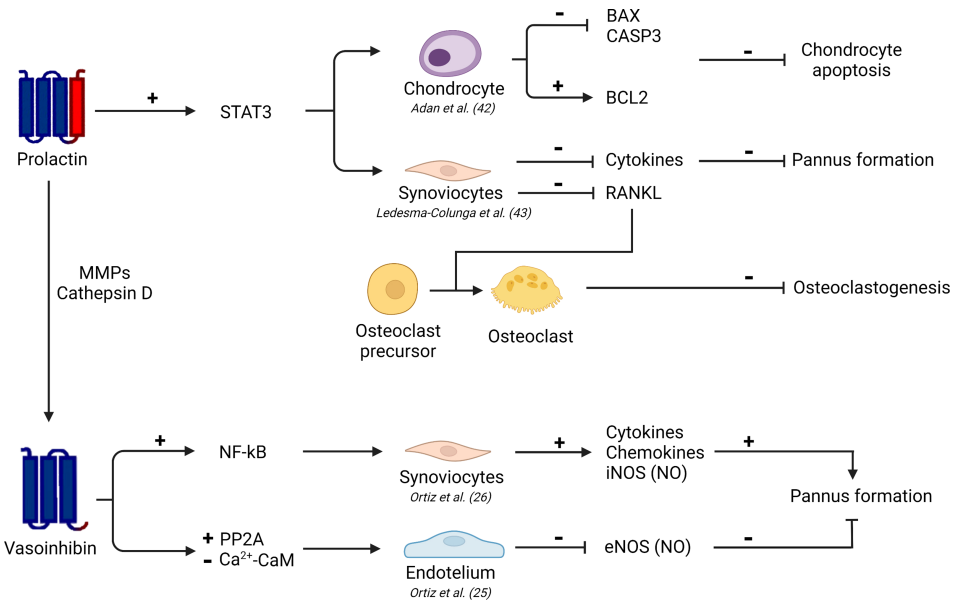

FIGURE 1 | Schematic representation of PRL and vasoinhibin signaling in various cells of the joint. MMPs, matrix metalloproteases; STAT3, signal transduction activator of transcription 3; NF-kB, nuclear factor kappa-B; PP2A, protein phosphatase 2A; Ca^{2+}- CaM, calcium-calmodulin complex; BAX, BCL2 associated X-protein; Bcl-2, B-cell lymphoma 2; CASP3, caspase 3; RANKL, receptor activator of nuclear factor κB ligand; iNOS, inducible nitric oxide synthase; NO, nitric oxide; eNOS, endothelial nitric oxide synthase. Scheme created with Biorender.com.

κB ligand (RANKL), a major promoter of osteoclastogenesis in RA (43) (**Figure 1**).

In contrast to PRL, vasoinhibin acts on synovial fibroblasts to promote inflammation. Vasoinhibin activates the NFκB signaling pathway in cultured synovial fibroblasts to upregulate proinflammatory mediators, chemokines, and iNOS-mediated NO production (26) (**Figure 1**). However, like PRL, vasoinhibin inhibits inflammation albeit through the inhibition of vascular endothelial cells. Vasoinhibin signals on synovial endothelial cells to stimulate protein phosphatase 2A and inhibit the Ca^{2+}-calmodulin binding that leads to blockage of the VEGF-induced endothelial NOS (eNOS) activation required for pannus vasopermeability and angiogenesis (25, 53) (**Figure 1**).

Dual actions on inflammation illustrate the complex balance of the inflammatory response. As PRL and vasoinhibin, major proinflammatory cytokines (INFγ, IL-2, IL-6, TNFα) function as anti-inflammatory mediators and classical anti-inflammatory factors (IL-10, TGFα, glucocorticoids) exhibit proinflammatory effects depending on cytokine concentration, the stage of the disease, and the combination with other cytokines (54, 55). Major questions are how and when PRL and vasoinhibin opposing actions operate and mechanistically interact to influence arthritis progression (**Figure 2**). Current data suggest that vasoinhibin generation is dependent on hyperprolactinemia and that the proinflammatory action of vasoinhibin on synovial fibroblasts may occur during the early mild phase of arthritis, whereas the anti-inflammatory effect, *via* inhibition of synovial vascular cells, manifest at a later, more severe stage (26). We hypothesize that these opposing actions work in concert to prevent infection and limit destruction of joint tissues.

CLINICAL IMPLICATION

Animal models of RA are of limited therapeutic information since none of these models are truly RA. However, murine inflammatory arthritis has been extensively used for drug development (38) and has provided insights into the influence of the PRL/vasoinhibin axis on joint inflammation. Experimental studies showed that increasing prolactinemia, either by PRL infusion or treatment with the dopamine D2 receptor blocker haloperidol, ameliorates the severity of arthritis, either directly (42) or *via* the PRL conversion to antiangiogenic vasoinhibin (25) (**Figure 2**). Of note, a pilot clinical trial carried out 40 years ago showed that haloperidol improved the evolution of RA (56) and a recent observational study described the potential inverse association between haloperidol and RA (57). Also, inhibition of angiogenesis is a promising therapy for RA (50) and vasoinhibin itself may represent a therapeutic opportunity by virtue of its antiangiogenic and anti-vasopermeability properties. Translation of vasoinhibin into the clinic has been hampered by difficulties in its recombinant production (58). However, the barrier of using vasoinhibin as therapeutic agent was recently removed by showing that seven amino acid peptides containing the anti-angiogenic motif (HGR) of vasoinhibin inhibit angiogenesis and vasopermeability with the same potency as the whole protein (37). Oligopeptide optimization to target vascular and not non-vascular actions in arthritis represents

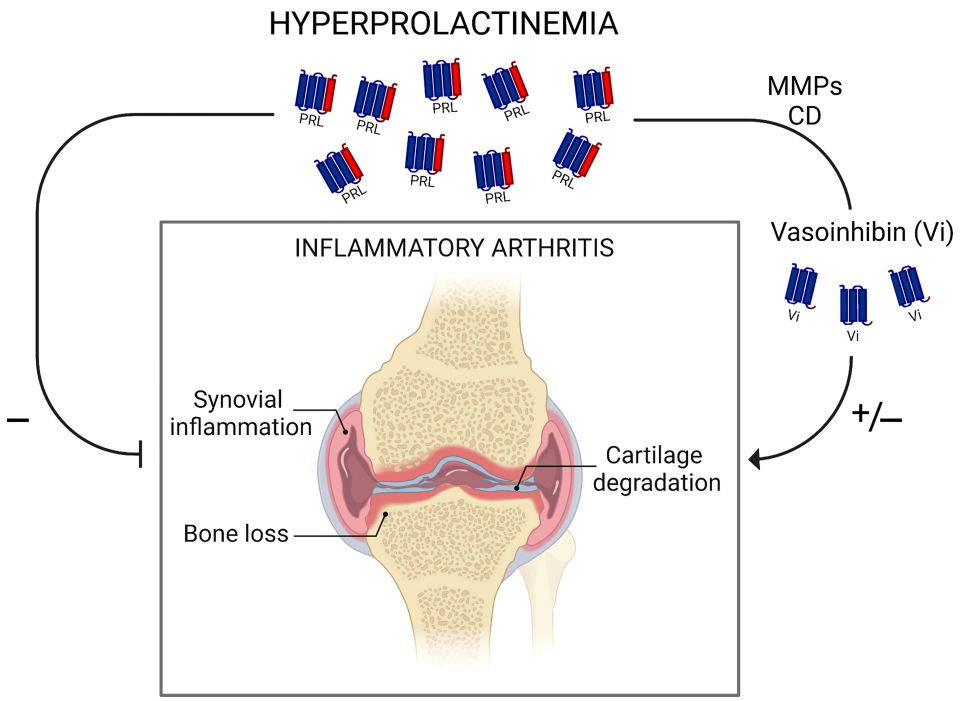

FIGURE 2 | Schematic representation of findings in rodent models showing dual actions of hyperprolactinemia in inflammatory arthritis. Hyperprolactinemia inhibits synovial inflammation, cartilage degradation, and bone loss directly or *via* its proteolytic cleavage by matrix metalloproteases (MMPs) and cathepsin D (CD) to antiangiogenic vasoinhibin. PRL conversion to vasoinhibin may also worsen inflammatory arthritis by a vasoinhibin pro-inflammatory effect. Understanding how and when PRL and vasoinhibin actions operate and mechanistically interact to influence arthritis progression warrants further research. Scheme created with Biorender.com.

a promising therapeutic approach and an important tool for guiding future research. Nonetheless, dichotomous actions of the PRL/vasoinhibin axis expose its intricate role in inflammatory arthritis and demand further research to better understand its role and therapeutic application in RA.

CONCLUSIONS

The role of PRL in RA remains poorly defined but hyperprolactinemia is emerging as a protective influence. Evidence supporting the beneficial impact of physiological hypeprolactinemia (in pregnancy and after breastfeeding) on RA is reinforced by experimental studies showing that sustained PRL administration or genetic deletion of the PRL receptor ameliorates or worsens the severity of inflammatory arthritis, respectively. PRL signals on arthritic joint tissues (chondrocytes and synovial fibroblasts) to inhibit cartilage degradation, synovial inflammation, and osteoclastogenesis. Hyperprolactinemia promotes the conversion of PRL to vasoinhibin, a PRL fragment that directly stimulates and indirectly inhibits (via an antiangiogenic mechanism) joint inflammation in a context- and cell type-dependent manner. Understanding the mechanisms governing the regulation and action of the PRL/vasoinhibin axis in inflammatory arthritis should help clarify the role of PRL in RA to ultimately develop novel therapeutic interventions that can be tested in patients.

AUTHOR CONTRIBUTIONS

CC wrote the manuscript. GO, JG-R, ML-C, FM-D, NA, and GME edited, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Prolactin: The Third Hormone in Breast Cancer

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Prolactin coordinates with the ovarian steroids to orchestrate mammary development and lactation, culminating in nourishment and an increasingly appreciated array of other benefits for neonates. Its central activities in mammary epithelial growth and differentiation suggest that it plays a role(s) in breast cancer, but it has been challenging to identify its contributions, essential for incorporation into prevention and treatment approaches. Large prospective epidemiologic studies have linked higher prolactin exposure to increased risk, particularly for ER+ breast cancer in postmenopausal women. However, it has been more difficult to determine its actions and clinical consequences in established tumors. Here we review experimental data implicating multiple mechanisms by which prolactin may increase the risk of breast cancer. We then consider the evidence for role(s) of prolactin and its downstream signaling cascades in disease progression and treatment responses, and discuss how new approaches are beginning to illuminate the biology behind the seemingly conflicting epidemiologic and experimental studies of prolactin actions across diverse breast cancers.

Keywords: prolactin (PRL), breast cancer, mammary cancer, luminal breast cancer, HER2+ breast cancer, STAT 5 transcription factor, triple negative breast cancer

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1 INTRODUCTION

Factors that regulate cell-specific proliferation and differentiation repeatedly have been shown to be significant actors in oncogenesis and potential therapeutic targets in established cancers. Prolactin (PRL) cooperates with the ovarian steroids, estrogen and progesterone, to orchestrate the cycles of mammary development and differentiation that lead to successful lactation, providing nourishment for the offspring. PRL-initiated signals that expand alveolar cells during pregnancy and coordinate their differentiation at the time of birth have been mechanistically defined [(1–4) and references therein]. The essential actions of PRL in these physiological processes have suggested roles in breast

Abbreviations: APC, adenomatous polyposis coli; COL1A1, collagen type I alpha 1; DCIS, ductal carcinoma in situ; ECM, extracellular matrix; ER, estrogen receptor alpha; FAK, focal adhesion kinase; GH, growth hormone; JAK2, janus kinase 2; MHT, menopausal hormone therapy; MMP, matrix metalloprotease; MMTV, mouse mammary tumor virus; NOD, non-obese diabetic; NR4A, nuclear receptor subfamily 4 group A member 1; NRL, neu-related lipocalin; NSG, NOD SCID gamma; OHT, hydroxytamoxifen; PDX, patient-derived xenograft; PGE2, prostaglandin E2; PRL, prolactin; PRLR, prolactin receptor; PTGS2, prostaglandin-endoperoxide synthase 2; RCAS, replication-competent ASLV long terminal repeat (LTR) with a splice acceptor; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription; TNBC, triple negative breast cancer.

cancer [(5–14) and references therein], by analogy to the recognized roles of the two other major hormones that regulate mammary development and function, estrogen and progesterone. Yet understanding its activities and consequences across diverse clinical breast cancers in order to develop prevention or treatment strategies has been elusive.

While control of PRL expression by pituitary lactotrophs during pregnancy and lactation is well understood [reviewed in (15)], its expression outside of pregnancy has received less attention. Pituitary PRL secretion is influenced by many factors, and circulating levels in nonpregnant women vary considerably (16–18). In addition to physiological stimuli, estrogen-progestin menopausal hormone therapy (MHT) raises circulating PRL (18), and anti-psychotics that antagonize dopamine induce hyperprolactinemia (19, 20). Further, PRL also can be expressed by non-lactotrophs, including within the mammary gland (21–23), and by breast cancer cells themselves (24–27). COX-2 (PTGS2) can induce PRL expression in fibroblasts, including at potential metastatic sites, mediated by PGE2 induction of NR4A (28). Moreover, in contrast to growth hormone (GH) in nonprimates, hGH is also a potent PRL receptor agonist (29, 30). Like PRL, it can be produced locally by breast cancer cells (26), and hGH and PRL receptors can heterodimerize (31). Thus, PRL receptors (PRLR) in the breast may be exposed to agonists from local and circulating systemic sources, even in the absence of pregnancy.

Here we review the epidemiologic evidence linking PRL to oncogenesis in the breast, and recent experimental studies implicating multiple underlying mechanisms. We then address the more controversial role(s) for PRL in established breast cancers. PRLR is highly expressed in many breast cancers across all different subtypes, and epidemiologic analyses and experimental studies are revealing that PRL can elicit both pro-differentiation and pro-aggression outcomes. We discuss how new approaches are illuminating the factors that determine the responses to PRL, including intrinsic tumor cell properties and the microenvironment, and point to directions for future studies that will integrate our understanding of this hormone in breast cancer progression and therapeutic responses.

2 PRL ACTIONS IN DEVELOPMENT OF BREAST CANCER

2.1 Epidemiological Studies

Multiple epidemiologic studies have examined the relationship between levels of circulating PRL and development of breast cancer [meta-analysis and review (32),]. Large prospective studies have linked higher levels of circulating PRL within the normal range to increased risk for breast cancers which express estrogen receptor alpha (ER+) in postmenopausal (16, 33), or premenopausal women (34). In the study nested within the Nurses' Health Study, PRL levels predicted breast cancer risk independent of estrogen (35). Additional analyses of this cohort found that the association of circulating PRL in the highest quartile in postmenopausal women ten years prior to diagnosis was strongest for aggressive ER+ breast cancer (36). Furthermore, epidemiologic studies have linked PRL to mammographic density (34, 37, 38), a potent independent contributor to increased breast cancer risk (39, 40). Incorporation of PRL in risk prediction models improves their efficacy (34, 41). Conversely, the reduced PRL levels in parous compared to nulliparous women may play a role in the long term protection conferred by pregnancy (16, 18, 34, 42).

2.2 Experimental Studies

2.2.1 *In Vivo* Models

The ability of PRL to stimulate mammary tumorigenesis in rodent models has been recognized for some time. Many early studies manipulated pituitary PRL (5–7), especially using pituitary isografts transplanted to the kidney capsule to chronically elevate circulating PRL by removing the inhibitory effects of dopamine (43). This approach reveals effects of PRL in combination with progesterone; in rodents, PRL also supports the corpus luteum (44).

More recently, genetically modified mice have permitted interrogation of mechanisms by which PRL may increase risk of breast cancer, apart from ovarian steroids. Transgenic PRL under the control of several promoters leads to mammary cancers [reviewed in (45)], as does transgenic mammary STAT5A, the canonical mediator of PRL signals (46). PRL drives development of mammary cancers in mice with germline ablation of *Stat1* secondary to somatic truncating mutations in *Prlr*, resulting in an alternatively spliced protein resembling the human "intermediate" isoform (47) (see Sections 3.1, 3.4.2). Our group generated the NRL-PRL mouse (48, 49), in which transgenic rat PRL is expressed by mammary epithelia, mimicking the local PRL synthesis in breasts of women (23). Unlike circulating PRL, this locally elevated PRL does not disturb estrous cycling, enabling study of the interactions of PRL with ovarian hormones, of particular importance when assessing models of pre- and post-menopausal breast cancer. Mammary glands of young adult NRL-PRL females exhibit elevated pERK1/2 and pAKT, in addition to pSTAT5 (50), reflecting the spectrum of PRL-initiated signaling cascades (22, 51). These mammary glands exhibit both ductal abnormalities (mammary intraepithelial neoplasias, resembling ductal carcinoma in situ, DCIS), and epithelial hyperplasias (48, 52). With age, nulliparous females spontaneously develop histologically diverse, metastatic ER+ carcinomas with long latencies, mirroring the epidemiologic link between PRL exposure and aggressive ER+ cancer (36). These tumors can develop without postpubertal ovarian steroids, similar to the observation that the increased risk conferred by PRL in women is independent from estrogen (16), although supplemental 17β-estradiol decreases tumor latency (50). Once established, tumors are no longer dependent on estrogen for growth, modeling clinical anti-estrogen resistant luminal B cancers (53–55). However, the ER remains functional; estrogen activity modulates tumor gene expression and behavior, including proliferation and cancer stem cell activity (54, 56).

In order to understand the molecular events underlying PRL-driven oncogenesis, we performed comprehensive genomic

profiling over the course of disease (57). Similar to clinical ER+ breast cancers (58–60), end stage tumors exhibited few nonsynonymous somatic mutations. However, nearly 80% of tumors showed alterations in the Ras pathway, including canonical activating mutations and copy number amplifications of *Kras*. Interestingly, many aggressive clinical ER+ breast cancers exhibit elevated Ras pathway activity as a result of mutations in the Ras proteins, or reduced expression or somatic loss of Ras-GAP tumor suppressors (61–64). Many of the remaining 20% of experimental PRL-induced cancers exhibited elevated pAKT, but not pERK1/2, consistent with driver mutations in the phosphatidylinositol-3-kinase pathway, common in many clinical ER+ cancers (63, 65, 66). Transcriptomic analyses showed that tumors expressed variable transgenic PRL compared to preneoplastic tissue, suggesting divergent PRL influence once tumors are established. These analyses also revealed marked alterations in cell-intrinsic processes and the tumor microenvironment, including immune activity. Consistent with low numbers of intratumoral lymphocytes, including CD8+ effector T cells, but large numbers of infiltrating macrophages, tumors contained strikingly reduced transcripts for many chemokines and indicators of anti-tumor immunity. This immunosuppressed environment resembles that of clinical ER+ breast cancers [reviewed in (67, 68)].

2.2.2 Direct Actions on Mammary Epithelia

Extensive studies of the direct actions of PRL on breast cancer cells *in vitro* have demonstrated increased proliferation and cell turnover [reviewed in (22)], and these effects are also observed in normal mammary epithelia in the dynamic *in vivo* environment in multiple murine models (2, 45, 49). In addition, recent studies have revealed that PRL powerfully influences the mammary epithelial hierarchy, both independent of ovarian steroids and in concert with these hormones (69) (**Figure 1**).

In the NRL-PRL model, local PRL increased epithelial stem/progenitor activity and dampened the regulatory networks which drive differentiation (69). In ovariectomized young adult females, transgenic PRL increased luminal progenitors; in combination with estrogen and progesterone, PRL increased bilineage progenitors, and raised stem cell activity associated with augmented canonical Wnt signaling. However, PRL opposed steroid-driven luminal maturation, associated with reduced *Gata3* and higher *Sox9* transcripts (69). A growing literature supports stem/progenitor cell populations as cancer cells of origin (70), and mammary luminal progenitors have been implicated as precursors for multiple subtypes of breast cancer [reviewed in (71)]. The ability of PRL to expand these epithelial subpopulations would contribute to increased cancer risk.

2.2.3 Effects on Non-Epithelial Cells

Multiple non-epithelial cells in the mammary environment have been reported to express PRLR (72, 73). Although few actions of PRL at stromal targets have been addressed experimentally in the context of breast cancer, data from physiologic and other pathologic states suggest the need for additional study. The critical roles of the immune system in mammary development, lactation and involution are increasingly appreciated (74–76). PRL, like the ovarian steroids, can influence mammary immune cell content and activity, both indirectly by altering epithelial cytokine secretion (77), as well as directly acting on both innate and adaptive immune cell subpopulations (78–83). Studies of lymphocyte activation *in vitro* showed a bimodal concentration-dependent response to PRL (84, 85), suggesting the intriguing possibility that mammary PRL synthesis may influence local immune activity. Together, these reports suggest that PRL may modulate inflammation and/or immunotolerance during tumorigenesis, with potential to contribute to a permissive environment for development of breast cancer.

PRL-induced synthesis of components of the extracellular matrix (ECM) by both epithelial and non-epithelial target cells may complement mitogenic effects of PRL on mammary epithelium to augment mammographic density, suggesting another mechanism by which PRL may raise risk (86). In this

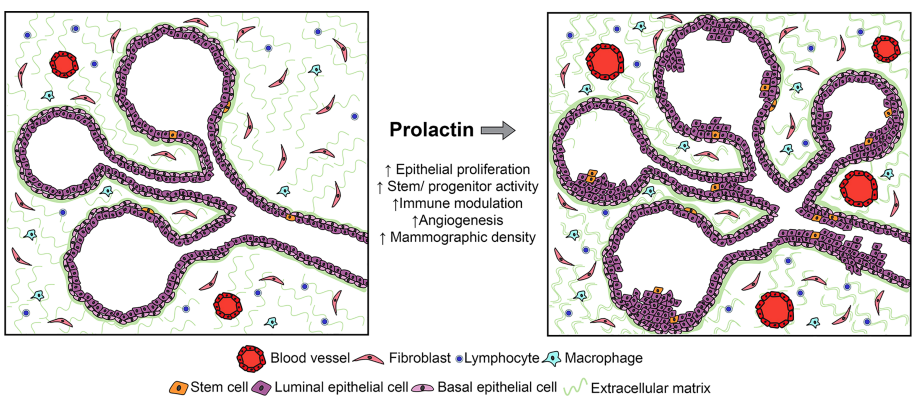

FIGURE 1 | Studies of human samples and experimental models have shown that PRL can act on multiple target cells within the mammary environment, including not only epithelia, but also stromal cells, including immune and fibroblastic cell subpopulations. Although its effects on epithelia are best understood in the context of breast cancer, its actions on stromal targets which have been defined in other systems would also be predicted to increase the risk for breast cancer. See Section 2.2 for details.

regard, the ability of PRL to stimulate macrophages to augment fibrosis in pancreatic cancer is of interest (87). Furthermore, PRL can modulate angiogenesis. As an intact protein, it can promote vascularization (88, 89); in contrast, its proteolytic products (vasoinhibins) impede this process (90). These activities have suggested roles in normal mammary function as well as breast cancer (91).

2.2.4 Contributions to Growth of Early Lesions

These actions of PRL on breast epithelia and potentially on other stromal cells could support development of breast cancers. Moreover, these activities would fuel early lesions regardless of the initiating event (**Figure 1**). Many clinical DCIS lesions express PRLR (92), and the PRL antagonist, Δ1-9-G129R-hPRL, inhibited the mammosphere-forming activity of primary DCIS samples (93). The rich literature elevating systemic PRL using pituitary isografts in mouse models demonstrates that PRL in combination with progesterone can promote carcinogen- and p53 null-induced tumors [e.g., (6,94)]. Conversely, germline genetic ablation of *Prl* or *Prlr* slowed growth of lesions induced by viral oncogenes (95, 96). Antipsychotics that act by antagonizing dopamine, thereby raising circulating PRL, promoted tumorigenesis in experimental models initiated by RCAS-caErbB2, RCAS-HrasQ61L and MMTV-Wnt-1 (97). A recent study found that patients using these drugs had a significantly increased risk of breast cancer (20). Together, these observations in patients and murine models indicate a role(s) for PRL in progression of early lesions.

2.2.5 Cooperation With Other Factors

2.2.5.1 Estrogen, Progesterone

In patients, PRL would act in concert with other hormones and potentially carcinogenic factors, as well as dysregulation of multiple pathways as disease progressed. Prior to menopause, PRL would interact with ovarian steroids; after menopause, estrogen/progestin MHT would continue this crosstalk. In the European Prospective Investigation into Cancer and Nutrition cohort, postmenopausal women with higher circulating PRL who had used combined estrogen/progestin MHT had the most significant increase in incidence of ER+ breast cancer (33). Estrogen in the absence of progestins also would be an actor in postmenopausal women receiving either estrogen only MHT, or in untreated women by extraovarian estrogen synthesis (98, 99). PRL cooperates with estrogen, a well-recognized risk factor for breast cancer, by multiple mechanisms, including reciprocal upregulation of the other's receptors (100, 101), and downstream crosstalk (102, 103). Supplemental estrogen accelerates PRL-driven mammary cancers in the NRL-PRL model (50). Furthermore, PRL induced pAKT and pERK1/2 can activate ERα in the absence of estrogen ligand *in vivo* as well as *in vitro* (104–106). PRL interaction with progesterone has been best studied in the context of pregnancy and lactation, where these hormones cooperatively drive expansion of alveolar cells during pregnancy, but oppose one another to initiate lactation (3). Outside of pregnancy, they regulate the other's receptors, and as observed above, work together to increase mammary stem cells (69, 107, 108).

2.2.5.2 Other Oncogenic Factors

Locally elevated transgenic PRL also has revealed potent collaboration with other oncogenic pathways. Crosses between NRL-PRL mice and other murine models of mammary cancer, including elevated TGFα, loss of p53, and mutagen with increased canonical Wnt signals conferred by an inactivating mutation in the tumor suppressor APC, dramatically reduced tumor latency or increased tumor incidence (52, 104, 109–111). Transgenic PRL not only enhanced carcinogenesis, but also markedly influenced the resulting cancers in ways that would impact treatment responses. For example, transgenic local PRL increased the proportion of claudin low tumors in the absence of p53 (110), and in the presence of mutated APC, elevated PRL resulted in tumors with Notch-dependent cancer stem cell activity, compared to the β-catenin-dependence observed in tumors with mutant APC alone (111). Further, transgenic PRL and TGFα in combination sustained activation of the ERK1/2 and AKT signaling cascades (104, 109), reflecting the potent cooperation of PRL with growth factor-initiated signals (112, 113). This further activates ERα in the absence of estrogen ligand *in vivo* (104, 106, 109), one mechanism which underlies resistance of ER+ breast cancers to anti-estrogens (114, 115) (Section 3.3 below). In contrast to the positive interactions between PRL and growth factors in these transgenic murine and breast cancer models, PRL and EGF have been reported to oppose one another in "normal" mammary cell lines, such as HC11 and NMuMG; the phenotype of the target cell is likely to be critical in dictating the outcome of PRL signals and crosstalk with other signals (112, 116).

3 ROLE OF PRL IN ESTABLISHED BREAST CANCERS

In contrast to the strong epidemiologic data supporting a role for PRL in development of breast cancer, particularly of ER+ tumors, its role in established cancers continues to be actively debated. Much of the discussion revolves around the extent of PRLR expression by the tumor parenchyma, including which breast cancer subtypes and which PRLR isoforms, and importantly, whether PRL fuels tumor aggression or fosters a more differentiated phenotype.

3.1 PRL Receptors in Clinical Breast Cancers

PRLR isoforms with distinct intracellular domains and consequent differing signaling capacities are generated by alternative splicing. The full length "long" PRLR isoform is best studied, but as noted below, expression of the "intermediate" PRLR isoform in breast cancers is also recognized. Homo- and hetero-dimerization of these PRLR isoforms not only influences the repertoire of potential signaling pathways, but also stability of the receptors [reviewed in (22, 117)]. These isoforms have further confounded detection of PRLR across breast cancer subtypes, which is already complicated by the specificity and sensitivity of historically

available antibodies (117, 118). However, multiple recent studies have reported PRLR protein expression in ER+, HER2+ and triple negative (TNBC) breast cancers, in sharp contrast to the epidemiologic link between PRL and development of only ER+ breast cancers. The relative proportion of tumors within each subtype that expressed PRLR varied with the cohort examined, antibody utilized [i.e., detecting the extracellular domain shared by most PRLR isoforms (119–121), intracellular domain of the full length "long" PRLR (92, 122), or unique intracellular domain of the "intermediate" PRLR isoform (117)], and other methodological differences. Given these variables, it is not surprising that the proportion of PRLR-expressing breast cancers varied from 25-83%.

Although PRLR expression was independent of ER (92, 119, 120, 122), PRLR levels were highest in ER+ tumors when analyzed (92, 123). Some of these studies found highest PRLR expression in more differentiated tumors in patients with longer metastasis free survival (92, 119). In contrast, Shemanko and her colleagues found that higher tumor PRLR protein levels correlated with a shorter time to bone metastasis, consistent with experimental PRL-induced osteoclast differentiation (120). Some of these reports included interrogation of relative transcript abundance and outcomes in various publicly available databases; with the caveat that transcript levels may not reflect protein expression, these results also differed (92, 123).

Although overall, TNBCs expressed less PRLR detected with an antibody to the intracellular domain of the "long" PRLR (92), a subset of these tumors expressed higher PRLR levels. This TNBC subset was found to be more differentiated, supporting the hypothesis that PRL drives a pro-differentiation program in these cancers (122). (*See review by Ali and colleagues elsewhere in this series*). Interestingly, however, Clevenger and colleagues reported that the "intermediate" isoform of the PRLR, which would not have been detected with the antibody used in the study above, was most highly expressed in TNBC (117). Moreover, they found that cancers with a high ratio of transcripts for the "intermediate" to the "long" PRLR isoform were associated with greater likelihood of distant metastases in the TCGA database. Experimentally, heterodimers of these PRLR isoforms were more stable, and less able to activate STAT5 (Section 3.4.2 below). Clinical TNBCs are very heterogeneous (124, 125); together, these reports suggest that different TNBC subsets may respond quite differently to PRL.

Although many of these studies correlated levels of PRLR protein or transcripts with prognosis, albeit with conflicting conclusions, the outcome of PRLR signaling has been directly addressed only in small Phase I/II studies of patients with advanced disease. A study of a PRLR neutralizing antibody as a monotherapy that included 34 breast cancer patients (all subtypes, but 75% ER+ cancers) found no significant effect on disease progression (126). In another small Phase II trial (20 breast cancer patients), the dopamine D2 receptor agonist, cabergoline, was used to inhibit secretion of pituitary PRL; two of these patients experienced extended disease control (127). The lack of definitive positive results dampened enthusiasm, although the small number of patients, the advanced stage of their disease, and extensive pretreatment regimens limit interpretation. However, interest in this area persists. Conjugates of other therapeutic agents to PRL antagonists or PRLR neutralizing antibodies are being developed, as discussed further in Section 3.3 below.

3.2 Experimental Studies Using Xenografts

Mouse PRL has little activity at the human PRLR (30), which has complicated experimental study of breast cancers *in vivo*. However, Rui and his colleagues have developed a mouse in which the mouse *Prl* gene has been replaced with the human *PRL* gene (NSG-Pro), resulting in physiologic regulation of hPRL expression (128). In these recipients, ER+ patient derived xenografts (PDXs) displayed a remarkable 15-20 fold higher transplantation rate than in wildtype NOD SCID gamma (NSG) mice. Moreover, the NSG-Pro mice facilitated metastatic dissemination and growth of distant lesions, genetic evolution and development of anti-estrogen resistance (128). These studies support an important role for PRL in the biology of ER+ tumors.

Well-characterized breast cancer cell lines modeling different breast cancer subtypes have been extensively studied *in vitro* to understand the outcomes of PRL actions, and to dissect its signals and mechanisms of interaction with other factors; these reports will not be further reviewed here. (*See reviews by Ali and Clevenger and their colleagues elsewhere in this series*). Some investigators have examined PRL responses in murine recipients of transplanted cell lines by providing another source of hPRL, with conflicting results. Primary tumors of transplanted MDA-MB-468 breast cancer cells that expressed hPRL grew more rapidly than tumors that did not in *nu/nu* (nude) mice (129). Reduction of the "long" PRLR isoform reduced pulmonary and hepatic metastatic burden in NOD-SCID recipients of HER2+ BT474 cells, which were supplemented with hPRL (130). In contrast, hPRL-treatment of NOD/SCID mice bearing xenografts of MDA-MB-453 breast cancer cells reduced tumor growth and dissemination (122), and growth of primary HER2+ SKBR3 tumors (131).

In a different approach, Ali and her colleagues used CRISPR/Cas9 to reduce PRLR expression in the ER+ MCF7 and HER2+ SKBR3 breast cancer cell lines (132). Loss of PRLR in MCF7 cells reduced ER expression, consistent with regulation of *ESR1* by PRL, and promoted less differentiated, more aggressive tumors upon transplantation to NOD/SCID mice. In HER2+ SKBR3 cells, loss of PRLR increased HER2 expression, and ability to colonize lungs of NSG recipients. These findings, together with associated *in vitro* analyses, indicate beneficial actions of PRLR in these models (132). (*See review by Ali and colleagues elsewhere in this series*).

The basis for the disparate responses to PRL in these xenograft studies is unclear. There are many differences among these experiments. The transplanted cancer cells, whether PDXs or different breast cancer cell lines (133, 134), are quite distinct. Moreover, the design of these studies differs markedly, including placement of the transplanted cells, the extent that the murine hosts are immunocompromised, and method of manipulating PRL activity. Additional studies are necessary to understand how these findings reflect diverse clinical breast cancers.

Few studies have been performed in syngeneic experimental models. However, the findings are intriguing. In a murine model of HER2+ cancer (MMTV-neu), the PRL antagonist, G129R-hPRL, reduced metastases after removal of the primary tumor (135). In a subsequent study, the effects of G129R-hPRL on HER2 signaling in this model were found to be dependent on cancer associated fibroblasts (136), supporting the importance of study of complex systems with multiple cell types. Systemically reducing expression of the "long" PRLR isoform using a novel method reduced metastases in the aggressive 4T1 model, and PRL-supported immunosuppressive Tregs were identified as a major target (83, 130). These observations underscore the drawbacks of xenograft models. Most notably, currently available xenograft recipients are severely immunocompromised, lacking critical components of the host response. Ongoing efforts to develop mice with "humanized" immune systems will address this shortcoming. In addition, subtle differences in the structures of mouse/human proteins can obscure paracrine/systemic communication between tumor and stromal cells, e.g., PRL itself (30).

3.3 PRL Interactions With Other Treatment Approaches

Although PRL/PRLR inhibitors have not shown robust promise as monotherapies, there has been long term interest in their interaction with other treatment modalities, especially with anti-estrogens in ER+ breast cancers. As noted in Section 2.2.5, PRL cooperates with estrogen by multiple mechanisms, which have been dissected primarily in the well-differentiated ER+ breast cancer cell line, MCF7 (100–103). Not surprisingly, as for other aspects of cancer biology, this relationship evolves with disease progression. In an experimental rat model of hormonally-responsive ER+ mammary cancer, concomitant inhibition of PRL and aromatase cooperatively reduced tumor growth (137). Similarly, in therapy naïve ER+ PDXs transplanted to NSG-Pro recipients, PRL initially supported anti-estrogen responsiveness, but with time, the PRL environment facilitated development of resistance to tamoxifen (128). This was associated with increased growth factor signals, including ligand independent activation of ER, a potent outcome of PRL-growth factor crosstalk (See Section 2.2.5), and activation of the ERBB2 pathway (128). This relationship between PRL and resistance to anti-estrogens is reflected in some but not all small clinical studies [reviewed in (16)]. Interestingly, LAT1/SLC7A5, a transporter for branched chain amino acids which is regulated by PRL during lactation (138), is highly expressed by tamoxifen resistant cancers (139–141). The growing recognition of the importance of tumor metabolism, and role of PRL in regulation of metabolism during lactation, points to this area for further study.

The potent crosstalk of PRL with growth factor-initiated signals observed both in breast cancer cell lines (112, 142), and anti-estrogen resistant ER+ PDXs (128) has suggested that targeting PRL signaling in combination with these pathways may be an efficacious therapeutic strategy. PRL can initiate phosphorylation of HER2 in SKBR3 and BT474 breast cancer cells *in vitro* and in a murine MMTV-neu-derived tumor ex vivo (136, 143, 144). In light of the apparent conflict of these *in vitro* observations with the results of some but not all xenograft studies as noted in Section 3.2, this deserves additional investigation.

Several small clinical studies suggested that reduction of PRL might improve responses to chemotherapies [reviewed in (16, 145)]. The ability of PRL to promote survival of breast cancer cells *in vitro* has been appreciated for some time [reviewed in (22, 145)]. In breast cancer cell lines representing different cancer subtypes, PRL promoted to resistance to chemotherapies, including doxorubicin, paclitaxel, and cisplatin (93, 146, 147). Several related mechanisms have been identified, including PRL-induced expression of anti-apoptotic proteins (148), transcription of the multidrug resistance transporter ABCG2 (149), and activation of glutathione-S-transferase (147). Interaction with chemotherapies has not been directly revisited clinically, but these actions may contribute to the efficacy of compounds conjugated to anti-PRL agents, as noted below.

The relatively low toxicity of PRL antagonists and PRLR neutralizing antibodies and widespread PRLR expression across different breast cancer subtypes have prompted their development as delivery vehicles for other therapeutic agents, including cytotoxic compounds (121, 135, 150, 151), anti-HER2 (152), and immunomodulators to attract and/or activate CD8+ T cells (135, 153). In addition to targeting delivery of other treatments, testing of these molecules will also provide information on the efficacy of concomitant inhibition of PRL signals.

3.4 PRL Initiated Signals

3.4.1 Canonical JAK2-STAT5 Pathway

As described above, most studies have examined the outcome of the sum of all PRL-initiated signals in different breast cancer settings. However, it has long been appreciated that PRL can activate multiple signaling cascades (22, 51), with potentially different outcomes. The JAK2-STAT5A pathway mediates PRL-driven proliferation and differentiation that is essential for successful lactation (2, 154, 155), and binding of STAT5A to regulatory enhancer regions initiates chromatin remodeling, coordinating tissue specific gene expression (4, 156). (*See review by Clevenger and colleagues elsewhere in this series*). Transgenic overexpression of STAT5A leads to mammary carcinomas in the absence of other oncogenes (46). However, evidence for high STAT5A activity in clinical breast cancers has been repeatedly associated with more differentiated cancers and better prognoses [(154, 157, 158) and references therein]. Consistently, the JAK2-STAT5A pathway also has been linked to PRL-induced pro-differentiation activities in various experimental models (53, 56, 159–161) (**Figure 2**). Of particular interest for premenopausal breast cancer, PRL-activated STAT5 suppressed a progestin-induced progenitor population in T47D breast cancer cells (162).

The highly homologous STAT5B remains a complication in these studies. In contrast to STAT5A, STAT5B is not associated with a more favorable prognosis in patients (158), and *in vitro*, STAT5B drives aggressive behavior in several models (56, 159, 161). Activities distinct from STAT5A are supported by different

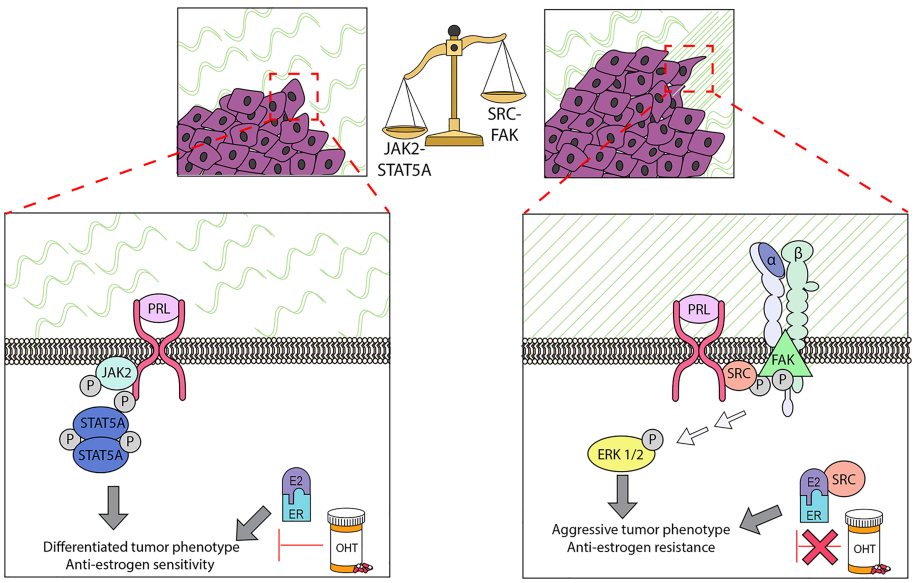

FIGURE 2 | PRL can initiate multiple signaling cascades in established cancers, which can result in different biological outcomes. Determination of the repertoire of PRL signals can be modulated by multiple factors, including properties of the ECM. In ER+ cell lines, the stiffness and density of the extracellular matrix (ECM) strongly influences the balance of these signals: in stiff matrices, PRL signals are shifted away from the canonical JAK2/STAT5A pathway, and toward FAK/SFK/ERK1/2. This shift permits PRL to drive proliferation, invasion, and resistance to tamoxifen (OHT), and further remodel collagen fibers in the ECM. These experimental findings support the clinical observations that ER+ cancers with regions of aligned collagen perpendicular to the tumor boundary have a worse prognosis, and that activated STAT5A is strongly linked to more differentiated cancers and tamoxifen sensitivity. See Section 3.4 for details.

target genes (56, 158, 163), and divergent regulation by estrogen activity (56). Additional studies with more specific reagents are needed to resolve the roles of the STAT5 isoforms in PRL actions in breast cancer.

3.4.2 Other Signaling Cascades

PRL can also initiate activation of src family kinases, and multiple additional mediators, including AKT and MAP kinases (164–168). Interestingly, the alternatively spliced PRLR isoforms with distinct intracellular domains are less able to activate the JAK2/STAT5 pathway than the well-studied "long" PRLR isoform. Indeed, heterodimerization of the "long" PRLR isoform with the "intermediate" isoform, which was recently reported to be highly expressed in a subset of TNBC (see Section 3.1), inhibits phosphorylation of STAT5, without impacting other PRL-activated signaling pathways (117). AKT and MAP kinase cascades are linked to tumor progression for many cancers (169–173). Moreover, as noted above, they can activate ERα in the absence of ligand (105, 106), with implications for therapeutic responses to anti-estrogens in ER+ breast cancers. Importantly, they are also potent sites of cooperation with other oncogenic factors, including growth factors (112, 142). Together, these observations point to the potentially divergent outcomes of different arms of PRL signals, and raise the question of determinants of the repertoire of signaling options for PRL. Clearly intrinsic differences in tumor cells themselves, including relative levels of PRLR isoforms and other signaling components play a role; different breast cancer cell lines, even of the same breast cancer subtype, exhibit different spectra of PRL activated signals *in vitro* [e.g., (137, 174)]. In addition, as discussed below, environmental factors also can powerfully modulate the balance of PRL signals.

3.4.3 Features of the Extracellular Matrix Shift PRL-Initiated Signals in ER+ Tumor Cells and Alter Sensitivity To Anti-Estrogens

Accumulating data underscore the importance of the ECM in normal mammary function and tumor behavior (86, 175). A mechanically stiff matrix increases signaling through focal adhesions (176, 177). Aligned collagen fibers oriented perpendicularly to the tumor boundary have been linked to a poor prognosis, particularly in ER+ breast cancer (178). We have demonstrated that ECM structure can strongly influence the spectrum of PRL signals and PRL-estrogen crosstalk in ER+ breast cancer cells, and reciprocally, that these hormones can modify ECM structure (**Figure 2**). When well-characterized ER+ breast cancer cell lines were cultured in stiff ECM *in vitro* (MCF7 and T47D cells in 3-dimensional collagen cultures and tunable polyacrylamide substrates), PRL was less able to activate JAK2/STAT5, but more strongly activated FAK-SRC-ERK1/2, associated with increased localization of PRLR in focal adhesions (167, 179). These conditions augmented PRL-driven invasion and re-orientation of collagen fibers *in vitro* (167), and intravasation and metastasis of PRL-initiated ER+ tumors in a syngeneic model of increased COL1A1 density/stiffness *in vivo* (180). Moreover, a stiff/dense matrix enhanced PRL-estrogen crosstalk to increase invasion, reduce responsiveness to tamoxifen, and further modify ECM structure *in vitro* (181);

these findings were supported using the syngeneic ER+ model above (55). Moreover, progesterone further augmented PRL induction of *MMP3* RNA in stiff matrices (107). These studies indicate that desmoplasia, a feature of the microenvironment of many tumors, can alter the repertoire of PRL-initiated signals to favor pro-tumor pathways and anti-estrogen resistance, thus illuminating one mechanism underlying the apparent disparate reports of the outcomes of PRL signals in ER+ breast cancers. Extension of these studies of the effect of ECM characteristics on PRL signals to other breast cancer subtypes may further resolve some of the apparently contradictory reports.

4 SUMMARY AND FUTURE DIRECTIONS

Strong epidemiologic data linking higher levels of circulating PRL to increased risk for ER+ breast cancer are supported by multiple lines of experimental evidence. Independently from ovarian steroids, PRL can modulate the epithelial hierarchy and increase progenitor populations, drive development of ductal and alveolar abnormalities, and with time, promote aggressive metastatic ER+ carcinomas. PRL engages in complex crosstalk with estrogen and progesterone, cooperating with them by multiple mechanisms, but also opposing steroid-driven differentiation. Further understanding of these interactions apart from the hormonal milieu of pregnancy will provide additional insight into the impact of PRL on increased breast cancer risk in premenopausal women and postmenopausal women treated with estrogen-progesterone MHT (107, 162, 182). As disease progresses with dysregulation of multiple pathways, intrinsic tumor cell properties and the stromal environment are likely to alter the responses of target cells to PRL and its interactions with other potential oncogenic factors. Although not well understood, the literature suggests that PRL also may act directly on multiple mammary stromal cell types including immune cell subpopulations and/or modulate their activity *via* paracrine signals, which may further increase risk. The high PRLR expression in clinical DCIS and preneoplastic structures in preclinical models is reminiscent of ER expression in many of these lesions [reviewed in (183)], and suggests a role for PRL at this early stage of the disease process.

In contrast, the role(s) of PRL in the biology of established clinical breast cancers remains unclear. Although PRLR is highly expressed by many tumors across breast cancer subtypes, data from small clinical trials inhibiting PRL action are difficult to interpret, and studies of xenografts, particularly of breast cancer cell lines, are conflicting. Responses of phenotypically diverse heterogeneous cancers are complicated by different levels of PRLR isoforms with distinct signaling capabilities, selection and genomic evolution as tumors progress and respond to initial therapies, and environmental context, including site-specific responses of the metastatic niche [e.g., bone (120)], ECM properties and the steroid hormone and growth factor milieu (**Figure 3**). The emerging data support complex actions of PRL in breast cancer biology, resembling the major recognized hormonal actor in breast cancer, estrogen (184–186). This is illustrated in ER+ cancers, the breast cancer subtype in which PRL actions are currently best understood. Experimental

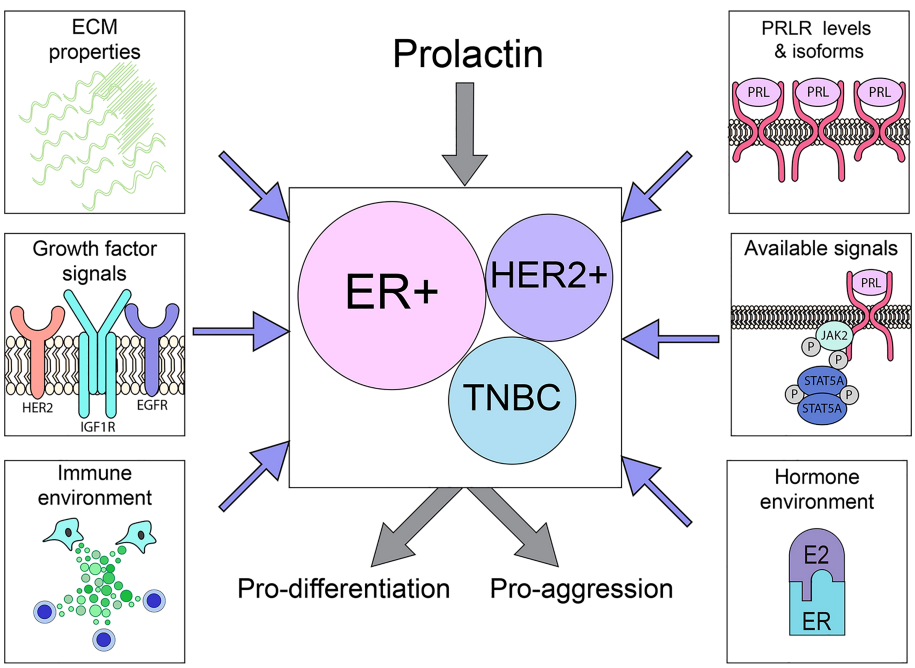

FIGURE 3 | PRLR is expressed on a substantial subset of breast cancers across the major subtypes. The outcome of PRL signals may vary considerably, driving either differentiation or aggression, depending on tumor cell intrinsic properties (PRLR expression, available downstream signaling cascades) as well as extrinsic factors (ECM characteristics, hormone/growth factor milieu), as illustrated above. See Section 3 for details.

evidence shows that PRL can activate STAT5A-driven differentiation, and maintain ERα expression, thereby facilitating anti-estrogen responsiveness (56, 128, 132, 159, 161). However, PRL can also drive proliferation and invasion, and support development of resistance to anti-estrogens (128, 181). Within the heterogeneous TNBCs, evidence indicates distinct subgroups which exhibit divergent responses to PRL (117, 122). Conflicting outcomes in a very limited number of different HER2+ breast cancer cell lines suggest similar possibilities. Together, these reports paint a more nuanced picture of PRL action in established cancers, and potential for very different outcomes depending on context. They underscore the need for additional study of PRL in diverse clinical breast cancers, changes with disease progression and therapeutic pressure, and influences of the metastatic sites.

New technologies will assist in the resolution of these issues. The NSG-Pro mouse is a powerful tool to interrogate the actions of PRL in diverse clinical breast cancers in a dynamic *in vivo* environment (128). Already providing insights into ER+ cancers, this model will help resolve some of the conflicting studies observed with experimental xenografts of a relatively small number of breast cancer cell lines of other subtypes. It will enable dissection of the mechanisms underlying observed differences, and facilitate identification of biomarkers that predict beneficial or adverse responses to PRL and/or PRL inhibitors. Pending validation of humanized mice, syngeneic mouse models continue to be essential to reveal the impact of PRL as well as other agents on inflammation and suppression of anti-tumor immunity, a critical step toward employment of the promise of immunotherapies for hormonally responsive cancers. Further, as discussed in Section 2.2.3, many other stromal cell types which sculpt the tumor microenvironment are potential PRL targets, motivating additional study in the context of breast cancers. In addition, the paucity of inhibitors to interrogate PRL actions in clinical samples and experimental models is now addressed by small molecule inhibitors (129), the technology to reduce specific PRLR isoforms *in vivo* (130), and renewed interest in PRLR neutralizing antibodies (Sections 3.1, 3.3). Development of selective inhibitors of the JAK2 and src family kinase-mediated signals of PRL, taking advantage of our understanding of the closely related growth hormone receptor, will advance these studies (142, 187, 188). Together, these approaches will unravel the complex actions of PRL, permitting a new understanding of the role of this third hormone in breast cancer, with implications for prevention and treatment.

AUTHOR CONTRIBUTIONS

Both LS and KO'L contributed to the original drafts and editing process. All authors have read and agreed to the published version of the manuscript.

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Terminal differentiation and anti-tumorigenic effects of prolactin in breast cancer

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Breast cancer is a major disease affecting women worldwide. A woman has 1 in 8 lifetime risk of developing breast cancer, and morbidity and mortality due to this disease are expected to continue to rise globally. Breast cancer remains a challenging disease due to its heterogeneity, propensity for recurrence and metastasis to distant vital organs including bones, lungs, liver and brain ultimately leading to patient death. Despite the development of various therapeutic strategies to treat breast cancer, still there are no effective treatments once metastasis has occurred. Loss of differentiation and increased cellular plasticity and stemness are being recognized molecularly and clinically as major derivers of heterogeneity, tumor evolution, relapse, metastasis, and therapeutic failure. In solid tumors, breast cancer is one of the leading cancer types in which tumor differentiation state has long been known to influence cancer behavior. Reprograming and/or restoring differentiation of cancer cells has been proposed to provide a viable approach to reverse the cancer through differentiation and terminal maturation. The hormone prolactin (PRL) is known to play a critical role in mammary gland lobuloalveolar development/remodeling and the terminal differentiation of the mammary epithelial cells promoting milk proteins gene expression and lactation. Here, we will highlight recent discoveries supporting an anti-tumorigenic role for PRL in breast cancer as a "pro/forward-differentiation" pathway restricting plasticity, stemness and tumorigenesis.

KEYWORDS

Prolactin/prolactin receptor, breast cancer, stem cells, plasticity, single cell analysis, JAK/STAT, differentiation

Abbreviations: A/B, apical/basal; ALDH; aldehyde dehydrogenase gene; ATAC, Assay for Transposase-Accessible Chromatin; BCSCs, breast cancer stem cells; BL, basal-like; BRD4i, bromodomain-containing protein 4 inhibitor; CK5, cytokeratin-5; DT, differentiation therapy; EGF, epidermal growth factor; EMP, epithelial-mesenchymal-plasticity; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; GOBO, Gene expression- based Outcome for Breast Cancer Online; HDACi, histone deacetylases inhibitor; HER2, human epidermal growth factor receptor-2; HER2-E, HER2-enriched; hPRL, human prolactin; LAR, luminal-androgen receptor; MaSC, mammary stem cell; NMI, N-myc interactor; PPARγ, peroxisome proliferator- activated receptor gamma; PR, Progesterone receptor; PRL, Prolactin; sc, single cell; TNBC, Triple negative breast cancer.

Introduction

Cancer is a complex disease caused by both genetic and epigenetic mutations/alterations promoting uncontrolled growth and ultimately ensuring the dysregulation of control mechanisms of normal tissue differentiation and homeostasis (1, 2). Recent advances in our understanding of the process of tumorigenesis have indeed emphasized tumor plasticity (encompassing dedifferentiation, blocked differentiation, and/or trans-differentiation) and enrichment of stem-like cell population(s) underlie tumor heterogeneity, progression and therapy failure and resistance. Just recently, tumor cellular plasticity was recognized within the "hallmarks" of cancer, initially proposed in 2000, as an enabling feature promoting tumor evolution and progression (2, 3). Thus, reprograming and/or restoring differentiation of cancer cells has been proposed to provide a viable approach to reverse the cancer phenotype through differentiation and terminal maturation (4). Importantly, while differentiation-based therapeutic approaches have already been employed and shown success in the treatment of hematological malignancies, their application to solid tumors including breast cancer is yet to be fully developed and is an area of intense investigation (5–8). Thus, it is evident that characterizing mechanisms/pathways promoting differentiation in breast cancer is fundamental and will help generate novel differentiation-based reagents and approaches to better manage and serve patients stricken by this aggressive disease. In this review we will summarize knowledge gained from exploring the impact of the mammary differentiation hormone PRL in the context of suppression of breast tumorigenesis through restoration of differentiation and suppression of stemness.

Breast cancer differentiation state illustrates good prognosis vs poor prognosis

Tumor differentiation state in breast cancer is classically determined by the tumor grade established based on the use of certain histological and morphological criteria, such as nuclear pleomorphism, gland or tubule formation and number of dividing cells, and has long been used as predictive of cancer behavior where immature tumor (not resembling the tissue of origin) is more aggressive than the more differentiated counterpart (9–11). Findings emanating from a large study examining tumor grade and patient outcome indicated that high-grade (grade 3) breast cancers tend to recur and metastasize early following diagnosis and show poor prognosis, whereas low-grade tumors (grade 1) tend to show a very good outcome and grade 2 tumors show an impaired outcome in the long term (12, 13).

Moreover, the correlation of breast cancer differentiation state with tumor behavior and patient outcome can also be gleaned from the current classification schemes of breast cancer whether based on evaluating the histological expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2), or classifications based on intrinsic gene expression and genomic profiling (PAM 50) (14, 15). Largely, breast cancers can be categorized into molecularly distinct subtypes including, luminal A, luminal B, HER2-enriched (HER2-E) and basal-like and claudin low (representing triple negative breast cancer [TNBC]: ER^-, PR^-, $HER2^-$) (16–18). Among the different breast cancer subtypes, the most differentiated breast tumors are those of the luminal A subtype which tend to be of low grade showing epithelial-like differentiation and interestingly have the least aggressive tumor biology and the most favorable prognosis. In contrast, luminal B, HER2-E and TN are considered 'aggressive' subtypes, characterized by a tumor biology showing generally high grade, high mitotic/proliferation index, and a greater risk of local recurrence, metastasis and poor survival outcomes (19–21). In agreement, recent studies using single cell (sc) approaches have further emphasized the phenotypic and cellular diversity of breast tumors (22, 23). Importantly, tumor cellular phenotypic abnormalities linked to deviation from the juxta-tumoral area were found to be higher for tumor cells of luminal B, luminal B-HER2+, TN, and grade 3 tumors than for luminal A and lower grades tumors. Moreover, phenotypically abnormal cells were also correlated with hypoxic phenotype and proliferation marker expression which were previously linked to poor differentiation in breast cancer (24). Moreover, sc-analyses of the heterogeneous TNBC subtype showed that TNBC tumors of the basal-like phenotype as exhibiting high proliferation index compared to the TNBC subtype showing luminal-androgen receptor (LAR)-differentiation phenotype (23, 25).

Additionally, over the past two decades studies evaluating the breast cancer cell-of-origin and the cancer stem cell hypothesis have emphasized a link between the mammary stem cell (MaSC) hierarchy, breast cancer stem cells (BCSCs) and the inter- and intra-tumoral heterogeneity of breast cancer (26–28). These studies highlighted that essentially breast cancer originate from a mammary luminal progenitor population and indicated the presence of rare populations of cancer cells within breast tumors that exhibit high tumorigenic capacity and resistance to chemotherapy with a stem-like phenotype capable of self-renewal and tumor repopulation. These aggressive BCSCs are found to be mostly enriched in the aggressive breast tumors such as TNBC as well as HER2-E tumors (29). In summation, there is extensive literature implicating loss of tumor differentiation, and the accumulation of dedifferentiated immature cancer cells endow breast cancer with aggressive features and is predictor of poor prognosis.

PRL regulation of alveolar differentiation and apical/basal polarity

The hormone PRL is best known as a lactation hormone critical for mammary gland lobuloalveolar development/remodeling and the terminal differentiation of the mammary epithelial cells promoting milk proteins gene expression and lactation (30–32). PRL mediates its effects by binding to its specific receptor (PRLR), resulting in receptor dimerization and activation of different intracellular signaling cascades, most well studied is the Jak2/Stat5 pathway (33). Importantly, PRL, PRLR, Jak2 and Stat5 knockout mouse models have all shown defects in mammary gland development and lactation, clearly highlighting the prominent role of PRL in the normal development and functional differentiation of the mammary gland (34–38). Indeed, during the pregnancy/lactation cycle the mammary gland undergoes a complex growth and remodeling characterized by the establishment of the secretory alveolar units. These mammary alveoli consist of a layer of terminally differentiated luminal mammary epithelial cells attaining apical/basal (A/B) polarized architecture with closed tight junctions and well-established adherence junctions. Their main function is to allow for the synthesis and directional secretion of milk proteins and solutes into the lumen of the alveolar unit to the mammary ductal system upon suckling of the infant (39, 40). In agreement with the above work and crucial to the differentiation role of PRL in the breast, using a well-established *ex vivo* mammary 3D cell culture model, PRL signaling through Jak2 was found to induce A/B polarity and to organize the mammary epithelial cells around a single hollow lumen (41, 42). Recently, PRL regulated gene Pre-B-Cell Leukemia Transcription Factor-Interacting Protein 1 (PBXIP1/HPIP) was also found to play a role in PRL-mediated mammary epithelial cell differentiation and acini morphogenesis (43). Moreover, studies from our laboratory also highlighted that PRL indeed limits the proliferative capacity of the mammary epithelial cells and provided resistance to the proliferative effects of EGF (42, 44). Previously, PRL was shown to be part of a cooperative signaling network with EGF promoting alveolar survival, morphogenesis, and functional differentiation (45, 46). Our studies however, highlighted an important negative cross-talk between PRL/Jak2-differentiation axis and the EGF-Erk1/2-proliferative pathway (44). Together, these results expand on the vital role for PRL in deriving the normal differentiation program of the mammary cells and constrains the proliferative effects of growth factors (Figure 1).

PRL regulation of the MaSC hierarchy and terminal differentiation

Extensive research has been devoted to characterizing the breast epithelium delineating the mammary stem cell (MaSC) hierarchy and its relevance to breast cancer inter-tumor heterogeneity with the interest of identifying new therapeutic targets in breast cancer (27, 47). Studies have described a MaSC hierarchy consisting of different cell populations based on expression of cell surface markers into: basal ($EpCAM^{low/-}$/$CD49f^{high/+}$), luminal progenitor ($EpCAM^{high/+}$/$CD49f^{high/+}$), and mature luminal cells ($EpCAM^{high/+}$/$CD49f^{low/-}$) (48). With advances in sc-analyses, recent studies have indeed expanded on this model and highlighted a more complex mammary lineage hierarchies and cell states within the mammary epithelium (49–51). Still, these studies confirmed that the epithelium in mouse and human samples are mainly divided into three major clusters, namely basal cells, luminal progenitors, and mature hormone-sensing luminal cells. We previously investigated the contribution of PRL to the differentiation program of the MaSC hierarchy. Mammary epithelial cells isolated from mid-pregnant mice showed two distinct cellular sub-populations based on the expression profile of EpCAM and CD49f. One population featured a surface marker signature with $EpCAM^{high/+}$/$CD49f^{high/+}$ defining the luminal progenitor cells and another with $EpCAM^{high/+}$/$CD49f^{low/-}$ defining mature luminal cells. Comparing with EGF treated cells, treatment with PRL resulted in a shift in the luminal progenitor ($EpCAM^{high/+}$/$CD49f^{high/+}$) cells into the mature luminal ($EpCAM^{high/+}$/$CD49f^{low/-}$) cells suggesting that PRL derives the terminal differentiation of the mammary epithelial cells (42). This proposition is also supported by the sc-studies described above where PRLR expression was found to be enriched in the most differentiated hormone sensing cells and least expression was found in the basal compartment (49). As well, PRL-target milk proteins (e.g. Wap, Csn2) were expressed exclusively in cellular clusters composed of cells from gestation and lactation defining them as differentiated secretory alveolar cells. Interestingly, Assay for Transposase-Accessible Chromatin (ATAC) analyses pointed to a strong correspondence between high FOXA1 transcription factor, known regulator of luminal differentiation and an antagonist of the epithelial-to-mesenchymal transition (EMT), motif accessibility, and gene expression in the hormone-responsive luminal cells (52). Interestingly, we have previously found that there is positive correlation of expression between PRLR and FOXA1 in breast cancer cases (53). Altogether, these results suggest that PRL/PRLR derives the terminal maturation of the mammary stem cells into a differentiated hormone sensing cells and differentiated alveolar cells. These results also highlight the close association between FOXA1 and the PRLR in the differentiated hormone sensing luminal cells that is maintained in breast cancer.

Evidence of anti-tumorigenic functions of PRL/PRLR pathway in breast cancer

While the role of PRL as a differentiation factor in the mammary gland is well known, its role in breast cancer is still

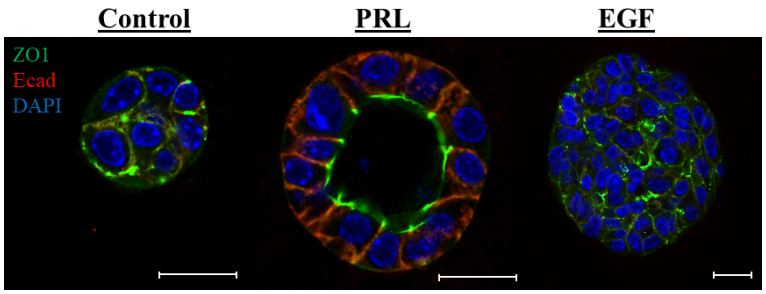

FIGURE 1
PRL induces mammary A/B polarity and acini morphogenesis: Primary mouse mammary epithelial cells grown in 3D culture were stained with antibody to ZO1 (green) and Ecad (red). Nucleus was counter stained with DAPI (blue). Scale bar, 20 μm. The morphology of the colonies was evaluated following different treatments: (1) Control: 2% FBS, (2) PRL: 2% FBS + 2 μg/mL ovine PRL or (3) EGF: 2% FBS + 10 ng/mL EGF. In contrast to control or EGF treated cells, PRL treated mammary epithelial cells organize around a single lumen showing apical localization of the tight junction protein ZO1 and basal/lateral localization of the adhesion protein E-cadherin.

not fully characterized. Several studies using *in vitro* cell culture approaches as well as transgenic and knock-out mouse models have highlighted a pro-tumorigenic role for PRL in breast cancer promoting tumor initiation, development and metastasis (reviewed elegantly in this series Schuler, LA and O'Leary, KA as well as previously (54)). These findings prompted interest in developing strategies to block PRL as a treatment modality in breast cancer. Most recent and indeed direct approach was the generation of humanized antibodies to block PRLR as a targeted therapy in breast cancer (55, 56). Following extensive characterization of these antibodies, their therapeutic value was assessed. Indeed, these agents failed to show any antitumorigenic effects in a landmark multicenter clinical trial performed in PRLR expressors breast cancer patients (Novartis, 2016) (USA, Belgium, Italy and Spain), despite effective blockage of the PRLR, resulting in the termination of the trial (57, 58). The lack of anti-tumorigenic effects of blockers of PRLR suggests that the described pro-tumorigenic role of PRL in breast cancer BC is not of clinical value. Also, these results indicate that PRL role in breast cancer needs further evaluation.

Epidemiological studies examining the normal physiological levels of circulating PRL (2-29 ng/mL) have implicated PRL as a risk factor and is involved in breast cancer etiology (59–63). However, later extended follow-up analyses showed either modest association, that is limited to patients who were on hormone replacement therapy or no significant associations (60, 61, 64, 65). Importantly, no differences in mean serum PRL levels in premenopausal (~21 ng/mL) or postmenopausal (~13 ng/mL) breast cancer cases compared with normal cases was reported (65). This finding suggests that serum PRL is not a breast cancer risk factor. In addition, studies of patients with conditions that result in high circulating PRL levels such as prolactinomas or the use of antipsychotics showed no causal link to breast cancer (66, 67). In fact, other conditions that lead to high circulating levels of PRL (~200 ng/mL) such as breastfeeding have been linked to reduced risk of breast cancer. A seminal study (2002) that examined 50,000 breast cancer cases from 47 epidemiologic studies in 30 countries, reported that the relative risk for breast cancer is reduced by 4.3% for every 12 months a woman breastfeed (68). Another study reported a 14-28% lower risk of developing breast cancer in parous women who ever breastfed compared with parous women who never breastfed (69). Furthermore, little-to-no breastfeeding correlated with increased risk of developing aggressive types of breast cancer (70–72). While studies have emphasized the local/autocrine PRL and not the circulating endocrine PRL as contributing to mammary tumorigenesis and breast cancer development, however, other studies using large breast cancer patient data and cell lines provided different conclusions. PRL mRNA expression was found to be either very low or undetectable in the majority of samples representing 144 breast cancer patients and in many breast cancer cell lines and the study concluded that autocrine PRL signaling is unlikely to be a general mechanism promoting tumor growth in breast cancer (73). We have also analyzed PRL protein and mRNA levels in breast cancer cases (74). Interestingly, our results agreed with the above report and showed a significant down regulation of PRL expression in breast cancer compared to normal tissue. Moreover, inline with the differentiation role of PRL in the breast, expression of PRL mRNA was associated with more differentiated tumors, early stage, smaller tumor size and absence of distant metastasis with higher PRL mRNA levels correlating with prolonged relapse free survival (74). Importantly, in preclinical xenograft mouse models of TNBC and HER2-E breast cancer types, PRL was found to cause tumor downstaging as measured by tumor volume/growth and expression of the proliferative marker Ki67 (53, 75, 76). Also, PRL was found to suppress induction of the cytokeratin-5 (CK5)-positive stem-like population in breast cancer cells both *in vitro* and *in vivo* (77, 78). As well, PRL was recently found to sensitize ER+ breast cancer cells to tamoxifen in a xenograft mouse model

expressing hPRL gene (79). Altogether, these findings implicate that PRL of endocrine or tumor source is not a risk factor in breast cancer but rather a marker of more differentiated and less aggressive tumors and is a potential therapeutic agent.

Assessing the expression levels of PRLR in breast cancer cases is vital to further define the role of PRL in breast cancer. Whereas short forms of the human PRLR generated by alternative splicing or as mutant truncation forms have been described, the long form of the PRLR is considered as the signaling hub for PRL (80–82). Previous reports have examined PRLR expression and have reported a widespread expression in breast cancer samples (83). More recent findings contradict these observations and implicate that PRLR expression is generally downregulated in breast cancer. For example, it was reported that using specific anti-human PRLR antibodies in a screen of 160 mammary adenocarcinomas demonstrated significant immunoreactivity in only 4 tumors (ie less than 3% expression). This led the authors to conclude that PRLR is generally not strongly upregulated in human breast cancer (84). We previously used human breast cancer cases organized in tissue microarrays as well as bioinformatics analyses and datasets to assess the expression of PRLR in breast cancer. We found that PRLR expression to be significantly downregulated in invasive breast cancer, only 21% of invasive cases showed detectable expression of the PRLR in comparison with normal/benign (80%) and *in situ* carcinoma (60%) (85). In addition, gene expression level of PRLR was also evaluated in relation to intrinsic molecular subtypes, tumor grade, and patient outcome using GOBO database for 1881 breast cancer patients. PRLR expression was found to associate with less aggressive clinicopathological parameters such as lymph node negativity and low-grade well-differentiated tumors. Also, among the different breast cancer subtypes, PRLR mRNA levels were highest in luminal A subtype and least expression was detected in the most aggressive TNBC basal-like subtype. Furthermore, PRLR expression was significantly associated with better survival outcome in breast cancer cases (85). Interestingly, within the TNBC subtypes, PRLR gene expression positively correlated with luminal and epithelial metagenes (LAR and Epithelial Cell-Cell adhesion), whereas it negatively correlated with metagenes defining the aggressive TNBC basal-like (BL) and mesenchymal stem-like subtypes (MSL) (25, 53). A subsequent study also found that PRLR expression defined a patient population with better prognosis showing lower recurrence and higher overall survival in TNBC patients (86). Interestingly, reports have shown that expression of truncated forms of the PRLR long form resulted in initiation of mammary tumorigenesis in mouse models of ER+ breast cancer as well as in human MCF10A xenograft model (87, 88). Similarly, direct knock out of the PRLR in ER+ and HER2-E breast cancer cell lines led to enhanced tumorigenic and metastatic phenotype as well as resistance to conventional therapies (89). Altogether, these results implicate that loss of PRL/PRLR expression contributes to the initiation and progression of breast cancer and argues against a role for PRL/PRLR in promoting breast tumorigenesis.

In agreement with the above data showing PRL/PRLR as favorable markers of tumor differentiation and suppressors of tumorigenesis, other groups have demonstrated that expression/activation of the PRL effector molecule-Stat5a in breast cancer promotes adhesion and inhibits invasion of breast cancer cells (90). As well, Stat5a expression in breast cancer clinical cases was found to associate with histologic differentiation (low grade) and favorable prognosis, whereas loss of Stat5a expression was associated with tumor progression, unfavorable prognosis and increased risk of failure to antiestrogen therapy (90–94). Recently, Stat5a-N-myc interactor (NMI)-signaling also further supported an anti-tumorigenic role for Stat5a. It was reported that this signaling axis is downregulated in breast cancer and its expression is distinctive for less frequent metastasis and good prognosis (95). Additionally, examining expression of PRL signaling pathway-based gene signature composed of PRL, PRLR, Jak2 and Stat5a showed a significant association with more differentiated tumors and prolonged survival (74). Interestingly, PRL-responsive milk proteins were also shown to inhibit tumorigenesis and invasion of breast cancer cells (96–98). Moreover, global gene profiling of prolactin-modulated transcripts in ER+ human breast cancer xenotransplant model revealed that PRL-upregulated genes were enriched in pathways involved in differentiation and a gene signature based on PRL-upregulated genes was associated with prolonged relapse-free and metastasis-free survival in breast cancer patients (99). Interestingly, gene profiling of PRL stimulated mammary epithelial cells also defined a gene signature derived from PRL-upregulated target genes to be associated with well differentiated tumors, whereas expression of a gene signature composed of PRL-downregulated genes showed a significant association with shortened distant metastasis free survival (74). Importantly, functional investigations of these PRL-downregulated genes identified novel players in breast cancer. Indeed, PRL-downregulated genes were found to be drivers of oncogenic processes including the epigenetic A-to-I RNA editing process and the metastatic and stemness epithelial-mesenchymal-plasticity (EMP) process (77, 78, 100, 101). Altogether, there is now a large body of evidence implicating PRL/PRLR pathway as a clinically relevant anti-tumorigenic pathway in breast cancer.

PRL/PRLR and the cancer cell-of-origin

The molecular classification of breast cancer subtypes based on global gene expression profile had a fundamental impact on the current understanding of inter-tumor heterogeneity. Studies have also highlighted the link between the mammary stem cells hierarchy serving as the cell of origin for malignant

transformation giving rise to the various tumor subtypes (16, 17, 102, 103). Direct comparison of the gene expression profiles of normal mammary epithelial subsets described above (i.e. basal/MaSC, luminal progenitor, and mature luminal cells) to those of breast tumors based on the molecular subtype classifications were performed (104). Interestingly, luminal A and B subtypes showed high similarity to the mature luminal cell population EpCAM $^{high/+}$/CD49f$^{low/-}$. The luminal progenitor gene expression signature was very similar to the basal-like subtype showing expression of basal-like markers; including cytokeratins14 and 5/6 (105). On the other hand, the MaSC-signature exhibited high association with the claudin-low subtype (106). Clinically, the detection of EpCAM$^{low/-}$/CD49f$^{high/+}$ in breast tumors was shown to be associated with poor clinical prognosis (107). Studies have also linked the MaSC hierarchy with the profile of tumor initiating cells/BCSCs characterized by CD44^{+}/CD24^{-} and ALDH^{+}, where, CD44^{+}/CD24^{-} correspond to the MaSC population (EpCAM$^{low/-}$/CD49f$^{high/+}$) and ALDH^{+} correspond to the luminal progenitor (EpCAM$^{high/+}$/CD49f$^{high/+}$) cells (29). Moreover, activation of the EMT program is well known to be a deriver of phenotypic plasticity and stemness in breast cancer (108, 109). Interestingly, our original work investigating the role of PRL in breast cancer BC revealed PRL to act a potent suppressor of the EMT process, further inhibiting the invasive capacity of breast cancerBC cells. This effect of PRL was found to be linked to the negative-crosstalk between PRL-induced signaling cascade and the two major pro-metastatic pathways MAPK-Erk1/2 and TGFβ (110). Subsequently, we have accumulated compelling evidence and notably, we found that treatment of breast cancer cells representative of the TNBC subtype or of the HER2-E subtype significantly depleted the highly tumorigenic CD44^{+}/CD24^{-} and ALDH^{+} BCSC subpopulations and induced their differentiation into the least tumorigenic phenotype (ie CD44^{-}/CD24^{-} and ALDH^{-} resulting in suppression of their tumorsphere formation/self-renewal capacities (53, 76). On the other hand, loss of expression of the PRLR in ER+ and HER2-E breast cancer cells resulted in the enrichment of these BCSC populations. Clinically, *Prlr* gene expression was also found to have inverse relationship with *CD44* gene expression in TNBC patients (76). Moreover, in RNA-seq data of breast cancer patients, PRLR expression correlated negatively with the mRNA levels of a number of genes (including *Aurkb*, *Ccna2*, *Scrn1*, *Npy*, *Atp7b* and *Chaf1b*) that are related to stemness, resistance to therapy and poor patient outcome (111). Among the multiple isoforms of ALDH, ALDH1A1 and ALDH1A3 are known to be associated with cancer stem cells (112, 113). Interestingly, PRL treatment of HER2-E breast cancer cells was found to suppress the expression levels of both ALDH1A1 and ALDH1A3 mRNA expression. Recent sc- analyses of mammary epithelial cells also identified ALDH1A3 as a marker of luminal progenitor cells having its levels gradually decreased as cells progressed away from their common origin and differentiated to express higher levels of PRLR either in the in hormone sensitive differentiated cells or the alveolar differentiated trajectories (49). In summary, PRL imparts significant anti-tumorigenic effect in breast cancer through differentiation and terminal maturation (Figure 2).

Outlook

In view of our improved understanding of the contribution of tumor cellular plasticity and loss/defects in normal tissue differentiation mechanisms to cancer progression and tumor evolution, significant efforts are directed at exploiting differentiation pathways as therapeutic avenues in cancer. The premise of differentiation therapy (DT) in cancer is a strategy that aims at engaging-forward differentiation and cellular reprograming restricting the proliferative, tumor repopulation,

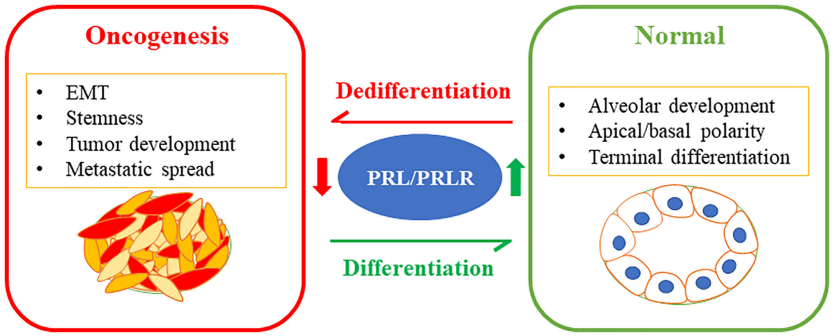

FIGURE 2
PRL/PRLR signaling pathway in breast cancer differentiation limiting tumorigenesis: The PRL/PRLR pathway is a fundamental pathway promoting mammary gland development, morphogenesis, and terminal differentiation of the mammary epithelial cells. Loss of this hormonal pathway is a marker of aggressive breast cancer characterized by poor differentiation promoting stem-like phenotype, tumor development and metastatic spread.

stemness, EMT and metastatic capacities of tumor cells leading to the cessation of the aggressive tumor phenotype and offering the cancer patients improved survival for decades (6, 114–116). Interestingly, the concept of DT was first proposed by Pierce in 1961, reporting on the differentiation of aggressive forms of teratocarcinoma into benign forms and in 1984 was the first clinical application of DT when the use of all-trans retinoic acid was approved for acute promyelocytic leukemia (117). Currently, still under development, several highly promising candidate differentiation and cellular reprograming targets encompassing epigenetics, transcription factors, metabolic and modulators of the cancer stem cells are being evaluated preclinically and clinically as anti-cancer therapeutics (i.e., inhibitors of histone deacetylases (HDACi) (118), micro-RNAs (119) peroxisome proliferator-activated receptor-γ (PPARγ) pathway (120–122), inhibitors of bromodomain-containing protein 4 (BRD4i) (123) among others (115)). Indeed, whereas significant advances have been achieved in treatment options for patients with hormone receptor positive tumors including anti-endocrine-based therapies, and more recently CDK4/6 inhibitors (124), and for HER2-E subtype targeting HER2 (trastuzumab (Herceptin), lapatinib, pertuzumab and trastuzumab emtansine TDM-1) no effective treatment options besides chemotherapy is available for patients with TNBC (125, 126). Notably, none of these approaches are differentiation-based therapeutics. Therefore, identifying drivers and mechanisms of tumor cellular differentiation in breast cancer are urgently in need in our pursuit to limit aggressive malignant changes of tumor progression and to develop new generation of biomarkers and anti-cancer therapies centered on the "pro/forward-differentiation" concept. Collectively, in breast cancer accumulating data implies PRL/PRLR as a clinically relevant potent differentiation pathway limiting the tumorigenic phenotype and thus may serve as a potential pro-differentiation therapeutic candidate.

Author Contributions

SA: formulation and writing of the manuscript DH: contributed to the writing of the manuscript and generated Figure 2 XL: Contributed to the writing of the manuscript and generated Figure 1 JJL: Contributed to the formulation and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Prenatal health behaviours as predictors of human placental lactogen levels

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Placental lactogen (hPL) is a key hormone of pregnancy responsible for inducing maternal adaptations critical for a successful pregnancy. Low levels of placental lactogen have been associated with lower birth weight as well as symptoms of maternal depression and anxiety. Lower placental lactogen has been reported in women with higher body mass index (BMI) but it is unclear whether prenatal health behaviours predict hPL levels or if hPL is associated with infant weight outcomes. This study utilised data from the longitudinal Grown in Wales cohort, based in South Wales. Participants were recruited at the pre-surgical appointment for an elective caesarean section. This study incorporates data from recruitment, post-delivery and a 12 month follow-up. Measures of maternal serum hPL were available for 248 participants. Analysis included unadjusted and adjusted linear and binary regression. Unadjusted, prenatal smoking and a Health Conscious dietary pattern were associated with hPL levels, however this was lost on adjustment for BMI at booking, Welsh Index of Multiple Deprivation (WIMD) score and placental weight. When stratified by maternal BMI at booking, a Health Conscious dietary pattern remained associated with increased hPL levels in women with a healthy BMI (p=.024, B=.59. 95% CI=.08,1.11) following adjustment for WIMD score and placental weight. When adjusted for a wide range of confounders, maternal hPL was also associated with increased custom birthweight centiles (CBWC) (p=.014, B=1.64. 95% CI=.33,2.94) and increased odds of large for gestational age deliveries (p=<.001, Exp(B)=1.42. 95% CI=1.17,1.72). This study identified that consuming a Health Conscious dietary pattern in pregnancy was associated with increased hPL, within women of a healthy BMI. Moreover, higher hPL levels were associated with increased CBWC and increased odds of delivering a large for gestational age infant. This improves the current limited evidence surrounding the nature of hPL in these areas.

KEYWORDS

placental lactogen, birth weight, maternal depression, health-conscious diet, body mass index

Introduction

During pregnancy the mammalian mother undergoes substantial adaptations to support fetal development and to prepare for nurturing her offspring once they are born (1–5). Playing an essential role in these maternal adaptations are the hormones produced by, or dependent on, the fetally-derived placenta. Placental hormones ensure sufficient nutrients are available to support fetal and placental growth by increasing maternal appetite, decreasing her activity and driving metabolic adaptations throughout pregnancy (6, 7). Placental hormones are also involved in inducing behavioural changes in the mother during pregnancy priming her to respond expeditiously to her offspring when they are born (8–13). Consequently, constraints in the production of placental hormones can have wide reaching consequences for fetal growth, maternal metabolism and maternal behaviour potentially contributing to the co-morbidity of common complications of pregnancy.

Human placental lactogen (hPL) is one of the key hormones of pregnancy, and the most highly expressed peptide hormone of the human placenta (14). hPL is collectively composed of two identical placental lactogen peptides encoded by *CHORIONIC SOMATOMAMMOTROPIN HORMONE 1* and *2* (aka *HPL-A* and *HPL-B*) (15). Placental lactogens are evolutionarily related to the pituitary hormone prolactin (15) and signal *via* the prolactin receptor to mediate their activity at target sites around the body (15, 16). During pregnancy hPL is synthesised in increasing amounts by the syncytiotrophoblast and extravillous trophoblast lineages of the human placenta with levels reaching 5–7 μg/ml in maternal blood at term, exceeding that of any other peptide hormone (14, 15, 17). While there are rare cases of pregnancy proceeding in the apparent absence of hPL (18), several studies have reported associations between lower than normal levels of hPL and pregnancy complications (19). For example, reduced levels of maternal serum hPL levels and placental *CSH1/2* mRNA expression have been associated with fetal growth restriction (20–22) while positive correlations have been reported between hPL and birthweight (23, 24). One study reported reduced serum hPL in gestational diabetes (25) while another reported significantly reduced placental *CSH1/2* associated with pre-eclampsia (26). We reported an association between lower placental *CSH1/2* at term and both clinically diagnosed depression and questionnaire reported symptoms of depression in pregnancy (27). More recently, we reported low serum placental lactogen at term was associated with symptoms of both depression and anxiety for up to ten weeks after birth (28). In this same study we noted a positive association between serum hPL and birthweight (g), placental weight and head circumference consistent with previous studies. While these studies in human populations do not demonstrate a causal relationship between placental lactogen and birthweight, gestational diabetes or maternal mental health, data from rodent models supports such a conclusion. For example, transgenic overexpression of mouse placental lactogen targeted to the beta cells of the pancreas increases the proliferation rate of these cells in mice, and drives both fasting and postprandial hypoglycaemia (29) while targeted deletion of the prolactin receptor provides indirect evidence that placental lactogens drive pancreatic β-cell expansion (30). Infusion of placental lactogen into the non-pregnant female rodent brain stimulates maternal caregiving behaviour (31, 32) while ablation of the maternal prolactin receptor disrupts maternal caregiving (33–37). Disruption of signalling *via* this receptor has also been linked to increased postpartum anxiety (38). Our work on mice with placental endocrine insufficiency driven by genetically modified changes in the expression of imprinted genes further demonstrates a role for placental hormones in regulating birthweight with a reduction in the number of placental endocrine cells linked to low birthweight in several models (39–41). We also reported both maternal neglect and maternal anxiety in response to the loss of placental endocrine lineages (42, 43) with the mouse offspring exhibiting anxiety-like behaviours later in life (44). Together, these data highlight the importance of placental hormones, and more specifically placental lactogens, for pregnancy health. Moreover, in addition to genetic drivers of placental endocrine insufficiency, a number of environmental stressors in pregnancy have been linked to changes in the expression of placental hormones and alterations in maternal behaviour (45) identifying a mechanism with potential to link early life adversity to a variety of poor health outcomes.

Given the importance of placental lactogen for a healthy and successful pregnancy, it is vital that we identify factors that positively or negatively influence the production of this hormone. Previously pre-pregnancy obesity has been linked to significantly lower placental expression of *CSH1/2* (46–48). Similarly, we have reported an association between maternal BMI at booking (week 12-14 of pregnancy) and serum hPL at term (28). In addition, we noted an association between serum hPL and the Welsh Index of Multiple Deprivation (WIMD) score. WIMD is the Welsh Government's official measure of relative deprivation for small areas in Wales calculated from anonymised postcodes (http://wimd.wales.gov.uk). The small areas used to construct the index are known as Lower Super Output Areas (LSOAs) with an average population of 1,600 people. There are 1,909 LSOAs in Wales - the most deprived area is given a rank of 1 and the least deprived a rank of 1,909 therefore lower scores are indicative of higher levels of deprivation. The WIMD is composed of a number of indicators which include income, employment, health, education, access to services, housing, community safety and physical environment. We have previously reported that WIMD

scores were significantly positively associated with a 'Health Conscious' dietary pattern which in turn was significantly associated with increased custom birthweight centile (CBWC) (49). Together, these observations suggest that factors in addition to maternal BMI may influence hPL levels. Here we explored the association between a variety of modifiable health behaviours in pregnancy and term serum hPL, as well as the influence of hPL on a range of infant weight outcomes, using data from the Grown in Wales Study.

Material and methods

Cohort

This study analysed data from the Grown in Wales (GiW) cohort, a pregnancy cohort recruited in South Wales, UK with a focus on maternal mental health (50). Full ethical approval for the GiW study was obtained by the Wales Research Ethics Committee (REC2 reference 15/WA/0004). Research was carried out employing the principles of the Declaration of Helsinki as revised in 2008. Recruitment occurred between September 2015 and November 2016 at the University Hospital of Wales (UHW) with women providing written consent to the study. Women were recruited by two trained research midwives at their morning pre-surgical appointment in advance of an elective caesarean (ELCS), one to four days before delivery. ELCS was chosen to maximise the potential for collecting biological samples. At UHW women routinely provide blood samples at their pre-surgical appointment before their surgery facilitating the collection of maternal blood for this study. The planned surgery took place during the working week when the research midwives were available to collect placental biopsies and cord blood samples. Recruitment criteria consisted of women being between 37 weeks and 42 weeks of pregnancy, aged between 18 and 45, having a singleton birth without fetal abnormalities or infectious diseases. Participants have been followed up within one week of birth, ten weeks and one year postnatally and most recently at four years postpartum.

Participants

355 women were initially recruited and seven later withdrew. Of these, hPL measures were available 272 participants within the overall cohort. The current analysis focused on participants who delivered at term (≥ 37 weeks) and those of Caucasian ethnicity. This selection was required as the dietary patterns were previously developed for these participants (49). This was due to the recruitment small number of participants of other ethnicities whose inclusion greatly influenced findings through the introduction of variation, an issue especially relevant for smaller cohorts (51, 52). Following the exclusion criteria, hPL data was available for 248 participants.

Human placental lactogen

Maternal venous serum samples were obtained at recruitment from blood taken as part of a standard anaesthetic review one to four days prior to surgery. Serum was obtained by centrifugation of maternal venous blood which was then frozen at −80°C. hPL levels were assayed in duplicate using the Leinco Technologies Human Placental Lactogen (HPL) Micro-ELISA test kit (Universal Biologicals product code T115-96 tests). Assays were performed by the NIHR Cambridge Biomedical Research Centre, Core Biochemical Assay Laboratory. Average value = 8.3 μg/mL ± 2.75.

Demographic and biological data

Demographic data such as a participants education level and income were obtained from the maternal questionnaire completed at recruitment. Participant postcodes were also collected and anonymised which enabled the calculation of Welsh Index of Multiple Deprivation (WIMD) 2014 scores (http://wimd.wales.gov.uk). The maternal questionnaires also contained the Edinburgh Postnatal Depression Scale (EPDS) (53) and the State-Trait Anxiety Inventory (STAI) (54) which provided data on maternal mental health. Biological data such as participants ethnicity, age, parity, weight and BMI at booking as well as data on mode of delivery, placental weight and infant sex were collected from the midwife recorded notes following delivery. Gestational weight gain was calculated from data on pre-pregnancy weight and weight at booking.

Prenatal health behaviours

Data on maternal prenatal smoking, alcohol intake and exercise were acquired from the maternal questionnaire completed at recruitment. Dietary patterns were identified from data collected through a food frequency questionnaire (FFQ), also completed at recruitment. The dietary patterns within the GiW cohort were Western and Health Conscious, with the process for obtaining the dietary patterns outlined in detail in (49). Briefly, the dietary pattern scores were obtained *via* the regression method following Principal Component Analysis. Each participant has a score for both dietary patterns. These scores are typically centred around zero, with greater positive scores indicating higher adherence to a dietary pattern and greater negative scores indicating lower adherence to a dietary pattern.

Infant weight outcomes

Data on birthweight (g) was obtained from the midwife recorded notes following delivery. CBWC were later calculated *via* the GROW bulk centile calculator (55) utilising the following data from the midwife notes; maternal height, weight, ethnicity and parity as well as infant gender, birthweight and gestational age. This enabled the classification of infant birthweight as small for gestational age (SGA), average for gestational age (AGA) or large for gestational age (LGA). Data on infant weight at one year of age was obtained from a maternally completed questionnaire.

Statistical analysis

All statistical analyses were undertaken utilising IBM SPSS Statistics Version 27. Normality for relevant variables was assessed *via* Kolmogorov-Smirnov test, Shapiro-Wilk test, normal Q-Q plots and histograms. All relevant variables were determined to be non-parametric, thus demographic statistics were displayed as median (IQR) or % (n) as appropriate. Health behaviour predictors of hPL were assessed utilising both unadjusted and adjusted linear regression. In the adjusted analysis the significant predictors were entered together in the model, with maternal BMI at booking (continuous), WIMD score and placental weight (g) selected as potentially confounding variables. Variables were selected as confounding variables if a previous GiW study identified them to be associated with hPL (28) or if the variables were found to be associated with the outcome variables in a univariate analysis (Supplementary Table 1). In light of the highly influential nature of maternal BMI, the association between predictors and hPL was also assessed when stratified by maternal BMI at booking, with the exception of the underweight BMI category due to low numbers. Unadjusted and adjusted linear and binary regression were undertaken to assess the influence of hPL on infant weight outcomes. The analysis of birthweight (g) was adjusted for the following potentially confounding variables; BMI at booking (continuous), WIMD score, maternal age, gestational weight gain fetal sex, placental weight (g), gestational age, smoking and a Health Conscious dietary pattern. Education and income were not included as the WIMD score incorporates these measures. The same variables were utilised for analyses of CBWC derived variables, with the exception of fetal sex and gestational age which are already accounted for within this birthweight measure.

Results

Demographic data for the 248 participants involved in the analysis is provided in Table 1. Categorical data is displayed as % (n) and continuous data as median (IQR).

Health behaviour predictors of hPL

Linear regression was utilised to investigate if prenatal maternal health behaviours influence levels of maternal serum hPL (Table 2). Unadjusted univariate regression identified that both smoking at any point in pregnancy and a Health Conscious dietary pattern were associated with hPL measures. Specifically, smoking compared to not smoking was associated with a decrease in hPL of 1.24 μg/mL, whilst a one unit increase in Health Conscious dietary pattern score was associated with an increase in hPL of.40 μg/mL. These significant health behaviours were adjusted for: maternal BMI at booking, WIMD score and placental weight (g). Following adjustment, no prenatal health behaviours remained significantly associated with hPL measures. To understand the highly influential effect of BMI further, the relationship between health behaviours and hPL was examined when stratified by maternal BMI at booking (Table 3). It was determined that a Health Conscious dietary pattern was significantly associated with hPL measures in women classified as having a healthy BMI at booking. This association remained after adjustment for WIMD score and placental weight (g). Specifically, for women with a healthy BMI at booking, a one unit increase in Health Conscious dietary pattern score was associated with an increase in hPL of .59 μg/mL equivalent to an increase of 8% of the average value.

hPL & infant weight outcomes

Linear regression was again utilised to investigate the relationship between hPL and a range of infant weight measures, collected both at birth and at one year of age (Tables 4, 5). The relationship between hPL and both birthweight and CBWC is displayed in Figures 1, 2. At the unadjusted level, hPL was significantly associated with all measures of weight with the exception of infant weight at 12 months of age. These significant associations were adjusted for the potentially confounding variables that included maternal BMI at booking, maternal age, gestational weight gain, fetal sex, placental weight, gestational age, smoking at any point in pregnancy, Health Conscious dietary pattern score and WIMD score. Following adjustment, hPL was no longer significantly associated with birthweight (g) or the odds of being born SGA compared to LGA. However, hPL remained significantly associated with CBWC, with a one unit increase in hPL associated with an increase in CBWC of 1.64 units. Additionally, hPL was associated with being born LGA compared to AGA, with a one unit increase in hPL associated with increased odds of delivering an LGA compared to AGA infant by a factor of 1.42.

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TABLE 1 Demographic data for the eligible GiW participants.

	% (n) or median (IQR)
Maternal BMI at booking - overall	26.33 (7.23)
Maternal BMI at booking % (n)	
Underweight	.40 (1)
Healthy	38.20 (89)
Overweight	35.60 (83)
Obese	25.80 (60)
Maternal age at booking	33.00 (6.00)
Parity, *% (n)*	
Multiparous	81.90 (203)
Nulliparous	18.10 (45)
Gestational weight gain (kg)	15.07 (7.88)
GDM % (n)	
Yes	5.30 (13)
No	94.70 (230)
Hypertension % (n)	
Yes	3.70 (9)
No	96.30 (236)
Fetal sex, *% (n)*	
Female	54.40 (135)
Male	45.60 (113)
Placental weight (g)	655 (183)
Gestational age (weeks)	39.00 (0)
Birthweight (g)	3500.00 (650.00)
Birthweight classification	
LBW	2.60 (8)
ABW	79.20 (247)
HBW	18.30 (57)
CBWC	57.85 (50.05)
Size for gestational age *% (n)*	
SGA	6.90 (17)
AGA	80.60 (200)
LGA	12.50 (31)
Smoking in pregnancy[a], *% (n)*	
No	89.80 (220)
Yes	10.20 (25)
Alcohol in pregnancy[a], *% (n)*	
No	59.30 (144)
Yes	40.70 (99)
Strenuous exercise, *% (n)*	
No	81.60 (200)
Yes	18.40 (45)
Western dietary pattern	-.03 (1.28)
Health Conscious dietary pattern	.05 (1.50)
Highest education level, *% (n)*	
Left before GCSE	5.90 (14)
GCSE & Vocational	22.90 (54)
A-level	12.70 (30)
University	30.90 (73)
Postgraduate	27.50 (65)
Family income (£), *% (n)*	
<18,000	7.50 (18)
18 – 25,000	10.00 (24)
25-43,000	19.70 (47)
>43,000	52.30 (125)
Do not wish to say	10.50 (25)
WIMD	1270.00 (1211.00)
A1 EPDS total	7.00 (6.00)
A1 STAI total	34.00 (13.00)

IQR, Interquartile range; BMI, body mass index; GDM, gestational diabetes mellitus; LBW, low birthweight; ABW, average birthweight; HBW, high birthweight; CBWC, custom birthweight centile; SGA, small for gestational age; AGA, average for gestational age; LGA, large for gestational age; WIMD, Welsh Index of Multiple Deprivation; EPDS, Edinburgh Postnatal Depression Scale; STAI, State-Trait Anxiety Inventory.
[a]At any point in pregnancy.

Discussion

This study aimed to investigate both the health behaviour predictors of hPL and the influence of hPL on infant weight outcomes. It was determined that, at the unadjusted level, both smoking at any point in pregnancy and consuming a Health Conscious dietary pattern were associated with hPL levels. However, this was lost following adjustment for the confounding variables of WIMD score, maternal BMI at booking and placental weight. Given that BMI is known to strongly influence maternal hPL, this association was also examined when stratified by BMI. Following adjustment for WIMD score and placental weight, consuming a Health Conscious dietary pattern in pregnancy was associated with increased hPL levels in participants with a healthy BMI at booking. Regarding infant weight outcomes, prior to adjustment hPL was associated with all weight outcomes with the exception of infant weight at 12 months. This analysis was adjusted for a range of confounding variables including maternal BMI at booking, maternal age, gestational weight gain, fetal sex, placental weight, gestational age, smoking at any point in pregnancy, Health Conscious dietary pattern score and WIMD score. After adjustment, maternal hPL was associated with increased CBWC as well as increased odds of delivering an LGA compared to AGA infant.

The associations between the modifiable prenatal health behaviours of maternal smoking and adhering to a Health Conscious diet on hPL were identified but did not remain associated once adjusted for BMI at booking, WIMD score and placental weight. However, given that BMI is known to be highly influential for hPL levels, this analysis was stratified by BMI at booking. When stratified, the association between a Health Conscious dietary pattern and hPL remained significant

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TABLE 2 Unadjusted and adjusted linear regression indicating the association between maternal prenatal health behaviours and hPL (µg/mL).

		p	B	95% CI
Unadjusted	Smoking	**.036**	-1.24	-2.40, -.08
	Alcohol	.359	.33	-.38, 1.04
	Exercise	.119	.72	-.19, 1.63
	Western dietary pattern	.640	-.09	-.47, .29
	Health Conscious dietary pattern	**.029**	.40	.04, .76
Adjusted	Smoking	.706	-.23	-1.42, .97
	Health Conscious dietary pattern	.618	.09	-.26, .44

CI, confidence interval. Bold values are significant at p < .05.

for women within the healthy BMI category. This finding supports the important influence of maternal diet in relation to hPL levels. Moreover, there is potential for this to be a direct relationship with studies in several experimental animal models reporting that both overnutrition and undernutrition reduce the expression of placental hormones (45). However, while this relationship was evident in women of a healthy BMI, it was not apparent in women with an unhealthy BMI. BMI is already known to have an influential effect on maternal hPL serum levels with structural changes in the placental *hPL* gene locus reported in women with higher BMI compared to those on the normal range (48). Together, these findings suggest that BMI has a stronger influence on hPL levels than maternal diet. The caveat is that, in our cohort, BMI and diet are linked with increasing BMI associated with decreasing Health Conscious dietary pattern score (49). Similarly in the majority of animal models of overnutrition, both weight gain and exposure to diet occur concurrently in pregnancy. Distinguishing direct and indirect relationships consequently presents a challenge.

We have previously reported a positive relationship between term serum hPL and infant birthweight (g), head circumference and placental weight (g) (28) consistent with a number of previous studies (20, 21). This study went further by examining additional weight measures. CBWC and the associated classifications of SGA, AGA and LGA have several advantages over the traditional population based weight measures (55, 56) and have been recommended for use in the UK by the Royal College of Obstetricians and Gynaecologists since 2002 (57). In our cohort nearly twice the number of infants would be classified as growth restricted by the CBWC criteria (Table 1). However, these measures are rarely utilised in research. We are unaware of any other studies reporting the influence of hPL on these birthweight measures. As such, this research strengthens and supports the current evidence base that

TABLE 3 Unadjusted and adjusted linear regression indicating the association between maternal prenatal health behaviours and hPL (µg/mL) when stratified by maternal BMI at booking.

			p	B	95% CI
Unadjusted	Healthy	Smoking	.179	-1.43	-3.53, .67
		Alcohol	.259	.66	-.49, 1.81
		Exercise	.264	.72	-.56, 2.00
		Western dietary pattern	.227	-.36	-.95, .23
		Health Conscious dietary pattern	**.020**	.65	.11, 1.19
	Overweight	Smoking	.321	-1.29	-3.85, 1.28
		Alcohol	-1.14	.23	-1.14, 1.61
		Exercise	.837	.22	-1.93, 2.38
		Western dietary pattern	.720	-.14	-.88, .61
		Health Conscious dietary pattern	.338	.35	-.37, 1.07
	Obese	Smoking	.448	-.66	-2.40, 1.08
		Alcohol	.914	.07	-1.28, 1.43
		Exercise	.161	1.39	-.57, 3.34
		Western dietary pattern	.214	.45	-.27, 1.17
		Health Conscious dietary pattern	.404	-.33	-1.10, .45
Adjusted	Healthy	Health Conscious dietary pattern	**.024**	.59	.08, 1.11

CI, confidence interval. Bold values are significant at p < .05.

TABLE 4 Unadjusted and adjusted linear regression indicating the association between hPL (μg/mL) and infant weight outcomes.

		p	B	95% CI
Unadjusted	Birthweight (g)	**<.001**	52.01	30.20, 73.82
	CBWC	**<.001**	3.82	2.62, 5.01
	12 month weight (kg)	.967	.00	-.17, .17
Adjusted	Birthweight (g)	.090	16.56	-2.64, 35.75
	CBWC	**.014**	1.64	.33, 2.94

CI, confidence interval; CBWC, custom birthweight centile. Bold values are significant at p < .05.

TABLE 5 Unadjusted and adjusted binary regression indicating the association between hPL (μg/mL) and infant weight categories.

		p	Exp (B)	95% CI
Unadjusted	SGA	**.008**	.70	.53, .91
	LGA	**<.001**	1.31	1.15, 1.49
Adjusted	SGA	.090	.72	.49, 1.05
	LGA	**<.001**	1.42	1.17, 1.72

CI, confidence interval; SGA, small for gestational age; LGA, large for gestational age. Bold values are significant at p < .05.

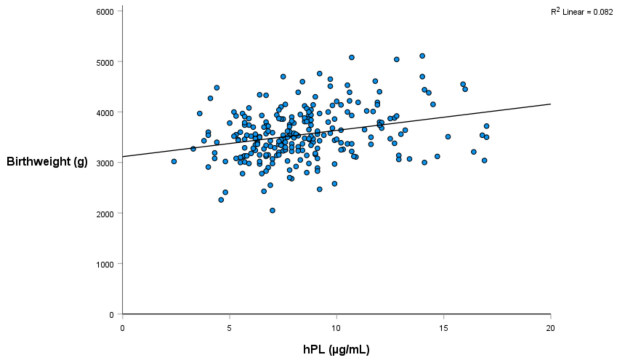

FIGURE 1
The relationship between hPL (μg/mL) and infant birthweight (g).

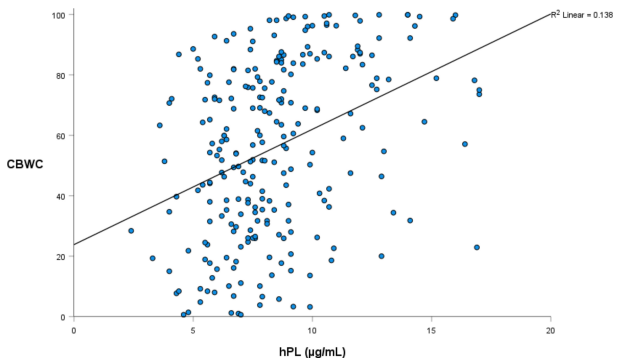

FIGURE 2
The relationship between hPL (μg/mL) and infant custom birthweight centile.

hPL is associated with birthweight outcomes. As there was no association between hPL and infant weight at 12 months, this also suggests that the influence of hPL on infant weight is short term in nature.

There are several potential limitations to consider regarding this study. Firstly, dietary patterns were originally identified using data from Caucasian participants, a demographic which forms the majority of the Grown in Wales study cohort (91%). As such, the generalisability of the study to other ethnicities may be limited and future research should be conducted with diverse populations to validate the findings. Secondly, our population were recruited to explore the impact of maternal depression on the placenta and therefore focused on recruiting women booked for ELCS. This selective process is both a limitation – due to the restricted nature of the cohort – and an advantage since hPL measures were all taken 1-4 days prior to birth by the same two research midwives on the morning of the participants surgical assessment. This focused timing in the collection of samples and the somewhat homogenous nature of the cohort means that we are able to detect subtle relationships in our relatively small pregnancy cohort. However, an important question remains unanswered which is the timings of the relationships. We have a single measure of hPL at near term. Determining a more precise timeline will be important.

In conclusion, we have established that there is a positive association between a healthy maternal diet and hPL, a key hormone of pregnancy, at least within women with a healthy BMI category. Moreover, hPL is associated with birthweight outcomes. While we have not established the extent to which this is a direct relationship, it is clear that consuming a healthy diet in pregnancy reduces the risk of a number of complications of pregnancy and is likely to protect offspring from the longer term problem association with exposure to early life adversity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, upon request.

Ethics statement

The studies involving human participants were reviewed and approved by Wales Research Ethics Committee REC2 reference 15/WA/0004. The patients/participants provided their written informed consent to participate in this study.

Author contribution

RJ: conceptualisation, funding acquisition, project administration, resources, writing – original draft, supervision. SG: data curation, formal analysis, investigation, writing – original draft. LS: data curation, writing – review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.946539/full#supplementary-material

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Prolactin and vasoinhibin are endogenous players in diabetic retinopathy revisited

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Diabetic retinopathy (DR) and diabetic macular edema (DME) are major causes for visual loss in adults. Nearly half of the world's population with diabetes has some degree of DR, and DME is a major cause of visual impairment in these patients. Severe vision loss occurs because of tractional retinal detachment due to retinal neovascularization, but the most common cause of moderate vision loss occurs in DME where excessive vascular permeability leads to the exudation and accumulation of extracellular fluid and proteins in the macula. Metabolic control stands as an effective mean for controlling retinal vascular alterations in some but not all patients with diabetes, and the search of other modifiable factors affecting the risk for diabetic microvascular complications is warranted. Prolactin (PRL) and its proteolytic fragment, vasoinhibin, have emerged as endogenous regulators of retinal blood vessels. PRL acquires antiangiogenic and anti-vasopermeability properties after undergoing proteolytic cleavage to vasoinhibin, which helps restrict the vascularization of ocular organs and, upon disruption, promotes retinal vascular alterations characteristic of DR and DME. Evidence is linking PRL (and other pituitary hormones) and vasoinhibin to DR and recent preclinical and clinical evidence supports their translation into novel therapeutic approaches.

KEYWORDS

vasoinhibin, PRL, diabetic retinopathy, diabetic macular edema, diabetes, levosulpiride

Diabetic Retinopathy is a common cause of vision loss and blindness

Most patients with longstanding diabetes mellitus develop microvascular complications of diabetes, namely nephropathy, neuropathy, and retinopathy. DR is a highly specific neurovascular complication of diabetes and is the most frequent cause of new blindness among adults aged 20-74 years in developed countries (1, 2). DR advances from mild nonproliferative abnormalities with increased vasopermeability and microaneurysms to moderate and severe stages characterized by the growth of new blood vessels in the retina

and the posterior surface of the vitreous. Fibrous tissue may exert tension on the retina and cause retinal detachment. The new blood vessels may bleed and cause preretinal and vitreous hemorrhage. A macular edema causing central vision impairment may occur because of increased vasopermeability and capillary nonperfusion (3). Major risk factors include the duration of diabetes, HbA1c levels, and blood pressure (3, 4). The onset of puberty and pregnancy increase the risk of progression of DR. Tertiary prevention of DR includes laser photocoagulation for proliferative diabetic retinopathy (PDR), anti-VEGF therapy for DME and PDR, and vitrectomy in advanced DR (5). Various pathophysiological and pathobiochemical pathways directly linked to chronic hyperglycemia which lead to a disorganization and breakdown of the blood-retinal-barrier are involved in the manifestation of DR and DME, including an activation of protein kinase C (6) and the accumulation of advanced glycation end products (7). However, there are patient populations with type 1 diabetes of extreme duration who do not develop diabetic complications and appear to be protected by unknown factors (8, 9). This contrasts with other studies, which usually report that >90% of patients with type 1 diabetes will eventually develop retinopathy (10). Also, there was a lack of association between glycemic control and prevalence of reported microvascular complications (11). Consistently, the total glycemic exposure (A1C and duration of diabetes) explained only 11% of the variation in risk in the Diabetes Control and Complications Trial (DCCT) cohort, where retinopathy progression was studied in conventional and intensive treatment groups (12). It is thus acknowledged that significant numbers of patients with diabetes can live without severe complications, likely due to factors that can neutralize the adverse effects of hyperglycemia or other unknown protective factors which prevent the development of diabetic complications (11). Hormonal factors are predisposed to confer protective effects against microvascular complications through their effects on organ function, repair and maintenance of homeostasis, the control of growth, and their capacity to adapt their levels and action in response to demand or to pathologic stimuli. The investigation of pituitary hormones is therefore warranted.

Pituitary infarction revealed an involvement of pituitary hormones in diabetic retinopathy

A role of pituitary hormones in the etiopathology of DR emerged soon after the observation that infarction or insufficiency of the anterior lobe of the pituitary, can result in hypoglycemia and high sensitivity to administered insulin, known as the Houssay-Biasotti phenomenon. In fact, infarction, or insufficiency of the pituitary gland, also known as Simmond's disease, can lead to terminal hypoglycemia, as reported in a series of early case studies (13, 14). Pituitary infarction can also occur after severe peri- or postpartum hemorrhage, as described by Sheehan (Sheehan's syndrome). In all instances, examples of cessation or regression of diabetic retinopathy was observed. Soon thereafter, pituitary ablations, stalk sections, and destruction by irradiation were introduced for treating diabetic retinopathy but became obsolete in the face of the harmful effects that were associated with these procedures and the following anterior pituitary insufficiency. The beneficial effects of pituitary insufficiency were attributed to the cessation of growth hormone secretion and consecutively lower insulin-like growth factor I (IGF-I) levels, however, the overall resumé of repeated cross-sectional, longitudinal, and prospective studies on the relationship between circulating IGF-I levels and DR did not establish a clear role for the GH/IGF-I axis (15). Patients with acromegaly and diabetes mellitus do not have a higher prevalence of DR (16) and patients with diabetes and congenital IGF-I deficiency (Laron syndrome) or GH gene deletion can develop DR (17, 18). Disparate data are available on circulating IGF-I levels and DR progression during pregnancy, with studies finding or not finding an association of IGF-I levels with DR during pregnancy (19, 20). On the other hand, it is known that an acute reduction of chronic hyperglycemia can accelerate DR, and that this deterioration is preceded by an upregulation of serum IGF-I (21). Both, GH, and IGF-I are present in the vitreous and the levels of IGF-I are higher in the vitreous of patients with retinal neovascularization (22, 23). Mechanistically, IGF-I has mitogenic and differentiating effects on cultured retinal endothelial cells (24) and on retinal capillaries (25), and can induce neovascularization in the avascular rabbit cornea (26). IGF-I and its receptor, as well as IGF binding proteins are distributed throughout the retina, and IGF-I mRNA has been detected in the ganglion cell layer, the inner nuclear layer and in the outer limiting membrane (27, 28). The total IGF-I distribution in ocular tissues is therefore a combination of local expression and systemic uptake. Altogether, the contribution of local and circulating IGF-I in diabetic retinopathy remains to be understood, can be interpreted as rather "permissive" than causal (17) and therapeutic interventions into the GH/IGF-I axis did not yield sufficient evidence in clinical studies to be considered in the current treatment recommendations for DR (5). Attesting to the heterogeneity and variation in pathomechanisms of proliferative retinopathies across the lifespan, ample evidence demonstrates the key role of IGF-I in retinopathy of prematurity (29–32).

Circulating PRL levels change in diabetes

Another pituitary hormone which attracted attention in respect to its involvement in DR is PRL. Not long after the

radioimmunoassay for PRL became available, which allowed the measurement of circulating PRL concentrations (33, 34), PRL was evaluated in patients without DR and DR at various stages. Early reports found higher PRL levels in patients with diabetes but without severe DR and hypothesized about the potential function of PRL as a protective factor in DR, and about some potential treatment based on the stimulation of PRL secretion (35, 36). Indeed, pituitary stalk section results in minimized GH secretion with subsequent decline of IGF-I levels but result in higher PRL-secretion due to a disinhibition of lactotroph PRL secretion by the disruption of dopamine transport through the pituitary stalk (37). The beneficial effects of pituitary stalk sections could therefore have been not only due to the reduction of IGF-I levels, but also due to an increase in circulating PRL. Comparable with IGF-I levels, various results were reported in which the association of PRL levels with DR presence and severity was not confirmed (38–41). A mechanism of action for protective effects of PRL levels was also missing. PRL exerts a diverse array of biological functions beyond its essential role in lactation (42–44), a fact which has received little attention in clinical medicine in the past, where the relevance of PRL is acknowledged in prolactinoma and secondary amenorrhea. Regarding diabetes and its complications, there is a new trend towards the recognition of PRL as an important metabolic hormone, directly involved in beta-cell function and survival, and the regulation of insulin sensitivity and resistance, respectively (45). Higher PRL levels are associated with higher insulin sensitivity and a lower incidence of type 2 diabetes mellitus, which led to a re-evaluation of current thresholds for normal PRL levels and hyperprolactinemia (45). It was proposed to re-define the interpretation of PRL levels beyond the upper threshold of 25 ng/ml where a homeostatic functionally increased transient hyperprolactinemia (homeoFIT) can be assumed, the suggested term for an elevation of PRL levels which may constitute a physiological response to increased metabolic demand (reviewed in ref. 45).

The PRL/vasoinhibin axis controls ocular angiogenesis and vascular function

A new perspective on the role of PRL in DR began to evolve when the antiangiogenic effects of an enzymatically cleaved 16 kDa N-terminal fragment of human PRL were discovered (46), and a direct pathophysiological implication towards the regulation of blood vessel growth emerged. It became evident that the 16 kDa N-terminal fragment is not the only fragment with antiangiogenic effects, and that multiple isoforms with a large variation in molecular mass exist, their size being determined by the PRL-cleaving enzyme and its cleavage site location within the PRL molecule. The isoforms were collectively called vasoinhibin (47–49), including similar proteins generated by the proteolytic cleavage of GH and placental lactogen (PL) (50, 51). A strong role of vasoinhibin as a regulator of ocular angiogenesis and vascular function evolved, and with reference to existing reviews (52–55), and 11 years after PRL and vasoinhibin were first portrayed as endogenous players in DR (56), the following discussion will focus on key principles and significant developments in the recent years (Table 1). The new understanding of circulating PRL levels in terms of homeoFIT-levels is relevant when considering the role of PRL and vasoinhibin in DR, as in partial disagreement to the early studies between 1970 and 1985, there appeared to be an association between circulating PRL levels and DR, reported by Arnold et al. in 2010 (62). The PRL levels were higher in patients with diabetes and no retinopathy (compared to healthy controls) and higher in patients with diabetes and non-proliferative DR than in patients with PDR (62). The PRL levels in the patients with diabetes were above the conventional threshold of 25 ng/ml, and therefore in the homeoFIT-range. In addition to answering to increased metabolic demand, PRL levels in the homeoFIT-range may also, through their proteolytic conversion to vasoinhibin, contribute to control the function and growth of ocular blood vessels. Interestingly, uncleaved PRL is protective in the retina and required for maintaining retinal functionality in mice during aging and has potential therapeutic value against age-related retinal disorders (68, 69). Short PRL isoforms are expressed in the canine retina undergoing retinal degeneration (70). A clinical study in patients with a prolactinoma using optical coherence tomography revealed a reduced thickness of the chorioretinal layers in patients with prolactinoma compared to controls (71). Patients with DR have a higher renal elimination of PRL (72) and the circulating levels of vasoinhibin are reduced in patients with DR (63).

The principle underlying vasoinhibin accumulation in the retina – or in other tissues – is that of an endocrine axis in which the levels of vasoinhibin are controlled by regulatory mechanisms at the hypothalamo-, the pituitary-, and the local level. The vasoinhibin levels depend on the availability and amount of secreted and circulating PRL (hypothalamo-pituitary level), and on the hypothalamo, pituitary, and peripheral tissue distribution and activities of PRL-cleaving proteases (local level). This hormonal axis was described as the PRL/vasoinhibin axis of which the vasculature is a major target tissue (53, 67). The cleavage sites in PRL through which vasoinhibin is generated are conserved in vertebrates (47, 67, 73) and high affinity cleavages sites evolved, most likely as a gain of function under positive selection, as a unique feature of higher primates (74). The cleavage of PRL to generate vasoinhibin occurs in the wider context of a hormone-metabolism junction, through which specifically cleaved hormones regulate essential functions to maintain homeostasis at the organismal, tissue, or organ levels (75, 76). The PRL/vasoinhibin axis contributes to maintaining corneal avascularity (66), restricts retinal vasculature (65), and is disrupted in retinopathy of

TABLE 1 Landmark original research articles and reviews highlighting the involvement of the prolactin/vasoinhibin axis in diabetic retinopathy.

Brief description	Year	Ref.
ORIGINAL RESEARCH ARTICLES		
Sulpiride-induced hyperprolactinaemia inhibits the diabetes- and VEGF-mediated increase in retinal vasopermeability by promoting the intraocular conversion of endogenous PRL to vasoinhibin	2022	(57)
Levosulpiride increases the levels of PRL in the vitreous of PDR patients and promotes its MMP-mediated conversion to vasoinhibin, which can inhibit angiogenesis in DR	2020	(58)
Study protocol of a prospective, randomized, double-blind, placebo-controlled trial enrolling male and female patients with type 2 diabetes having DME, randomized to receive placebo or levosulpiride	2018	(59)
AAV2 vasoinhibin vector decreases retinal microvascular abnormalities in rats	2016	(60)
AAV2-vasoinhibin vector in rats prevents pathologic retinal vasopermeability and suggest it could have therapeutic value in patients with DR	2011	(61)
Circulating PRL influences the progression of DR after its intraocular conversion to vasoinhibin. Inducing hyperprolactinemia may represent a novel therapy against DR	2010	(62)
Patients with diabetes mellitus and DR have lower circulating levels of vasoinhibin, compared to healthy patients	2009	(63)
Vasoinhibin blocks retinal vasopermeability in diabetic rats and in response to intravitreous injection of VEGF or of vitreous from patients with DR	2008	(64)
Vasoinhibin is a natural inhibitor of angiogenesis in the retina	2005	(65)
Vasoinhibin is a natural inhibitor of corneal vascularization	1999	(66)
Speculations whether stimulating PRL-release in patients with DR might be benefitial	1976	(36)
REVIEW ARTICLES		
Pharmacological interventions into the prolactin/vasoinhibin axis for the treatment of diabetic retinopathy	2017	(52)
Introduction of the prolactin/vasoinhibin axis and its pathophysiological significance including DR	2015	(67)
Review of the regulation of blood vessel growth and function by vasoinhibin	2015	(53)
Portray and review of PRL and vasoinhibin as endogenous players in DR	2011	(56)
Introduction of vasoinhibin as a novel inhibitor of ocular angiogenesis	2008	(55)

prematurity (77, 78). In rodents, hyperprolactinemia leads to vasoinhibin accumulation in the retina and reduces both VEGF-induced and diabetes-induced retinal vasopermeability (57, 62, 64); an effect also demonstrated by vasoinhibin gene transfer which not only prevented (61) but also reversed (60) excessive retinal vasopermeability and oxygen-induced retinal angiogenesis (79).

The bioactive site in vasoinhibin, through which the antiangiogenic and antivasopermeability effects of the molecule are mediated, is a short, conserved three-residue motif consisting of residues His46-Gly47-Arg48 which becomes active after the proteolytic cleavage of PRL to vasoinhibin (80). Molecular dynamics simulations predicted the three-dimensional structure of vasoinhibin comprising a three-helix bundle with a tendency to form dimers or multimers, which also complicated the experimental resolution of the vasoinhibin three-dimensional structure (73, 81, 82). Vasoinhibin signals through various binding partners such as a specific high affinity binding site on endothelial cells (83), integrin alpha5 beta1 (84), or plasminogen activator inhibitor 1, urokinase, and urokinase receptor multicomponent complex (85) to trigger intracellular signaling pathways that result in its effects on endothelial cells but a classical hormone receptor has not been identified. The circulating levels of vasoinhibin are unknown due to the absence of a quantitative vasoinhibin assay for human serum, which is why immunoprecipitation followed by SDS-PAGE and Western blotting is still the only more frequently used method for the evaluation of vasoinhibin in clinical samples (77). Alternative methods using a lab-on-a chip technology or mass spectrometry were reported (63, 86, 87) but did not establish themselves thereafter. The lack of monoclonal anti-vasoinhibin antibodies able to discriminate between PRL and vasoinhibin prevented attempts to develop a sandwich enzyme-linked immunosorbent assay (ELISA). Fortunately, monoclonal antibodies were recently developed, and their evaluation for an ELISA by which the levels of vasoinhibin could be quantified is underway (88). However, Western blot evaluation of vasoinhibin in clinical samples is supported by the measurement of its antiangiogenic properties in the presence or absence of anti-PRL antibodies that neutralize vasoinhibin action (58, 89).

A clinical trial investigates the elevation of PRL-levels in patients with diabetic retinopathy

Increased, hypoxia-driven expression of VEGF, produced by the retinal pigment epithelium, by endothelial cells, pericytes and other retinal cells, with consecutive enrichment in the retina and vitreous is a major driver of DME and PDR as it contributes to rupturing the blood-retinal barrier and induces angiogenesis which results in pathological neovascularization. The healthy vitreous is one of the few naturally avascular structures but is invaded by blood vessels in PDR. Not only the elevation of

growth factors facilitates its invasion by neovessels, the impaired production or insufficient upregulation of natural blood vessel inhibitors responsible for maintaining the avascular state of the vitreous are relevant as well (90). The healthy vitreous humor as such is antiangiogenic and inhibits tumor neovascularization (91), and angiogenesis in various other models, for example the retinal-extract induced angiogenesis in the chick chorioallantoic membrane (CAM) assay (92).

As mentioned, hyperprolactinemia leads to vasoinhibin accumulation in the retina of rats and prevents and reverses diabetes-induced blood retinal barrier breakdown and ischemia-induced angiogenesis by inhibiting vasopermeability and by targeting the retinal pigment epithelial cells in the outer blood retinal barrier (62, 93). These insights triggered the development of a randomized clinical trial, in which levosulpiride is evaluated as a medical treatment in patients with PDR and DME (59) (Figure 1). Levosulpiride is a dopamine D2 receptor blocker which is used as a prokinetic drug in patients with diabetic gastroparesis, where enteric inhibitory dopaminergic D2 receptor antagonism can have prokinetic effects. At the pituitary level D2 receptor antagonism with levosulpiride evokes hyperprolactinemia (94). One arm of the clinical study includes patients with PDR undergoing vitrectomy, with and without prior treatment with levosulpiride and subsequent laboratory evaluation of the vitreous fluid. Levosulpiride treatment increased PRL and vasoinhibin in the vitreous, and the vitreous from levosulpiride-treated patients with PDR, but not from placebo-treated patients with PDR, inhibited the basic fibroblast growth factor (bFGF) and VEGF-induced proliferation of endothelial cells in culture (58). The conversion of PRL to vasoinhibin was mediated by matrix metalloprotease (MMP) present in the vitreous fluid and was higher in patients without diabetes than in patients with PDR (58). This result is the first partial outcome of the clinical study which provided a proof-of-concept that treatment with levosulpiride is appropriate to elevate intraocular PRL and vasoinhibin levels. Further proof-of-concept was shown by an *in vivo* study in rats with streptozotocin-induced diabetes, in which racemic sulpiride increased ocular vasoinhibin levels and inhibited retinal hypervasopermeability (57). The other arms of the trial that also comprise patients with DME are awaiting completion and the publication of the results are expected soon.

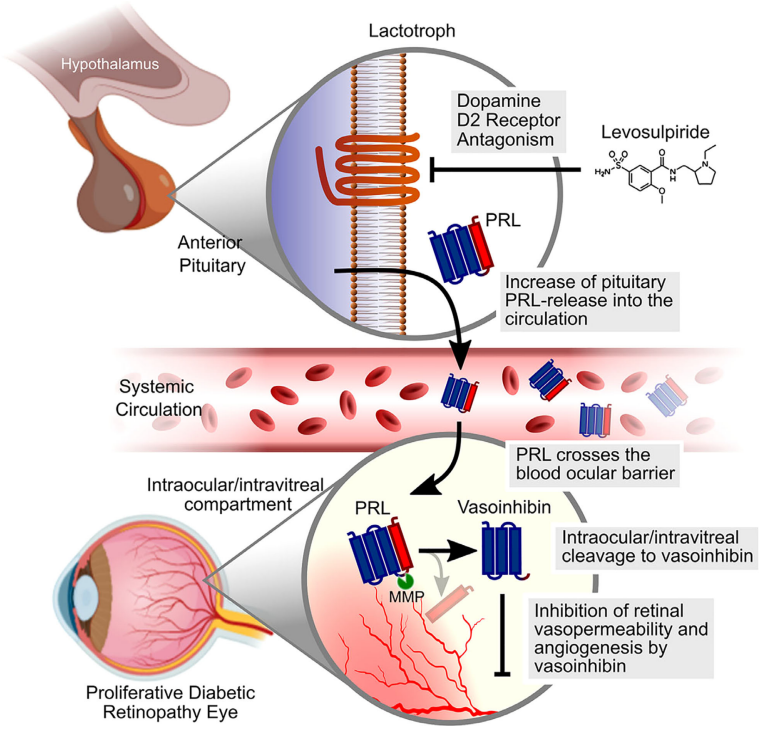

FIGURE 1
Schematic representation of the mechanism by which levosulpiride therapy could limit the progression of DME and DR. Levosulpiride, a dopamine D2 receptor antagonist, blocks dopamine D2 receptors located in the membrane of anterior pituitary cells that produce PRL (lactotrophs). Given that hypothalamic dopamine inhibits the release of PRL, levosulpiride leads to high levels of PRL in the circulation (hyperprolactinemia) which, in turn, favor PRL penetration across the blood–ocular barrier. MMPs in the intraocular/vitreous compartment cleave PRL to vasoinhibin, which can reduce retinal vasopermeability and angiogenesis in DME and DR. Scheme was partly created with Biorender.com. The original figure was published by Nunez-Amaro et al. (58) under the Creative Commons Attribution-Non-Commercial-NoDerivatives 4.0 International License (https://creativecommons.org/licenses/by-nc-nd/4.0/). The figure was not modified.

PRL and vasoinhibin are endogenous players in diabetic retinopathy with translational potential

By the providing the retina and the vitreous with PRL and antiangiogenic vasoinhibin, the PRL/vasoinhibin axis contributes to the physiological restricted and avascular states of the retina and vitreous body, respectively. The natural antiangiogenic capacity of the vitreous is impaired in DR, namely by the upregulation of factors stimulating blood vessel growth, but likewise by the downregulation of inhibitors. The downregulation includes a reduced MMP-mediated conversion of PRL to vasoinhibin in DR and facilitates an increase in retinal blood vessel permeability and neovascularization growing into the vitreous, with concurrent manifestation of edema, bleeding, tractional retinal detachment, and clinically loss of vision and blindness. Preclinical experimental and clinical proof-of-concept studies revealed the translational potential of raising systemic PRL levels to elevate ocular PRL levels and enhance the generation of vasoinhibin in the vitreous. The PRL/vasoinhibin axis and its regulation in diabetes is among the factors beyond glycemic exposure which may determine the risk of DME, and DR. Therapeutic interventions are currently evaluated in a clinical trial and will show whether patients with diabetes benefit from raising circulating PRL levels. The new clinical perspective of PRL in metabolism and its contribution to the control of blood vessel growth and function *via* the PRL/vasoinhibin axis is attesting to the clinical significance of PRL beyond reproduction-associated functions.

Author contributions

JT wrote the manuscript, TB and CC edited the manuscript. All authors approved the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Prolactin receptor gene transcriptional control, regulatory modalities relevant to breast cancer resistance and invasiveness

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The prolactin receptor (PRLR) is a member of the lactogen/cytokine receptor family, which mediates multiple actions of prolactin (PRL). PRL is a major hormone in the proliferation/differentiation of breast epithelium that is essential for lactation. It is also involved in breast cancer development, tumor growth and chemoresistance. Human PRLR expression is controlled at the transcriptional level by multiple promoters. Each promoter directs transcription/expression of a specific non-coding exon 1, a common non-coding exon 2 and coding exons E3-11. The identification of exon 11 of PRLR led to finding of alternative spliced products and two novel short forms (SF) that can inhibit the long form (LF) of PRLR activity with relevance in physiological regulation and breast cancer. Homo and heterodimers of LF and SF are formed in the absence of PRL that acts as a conformational modifier. Heterodimerization of SF with LF is a major mechanism through which SF inhibits some signaling pathways originating at the LF. Biochemical/molecular modeling approaches demonstrated that the human PRLR conformation stabilized by extracellular intramolecular S–S bonds and several amino acids in the extracellular D1 domain of PRLR SF are required for its inhibitory actions on PRLR LF-mediated functions. Studies in breast cancer cells demonstrated that the transcription of PRLR was directed by the preferentially utilized PIII promoter, which lacks an estrogen responsive element. Complex formation of non-DNA bound ERα dimer with Sp1 and C/EBPβ dimers bound to their sites at the PRLR promoter is required for basal activity. Estradiol induces transcriptional activation/expression of the PRLR gene, and subsequent studies revealed the essential role of autocrine PRL released by breast cancer cells and CDK7 in estradiol-induced PRLR promoter activation and upregulation. Other studies revealed stimulation of the PRLR promoter activity and PRLR LF protein by PRL in the absence of estrogen *via* the STAT5/phospho-ERα activation loop. Additionally, EGF/ERBB1 can induce the transcription of PRLR independent of estrogen and prolactin. The various regulatory modalities contributing to the upregulation of PRLR provide

options for the development of therapeutic approaches to mitigate its participation in breast cancer progression and resistance.

KEYWORDS

prolactin receptor (PRLR), transcriptional regulation, gene structure, signal transduction, breast cancer, prolactin (PRL)

Introduction

Prolactin (PRL) is a multifaceted protein hormone produced and secreted by the anterior pituitary gland, and it is also found in extra-pituitary tissues, including the mammary gland, brain, decidua, gonads, pancreas, immune cells, liver and adipose tissue, where it exerts autocrine and paracrine functions [reviewed in (1–4)]. Prolactin directs several physiological functions, such as lactation, immunomodulatory actions, and glucose and lipid metabolism, and is involved in pathological modalities, such as prolactinoma, hypogonadism and several cancers [reviewed in (2–4)]. The highly diversified actions of PRL are mediated through its transmembrane prolactin receptor (PRLR), a member of the lactogen/cytokine receptor family, which is expressed ubiquitously and functions as a dimer activated by PRL. A short isoform of PRLR with 310 amino acids (aa) was initially cloned from rat liver (5). The long form (LF) of PRLR with 610 aa was isolated from human hepatoma and breast cancer cells (6) and from the rat ovary (7). The monomeric structure of human PRLR (hPRLR) was resolved using combined NMR and computational approaches (8). PRLR gene expression is controlled by multiple promoters that regulate sustained PRLR levels and function (9). In humans, there are several PRLR isoforms, including the LF, many short forms (SFs), an intermediate variant and a soluble isoform [(10), reviewed in (11)]. The expression of these isoforms of PRLR widely varies in different tissues and is required for specific functions of the organ system at specific times. Certain short isoforms of hPRLR can interfere with the essential signaling of the long isoform, thereby exerting inhibitory action [reviewed in (11, 12)].

PRL is structurally similar to growth hormone and can act as a growth factor, immunomodulator, or neurotransmitter in an autocrine and paracrine manner [reviewed in (2)]. PRL mediates its actions through PRLR, resulting in the activation of the janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase (MAPK) signaling pathways [reviewed in (2)]. PRL/PRLR has been implicated in the development of several cancers and tumor progression [reviewed in (13–20)]. Hence, PRLR signaling has emerged as a relevant target in breast cancer. PRL is normally secreted in a pulsatile fashion, and this is clinically important for diagnostics related to problems in lactation and female infertility. Additionally, elevated circulating or locally produced PRL levels are associated with the risk of breast cancer [reviewed in (17, 18)]. PRL, through its cognate receptor, stimulates various downstream signaling cascades involving STAT5, RAS and MAPK, and phosphatidylinositol 3-kinase (PI3K) have been implicated in mammary tumorigenesis [reviewed in (18, 19, 21)]. The gain-of-function mutations in PRLR reported in benign and malignant breast cancer patients may support the hypothesis that PRLR signaling cascades could participate in benign breast tumorigenesis (22). In this review, we provide an overview of the current understanding of the transcriptional regulation of PRLR and signal transduction in physiological and pathological modalities in mammary glands with special emphasis on the role of PRL/PRLR signaling in breast cancer.

PRL and PRLR

PRL and PRLR distribution and biological functions

Prolactin is a 23 kDa protein hormone containing 199 aa that is produced in the lactotroph cells of the anterior pituitary gland. Prolactin is also secreted in an extra-pituitary and autocrine/paracrine manner from different tissues, such as mammary glands, brain, decidua, gonads, pancreas, immune cells, liver, and fat [reviewed in (1–3)]. The gene encoding human PRL is ~10 kb with five exons, and four introns is present on chromosome 6. In addition to its normal expression in the anterior pituitary, it is also expressed in mammary epithelial cells, the decidua, brain, myometrium, endometrium, lacrimal gland, thymus, spleen, skin fibroblasts, sweat glands and immune system cells. PRL secretion is regulated by several factors. Ovarian steroids, specifically estrogens, modulate PRL synthesis and prolactin release while suppressing dopamine synthesis [reviewed in (1–3)]. Increased PRL secretion (prolactinomas) directly suppresses the secretion of GnRH and indirectly suppresses follicle-stimulating hormone and luteinizing hormone, thereby disrupting the HPG axis and the ovulatory cycle (reviewed in 1-2). PRL performs innumerable

physiological functions in the body, including mammary gland development, lactation, gonadal functions (luteal cycle, uterine actions, Leydig cell development), parental behavior, preadipocyte differentiation, osmoregulation, angiogenesis, immunomodulatory function, islet cell proliferation, adrenal steroidogenesis, bone and calcium homeostasis, anovulation, reduced stress responses, oxytocin secretion and lipid metabolism. Prolactin also promotes neurogenesis in maternal and fetal brains [reviewed in (2, 3)].

PRLR has been identified in numerous cells and tissues of adult mammals. Cellular proliferation is also an important function of PRL in mammals. The expression of receptors (short and long forms) tends to vary with the stage of the estrous cycle, pregnancy, and lactation (23). The expression of PRLR in the late gestational fetal rat using *in situ* hybridization and immunocytochemistry showed that the long and short isoforms of PRLR were expressed during late fetal development (days 17.5 to 20.5). These studies showed PRLR transcripts were widely expressed in tissues from all three germ layers, in addition to the classic target organs of PRL (24). The expression of PRLR mRNA in the fetal adrenal cortex, gastrointestinal and bronchial mucosae, renal tubular epithelia, choroid plexus, thymus, liver, pancreas, and epidermis was higher than that in other tissues (23).

PRLR structure and isoforms

PRLRs belong to the lactogen/cytokine receptor superfamily, which mediates the various cellular actions of PRL in different target tissues. PRL binds to preexisting PRLR dimers and acts as a conformational modifier, which results in the activation of the JAK/STAT pathway and MAPK and SRC kinases, thus leading to the induction of PRL responsive genes [reviewed in (4, 18)].

PRLR structure

PRLR has three main domains: extracellular, transmembrane and intracellular. The extracellular domain is divided into two fibronectin domains, D1 and D2, and the WS motif in D2 acts as a molecular switch during ligand-bound activation of PRLR (25). PRLR has a single-pass transmembrane chain, and the receptor chain does not possess kinase activity. The receptor chain is dependent on the associated kinases to transduce phosphorylation-based signal cascades. The intracellular domain includes proline-rich sequence-mediated JAK2 association to the prolactin receptor is required but not sufficient for signal transduction Box-1/2 sub-domains. Box-1 is known to interact with JAK2 and SRC family kinases such as FYN [reviewed in (4)]. The intracellular domain of PRLR-LF is intrinsically highly unstructured/disordered and binds to negatively charged lipids of the inner plasma membrane through conserved motifs resembling immuno receptor tyrosine-based activation motifs. However, this lipid association of the PRLR intracellular domain is not accompanied by induced folding and is independent of specific tyrosine phosphorylation. These attributes may contribute to regulating intracellular signaling (26). There are two short isoforms of hPRLR generated by alternative splicing to exon 11 (10).

PRLR gene and isoforms

The genomic organization of the hPRLR gene (>200 kb) is complex and subject to alternative splicing, which results in several isoforms of the receptor. The gene resides on chromosome 5p14-13. The hPRLR gene contains eleven exons, where exon 1 consists of six non-coding sequences ($hE1_3$, $hE1_{N1\text{-}5}$) that are alternatively spliced to a common non-coding exon 2, and only exons 3 to 11 are coding exons [(9); Figure 1]. The LF of hPRLR is encoded by exons 3-10 (Figure 2). Additionally, an intermediate form (412 aa) with partial deletion of 198 aa within the cytoplasmic domain in exon 10 of the LF was isolated from the rat Nb2 lymphoma cell line (28) and human breast cancer cells (29). In addition, a soluble PRLR lacking transmembrane and cytoplasmic regions was isolated from the rat ovary (7). The transmembrane domain is encoded by Exon 8, while most of the intracellular domain is encoded by Exon 10 (Figure 2). Alternative splicing of exons 10 and 11 with a truncated intracellular domain resulted in two novel SFs of hPRLR, S1a and S1b, which inhibit the LF signal induced by PRL [(10); Figures 2, 3]. In addition, a unique spliced variant designated S1c, which completely lacks exon 10, has been identified in human spermatozoa (30). These short forms S1a and S1b are expressed as cell surface transmembrane receptors with a reduced cytoplasmic domain and unique C-termini S1b was far more effective in inhibiting the PRL-induced activation of the β-casein gene promoter mediated by LF (31, 32). A subsequent study demonstrated a naturally occurring ΔS2 deletion variant of SF in normal and cancerous human cells. These studies have also demonstrated that removal of the S2 extracellular subdomain can alter the conformation of the intracellular signaling region of the LF and both SFs (S1a and S1b), thereby supporting the concept that the conformation of the ECD can affect the conformation of the intracellular domain (33).

Our studies on human LF, S1a and S1b have revealed the existence of constitutive LF and SF homodimers and heterodimers (LF/S1a or LF/S1b) under non-reducing conditions in the absence of PRL that acts as conformational modifier (31). Both LF and SFs (S1a and S1b), as dimers, are capable of ligand binding and PRL-induced phosphorylation of JAK2, but only LF can activate downstream STAT5 signaling. S1a and S1b cannot induce the downstream activation of STAT5 due to the lack of an extended cytoplasmic domain. PRL signaling through the SF of PRLR in mouse ovaries actively regulates the expression of several genes and can profoundly affect follicular survival. SF can mediate the activation of MAPK

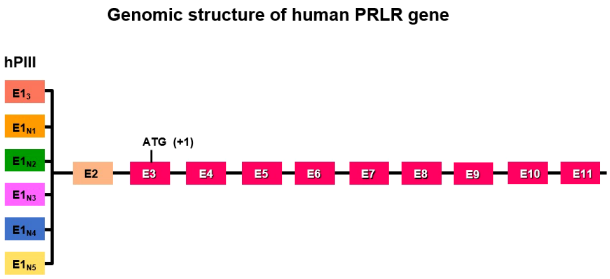

FIGURE 1
Schematic representation of multiple exons 1 (hE1$_3$, hE1$_{N1-5}$) driven by individual promoters and alternative splicing to common exon 2 of human PRLR. Exon 3 has initiation codon. PIII/hEI3 is the predominantly utilized generic promoter in addition to five specific exon 1/promoters (hE1N1 to hE1N5).

and PI3K pathways [reviewed in (34)]. In breast cancer, the ratio of SFs (S1a and S1b) to LF is markedly reduced compared to that in adjacent tissue, which indicates that the loss of inhibitory regulation of LF could increase tumor cell proliferation (35). Two intramolecular disulfide bonds within the extracellular D1 domains are essential for the inhibitory function of S1b on LF. Additionally, the JAK2 association was disrupted. S–S bond disruption of S1b (S1bx) affects the dimerization interface, thus causing a significant decrease in LF heterodimerization with S1bx and an increase in homodimerization of S1bx. Therefore, stability of the PRLR structure by intramolecular S–S bonds is required for the inhibitory action of S1b on LF-mediated function (36). Additionally, mutations in E69 of the D1 domain of S1b and neighboring amino acid residues (R66, E67, E42) close to its surface binding domain cause a loss of its inhibitory effect, while those away from this region or mutants in the D2 domain have no effect. These findings underscore the significant role of extracellular D1 on the S1b conformation and its inhibitory action in PRL-induced LF function [(12); Figure 4]. In addition, PRL signaling through SF of mouse PRLR can either stimulate or inhibit a substantial number of transcription factors in the decidua as well as ovary. Few transcription factors have been shown to be similarly regulated in both tissues, while most transcription factors are oppositely regulated by PRL (37). Additional studies are needed to better understand the role of alternatively spliced PRLR isoforms and the manner in which such splicing is regulated in breast cancer.

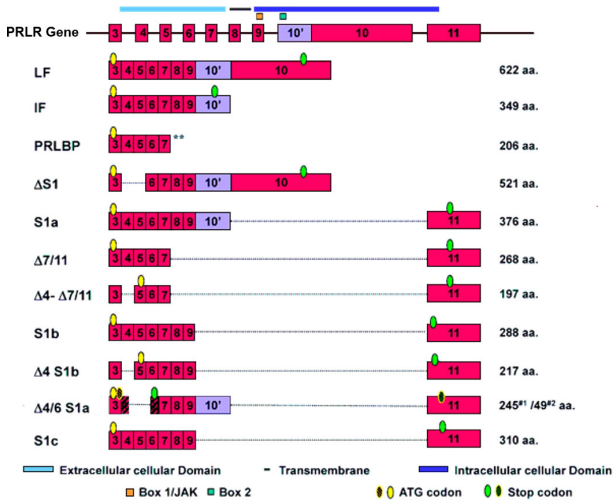

FIGURE 2
Schematic representation of human PRLR isoforms generated by alternative splicing [adapted from reference (27)] PRLR (prolactin receptor) Atlas Genet Cytogenet. ** indicates soluble form of PRLR.

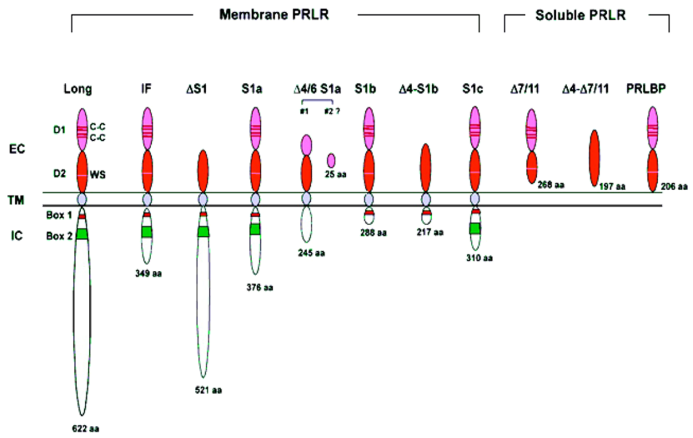

FIGURE 3
Structure of human PRLR variants. LF, long form; IF, intermediate form; S1a, S1b & S1c, short forms; PRLBP, prolactin binding protein; LFΔS1, long form lacking D1 domain; LFΔS2, long form lacking D2 domain; Δ, deleted exon. D1, D2, N-terminal subdomain; WS, WSXWS motif; C, cysteine; Y, tyrosine; EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain [adapted from reference (27)].

Transcriptional regulation of PRLR

The expression of PRLR is controlled by multiple promoters using a complex regulatory transcriptional network. In the rat these promoters are PI (gonad specific and SF1 dependent), PII (liver specific induced by HNF4), and PIII a widely expressed in all tissues and requires CCAAT/enhancer binding protein-β (C/EBPβ) and specificity protein 1 (Sp1) for its activation. Promoter PI is inoperative in mice due to disruption of the SF1 motif. These multiple promoters have been shown to direct tissue-specific expression in the ovary, liver and mammary gland in rats [(38), reviewed in (39)]. In humans, hPIII is a universal/generic promoter similar in structure and regulation to one of those found in rodents (PIII). This promoter in humans directs transcription in all PRL-responsive tissues as well as other promoter(s) of less known function (9). The promoters

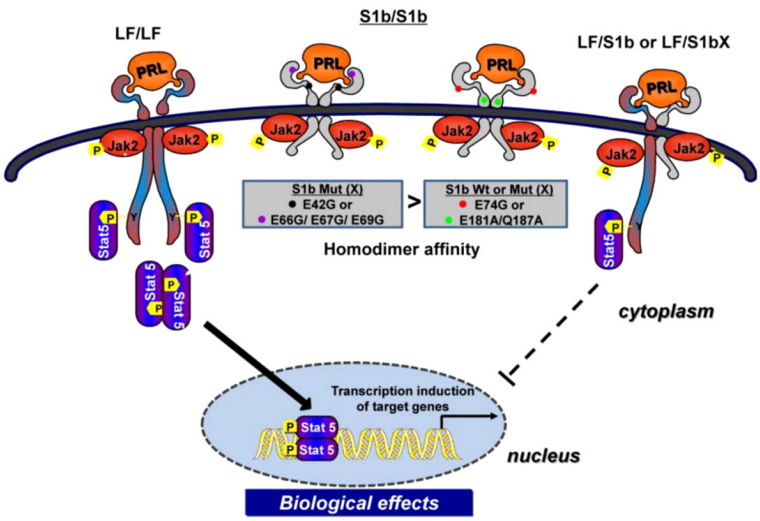

FIGURE 4
Mechanism of inhibitory action of short form S1b PRLR on PRL-induced long-from (LF) receptor signaling (reproduced from reference 12). Homodimer of the hPRLR long form (LF) mediates PRL stimulated JAK2/Stat5 signaling required for transcription/expression PRL/PRLR target genes which are essential for the various biological effects of the hormone. The inhibitory action of short form S1b on LF's function induced by PRL results from LF/SF heterodimer formation and marked reduction of LF/LF homodimers which are required by Stat5 activation.

include the predominantly utilized generic promoter 1/exon 1 (PIII/hEI3), which is also present in rats and mice, and five human-specific exon 1/promoters ($hE1_{N1}$ to $hE1_{N5}$) [(9, 39); Figure 1]. The PRLR promoters belong to the TATA-less/initiator class and are activated by estradiol 17β (E2). The preferentially utilized human promoter III (hPIII) promoter contains Sp1 and C/EBP elements that bind to Sp1/Sp3 and C/EBPβ, which are required for basal transcriptional activity (Figure 5). These promoters were found to be utilized in breast cancer tissue and cell lines, including MCF7 and T47D, and variably used in other tissues (40). Among these promoters, the generic hPIII (the human counterpart of rodent PIII), which drives the universal human $E1_3$ exon (the human counterpart of EIII in rodents), was functionally characterized in breast cancer cells, while the specific human exon hE_{N1} directed by promoter hP_{N1} is driven by domains containing an ETS element and a nuclear receptor NR half-site. The promoters for the specific human exons, i.e., hE_{N2-5}, remain to be identified (39).

E2 can induce an increase in hPRLR mRNA transcripts directed by the hPIII promoter *via* a non-classical ERE independent mechanism in breast cancer cells [(40); Figure 5]. The association of ERα with DNA-bound Sp1 and C/EBPβ is essential for E2-induced hPRLR gene transcription (Figure 5). The additional interaction between zinc fingers of Sp1 and leucine zipper of C/EBPβ stabilizes the ERα-Sp1-C/EBPβ complex. The enhanced complex formation of the ERα dimer (DNA binding domain) with Sp1 (zinc finger motifs) and C/EBPβ (basic region and leucine zipper) by E2 plays an essential role in the transcriptional activation of the PRLR gene [(40, 41); Figure 5]. An autocrine/paracrine loop increases PRLR mRNA expression *via* its ligand PRL in breast cancer cells. Similarly, in MCF7 cells overexpressing PRL, upregulation of PRLR was observed in response to endogenous PRL but not exogenous PRL. Furthermore, there was an increase in ER levels and estrogen responsiveness in these MCF-7 cells. Owing to the positive effect of estrogen on PRLR transcription, this reciprocal regulation amplifies both ER and PRLR signaling in breast cancer (42). Another steroid hormone that can also regulate PRLR transcription is progesterone through its receptor (Figure 5). Progesterone participates in the menstrual cycle, pregnancy, and embryogenesis and can be involved in tumorigenesis as well as in normal growth. It has been reported that the progesterone receptor lacks the consensus sequence or half-sequence response element in the PRLR gene PIII promoter and demonstrated that progesterone induces an increase in PRLR mRNA in a non-classical manner by inducing the expression of PRLR through the cooperative activation of Sp1 and CEBPβ at the PIII promoter in mouse cells and T47D breast cancer cells (43).

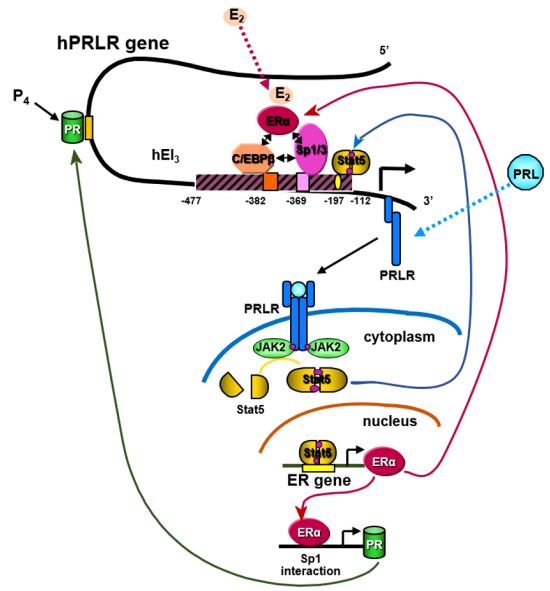

FIGURE 5
Transcriptional regulation of hPRLR induced by E2. E2 induces ligand mediated ERα-Sp1-C/EBPβ complex formation and binding to the hPIII promoter of the hPRLR gene (38, 39). This results in increased transcription of PRLR. PRL/PRLR activates JAK/STAT that can induce the transcription of ERα. P4 (progesterone)/Prog receptor can induce transcription of PRLR through association with Sp1and C/EBPβ through promoter hPIII.

PRLR Signal Transduction

JAK-STAT pathway

This is the most classical and well-studied downstream signaling pathway induced by the binding of PRL to PRLR. This pathway appears to mediate most of the PRL actions in lobuloalveolar development and lactation (Figure 6). The intracellular domain of PRLR is devoid of any intrinsic enzymatic activity; however, ligand-mediated activation of PRLR results in tyrosine phosphorylation of numerous cellular proteins, including the receptor itself [reviewed in (2, 4)]. Binding of PRL to PRLR results in conformational induction of preformed dimers and activation of JAK2 by transphosphorylation, which brings two JAK2 molecules close to each other [reviewed in (2, 4)]. JAK2 kinases are involved in the phosphorylation of Tyr residues of the PRLR itself, and the phosphotyrosines serve as potential docking sites for transducer molecules containing SH2 domains [(45), reviewed (4)]. The phosphorylation of Tyr residues of PRLR occurs in all isoforms except short isoforms of PRLR [reviewed in (4)]. The LF of PRLR mediates several processes upon receptor activation due to the phosphorylation of several Tyr residues present in PRLR (45).

The STAT family of proteins are the major transducers of cytokine receptor signaling, which contains eight members. STAT1/3 and STAT5a/5b have been identified as transducer molecules of PRLR. STAT contains five conserved domains: DNA-binding, SH3-like, SH2-like, and NH_2- and COOH-

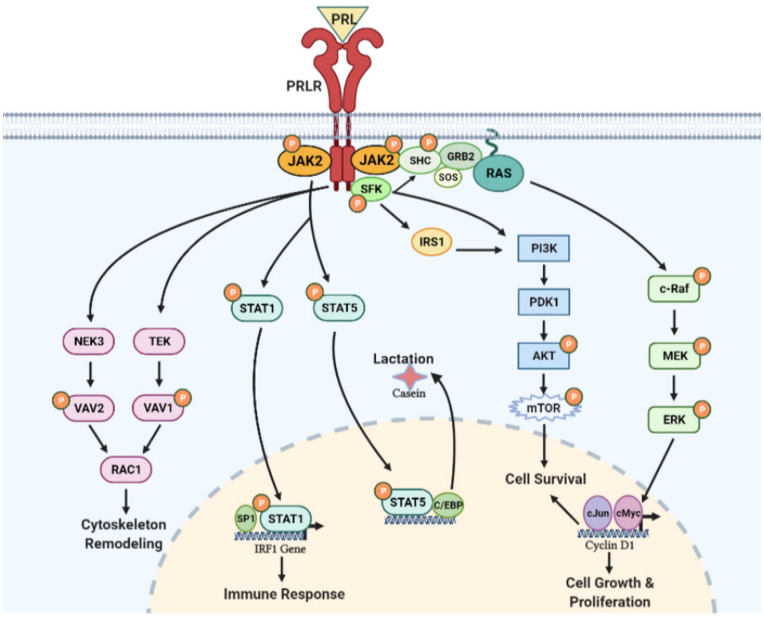

FIGURE 6
PRL/PRLR signaling pathways. PRL through its receptor PRLR induces multiple signaling cascades which include participation of JAK, SFK, PI3K/AKT, NEK3/VAV2, TEK/VAV1 kinases and STAT transcription factors. These signaling pathways are involved in lactation, immune response, cytoskeleton remodeling and cell growth, proliferation & survival [adapted from reference (44)].

terminal transactivating domains [(46), reviewed in 4)]. As per the consensus model of STAT activation, a phosphorylated Tyr of the activated receptor interacts with the SH2 domain of STAT. Then, STAT is phosphorylated by receptor-associated JAK kinase. The phosphorylated STAT dissociates from the receptor and undergoes homodimerization or heterodimerization through an interaction involving the phosphotyrosine of each monomer and the SH2 domain of another phosphorylated STAT molecule. The STAT dimer translocates to the nucleus and activates a STAT DNA-binding motif in the promoter of target genes such as β-casein, IRF1, c-Myc, and cyclin-D (46). The consensus DNA motif (TTCxxxGAA), termed GAS (γ-interferon activated sequence), is recognized by STAT1, STAT3, and STAT5 homo or heterodimers [reviewed in (47, 48)].

RAS/RAF/MAP kinase pathway

In addition to JAK/STAT signaling initiated by the activation of PRLR, several reports implicate PRLR in the activation of the mitogen-activated protein (MAP) kinase cascade (49, 50). Phosphotyrosine residues of PRLR can serve as docking sites for adapter proteins (Shc/Grb2/SOS) connecting the receptor to the RAS/RAF/MAPK cascade [(49, 50); Figure 6]. Although the JAK/STAT and MAPK pathways were initially regarded as independent or parallel pathways, results suggest that these pathways are interconnected [reviewed in (51)].

Other kinases: c-SRC and FYN

Several reports indicate PRL-induced activation of members of the Src kinase family, c-SRC (reviewed in 4) and FYN (52). PRL-induced rapid Tyr phosphorylation of insulin receptor substrate-1 (IRS-1) and a subunit of PI3K have been described. Both IRS-1 and PI3K seem to be associated with the PRLR complex. PRL-induced activation of PI3K has been proposed to be mediated by FYN (52). PRL/PRLR can also induce TEC-VAV1 and NEK3-VAV-1/VAV-2 signaling cascades and function in the regulation of the cytoskeleton (Figure 6). NEK3 kinase has been shown to regulates PRL-mediated cytoskeletal reorganization and motility of breast cancer cells (53, 54).

Role of PRL/PRLR signaling in breast cancer

PRL/PRLR induced signaling cascades promote breast development and progression

PRL plays a crucial role in mammary gland development and in the etiology and progression of breast cancer. Considerable supporting data indicate that PRL/PRLR hyper signaling contributes to the initiation of breast cancer. A strong correlation

between breast cancer with increased PRL and PRLR has been reported in several studies (17, 55–58). Higher levels of PRL in postmenopausal women may eventually lead to an increased risk of breast tumors and metastatic cancer (58–60). In premenopausal women with breast cancer, there are higher-than-average levels of serum PRL together with elevated PRLR expression. These are associated with an increased risk of tumor progression and invasion (60–62). An association is observed between invasive breast cancer risk in postmenopausal women with high circulating PRL, particularly for ER-positive disease. PRL/PRLR is expressed in 95% of mammary tumors and 60% of male breast carcinomas (63). These findings were replicated in transgenic mice overexpressing PRL that develop mammary tumors and in *in vitro* studies where PRL played a role in the proliferation of breast cancer cells (64, 65). A direct correlation is observed between single nucleotide polymorphisms (SNPs) in the *PRLR* gene and benign breast tumor incidence. The two SNPs PRLR-I76V and I146L demonstrate constitutive receptor activity, and one of the SNPs (PRLRI146L) correlates with benign breast disease in a patient cohort, but these patients did not have high levels of serum PRL (22, 66). However, in subsequent study these SNPs in the PRLR gene were found not to be associated with breast cancer and multiple fibroadenoma (67). In another study, SNPs were found in *PRL*, and *PRLR* genes were associated with breast cancer metastasis in Taiwanese women (68). Studies from our lab and others in T47D and MCF-7 breast cancer cells have shown that the PI3K/AKT and RAF/MEK/ERK pathways are activated in parallel following PRL treatment, which leads to profound cell proliferation and survival (69, 70). PRLR can also induce the MAPK/ERK signaling cascade *via* the PI3-kinase-dependent RAC/PAK/RAF/MEK pathway, which is in turn controlled by JAK2, SRC family kinases and focal adhesion kinase (FAK) (71). In addition to the role of the predominant LF of PRLR in breast cancer, a recent study showed that the human intermediate PRLR (alternatively spliced isoform) is a mammary proto-oncogene capable of stimulating cell survival and proliferation (29). Many breast tumors are characterized by reduced STAT5 and high levels of PRLR expression and MAPK signal components, including AP-1 and pro-invasive matrix metalloproteinases [reviewed in (56)]. MMPs are highly invasive agents and are associated with resistance to chemotherapy and anti-estrogen treatments (72). Extracellular matrix components in the breast tumor microenvironment can also influence PRL/PRLR signaling (73, 74). In invasive breast cancer, there is a shift in PRL signaling from STAT5-mediated pathways to focal-adhesion kinase and MAPK pathways, thereby favoring proliferation (73, 74). PRL signaling in high-density stiff collagen matrices increases MMP expression, thereby promoting cellular motility. Therefore, the tumor microenvironment may be responsible for favoring one signaling pathway over another (73, 74). There is a complex interplay between PRLR and estrogen receptor (ERα), and there is an important role for the tumor microenvironment. The co-expression of PRLR and ERα in a non-compliant, rigid matrix is associated with increased tumor invasiveness and reduced responsiveness to estrogen antagonists (75). PRLR signaling can also induce motility and invasion of T47D breast cancer cells by activating downstream effectors such as TEC and NEK3 kinases, thus leading to cytoskeletal and focal adhesion reorganization (54, 76). Several studies using breast cancer cells have shown that PRL activates unliganded ERα through phosphorylation at the Ser118 and Ser167 residues. The activation of ERα promotes ligand-independent transcriptional initiation of ERE-dependent target genes, which seems to be an important factor in the proliferative and transcriptional actions of PRL in breast cancer cells (70, 77, 78). The most significant transcription factor in PRL/PRLR signaling is STAT, which regulates the growth, differentiation, and survival of mammary tissue. STAT3 and STAT5 are activated/overexpressed in several types of cancers, including breast cancer [reviewed in (79, 80)]. STAT5 can act as both a tumor suppressor and an oncogene in breast cancer under different circumstances. In ER-positive breast cancer, STAT5 expression enhanced the response to hormone therapy and increased the overall survival of patients [reviewed in (81)]. Recent studies have shown that phosphorylation of STAT5a serine residues (S726 and S780) may regulate its activity to promote cell proliferation in MCF-7 cells (82). Reports have indicated that STAT5 acts as a suppressor of breast cancer invasion and metastatic progression and can be used as a tumor marker of favorable prognosis [reviewed in (79)]. STAT5 is progressively inactivated with the progression to metastatic breast cancer due to enhanced regulation by tyrosine phosphatases (83). The activation of STAT5 in breast cancer cells promotes homotypic adhesion and inhibits the invasive characteristics of cells (84).

PRL and cyclin-dependent kinase 7 (CDK7) in estrogen-induced upregulation of PRLR in breast cancer cells

We demonstrated the essential role of endogenous PRL in the upregulation of the PRLR promoter, which involves the requisite participation of E2/ERα at the hPIII promoter along with STAT5a (Figure 5). Phosphorylated STAT5a, which associates with its functional element at hPIII, interacts with non-DNA-bound E2/ERα, which in turn associates in a complex with Sp1 and C/EBPβ bound to their cognate DNA sites at the PRLR hPIII promoter [(85); Figure 5]. We have shown in MCF-7 cells that E2 induces ERα phosphorylation at S118 *via* CDK7 kinase and greatly increases the recruitment of E2/ERα to the hPIII promoter over basal unliganded ERα (86). Phosphorylation of ERα at S118 is necessary for its association with the Sp1-C/EBPβ complex and its interaction with STAT5a. Inhibition by the specific CDK7 inhibitor THZ1 markedly reduced E2-induced ERα phosphorylation at S118, while the JAK2 inhibitor AG490 or MEK inhibitor U0219, which inhibits downstream JAK2-induced pathways known to phosphorylate unliganded ERα at S118 and S167, had no effect. Targeting CDK7 kinase, which is known to regulate both transcription and

the cell cycle, and ERα phosphorylation with the THZ1 inhibitor was found to effectively inhibit the transcription of PRLR and cell migration in breast cancer cells (85). Our studies may provide insights for therapeutic approaches that will mitigate the transcription/expression of PRLR and its participation in breast cancer progression fueled by E2 and PRL *via* their cognate receptors (Figure 5).

Crosstalk between PRLR and other receptors in breast cancer

Studies have indicated that PRLR signaling crosstalk with other receptors can influence signal transduction. PRLR interacts with integrin *via* the signal regulatory protein alpha transmembrane glycoprotein and SHP2 (87). PRL/PRLR and E2/ER synergistically can regulate the gene expression and proliferation of breast cancer cells (87). Furthermore, PRLR signaling tends to activate the unliganded ER (70, 77). PRL and estrogen cooperatively induce phosphorylation of ERK1/2 and enhance prolonged activation of AP-1 in breast cancer cells (88). This type of signaling pathway crosstalk can promote breast cancer progression and chemotherapeutic resistance. Crosstalk occurs between PRLR and EGFR/HER2 (Figure 7). PRLR can activate HER2 signaling *via* JAK2 (70, 85). We have demonstrated that PRL/PRLR induces HER2 phosphorylation at Tyr residues 1221 and 1222 through JAK2, thereby activating downstream PI3K/AKT pathways in both MCF-7 and T47D cells (70). This crosstalk between PRLR and HER2 signaling further facilitates the phosphorylation of ERα, its recruitment to the PRLR promoter and upregulation of PRLR transcription. Interestingly, we also found that EGF/EGFR in MCF-7 and T47D cells can induce PRLR transcription *via* downstream MAPK and PI3K signaling pathways (Figure 7). Crosstalk between the PRLR and progesterone receptor (PR) signaling pathways has been shown to be relevant to both breast development and progression. Both PR and STAT5a are key transcription factors in these pathways and have been shown to be mediators of breast cancer stem cell outgrowth (89). This evidence, coupled with their established function in the same transcriptional complexes at phospho-PR-target genes with high cancer relevance, supports the importance of PR-PRLR crosstalk [reviewed in (90)].

Resistance to endocrine therapy in breast cancer and future perspectives

Endocrine therapy is one of most effective forms of targeted adjuvant therapy for hormone receptor-positive breast cancer. Adjuvant therapy has been well established with different types of antiestrogens, including selective ER modulators (tamoxifen, raloxifene), which block the activity of ER, selective ER downregulators, such as fulvestrant, which causes destabilization and degradation of ER, and the third generation of aromatase inhibitors (anastrozole, letrozole and exemestane), which reduce the production of E2 in tumors [(91), reviewed in (92)]. Although these endocrine therapies for women with ER+/PR+ breast cancer have led to substantial improvements, a significant number of cancer patients develop either intrinsic resistance or acquired resistance, which often results in tumor relapse. There can be multiple reasons for this endocrine resistance which includes mutations of ER, enhanced MAPK and PI3K/mTOR signaling pathways. Other aspects of endocrine resistance could result from overexpression of HER2, and crosstalk of ER with bypass signaling pathways, such as the EGFR/HER2 and PRLR signaling pathways [reviewed in (93–96)]. To overcome HER2 hyperactivation, trastuzumab is still being used as the most effective form of treatment for ER+ and HER+ breast cancer patients. However, some cancer patients develop resistance to trastuzumab and tumor relapse within one year of treatment. Hyperactivation of HER2-induced downstream PI3K/AKT signaling is often observed in trastuzumab-resistant breast cancer patients [reviewed in (97, 98)]. HER2-targeted therapies have been established in recent years, including tyrosine kinase inhibitors, such as lapatinib, neratinib, tucatinib, and pyrotinib (99). Together, these drugs targeting multiple receptors, such as HER2, EGFR and HER4, were studied in the early and advanced stages of breast cancer and revealed some promising outcomes. Furthermore, in clinical trials, the combination of HER family inhibitors with endocrine therapy has been shown to have better results [reviewed in (100)]. PRLR and EGFR/HER2 crosstalk, which greatly increases the activation of the RAS/ERK and PI3K/AKT pathways, are associated with poor prognosis and therapeutic resistance in breast tumor patients. In the case of PRLR, only a few attempts have successfully developed a potential therapeutic small molecule inhibitor or monoclonal antibody (LFA102) to block PRLR signaling induced cell proliferation in breast cancer cell lines (101). Therefore, simultaneous treatments targeting both the HER2 and PRLR signaling cascades may offer better outcomes by efficiently hindering breast tumor progression and ameliorating endocrine resistance. A study using G129R (PRLR antagonist) and trastuzumab (monoclonal antibody targeting HER2) as a combination therapy to inhibit HER2+ breast cancer cells and a nude mouse xenograft model showed inhibition of cell proliferation (102). Additionally, combining PI3K/AKT/mTOR pathway inhibitors with endocrine therapy has been shown to potentially reverse resistance to trastuzumab in HER2+ patients and metastatic breast cancer in early clinical trials. A rational combination of therapeutic agents based on the disease profile would be more beneficial to breast cancer patients [reviewed in (103)].

Concluding remarks

PRL is a pleiotropic hormone that plays a crucial role in mammary gland development, lactogenesis, reproduction and

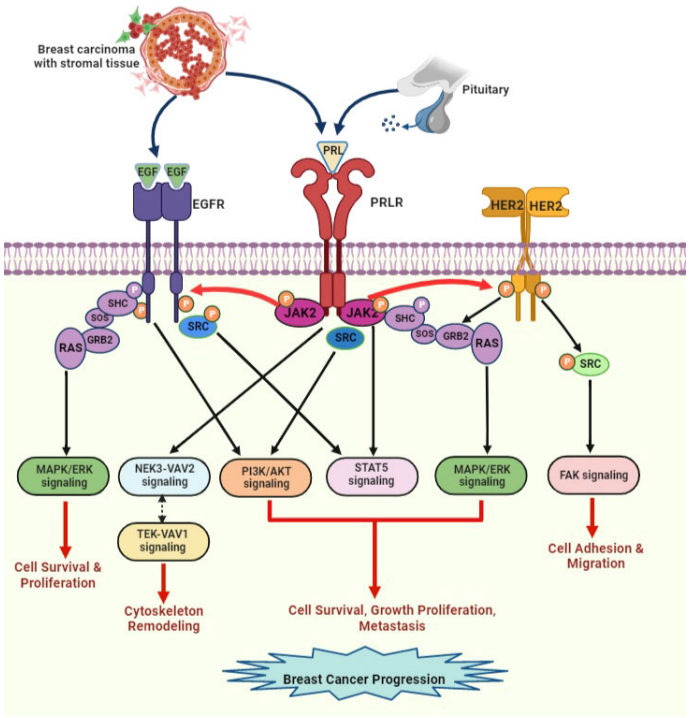

FIGURE 7
PRLR and EGFR/HER2 signaling crosstalk in breast cancer. EGF released by stromal microenvironment surrounding the breast tumor activates signaling cascades that overlap with PRLR signaling cascades upon activation with PRL secreted by breast tumor cells. PRL stimulates HER2 and EGFR signaling pathways via JAK2. EGF/EGFR also activates STAT5 signaling indirectly via s-SRC. This crosstalk between receptors can increase progression of breast tumor and endocrine resistance [adapted from reference (44)].

immune function. It mediates its actions through PRLR, a member of the lactogen/cytokine receptor family. PRLR activation induces JAK/STAT and mitogen-activated protein kinase signaling pathways implicated in the development of mammary glands and etiology of breast cancer. In this review, we provide an overview of the current understanding of the complex organization of the human PRLR gene and its transcriptional regulation. Preclinical data, epidemiological studies, and patient tumor tissues analyses strongly support the contribution of PRL/PRLR to breast tumorigenesis and cancer progression. This review also stresses the importance of signal transduction pathways (PI3K/AKT, RAF/MEK/ERK, FAK, and SFK) activated by PRL/PRLR in breast cancer. We have summarized how steroid hormones (E2 and PR) and growth factors (EGF/ERBB1 and HER2) can induce the transcription of PRLR, thereby increasing its expression in breast cancer cells and promoting cell proliferation. Therefore, in this era of precision medicine, we conclude that combination therapy involving pathway-selective kinase inhibitors and PRLR inhibitors depending on the status of the breast cancer can provide better outcomes in clinical studies.

Authors contributions

RK and MD were responsible for manuscript writing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The beneficial metabolic actions of prolactin

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The role of prolactin (PRL) favoring metabolic homeostasis is supported by multiple preclinical and clinical studies. PRL levels are key to explaining the direction of its actions. In contrast with the negative outcomes associated with very high (>100 μg/L) and very low (<7 μg/L) PRL levels, moderately high PRL levels, both within but also above the classically considered physiological range are beneficial for metabolism and have been defined as HomeoFIT-PRL. In animal models, HomeoFIT-PRL levels counteract insulin resistance, glucose intolerance, adipose tissue hypertrophy and fatty liver; and in humans associate with reduced prevalence of insulin resistance, fatty liver, glucose intolerance, metabolic syndrome, reduced adipocyte hypertrophy, and protection from type 2 diabetes development. The beneficial actions of PRL can be explained by its positive effects on main metabolic organs including the pancreas, liver, adipose tissue, and hypothalamus. Here, we briefly review work supporting PRL as a promoter of metabolic homeostasis in rodents and humans, the PRL levels associated with metabolic protection, and the proposed mechanisms involved. Finally, we discuss the possibility of using drugs elevating PRL for the treatment of metabolic diseases.

KEYWORDS

prolactin levels, homeoFIT-PRL, metabolically healthy and unhealthy obesity, metabolic homeostasis, insulin resistance, homeorhetic response

Introduction

Defining the role of prolactin (PRL) in metabolism has been challenging due to contrasting findings demonstrating positive and negative effects of PRL on metabolic homeostasis. This contradiction is disentangled after realizing that PRL levels and the physio-pathological context influence the direction of PRL action (1). Low and very high PRL levels are deleterious to the metabolism, whereas medium and moderately high levels are usually beneficial.

PRL action is necessary to maintain metabolic homeostasis, as the absence or reduction of PRL signaling due to the lack of PRL receptors (PRLR) or low PRL levels associate with exacerbated metabolic alterations, particularly in the context of a metabolic challenge or disease. In humans, low PRL levels associate with increased prevalence of metabolic diseases (1). In contrast, patients with overweight and obesity (OW/OB) having elevated PRL levels

show better metabolic profiles than BMI-matched patients with lower PRL values (2–6), to imply that elevated PRL is a mechanism dealing with metabolic challenge.

The mechanisms by which PRL promotes metabolic homeostasis involves actions in different metabolic organs. A detailed description of the levels of PRL and their cellular and molecular mechanisms mediating metabolic benefits warrant further research. Also, a careful evaluation of drugs that elevate PRL levels is needed in the context of metabolic diseases.

Prolactin promotes metabolic homeostasis in rodents

Serum PRL decreases in rodents with obesity, diabetes, and insulin resistance (2, 7–10), suggesting a role for reduced PRL levels in the pathophysiology of metabolic diseases. As a proof of concept, PRL treatment in mice and rats with streptozotocin (STZ)-induced diabetes or diet-induced obesity improves their metabolic profile (2, 11, 12), whereas PRLR null mice with STZ-induced diabetes or diet-induced obesity show a more severe disease phenotype (2, 13). Moreover, mice lacking PRLR in the liver become insulin resistant, whereas insulin resistant obese mice (db/db mice lacking leptin receptors) overexpressing the PRLR in the liver show improved insulin sensitivity (14).

In addition, PRL action is required to deal with the metabolic challenges of pregnancy, a state characterized by hyperphagia, excessive adiposity, and physiological insulin resistance to redirect nutrients towards the fetus (15–17). Pregnant mice null for the PRLR in the pancreas, specifically in β-cells, develop gestational diabetes (18–20), due to deficient pancreatic β-cell hyperplasia and hyperinsulinemia (21).

Moreover, PRL reduces metabolic alterations in lactating pups nursed by dams consuming a high fat diet (HFD) during lactation. The obesogenic milk from HFD-fed dams has 50% less PRL compared to the milk from dams fed a chow diet (22). Pups consuming the obesogenic-hypoprolactinemic milk develop obesity, excessive adiposity, severe insulin resistance, and fatty liver at weaning; whereas when their HFD-fed mothers or themselves receive exogenous PRL during lactation, metabolic alterations are ameliorated (22). These findings support PRL in maternal milk exerting beneficial metabolic effects in lactating pups, and low PRL levels in milk contributing to the maternal obesogenic diet-induced metabolic disease in pups.

Elevated prolactin levels as a mechanism to counteract metabolic alterations in humans

Low PRL levels associate with a higher prevalence of type 2 diabetes (T2D), insulin resistance, glucose intolerance, metabolic syndrome (MS), adipose tissue (AT) dysfunction, β-cell dysfunction, non-alcoholic fat liver disease (NAFLD), and cardiovascular events, whereas moderately high PRL levels correlate with metabolic protection in all these instances (Table 1).

Moderately high PRL levels (16–35 μg/L) associate with lower prevalence of T2D and even predict a reduced incidence of T2D 10 years later (23). PRL levels in the 4th quartile correlate with lower incidence (23, 25, 29) or prevalence (24, 26–28, 30, 42) of T2D (Table 1), and PRL levels are inversely related to fasting glucose levels and glycosylated hemoglobin (HbA1c) values (4, 25, 26, 28, 31, 35, 36) in both men and women. Consistently, high serum PRL in pregnancy predicts a lower risk of postpartum prediabetes/diabetes (29), and in women with gestational diabetes mellitus, lower PRL levels at 6 to 9 weeks postpartum associate with a higher future risk of developing T2D in a 10-year follow up (30) (Table 1). T2D and other metabolic alterations derive from insulin resistance, i.e., the inability of insulin to activate a normal insulin response on its target cells. Moderately elevated PRL levels associate with increased insulin sensitivity in men (2, 3, 5, 26, 31), women (3, 5, 26, 31, 33, 34) and even children (32) (Table 1).

Insulin resistance can derive from AT dysfunction and occur in parallel to β-cell dysfunction. High PRL levels associate with reduced AT dysfunction and predict smaller adipocytes (reduced hypertrophy) in visceral AT (2, 3, 5, 6, 34), the type of fat that, in excess, associates with metabolic alterations and disease severity (43–46). Regarding β-cell function, pregnant women with high PRL levels have a lower postpartum risk of developing diabetes and β-cell dysfunction (29), and women with polycystic ovary syndrome (PCOS) with PRL levels in the 4th quartile show lower prevalence of β-cell dysfunction (33) (Table 1).

The MS represents a group of alterations that elevate the risk of cardiovascular disease, stroke, and T2D, and consists of high blood pressure, hyperglycemia, abdominal obesity, and abnormal cholesterol and triglyceride levels (47). Moderately high PRL levels associate with lower prevalence of MS in children (32) and in adult patients suffering from certain conditions, such PCOS in women (38), and sexual dysfunction (SD) in men (36, 37). Also, high PRL levels in men with SD are associated with protection from major cardiovascular events (40). However, in the general adult population a correlation between PRL and MS has not been found (3, 25). When only dyslipidemia is evaluated, an inverse association occurs between PRL levels and total cholesterol, LDL cholesterol, and triglyceride levels (4, 5, 38, 39).

Another parameter closely linked to metabolic disease is a proinflammatory environment. In subjects with obesity, moderately high PRL levels associate with lower levels of interleukin 6 in children (32) and tumor necrosis factor-α (TNF-α) in adults (4).

Most studies in humans show that moderately high PRL levels are not associated with obesity itself, the exception being a study in children (32). This observation can be explained by the fact that some subjects with obesity remain metabolically healthy (metabolically healthy obesity - MHO), or at least show fewer metabolic alterations. Indeed, subjects having MHO have

increased circulating PRL levels as compared to those with metabolically unhealthy obesity (MUHO) (4–6). Moreover, logistic regression analysis showed PRL as an independent predictor of MHO (6). Patients with obesity and high PRL (HP) levels displayed reduced blood glucose, total and LDL cholesterol, triglyceride, and TNFα levels than patients with obesity and normal PRL (NP) levels. Also, after sleeve gastrectomy, patients in the HP group showed reduced PRL levels, whereas those in the NP group have increased PRL levels (4). Similarly, patients with OW/OB with higher PRL levels had a better metabolic profile than those with lower PRL values. Interestingly, PRL levels decreased once metabolic parameters improved following bariatric surgery (5) (Table 1). These studies support that increased PRL levels are protective against metabolic diseases and return to basal values after the metabolic challenge is resolved (Figure 1).

Another metabolic disease associated with low PRL levels is NAFLD. Patients with NAFLD show lower PRL levels than control subjects and those with severe hepatic steatosis have even lower PRL values than patients with a mild to moderate disease (41) (Table 1). Moreover, PRL levels are part of a mathematical model to diagnose the presence and severity of NAFLD (48).

The association between low PRL levels and higher prevalence of metabolic diseases also stands for postmenopausal women and middle-aged and elderly men (23, 36), implying its independence from gonadal status. Because PRL levels may decrease with aging, it remains to be determined whether the HomeoFIT-PRL range differs between young vs. middle-age or elderly individuals.

The right prolactin levels for metabolic maintenance and protection – *not too much and not too little*

While low and very high PRL levels have deleterious metabolic consequences, a specific range of PRL values is beneficial for metabolism. This PRL range includes levels in the normal physiological range (7 to 25 µg/L) but also levels above (25 to 100 µg/L). The latter, previously claimed as hyperprolactinemia, have been defined as HomeoFIT-PRL (Homeostatic Functionally Increased Transient Prolactinemia) (1), since they occur in response to physiological or pathological challenges and respond to it by favoring metabolic homeostasis (Figure 1).

TABLE 1 Moderately high PRL serum levels associate with lower incidence of metabolic disease.

Metabolic disease	Population	PRL level associated with lower disease incidence or prevalence (µg/L)
T2D	Women	>15.8 (23), 18.4 (24)
	Women & men	>12.9 (25), >11.5 (26), Q4 (27, 28)
	Pregnancy	>115 Lower postpartum risk (29)
	Women w/GDM	>78.7 postpartum, lower risk of future T2D (30)
Insulin resistance	Men	≥12.0 (2)
	Women & men	≥12.0 (3), >11.5 (26)
		Inverse association with PRL levels (31)
	Children	7.9 (32)
	Women w/PCOS	>14.9 (33), Inverse association with PRL levels (34)
	Women & men w/obesity	Inverse association with PRL levels (5)
Fasting glucose levels & HbA1c	Women w/T1D	Inverse association with PRL levels (35)
	Women & men w/obesity	19.2 (6)
	Women & men	30.5 (4), >11.5 (26), >12.9 (25), Q4 (28)
		Inverse association with PRL levels (31)
MS	Children	7.9 (32)
	Men w/SD	>11.1-35 (36), Inverse association with PRL levels (37)
	Women w/PCOS	>7.0 (38)
Adipose tissue dysfunction	Women & men	≥12.0 (3)
	Men	≥12.0 (2)
	Women w/PCOS	Inverse association with PRL levels (34)
	Women & men w/obesity	19.2 (5, 6)
Metabolically unhealthy obesity	Women & men w/obesity	19.2 (5, 6)
	Women & men	30.5 (4)
Beta cell dysfunction	Pregnancy	>115 Lower postpartum risk (29)
	Women w/PCOS	>14.9 (33)
Dyslipidemia	Women & men	30.5 (4)
	Women & men w/obesity	Inverse association with PRL levels (5)
	Women w/PCOS	>7.0 (38), >15.9 (39)
Major CVE	Men w/SD	> 12 – 35 (40)
NAFLD	Women & men	>12.8 (41)

Clinical studies within the last 12 years showing an inverse association between PRL circulating levels and risk, prevalence or incidence of metabolic diseases. Abbreviations: Q, quartile; T2D, type 2 diabetes; GDM, gestational diabetes mellitus; PCOS, polycystic ovary syndrome; T1D, type 1 diabetes; HbA1c, glycosylated hemoglobin; MS, metabolic syndrome; SD, sexual dysfunction; CVE, cardiovascular event; NAFLD, non-alcoholic fatty liver disease.

In healthy individuals PRL levels are usually within the classical normal range <25 µg/L. However, some physiological challenges elevate PRL in a transient manner, such as intense exercise, acute stress, sleep, and sexual arousal (49). These conditions together with reproductive states (pregnancy and lactation) can be categorized as conditions that trigger a homeorhetic response, meaning the orchestrated or coordinated control of body metabolic tissues necessary to maintain a physiological state (defined by Bauman and Currie) (50). Moreover, the association between moderately elevated PRL levels and a beneficial metabolic phenotype supports elevated PRL levels in obesity as part of a homeorhetic response occurring both, under physiological and pathological challenges (Figure 1).

Altogether, PRL levels ranging from 7 to100 µg/L are beneficial for metabolism. PRL values are in the lower end of this range under healthy physiological conditions (outside reproductive states); however, in the context of a metabolic challenge they are likely to increase towards maintaining metabolic homeostasis and return to basal when the stressor/challenge is eliminated. Conversely, patients experiencing a metabolic challenge, such as obesity, that are unable to respond by increasing PRL levels, are more prone to suffer from metabolic alterations than those upregulating their PRL levels (Figure 1).

Elevated PRL levels derived from prolactinomas are not part of a response to a metabolic challenge, they result from a diseased state (tumor) and are not considered HomeoFIT-PRL (and are usually above 100 µg/L). It is expected that normalization of PRL levels in subjects with prolactinomas associate with a healthier metabolic profile, if the PRL levels achieved by the treatment remain in the healthy range (>7µg/L).

Mechanisms mediating the beneficial metabolic action of prolactin

PRL actions favoring metabolism are the result of its pleiotropic action reflected by the presence of the PRLR in almost every tissue in the body, including the main metabolic organs —pancreas, liver, adipose tissue, muscle, intestine, and hypothalamus— where beneficial metabolic actions and mechanisms of PRL have been described (51, 52).

Pancreatic β-cells

PRL stimulates the proliferation and survival of β-cells (53, 54), promotes glucose-induced insulin secretion (53), stimulates pancreas development during the perinatal stage (55), and is essential for β-cell expansion during pregnancy (18, 19, 56). The mechanisms that mediate PRL effects on β-cells involve increased osteoprotegerin synthesis, leading to the inhibition of receptor activator of NF-kB ligand pathway, an inhibitor of β-cell proliferation (57); increased survivin levels (58), elevated expression of the transcription factors Foxm1 and MafB, increased cyclin activity, and higher islet serotonin production

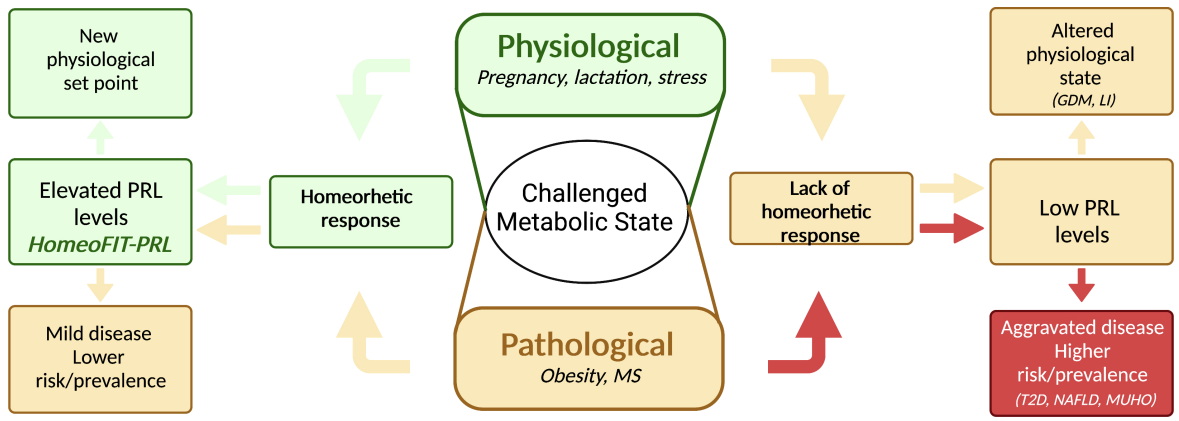

FIGURE 1
Elevated prolactin levels are part of a homeorhetic response upon metabolic challenges. A challenged metabolic state can be either physiological or pathological; in both cases a homeorhetic response includes elevated prolactin (PRL) levels, allowing a series of metabolic adaptations to deal with the physio-pathological demand. In a physiological challenge, such as pregnancy, lactation, or stress, this response leads to a new physiological set point (green arrows), whereas in a pathological challenge, such as obesity, it leads to a milder disease or protection from disease risk (yellow arrows, left side of figure). If the homeorhetic response fails, PRL levels do not rise and remain low instead, leading to altered physiological states (i.e., gestational diabetes mellitus, GDM, lactation insufficiency, LI, anxiety) (yellow arrows, right side of figure), or to aggravated disease with higher disease risk or prevalence (red arrows, right side of figure). MS, metabolic syndrome, T2D, type 2 diabetes, NAFLD, non-alcoholic fatty liver disease; MUHO, metabolically unhealthy obesity. Created in BioRender.com.

via Tph1 synthesis, all promoting β-cell proliferation (18, 56). Also, PRL leads to the inhibition of extrinsic and intrinsic apoptosis pathways (54) and improved glucose sensitivity through increased glucokinase and glucose transporter 2 expression (19, 59, 60) (Figure 2).

Liver

PRL regulates liver growth (61) and liver metabolic function. Increased PRLR expression in liver stimulates both liver and systemic insulin sensitivity, whereas reduced hepatic PRLR expression results in tissue and whole-body insulin resistance (14). Also, PRL reduces hepatic lipid accumulation by inhibition of the expression of the fatty acid transporter CD36 and the lipid synthesis enzyme, SCD1 (41, 62). Consistently, there is an inverse association between PRL levels and hepatic CD36 expression, and the PRLR decreases in the liver of patients with NAFLD (41). Thus, PRL prevents fatty liver disease. Mechanistically, the activation of STAT5 downstream of the PRLR mediates the insulin sensitizing effects of PRL (14). PRLR interacts with IRS1 (63) and promotes the phosphorylation of AKT (64), two key members of the insulin signaling pathway. Upregulating the hepatic PRLR in combination with systemic insulin treatment enhances the phosphorylation of the insulin receptor and of AKT in mouse liver, whereas reducing the expression of the PRLR by adenovirus-shRNA impairs insulin-induced liver phosphorylation of IR and AKT (14) (Figure 2). Moreover, the PRLR is regulated by the level of hepatic insulin resistance/sensitivity, i.e., it is downregulated in insulin resistant conditions and upregulated in insulin sensitive states (14).

Adipose tissue

PRL acts on the AT to regulate lipid metabolism and promote adipogenesis and healthy AT expansion (65). PRL inhibits lipid uptake *via* reduced lipoprotein lipase activity in human fat (66) and inhibits lipolysis in rat and human AT (67). PRL contributes to adipocyte differentiation in the adipocyte cell lines NIH-3T3 and 3T3-L1, by stimulating the activation of STAT5, and of the adipogenic transcription factors C/EBPb and PPARg (68, 69). PRL is essential for brown fat formation and activity in newborn mice, and for brown preadipocyte differentiation (70). The PRLR is present in AT from rodents and humans and PRL is secreted by human AT (65, 66, 71), while obesity decreases PRL release from human fat (67). In PRLR null mice, there is either decreased or no change in fat mass (2, 72–74) depending on age, fat depot, and genetic background. C57BL/6 PRLR null mice fed an HFD, show increased adiposity and exacerbated adipocyte hypertrophy in AT (2). In obese rats, PRL treatment stimulates the healthy expansion of AT by promoting adipocyte hyperplasia and reducing visceral adipocyte hypertrophy, *via* increased expression of transcription factors PPARg and Xbp1s, both favoring adipogenesis and insulin sensitivity (2) (Figure 2).

Hypothalamus

PRL promotes insulin sensitivity, at least in part, by central actions on the hypothalamus. Increased PRLR expression in the hypothalamus stimulates whole body insulin sensitivity, whereas reduced PRLR expression results in insulin resistance and glucose intolerance (75). PRL effects on the hypothalamus lead to vagal signals that promote increased liver insulin sensitivity (75). Also, in 90% pancreatectomized rats, intracerebroventricular infusion of PRL increases liver insulin sensitivity, inhibits β-cell apoptosis, and reduces body weight and adiposity by increasing hypothalamic dopamine levels and leptin signaling (76) (Figure 2).

Prolactin elevating drugs in the treatment of metabolic diseases

Several drugs elevate PRL circulating levels, mainly those that act as dopamine D2 receptor blockers, including first- and second-generation antipsychotics and medications treating gastrointestinal symptoms, antidepressants, antihypertensives, and others (77, 78). The use of antipsychotics has been associated to the development of metabolic alterations; however, a recent meta-analysis, evaluating the metabolic actions of 18 antipsychotics in around 26,000 patients with schizophrenia (79), showed a large variation in the metabolic side-effects of antipsychotics. Some drugs had clear adverse effects increasing body weight, triglyceride levels, cholesterol levels, and glucose levels (olanzapine, clozapine, and quetiapine), while others showed neutral or even positive metabolic outcomes, with very mild or no effects on body weight and triglyceride levels, and some reducing LDL cholesterol and glucose levels (aripiprazole, brexpiprazole, cariprazine, lurasidone, ziprasidone and amisulpiride). Regarding the effect of these drugs on PRL levels (77), some of the drugs exerting beneficial metabolic actions present a moderate to high risk for elevating PRL levels (77), whereas the drugs causing adverse metabolic actions have minimal to moderate risk for elevating PRL levels (77). This and other studies (80, 81) support those metabolic adverse effects derived from treatment with antipsychotic drugs not being associated with elevated PRL levels. Attention on drugs that exert beneficial metabolic effects by elevating PRL to HomeoFIT-PRL levels with negligible adverse actions is warranted.

One example is amisulpiride, a D2/D3 antagonist shown to reduce glucose levels in humans (79) and in diet-induced obese mice (82). The proposed beneficial metabolic action of

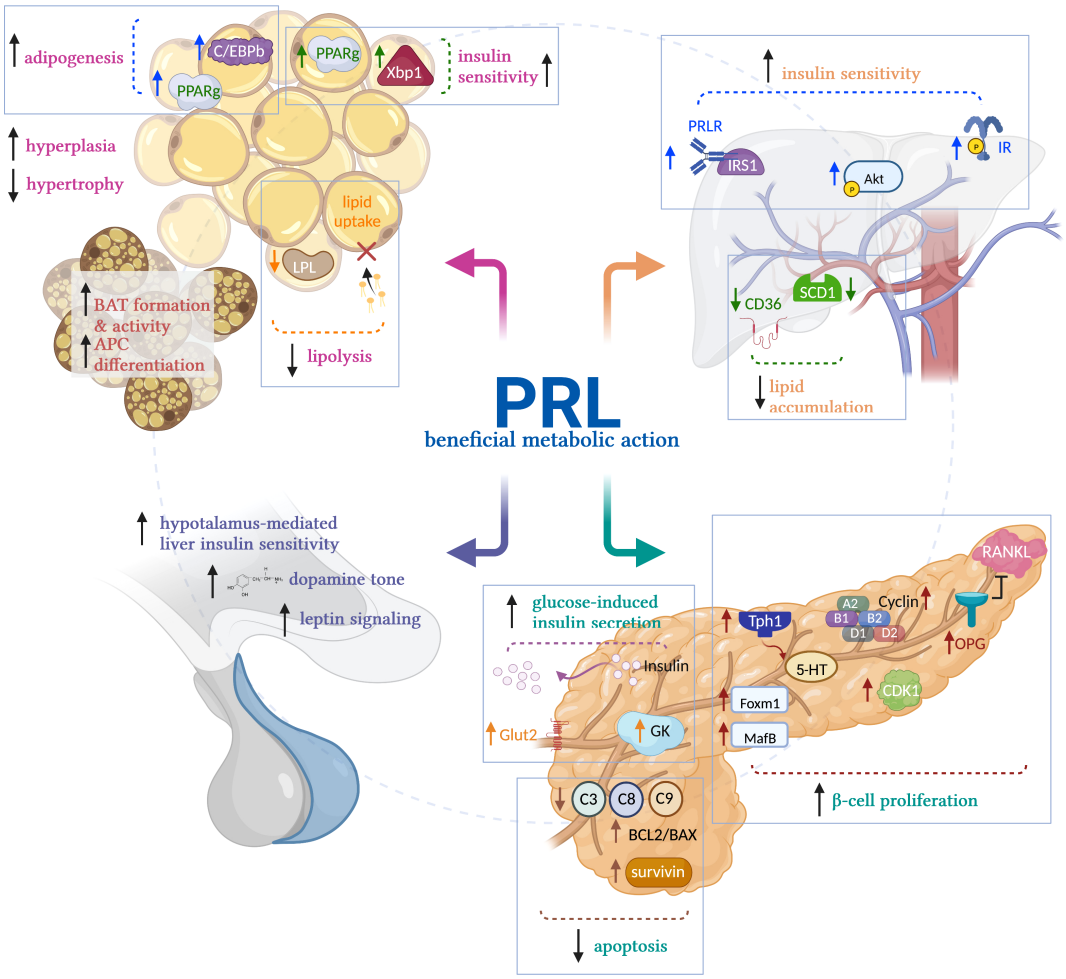

FIGURE 2
Mechanisms of prolactin's beneficial metabolic actions. Prolactin (PRL) promotes metabolic homeostasis acting on the main metabolic tissues. In white adipose tissue, PRL reduces adipocyte size by stimulating lipolysis and reducing LPL activity, preventing lipid uptake. Also, it stimulates insulin sensitivity by activating PPARg and Xbp1s and promotes adipogenesis by activating CEBP/b and PPARg, favoring the healthy expansion of adipose tissue by hyperplasia vs hypertrophy in obesity conditions. In brown adipose tissue (BAT), PRL promotes adipocyte differentiation and BAT formation and activity in newborns. In liver, PRL promotes insulin sensitivity by its canonical signaling STAT5, and by activation of IRS1 and AKT. PRL also reduces liver lipid accumulation by reducing the activity of SCD1 and CD36, preventing aggravated fatty liver in NAFLD. In pancreas, PRL promotes β-cell proliferation, inhibits their apoptosis, and elicits glucose-induced insulin secretion. In hypothalamus, PRL promotes dopamine release and stimulates leptin signaling, inducing hypothalamus-mediated liver insulin sensitivity. LPL, lipoprotein lipase; PPARg; peroxisome proliferator-activated receptor-g; Xbp1s, spliced form of X-box-binding protein-1; CEBP/b, CCAAT/enhancer-binding protein beta; PRLR, prolactin receptor; IR, insulin receptor; IRS1, insulin receptor substrate 1; AKT, Protein kinase B; SCD1, stearoyl-CoA desaturase 1; CD36, fatty acid translocase; Tph1, tryptophan hydroxylase 1; 5-HT, serotonin; OPG, osteoprotegerin; RANKL, receptor activator of NF-kB ligand; Foxm1, forkhead box M1; MafB, MAF BZIP transcription factor B. Created in BioRender.com.

amisulpiride at low doses involves increasing dopaminergic activity by preferentially blocking presynaptic D2/D3 receptors (83). Also, amisulpiride seems to stimulate insulin secretion by pancreatic β-cells (82). Therefore, given the positive metabolic effects of amisulpiride at low doses and its capacity to increase PRL levels, it is worth testing whether this and other benzamides can improve metabolic outcomes in obesity conditions.

Another benzamide, levosulpiride, is being tested in a clinical trial on patients with diabetic retinopathy and diabetic macular edema to elevate PRL levels and favor its conversion into vasoinhibin, the antiangiogenic, anti-vasopermeability PRL-derived fragment (84). The results of this clinical study raise the possibility to explore the potential therapeutic benefits of levosulpiride on obesity-derived metabolic alterations.

The fact that bromocriptine quick release (Cycloset), a PRL-lowering drug, is an FDA-approved treatment for T2D questions the association between low PRL levels and high prevalence of T2D. This controversy can be explained by the fact that dopamine and PRL act through different mechanisms to promote metabolic homeostasis. There is a morning surge of

dopaminergic activity in the central nervous system that lowers insulin resistance and hyperglycemia, and this surge is reduced in patients with T2D (85). Accordingly, by counteracting such reduction, treatment with bromocriptine benefits glucose homeostasis. Also, bromocriptine increases glucose tolerance in diet-induced obese mice that are PRL deficient (86). Whether normalizing PRL levels in bromocriptine-treated patients leads to further metabolic improvements is unclear and needs to be investigated.

Conclusions and future perspectives

PRL is present in the circulation throughout life and, particularly in humans, its levels are comparable between sexes, highlighting the role of PRL in physiology beyond reproduction. PRL senses the metabolic status of an individual, and upon physiological and pathological metabolic challenges its levels rise as part of an homeorhetic response, allowing organisms to adequately adjust to such demands. On the other hand, the inability to elevate PRL levels in challenged conditions aggravates metabolic diseases and alters physiological outcomes.

Key questions remain to be addressed such as: *1*) What are the signals that increase PRL levels in metabolically healthy individuals and what prevents such elevations in metabolically unhealthy individuals? *2*) Does the pharmacological elevation of PRL levels in metabolically unhealthy individuals improve their health outcomes? *3*) Are changes in PRL (either decreased or elevated levels) in metabolic diseases part of a larger cascade of altered responses? and, if so, what is the upstream or leading regulator of the cascade? *4*) What and how is the PRLR regulated in different physio-pathological conditions and a tissue-specific manner?

Future studies should focus on answering these questions, evaluating the benefit of PRLR-specific agonists, and carefully testing whether the current D2 receptor antagonists at low doses may be useful in the treatment of metabolic diseases due to their PRL-elevating properties. Understanding the underpinnings of PRL actions on metabolism in physiological and pathological conditions will help target this hormone to improve health outcomes.

Author contributions

YM wrote manuscript. XR-H prepared figures. XR-H, DV-C, GR-H, GE and CC reviewed, edited, and approved manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Is prolactin receptor signaling a target in dopamine-resistant prolactinomas?

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The hypothalamic neuroendocrine catecholamine dopamine regulates the lactotroph function, including prolactin (PRL) secretion, proliferation, and apoptosis. The treatment of PRL-secreting tumors, formerly known as prolactinomas, has relied mainly on this physiological characteristic, making dopamine agonists the first therapeutic alternative. Nevertheless, the group of patients that do not respond to this treatment has few therapeutical options. Prolactin is another physiological regulator of lactotroph function, acting as an autocrine/paracrine factor that controls PRL secretion and cellular turnover, inducing apoptosis and decreasing proliferation. Furthermore, the signaling pathways related to these effects, mainly JAK/STAT and PI3K/Akt, and MAPK, have been extensively studied in prolactinomas and other tumors as therapeutic targets. In the present work, the relationship between PRL pathophysiology and prolactinoma development is explored, aiming to comprehend the value of PRL and PRLR-associated pathways as exploratory fields alternative to dopamine-related approaches, which are worth physiological characteristics that might be impaired and can be potentially restored or upregulated to provide more options to the patients.

KEYWORDS

prolactinomas, PRL, PiNETs, PRL receptor, JAK/STAT, PI3K/AKT

1 Introduction

Pituitary neuroendocrine tumors (PitNETs), formerly pituitary adenomas, are systematized according to the 2022 WHO classification, accounting for the expression of transcription factors, hormones, and biomarkers. Lactotroph tumors (commonly referred to as prolactinomas) are a type of Pit-1-linage PitNET characterized by the presence of PRL, either in paranuclear dot-like expression ("Sparsely granulated lactotroph tumor") or a diffuse cytoplasmatic manner ("Densely granulated lactotroph tumor"). Other PitNET-expressing PRL includes the Mammosomatotroph tumor, the Mature plurihormonal PIT1-lineage, the Immature PIT1-lineage tumor, and the Acidophil stem cell tumor and Mixed somatotroph and lactotroph tumor (1).

The present review will discuss the relationship between PRL and the pathogenesis of PRL-related neuroendocrine tumors, focusing primarily on non-aggressive lactotroph tumors, aiming to identify targets for upcoming treatment strategies.

Lactotroph PiNETs are benign adenomas and constitute about 50% of pituitary tumors, with a prevalence that ranges from 25 to 63/100000, depending on the region reported (2), and an annual incidence of 4 new cases per 100,000 inhabitants (3).

The clinical consequences of lactotroph adenomas are concomitant hyperprolactinemia and mechanical compression effects at the brain level exerted by the presence of the tumor. These aspects have been previously reviewed, and the reader can refer to Melmed et al. (5)or Karavitaki (3) for further details.

The first-line treatment, dopamine receptor 2 (D2R) agonist administration, reduces PRL levels and tumor size. The Endocrine Society recommends administering cabergoline to treat hyperprolactinemia in patients presenting macroadenomas (4). However, 20%-30% of patients do not respond to treatment (2, 4–6).

The pathogenesis of PRL-secreting PiNETs has been extensively investigated. Two germline mutations induce familial prolactinomas: MEN1 and AIP mutations (5). However, spontaneous pituitary adenomas are the most prevalent, and these tumors' pathophysiology remains elusive. Being dopamine a natural inhibitor of PRL secretion and lactotrophproliferation; and the dopamine pathway a successful target, most efforts have been made to understand the pathophysiology of the dopamine and dopamine-associated pathways, such as the biology of dopamine receptors, extracellular-regulated mediators such as TGF-beta or intracellular signaling pathways such as ERK1/2 (7–9).

Nevertheless, in this interconnected network of neuroendocrine, endocrine, and local factors crosstalk, PRL is the precise outcome, but that could also be an initiator or intermediate player. So, this review will summarise the current knowledge about the relationship between PRL and pituitary physiology and aim to identify PRL's role in the pathophysiology of prolactinomas. Is PRL a pro or anti-prolactinoma factor?

2 Prolactin and prolactinomas: A retrospective viewpoint

Hypothalamic neurons of the tuberoinfundibular (TIDA) and tuberohypophysial dopamine systems express PRL receptors (PRLR), so they are sensitive to changes in circulating levels of this hormone. Circulating PRL reaches the arcuate nucleus and stimulates the synthesis and activity of the tyrosine hydroxylase in the TIDA neurons, which increases dopamine release to the portal system, inhibiting the secretory activity of lactotrophs. This way, PRL regulates its synthesis and release by controlling hypothalamic dopamine secretion.

At the pituitary level, PRL can induce a negative feedback control strategy. Prolactin inhibits its production and secretion (10), and as it will be discussed later, it inhibits cellular proliferation. Notably, this effect contrasts PRL in many other target tissues, such as the mammary gland, lymphoid cells, pancreas, or the prostate, where the main physiological action of PRL is pro-proliferative. Prolactin has been implicated in tumorigenesis in some tissues, like the mammary gland and prostate (reviewed in (11)), and others, such as glioblastomas.

So, in the context of prolactinomas, what has sounded intuitively comprehensive has been that PRL, being elevated in the pathological context, could be contributing to prolactinoma proliferation and creating a positive loop: high PRL levels lead to enhanced lactotroph activity and, thus, contributes to, at least, the prolactinoma progression.

Early studies proposed PRL as a growth factor in a somatotroph-derived cell line, GH3 (12). Later, it was demonstrated that PRL inhibits its transcription, controlling its production through an ultra-short feedback loop (10). Dopamine receptor 2 KO mice (D2RKO) develop pituitary hyperplasia and hyperprolactinemia. Consequently, it was proposed that those tumors were consequences of the increased levels of PRL in these animals, assigning PRL a proliferative action on pituitary cells, especially lactotrophs (13).

On the other hand, PRL Receptor KO mice (PRLRKO) present hyperprolactinemia and develop prolactinomas after 12 months of age with high penetrance (6). However, the seminal work by Schuff et al. showed that *in vivo*, constitutive double D2RKO/PRLRKO mice also exhibit prolactinomas, even significantly higher than single knockouts. This observation led to questions about whether there are independent actions of PRL on lactotroph cells (13).

The same group explored the effects of PRL in cultured lactotroph cells from wild-type and D2RKO mice, as they hypothesized a dopamine-independent PRL effect. They observed that PRL treatment reduces the proliferative index of lactotroph proliferation from wild-type female animals, whereas PRL has little effect in cultured lactotrophs derived from hyperprolactinemic D2RKO animals. Another exciting aspect is that although cabergoline restores circulating PRL levels in PRLRKO mice, it does not induce tumor reduction, suggesting that dopamine and PRL effects can be interplaying but also have separate actions (6).

Many years later, conditional deleting of the PRLR, specifically in lactotrophs, showed no effect on PRL levels, and the authors did not observe changes in pituitary size. The deletion was achieved in 20% of pituitary cells leading to a qualitative reduction in one of the PRLR-mediated signaling activation, pSTAT5. Interestingly, these mice presented an elevated dopamine tone, suggesting a strengthening in the inhibitory input as a compensatory mechanism of the constitutive deficiency of PRLR inhibitory effect in lactotrophs (14).

So far, all these backgrounds suggested that 1) PRL can exert an effect on lactotrophs inhibiting proliferation, 2) That effect is independent of dopamine, and 3) In a hyperprolactinemic context, this physiological mechanism could be impaired.

Apart from the knockout mouse models described above, other evidence suggested that PRL could be implicated in regulating lactotroph cell turnover. In rats, two-week treatment with estradiol leads to hyperprolactinemia. Although pituitary hyperplasia is observed in this animal model, the apoptotic rate of hyperprolactinemic estradiol-treated rats is higher than control ovariectomized females (15). Although the role of dopamine and estrogens themselves could not be excluded at the time, the presence of PRL and an elevated apoptotic rate was suggestive of a relationship between PRL and the regulation of pituitary turnover.

Nevertheless, a question remained elusive: Does PRL act directly on lactotrophs through PRLR activation?

3 Prolactin effects on pituitary lactotrophs: Evidence for direct effects

Apart from the knockout mouse model and the chronic estradiol treatment described above, other evidence suggests that PRL can be implicated in regulating lactotroph cell turnover *in vivo*. One is that the induction of acute hyperprolactinemia by PRL injection leads to a decrease in pituitary proliferation and an increase in the apoptotic rate, particularly in lactotroph cells. The same is observed when hyperprolactinemia is induced by acute treatment with a D2R antagonist. This evidence illustrates a possible dopamine-independent effect of PRL on lactotrophs (16).

The implication of a PRLR-mediated effect of PRL was further confirmed in male and female transgenic mice constitutively expressing a PRLR antagonist. Both males and females that lack PRLR activation either by the presence of a PRLR antagonist or by lacking PRLR (e.g., PRLR KO mice) present pituitary hyperplasia and altered proliferation and apoptotic rates (16, 17).

Interestingly, circulating hormones regulate anterior pituitary cell proliferation and apoptotic rates in female rodents. The proestrus seems to be an essential regulation point of cellular homeostasis at the pituitary level. Estradiol, TNF-Alpha, FasL, and dopamine induce apoptosis, particularly during this estrous cycle stage. The highest proliferative rate occurs in estrus, whereas the highest apoptotic rate occurs in proestrus, leading to a balance in the apoptosis/proliferation rate in the tissue. This apoptosis peak coincides with the PRL peak and is absent in PRLRKO females, even before tumor formation (around 6 months old), although hyperprolactinemia has been evident since early ages (6, 13). Thus, a cumulative lack of PRLR-dependent apoptosis could explain the later pituitary hyperplasia in this animal model (16).

The alteration of low but recurrent apoptotic rates was also observed in females where the PRLR was constitutively antagonized. These mice also present an altered proliferation rate and develop pituitary hyperplasia (16).

Studying autocrine factors can be challenging since adding the agonist to a system already exposed to that factor can mask some effects, pushing the system to non-physiological conditions. So, it was not until later, with the use of a PRLR antagonist, that question could be further clarified (18).

The inhibition of the PRLR activation by locally produced PRL showed that local PRL acts as a proapoptotic and antiproliferative factor in both primary cultures and the tumor-derived GH3 cell line (16, 17).

This body of evidence supports the physiological Role of autocrine/paracrine PRL in modulating cell turnover homeostasis and that alterations in this mechanism could lead to enhanced pituitary tumorigenesis.

4 Mechanism of action of PRL in Lactotrophs

PRL acts through a receptor belonging to the class I cytokine receptor group, a group of transmembrane-step proteins that share conserved sites in the extracellular and intracellular domain and do not possess intrinsic tyrosine kinase activity (19). Alternative processing of the primary transcript of the PRLR gene gives rise to different isoforms, which differ in the length of the amino acid chain of the intracellular portion but share identical extracellular portions and transmembrane domains (19–21). These isoforms are called long and short (or several types of short isoforms depending on the species) because of the length of their intracellular portion (358 and 57 amino acids, respectively) (19). The long isoform contains the *box* 1 and 2 regions, while the short isoforms lack the latter (22, 23).

The phosphorylation of PRLR depends on the binding of the intracellular portion of PRLR to intracytoplasmic kinases. PRLR is constitutively associated with proteins in the Janus kinase family, specifically, the JAK2 protein. Phosphorylated tyrosine residues possess the ability to bind transcription factors with SH2 domains, such as the family of transducer and transcription activator proteins (STAT, *signal transducer and activator of transcription*). After being phosphorylated, STAT proteins translocated to the nucleus and modulate the expression of specific genes (11, 20). The STAT family of proteins includes STAT 1, 3, and 5, and the latter is most often associated with the PRLR signaling pathway (18, 24). While all class I cytokine receptors can recruit proteins from the STAT family, the specificity of signaling occurring by binding a specific ligand to a given receptor is given by the subset of STAT proteins that each receptor recruits. Thus, it has been postulated that signaling through JAK2/STAT5 would be the specific pathway of the PRLR (24). Other proteins with the SH2 domain can be recruited by PRLR, such as the socs family proteins, SOC1-SOC7, and CIS (20). These PRL-induced proteins bind to and inhibit JAK2 activity by forming JAK-SOCS or JAK-SOCS-PRLR complexes. In addition, PRL induces the expression of the protein inactivator of *activated STAT* (PIAS). These proteins exert negative feedback by inhibiting the JAK/STAT signaling pathway, inhibiting PRL signaling. In addition to the JAK/STAT pathway, PRLR is very well known to activate other signaling pathways such as MAPK, Src (21), phosphoinositide-3 Kinase (PI3K)/Akt (25), or Nek3-vav2-Rac1 (22).

Since the JAK2 protein is associated with the intracellular portion proximal to the membrane, both LPRLR and SPRLR can bind to this enzyme. However, only the long isoform is phosphorylated by the activation of JAK2 since the tyrosine residues of the receptor susceptible to being phosphorylated in the terminal C portion of the PRLR are not present in the short isoform of the receptor (20). Therefore, PRL can activate or inhibit other pathways, such as MAPK and phosphatidylinositol 3 kinase (PI3K), without recruiting STAT proteins (21, 26, 27). In breast cancer cell-derived cell lines, PRL activates both Src family kinases and the JAK/STAT, as well as PI3K/Akt and MAPK signaling pathways. Whereas activation of MAPK occurs independently of STATs protein recruitment, it depends on JAK activation with PI3K as an intermediate cascade (26). In the ovary, PRL activates ERK1/2 and p38 MAPK independently of the JAK/STAT pathway by specific activation of the short isoform of the receptor (28). Hepatocytes express the PRLR short isoform in rodents (29, 30), and PRL inhibits the MAP3K-/c-Myc pathways in these cells Since the PRL action is mediated by that isoform of the PRLR (31), whereas other actions are mediated by the PRLR Long/JAK/STAT5 pathways (32, 33).

Adding to the complexity of the PRL/PRLR isoform and signaling puzzle, the expression of PRLR can be modulated by endocrine

factors. Apart from sex differences, in hormone-responsive tissues, the expression of PRLR is variable in either reproductive stages or along the sexual cycle (16, 24, 28, 29, 34–36).

The rat, mouse and human adenohypophysis express both isoforms of PRLR (16, 29, 37–39). While the ratio of LPRLR to SPRLR isoforms is approximately 13:1 in males, it is variable along the estrous cycle in females, and the PRLR expression is higher in diestrus, with changes in the ratio that varies from approximately 36:1 in diestrus to 1:1 in proestrus (16, 17, 29).

Since both LPRLR and SPRLR isoforms are expressed in the pituitary, either isoform could mediate the effect of PRL action in lactotrophs. In this regard, a study showed that mice lacking the LPRLR isoform present high serum prolactin levels. This indicates a partial impairment in the negative feedback mechanism acting in the hypothalamus and the pituitary, supporting a role for the long isoform of the PRLR in controlling PRL levels (22).

5 Prolactin, prolactin receptor, and signaling pathways associated with the control of cellular turnover

The lactotroph function is controlled by several intracellular pathways controlling hormone production, secretion, and cell survival.

Prolactin gene expression is modulated by various signals, stimulatory such as estradiol and inhibitory such as dopamine, that Converge in several signaling pathways such as the AMPc/PKA, PKC, or MAPK pathways (19, 40, 41). The secretion of PRL is another control point, regulated mainly through calcium-dependent-mechanisms (42, 43) which can depend on the cell's electrical activity, e.g., voltage-dependent calcium entry or signaling molecules such as IP3, initiated chiefly by $G_{q/11}$-coupled membrane receptors (44).

The specific intracellular signals that control lactotrophs' proliferation, death, and phenotype under physiological and pathological conditions also result from systemic, hypothalamic, and intrahypophyseal signals. Regardless of the signal trigger (estrogens (45–47), dopamine (8, 48), or TGF-β (9), for example), some intracellular signaling pathways have been identified as critical regulators of proliferation and apoptosis in both normal and tumoral lactotrophs. All these pathways are also susceptible to modulation by PRL.

The MAPK pathway is a pathway in which several extracellular signals converge, and particularly ERK is dysregulated in cell lines derived from prolactinomas (49, 50). The PI3K-Akt pathway is a proliferative pathway inhibited by dopamine, which also regulates the MAPK/ERK pathway, and both pathways work together, regulating cell proliferation (51). However, a Ras/MAPK mutation alone does not promote tumorigenesis in lactotroph cells (7). TGF-beta regulates transcription by recruitment of Smad proteins but also, through its so-called non-canonical pathway, regulates ERK1/2 and Jun kinases, PI3K, and Akt proteins (52, 53).

A balance between proliferation and apoptosis keeps the cell turnover. The evidence of factors controlling lactotrophs apoptosis has been less studied than the proliferative factors. Dopamine and estradiol have been extensively studied among the apoptosis factors for lactotroph cells. It was described that dopamine induces adenohypophysis cell apoptosis by activating p38 MAPK or oxygen-reactive species generated by dopamine metabolism (48, 54), by activation of the MEK/ERK1/2 pathways (55), and estrogens sensitize to cytokine-induced cell death by regulating transcription factor NFK-B (56) and protein balance of the Bcl-2 family (57). This apoptotic protein family is modulated by dopamine (58) and PRL.

The activation of PRLR leads to the phosphorylation of JAK and nuclear translocation of phosphorylated STAT5. Although PRLR-activated pathways are usually associated with cell differentiation or proliferative effects (11, 19–22), these pathways can also induce apoptotic effects. For example, STAT5 phosphorylation mediates the apoptosis of osteosarcoma-derived cells and cerebellar neurons by regulating the Bax/Bcl-2 ratio (59–61). The JAK2/STAT5-dependent balance towards proapoptotic Bax proteins leads to apoptosis in lactotroph cells (62).

PRLR downregulates MEK/Erk1/2 and PI3K/Akt pathways, leading to apoptosis and decreased proliferation (62). Furthermore, the mutation of a splicing factor, SF3B1, was associated with a bad prognosis. This mutation stimulates the PI3K/Akt pathway in prolactinomas, increasing tumor invasiveness (63). Similar pathways have been identified as therapeutic targets in prolactinoma by studying differentially expressed mRNA together with microRNAs (64).

In their recent review, Biagetti et al. identified potential therapeutic options related to relevant signaling pathways for the treatment of dopamine-resistant prolactinomas, highlighting the JAK/STAT3, PI3K-Akt-mTOR, MAPK/AMPK, and JAK2/STAT5 pathways. All of them are related to paracrine/autocrine PRL effects in the pituitary; for all, there are already described pharmacological modulators and thus are relevant pharmacological targets for potential aggressive prolactinomas. Nevertheless, no clinical trial currently assesses these therapeutic options (65).

6 Prolactin receptor expression and associated genetic alterations related to PRL-secreting adenomas

Suppose the PRLR mediates a physiological autocrine/paracrine control of the lactotroph population by PRL. In that case, mutations in this receptor are expected to be related to the formation, progression, or prognosis of PRL-secreting adenomas.

In 2013, a loss-of-function PRLR mutation was described in the extracellular domain-encoding region. The mutation was present in a family with autosomal dominant hyperprolactinemia. This mutation leads to an impairment in the JAK2/STAT5 signaling, and although no changes in the pituitary size were observed at the time of the study, this can indicate that the PRLR/JAK2/STAT5 activation can be a relevant control mechanism of lactotroph function in humans (66).

The first analysis of inactivating germline mutations of PRLR was not associated with prolactinomas concluding that most prolactinomas occur independently of germline changes in the PRLR gene (67). Nevertheless, in 2019, two germline PRLR intracellular domain variants were later associated with

prolactinoma manifestation. Interestingly, one of those variants results in the overactivation of the Akt-related pathways (68).

Although genetic mutations are not the leading cause of prolactinoma development, since PitNETs are mainly sporadic (4, 69), the studies mentioned above can shed light on the mechanisms that could be altered during the initial phases of prolactinoma development.

Since both loss-of-function and gain-of-function genetic alterations can lead to alteration in the lactotroph function, it is possible that a balance between PRLR cascades plays a role in the maintenance of lactotrophs homeostasis and that the lack of equilibrium in the intricated pathway network, as discussed previously, can lead to clinical manifestations. Given the complexity of the PRL and lactotroph turnover regulation, more efforts should be put into understanding the interconnections between receptors, isoforms, and signaling pathways to elucidate the physiological relevance of PRLR in the control of lactotroph function *in vivo*.

7 Discussion

Prolactin-secreting PiNETs that do not respond to standard treatments with dopamine agonists imply a large number of patients annually around the globe. It has been proposed that prolactinomas have a monoclonal origin (4), and although several oncogenes are overexpressed in these tumors, the pathophysiological processes that lead to the formation of prolactinomas have not yet been established (4, 7, 19). From the analysis of familial pituitary tumors, a series of oncogenes involved in tumor development have been proposed, but most prolactinomas (more than 95%) occur spontaneously, and these oncogenes do not explain their appearance (7, 70). Although progression to invasive and metastatic tumors is rare, lactotroph macroadenomas are one of the predominating types (71–73), and the mechanism leading to malignant transformation is currently unknown (74).

Since the adenohypophysis is a gland with high plasticity (75), alterations in the mechanisms that normally regulate adenohypophysis cell renewal could be involved in developing pituitary tumors (38).

The evidence presented here suggests a significant role of PRL in the pathogenesis of prolactinomas. Such implications can be considered in two main scenarios. In one scenario, alteration of PRLR-related actions locally at the pituitary level, either initiating or contributing to tumor development. The second is the effect of PRL at the hypothalamic level, controlling neuroendocrine functions, such as dopamine or potentially other hypothalamic factors, that further control the pituitary's cell physiology.

At the hypothalamic level, prolactin feedback onto TIDA neurons contribute to maintaining lactotroph homeostasis by negative feedback that restores dopamine inhibitory input to the pituitary (76). In the adenohypophysis, PRL possesses proapoptotic and anti-proliferative effects, which are critical for maintaining tissue homeostasis of the gland in rodent models, in an interplay with mainly hypothalamic factors (13, 14, 16, 17, 77). Deficiencies in PRLR signaling due to PRLR activity alterations or wrong intracellular pathway connectivity, crosstalk, or co-regulation exerted by other factors, such as hypothalamic or paracrine mechanisms, can lead to pituitary hyperplasia and eventual tumor development.

The intracellular signals that regulate the specific phenotype of lactotrophs, as well as the control of their proliferation and the death of these cells, are very little known in humans (7, 78). Approaching how prolactinomas develop from studying intracellular signaling pathways that regulate the proliferation and apoptosis of lactotrophs and the study of a physiological regulator of these pathways, PRL, is necessary to understand the pathophysiology of the development of tumors in this gland. Identifying therapeutic targets that contribute to the design of new treatments will be possible if new hypotheses are tested and efforts are currently required to understand the mechanisms in human pituitaries.

Prolactin, dopamine and other factors control lactotroph homeostasis (7, 19, 48, 52, 65). For patients where dopamine agonists are inefficient, it is worth consideringwhether the pathogenesis of those tumors is the same as in those responsive to dopamine. The field usually includes prolactinomas in a unique group in which, first, a dopamine agonist is administered, and in case of treatment failure, surgery and a very limited pharmacological toolbox are considered, although the probability of success is decreased (2, 79). Merely adding other players in the lactotroph physiological regulation may help to understand if tumors categorized as "refractory to treatment with dopamine agonists" involve a different pathophysiological mechanism.

If such factors can be identified, the exploration, for example, of PRLR or PRLR-associated pathways, not only in terms of mutations but also in gene expression regulation or modulatory molecules using high throughput technologies in patients, could help in designing a specific personalized therapy (63, 65, 67, 68),

The approach to the knowledge of how prolactinomas develop from studying physiological factors that control the intracellular signaling pathways that regulate the proliferation and apoptosis of lactotrophs is critical, and PRL is a promising candidate.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Prolactin receptor signaling: A novel target for cancer treatment - Exploring anti-PRLR signaling strategies

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Prolactin (PRL) is a peptide hormone mainly secreted from the anterior pituitary gland. PRL is reported to play a role in pregnancy, mammary gland development, immune modulation, reproduction, and differentiation of islet cells. PRL binds to its receptor PRLR, which belongs to a superfamily of the class I cytokine receptor that has no intrinsic kinase activity. In canonical signaling, PRL binding to PRLR induces downstream signaling including JAK-STAT, AKT and MAPK pathways. This leads to increased cell proliferation, stemness, migration, apoptosis inhibition, and resistance to chemotherapy. PRL-signaling is upregulated in numerous hormone-dependent cancers including breast, prostate, ovarian, and endometrial cancer. However, more recently, the pathway has been reported to play a tumor-promoting role in other cancer types such as colon, pancreas, and hepatocellular cancers. Hence, the signaling pathway is an attractive target for drug development with blockade of the receptor being a potential therapeutic approach. Different strategies have been developed to target this receptor including modification of PRL peptides (Del1-9-G129R-hPRL, G129R-Prl), growth hormone receptor/prolactin receptor bispecific antibody antagonist, neutralizing antibody LFA102, an antibody-drug conjugate (ABBV-176) of the humanized antibody h16f (PR-1594804) and pyrrolobenzodiazepine dimer, a bispecific antibody targeting both PRLR and CD3, an *in vivo* half-life extended fusion protein containing PRLR antagonist PrlRA and albumin binding domain. There have also been attempts to discover and develop small molecular inhibitors targeting PRLR. Recently, using structure-based virtual screening, we identified a few antipsychotic drugs including penfluridol as a molecule that inhibits PRL-signaling to inhibit PDAC tumor progression. In this review, we will summarize the recent advances in the biology of this receptor in cancer and give an account of PRLR antagonist development for the treatment of cancer.

KEYWORDS

PrlR, antagonist, small molecule inhibitor, immunotherapy, antibody-drug conjugate

1 Introduction

Prolactin (PRL) and its cognate receptor, prolactin receptor (PRLR), have been characterized in hundreds of biological functions, especially mammary gland development and lactation. PRL is a peptide hormone that resembles the growth hormone due to a conserved helix bundle composition. It is largely produced by the lactotrope cells of the anterior pituitary gland as a pro-hormone that undergoes proteolytic cleavage to produce a 199 amino acid active peptide (1). However, aberrant PRL levels are also observed in disease states, which may also be related to its synthesis from the affected tissues including the prostate, skin, adipose tissue, endometrium, myometrium, immune cells, brain, and breast tissues (2). It can therefore participate in paracrine and autocrine signaling functions related to cell homeostasis and growth (3). Composed of 4 parallel alpha helices, PRL, binds to PRLR *via* several residues, including Lys-69, Tyr-169, and H180 of Site 1, and Arg-24, Lys-124 within the Gly129 cavity and Glu-43 within the N-terminus of Site 2, stimulating dimerization of PRLR on the cell surface, leading to activation of canonical signaling *via* Janus kinase (JAK)-signal transducer and activator of transcription (STAT) (Figure 1) (4–8).

Extrapituitary prolactin is thought to be regulated primarily at the transcriptional and translational level. In contrast, lactotrope cells have large vacuolar stores of PRL, which can be released by calcium-dependent exocytosis. Transcription of PRL mRNA in tissues other than the pituitary is regulated by an alternative promoter upstream of the site utilized by lactotrope cells (9). Transcripts generated from alternative promotor driven transcription results in inclusion of an additional exon1a within the 5'untranslated region of the transcript. However, this does not alter the amino acids of the encoded protein (10). While pituitary PRL synthesis and release is sensitive to regulation by dopamine, typically extrapituitary PRL is not (11). An exception to this is in the context of adipocytes in which PRL is dependent on dopamine (12). The mechanisms that control expression of PRL at extrapituitary sites is poorly understood; however, the use of an alternate promoter indicates site specific regulation of PRL transcription to modulate expression, which warrants further study especially during tumorigenesis (13).

PRLR is a type 1 cytokine receptor, encoded by the PRLR gene on chromosome 5. Conserved homology permits binding by human growth hormone (GH) in addition to PRL. In humans, the PRLR gene contains 11 exons and is widely expressed throughout the body (14). PRLR can undergo alternatively splicing events resulting in the expression of several PRLR isoforms, with tissue specificity. These isoforms have modified cytoplasmic domains, but share identical extracellular domains that bind PRL. Moreover, PRLR lacks

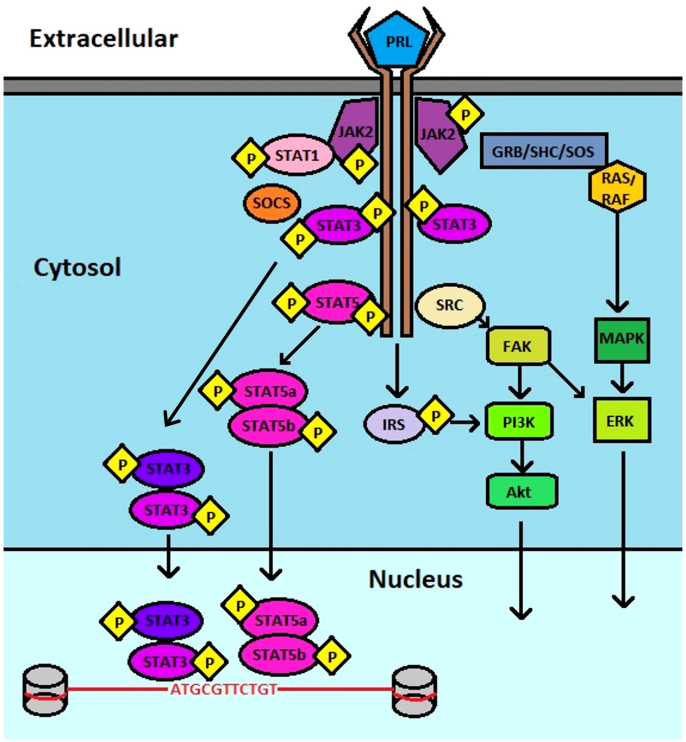

FIGURE 1
Schematic of PRL : PRLR signaling. PRL binds to PRLR, inducing JAK2 association that leads to downstream activation of multiple pathways that include STAT3, STAT5, PI3K, AKT, and ERK.

intrinsic kinase activity, thus necessitating dependency on associated kinases such as the Janus kinases (JAKs) to further transduce signaling. PRLR is a single pass transmembrane protein that has two conserved cytoplasmic regions, Box1 and Box2, which are responsible for association with JAK2 (15).

PRLR signaling plays a major role in numerous biological functions, primarily mammary gland development and lactation. However, due to the widespread expression of PRLR within tissues, aberrant activation of this signaling has been linked to progression of prostate, breast, cervical, ovarian, and pancreatic tumors (16).

High expression of PRLR and circulating PRL can drive the expression of genes involved in proliferation, migration, and invasion of cancer cells. In breast cancer, PRL-mediated JAK/STAT signaling contributes to endocrine therapy resistance in conjunction with elevated HER2, by activating oncogenic factors such as MYC, FOS, and JUN (17). This has been shown to be mediated, in part, by the estrogen independent activation of ERα by PRL, both *in vitro* and *in vivo* (18, 19). In particular, PRL has been shown to activate ERα through a PAK1 mediated mechanism, circumventing the mechanism of action of anti-estrogen therapies (20). Others have shown that PRL participates in endocrine therapy resistance through the activation of PRLR, and stimulating downstream signaling pathways that include STAT5, ERK1/2, and PI3K (20–22). With prostate cancer, PRL overexpression contributes to increased hyperplasia of prostatic tissues, thereby elevating the risk for developing adenocarcinomas. Epidemiologic studies have linked PRL and STAT5 with higher grade tumors and more aggressive disease (23). Enhanced PRLR signaling in gynecological, pancreatic, and colorectal tumors promotes metastatic potential, chemoresistance, and pro-survival signaling events (24–27). Briefly, preincubation of PRL for 1 hour abrogated cisplatin-induced apoptosis of ovarian and endometrial cancer cells, as determined by Annexin V/PI staining (27). The authors demonstrate significant activation of Ras signaling, as well as STAT3, ATF-2, MEK1, CREB, and p53 within 5 minutes of PRL stimulation (27). Interestingly, GH has been shown to induce the expression of ABC efflux transporters (ABCB1, ABCB5, ABCC1, ABCC2, ABCG1, and ABCG2), contributing to acquired drug resistance (28). Concurrently, PRL has been shown to induce the expression of ABCG2 through the activation of STAT5, leading to binding at consensus sequences upstream of the ABCG2 transcription start site (29). Moreover, the authors further demonstrated that STAT5 was required, but insufficient for PRL induced transcription, as MAPK and PI3K inhibitors also decreased PRL induced ABCG2 expression, without affecting STAT5 DNA binding (29). In our own studies with pancreatic cancer, we observed that PRLR signaling potentiated invasive cell behavior and stemness through JAK2/STAT3 and ERK phosphorylation (25). We had previously observed in colon cancer, that PRL enhanced stemness in a JAK2/STAT3/ERK dependent manner by modulating Notch signaling (26). Interestingly, in both pancreatic and colon cancer, we did not observe activation of STAT5 (25) As such, PRLR signaling plays an extensive role in human cancers, which has led to research directed towards developing therapeutic strategies to modulate activity.

Due to the strong evidence supporting the critical role of PRL and PRLR in human cancers, various approaches have attempted to modulate activity both by suppressing downstream signaling as well as by developing PRLR antagonists. These strategies will be discussed in more detail later. In brief, the use of a PRL antagonist peptide, G129R, was shown to block the PRL : PRLR signaling axis in ovarian cancer mouse models (30). This resulted in greater than 90% reduction in tumor weights compared to controls, when used in combination with the standard-of-care agent paclitaxel. A preclinical study of the anti-PRLR antibody REGN2878-DM1 suggested induction of cell-cycle arrest and apoptosis in PRLR expressing breast cancer cell lines, and also exhibited synergistic activity with fulvestrant (31). In preclinical studies with pancreatic cancer, we identified a small molecule Penfluridol to inhibit PRL induced JAK/STAT activation by competitively binding to PRLR. This resulted in suppression of cancer cell growth *in vitro* and *in vivo* (25). The efficacy of these preclinical studies demonstrates the validity of targeting PRLR while also establishing the critical role of PRL : PRLR signaling in human cancers.

In this review, we discuss the current research strategies directed towards PRL : PRLR inhibition. Due to the extensive expression of PRL and PRLR in various tissues, and the efficacy of preclinical inhibitory strategies, it is clear that the PRL : PRLR signaling axis is a critical pathway in human biology and cancers.

2 Novel approaches to target prolactin receptor

2.1 Competitive antagonists of the human prolactin

A class of inhibitors that was first developed to target prolactin-sensitive pathologies such as dopamine-resistant prolactinomas, as well as breast, prostate and pancreatic malignancies were designed to compete with endogenous PRL for PRLR binding (32). As such, these types of antagonists often require higher molar concentrations compared to endogenous PRL to ensure sufficient activity (33). Moreover, it is vital that any unintended agonistic properties are eliminated, particularly at high concentrations (33). As described previously, PRLR signaling is activated by the binding of PRL to a PRLR homodimer. This interaction is ternary in nature and has 3 intermolecular interactions referred to as sites 1-3. Site 1 and 2 interactions are between prolactin and each receptor, while site 3 is the interaction between two receptor units. Once active, this ternary complex induces various downstream signaling

pathways, including the JAK2-STAT3/STAT5 axis, MAP kinase, AKT, and Src kinase pathways (8, 34). It is this ternary interaction between PRL and PRLR that has served as the design template for the development of competitive PRLR antagonists, such as G129R-hPRL and Del1–9-G129R-hPRL, which will be discussed in detail below.

2.1.1 G129R-hPRL

G129R-hPRL was developed in the early 1990s as the result of a mutational screen of hPRL with the purpose of identifying and characterizing binding sites in PRLR. This was based on strategies that were utilized for growth hormone (GH) and its cognate receptor (GHR) that yielded the discovery of a potent GHR antagonist and drug, Pegvisomant (7, 35–39). G129R-hPRL was tested for its inhibitory activity in the NB2 rat cell proliferation assay, because PRL induces proliferation of these cells. Surprisingly, instead of being an antagonist, G129R-hPRL appeared to actually be a weak agonist, increasing the proliferation of NB2 cells rather than suppress it (7). Binding of G129-hPRL was confirmed by surface plasmon resonance; however, the affinity towards site 2 of PRLR was demonstrated to be decreased compared to WT hPRL (6, 7, 40). Based on these findings, it was concluded that the lack of antagonistic activity was due, at least in part, to poor affinity for site 2, leading to insufficient hindrance of ligand:receptor interaction. Shortly after these initial reports, several studies determined that detection of the competitive antagonistic properties of G129-hPRL was impacted by the bioassay used and species of origin (41–43). A PRL-responsive luciferase reporter assay was designed in human embryonic kidney fibroblasts (HEK293) that were transfected with a hPRLR long isoform expressing construct. Under these conditions, G129R-hPRL exerted potent antagonistic activity (6). Species specific discrepancies were also confirmed, as G129R-hPRL had reduced antagonism towards rat PRLR (6). These findings were validated by multiple groups using various hPRLR-mediated cell bioassays and breast cancer cell lines (44–47). However, conflicting results were obtained when studies were performed in Ba/F03 human cells stably transduced with hPRLR (Ba/F03-hPRLR). When stimulated with hPRL, Ba/F03-hPRLR cells exhibited increased proliferation, while G129R-hPRL failed to induce antagonistic effects, similar to results obtained previously in NB2 rat cells (48). As such, it was hypothesized that G129R-hPRL behaves as a weak antagonist/partial agonist in sensitive bioassays, while in low sensitivity assays where the levels of PRLR activation induced by G129R-hPRL is not sufficient to produce a biological effect, it acts as an antagonist (48). Despite these contradictory findings, many studies have since been performed demonstrating antagonistic activity, which are outlined below.

In a recent report, it has been shown that G129R-hPRL blocks the activity of PRL-PRLR signaling in ovarian cancer (30). The authors demonstrate that in orthotopic mouse models G129R-hPRL inhibits tumor growth in a dose-dependent manner. Moreover, prolonged treatment with G129R-hPRL at 100 µg/day resulted in a durable response, and reduced tumor weights by 50% compared to control, while in combination with paclitaxel produced more than a 90% inhibition (30). There was also no apparent off target toxicity with G129R-hPRL. In *in vitro* studies, the authors further demonstrated that G129R-hPRL did not inhibit proliferation or migration in 2-dimensional monolayer cultures of SKOV3 cells; however, in 3-dimensional spheroid cultures of HeyA8 and SKOV3 cells, G129R-hPRL abrogated cellular growth and induced apoptosis (30). Furthermore, G129R-hPRL attenuated PRL induced growth and activation of JAK2, STAT3, and STAT5 phosphorylation in HeyA8 cells, further supporting the antagonistic properties of G129R-hPRL (30).

Several groups have studied the role of PRLR in breast cancer, and in the process have observed antagonistic activity in cells treated with the G129R-hPRL analog. Chen et al. showed that G129R-hPRL treatment inhibited proliferation of T47D breast cancer cells and induced apoptosis within 2 hours of treatment at a dose of 50 ng/mL (45). In regard to PRLR signaling, Catalado et al. sought to determine the effect of G129R-hPRL on STAT3 activation, and identified that hPRL activated STAT3 preferentially compared to STAT5 in T47D breast cancer cells (47). Furthermore, the authors determined that G129R-hPRL inhibited STAT3 phosphorylation (47). This was further confirmed by others, in which G129R-hPRL attenuated PRL-induced activation of JAK-STAT and MAPK pathways (44). In breast cancer xenograft models, PRL was found to induce tumor growth of T47D and MCF-7 tumors, while G129R-hPRL inhibited growth (49). These findings provide evidence that targeting the PRL : PRLR signaling axis is feasible and that the G129-hPRL has antagonistic activity. As a result, further interest in targeting PRLR has led to several studies focused on developing G129R-hPRL fusion proteins as well as combinatorial therapeutic strategies.

A fusion protein of G129R-hPRL with Pseudomonas exotoxin A (PE40) was developed and found to competitively bind to hPRLR in T47D cells, further suppressing PRL-induced STAT5 phosphorylation and inducing caspase-independent cytotoxicity (50). In another study, G129R-hPRL was fused to endostatin, and was shown to inhibit PRL-induced signaling in T47D breast cancer cells, while further suppressing HUVEC cell proliferation, tube formation, and tumor formation of mouse 4T1 cells *in vivo* (51, 52). Tomblyn et al. have examined the combination of three G129R-hPRL based fusion proteins, which include G129R-hPRL fusions with endostatin (an angiogenesis inhibitor), interleukin 2 (immune modulator), and PE38KDEL (a truncated cytotoxin) in allografts of a mammary carcinoma cell line (McNeuA) derived from MMTC-neu mice (53). Treatment with these fusion proteins increased the number of cytotoxic CD8+ T cells in the tumor, while reducing recurrence and lung metastases (53). In similar studies conducted by Scotti et al, combining G129R-hPRL with Herceptin resulted in

suppression of STAT3 and STAT5 phosphorylation and reduced HER2 expression in T47D and BT474 breast cancer cells (54). The combination of G129R-hPRL with Herceptin also demonstrated an additive inhibitory effect on HER2 and MAPK activation and further suppressed tumor xenograft growth in athymic nude mice (54). Taken together, these studies demonstrate antagonistic activity of G129R-hPRL, despite previous confounding studies, and show the feasibility of inhibiting PRLR signaling to suppress cancer growth.

2.1.2 Δ1–9-G129R-hPRL

Due to confounding evidence of agonistic activity of G129R-hPRL, development of a second-generation PRLR antagonist was attempted, resulting in a competitive antagonist that is devoid of residual agonistic properties in cell culture and animal models (55). The Δ1–9-G129R-hPRL is a human prolactin core protein analog that has two modifications: 1) a deletion of nine N-terminal amino acid residues and 2) a glycine substitution by arginine at residue 129 (55). This second-generation PRLR antagonist was developed following findings from G129R-hPRL. Furthermore, crystal structures of ovine placental lactogen (PL), a polypeptide that shares high structural and functional similarities with PRL, and rat PRL binding protein (PRLBP) identified that the N-terminal region of PL is critical in site 2 binding of PRLR (56). This finding led to in depth analyses of the N-terminal domain in PRL biological activity (57). Multiple deletion constructs were developed including deletion of amino acids 1-9 (Δ1-9-hPRL) and 1-14 (Δ1-14-hPRL) (58). Interestingly, the Δ1-9-hPRL construct increased receptor binding affinity and biological activity, while the Δ1-14-hPRL construct decreased binding affinity and activity by modulating site 2 functionality (55, 58). Although the effects were modest, these deletion mutations were introduced into G129R-hPRL, intending to improve upon the antagonistic properties of the parent construct. Both of the double mutant analogs, Δ1-9-G129R-hPRL and Δ1-14-G129R-hPRL, exhibited similar dose-response curves in bioassays of PRLR activity. These new analogs failed to improve upon the antagonistic properties of the first-generation construct, G129R-hPRL; however, the authors did observe significant improvements related to agonistic activity. While G129R-hPRL displayed agonistic properties in sensitive bioassays, particularly Ba/F-LP and Nb2 cell proliferations assays, the new double mutant analogs failed to stimulate proliferation. These data demonstrate that the absence of agonistic activity markedly improved the second-generation antagonists.

Goffin et al. published a crystallographic structure of Δ1-9-G129R-hPRL to understand the structural and thermodynamic basis of PRLR antagonism (56). The authors reported no major structural changes compared to wild type hPRL, suggesting the pure antagonistic properties of Δ1-9-G129R-hPRL are due to intrinsic mutations and deletions (56). Moreover, they compared the physiochemical, structural, and biological properties of wild type hPRL and various variants including N-terminal or Gly129 mutations, either alone or in combination. The authors determined that human PRL activity was unaffected by N-terminal modifications; however, in the context of G129R mutants, N-terminal deletions eliminated residual agonist activity. Moreover, this was unrelated to site 1 affinity (56). Conversely, N-terminal alterations impacted biological activity only when site 2 binding was affected by G129 mutants (56). N-terminal deletions of PRL did have measurable decreases in site 2 affinity alone, as determined by SPR; however, these modifications were insufficient to eliminate biological activity indicating the critical nature of G129 to hPRL function (56). What this indicates is twofold: 1) that the N-terminus participated in site 2 binding and 2) that residual agonism of early PRL antagonists may be eliminated by further modifying the N-terminus interactions with site 2.

Several studies have employed the second-generation antagonist to dissect PRL : PRLR biology. Ferraris et al. studied the effects of Δ1-9-G129R-hPRL in the turnover of mouse anterior pituitary cells and PRLR expression *in vivo* using transgenic mice constitutively expressing the analog (59). The authors observed that the weight and proliferation index of the pituitary gland was elevated in transgenic mice expressing the antagonist compared to wild type mice (59). Moreover, *in vitro* studies showed that Δ1-9-G129R-hPRL enhanced proliferation while reducing apoptosis of GH3 cells, a somatolactotrope and primary rat anterior pituitary cells (59). These data suggest that PRL acts as an antiproliferative and pro-apoptotic factor in cells of the anterior pituitary gland. Dwivedi et al. identified hematopoietic PBX-interacting protein (HPIP) as a novel regulator of mammary epithelial cell differentiation, where Δ1-9-G129R-hPRL attenuated HPIP-mediated synthesis of PRL, activation of AKT, and synthesis of β-casein in cultured HC11 cells (60). Recently, synthesis and purification of Δ1-9-G129R-hPRL was performed by testing different activation temperature and chromatographic techniques including nickel-affinity chromatography, size-exclusion chromatography and high-performance size-exclusion chromatography (HPSEC) (61). Δ1-9-G129R-hPRL was extracted with more than 95% purity, enhanced solubility, correct folding, and without methionine, and has a significant potential in clinical application (61).

In the context of cancer, several groups have shown anti-tumor activity and suppression of PRLR signaling following treatment with the Δ1-9-G129R-hPRL antagonist. Treatment with Δ1-9-G129R-hPRL abolished the increase in nitric oxide production by prolactin-induced plasma membrane carboxypeptidase D in triple-negative breast cancer cell lines (62). It was further shown to inhibit prolactin-induced osteoclast differentiation and bone lysis in breast cancer cells (63). Similar inhibition of PRL-induced carboxypeptidase D was also seen in prostate cancer (64). In addition, Hou et al. demonstrated that while PRL increased oncogenic potential in breast cancer cells by

stimulating HOXA1, which in turn induced STAT5, ERK phosphorylation, and increased transcriptional activity of ELK1, SAP1A, STAT5A and B to increase cell proliferation, survival and anchorage dependent growth, following treatment with Δ1-9-G129R-hPRL (65). The effect of Δ1-9-G129R-hPRL induced PRLR antagonism was further studied by Howell et al. in multiple breast cancer cell lines (66). As a monotherapy Δ1-9-G129R-hPRL failed to demonstrate antiproliferative effects of the cell lines, but potentiated the effects of doxorubicin and paclitaxel when used in combination (66). Moreover, Δ1-9-G129R-hPRL inhibited the growth of colonies in soft agar and mammosphere formation supporting the rational for use in combination therapeutic strategies for breast cancer (66). Asad et al. have studied the effects of PRLR inhibition on glioblastoma multiforme (GBM) pathogenesis (67). The authors identified that PRLR was highly expressed and was further correlated with poor survival in GBM patients (67). Moreover, Δ1-9-G129R-hPRL treatment reduced the proliferation, colony formation, chemoresistance and migration in GBM cells suggesting potential for PRLR as a therapeutic target in GBM (67). Lastly, Δ1-9-G129R-hPRL treatment prevented early stages of prostate carcinogenesis by inhibiting STAT5 phosphorylation, proliferation, abnormal basal-cell pattern and grade of intraepithelial prostate neoplasia suggesting the application of PRLR-based therapies in prostate cancer (68). Collectively, these studies demonstrate antagonistic activity of Δ1-9-G129R-hPRL and further provide solid evidence for targeting PRLR in human malignancies.

2.1.3 Improving half-life of PRLR antagonists *In vivo*

While current PRLR antagonists have shown promise in pre-clinical applications, there remain challenges limiting their usage in clinic. PRL and current PRL antagonists have molecular weights of ~23 kDa, which are below the 60kDa cut-off values for glomerular filtration by the kidneys (69). Hence, these are quickly cleared from the blood following intravenous delivery. Hence, the half-life of PRL in the blood is ~41 minutes (70), and speculation towards PRLR antagonists would yield similar results. As such, their application in a clinical setting is limited.

To overcome this challenge, Yu et al. have developed a PRLR antagonist fusion protein designed around Δ1-9-G129R-hPRL, and several additional mutations (C11S, S33A, Q73L, G129R and K190R). In addition, the fusion protein included an albumin binding domain (ABD) from Streptococcal protein G, also known as ABD_{035}, which has 46 amino acids in a three-helix structure (71). Surface plasmon resonance of this fusion protein, called PrlRA-ABD determined the K_D to be 2.3 ± 0.2 vs 3.4 ± 0.5 nM of PrlRA alone, while PRL showed a KD value of 23 ± 4 nM (71). Furthermore, ABD-PrlRA and PrlRA both inhibited PRL-induced phosphorylation of STAT5 in U251-MG cells in a dose-dependent manner (71). To understand the changes in pharmacokinetics both PrlRA and ABD-PrlRA were injected subcutaneously in Wistar rats at a dose of 4 mg/kg. After 24 h, serum was analyzed for PrlRA and ABD-PrlRA concentration and determined to be 150 ng/ml and 15,000 ng/ml, respectively (71). This data suggests that addition of ABD to PrlRA enhanced its *in vivo* half-life by 100-fold, demonstrating the feasibility of *in vivo* applications.

Additional strategies that have been effective for hGH may also have implications for PRL antagonists. Pegvisomant is a PEGylated G120K protein analog of hGH, and was the first drug approved as a GHR antagonist (39). Much like PRL, hGH is readily cleared by kidney filtration. To slow clearance, polyethylene glycol (PEG) polymers were attached to hGH derivatives. The authors observed significant retention of PEG-hGH derivatives in serum compared to hGH, with measurable concentrations detected out to ~200 hours and 12 hours, respectively (37). Since PEGylation of hGH derivatives proved successful, we could speculate that these strategies may be useful for PRL antagonists as well; however there are challenges that must be overcome with the use of PEG based polymers. PEG chains can mask the protein binding sites, and thereby reduce affinity of biological activity (72). Therefore, design of the polymer is crucial to developing an effective PEGylated protein. Nevertheless, this may provide additional opportunities for improving PRL antagonist half-lives, and warrant further study.

2.2 Antibody-based PRLR antagonists

The use of antibody-based therapeutic agents has become attractive and one of the most successful strategies for the treatment of various diseases, including cancer (73). The use of monoclonal antibodies has achieved significant success in recent years, while antibody-drug conjugates have only recently been utilized for the treatment of solid tumors and lymphomas (73). The anticancer effects of these monoclonal antibodies can be due to direct receptor blockade, immune-activated cell killing, and specific defects of antibodies on cancer vasculature and stromal components as well as drug delivery (74–77). Specific examples of successful monoclonal antibody therapies targeted epidermal growth factor receptor (EGFR) (75, 78), C-MET (79), HER2 (80), fibroblast activation protein (FAP) (81), and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) (82). The ideal properties of monoclonal antibodies include high selectivity towards specific target antigens, activating immune cell responses, and modulating downstream signaling pathways (83). Hence, antibody design is critical for successful preclinical and clinical applications. The successful development of monoclonal antibodies for use in a clinical setting involves identification of the physiochemical properties of the antibody, analysis of specificity, study of immune response and signaling pathways as well as *in vivo* antibody localization, biodistribution, toxicity, and efficacy (73). Several monoclonal antibodies have

received approval by US Food and Drug Administration in the recent decade, which have been summarized previously (84–87). In the context of PRLR, the presence of a defined extracellular domain structure makes it an attractive target for designing monoclonal antibody based inhibitors and therapeutics. As such, several antibodies and antibody based constructs have been developed targeting PRLR and are being tested in preclinical and clinical studies, which will be summarized in detail in the following sections.

2.2.1 PRLR neutralizing antibodies

2.2.1.1 LFA102

Damiano et al. developed and characterized a neutralizing antibody LF102 targeting human PRLR, which was shown to inhibit the physiological functions of both autocrine and paracrine PRL (88). The authors first generated a parental hybridoma to LFA102 in mice immunized with recombinant PRLR extracellular domain, then LFA102 was prepared by humanizing the antibody (88). Using flow activated cell sorting, the authors demonstrated that LFA102 binds to PRLR in human breast cancer cell lines, in addition to primary breast cancer cells (88). Moreover, LFA102 was also found to bind to rat pre-T cell lymphoma cell line Nb2-11 suggesting this antibody has cross-reactivity to rat PRLR (88). To assess selectivity of LFA102, the authors used a PRLR-negative BaF3 cell line and re-expressed PRLR (BAF3-PRLR). LFA102 did not bind to PRLR-negative BaF3 cells but was found to bind to BaF3-PRLR (88). In addition, the antibody did not interact with cells expressing murine PRLR. To determine if LFA102 acted through a competitive or non-competitive mechanism with PRLR, the authors designed a ligand competition assay using Alexa647-labeled PRL (A647-PRL). The authors demonstrated that LFA102 did not affect A647-PRL binding to PRLR even at saturation concentrations of LFA102 (88), suggesting that LFA102 is not a ligand-competitive inhibitor. To determine whether LF102 affects PRL-mediated signaling, T47D breast cancer cells were treated with the antibody. There was significant attenuation of PRL-induced phosphorylation of STAT5, AKT, and ERK in a concentration-dependent manner (88). However, LFA102 failed to regulate PRLR signaling when treated alone demonstrating the absence of residual agonistic activity.

As proof of principle for *in vivo* activity, T47D-T2 xenografts were generated in NOD/SCID mice and LFA102 or as control, a human IgG1 was administered, followed by a bolus of PRL to stimulate PRLR. Mice treated with PRL alone showed increased levels of phosphorylated STAT5 in the tumors, while the treatment of LFA102 inhibited this PRL-induced phosphorylation suggesting the *in vivo* efficacy of LFA102 (88). In addition, LFA102 achieved a 30-56 μg/mL concentration in the serum of these mice. Detailed pharmacokinetic and pharmacodynamics studies of LF102 were subsequently performed. The clearance of LFA102 ranged from 1.45 to 0.92 mL/h/kg and 13.5 to 3.93 mL/h/kg in males and females, respectively. In addition, the mean estimated half-life was between 1.43 and 8.99 days and 0.12 to 4.23 days in males and females, respectively (88). Subsequently, to further understand the antitumor efficacy of LF102, a subcutaneous xenograft model in SCID mice was used. Mice were injected subcutaneously with luciferase expressing Nb2-11 cells to generate tumors. These mice were treated with a single dose of LFA102 (0.01-10 mg/kg) or control IgG antibody, and disease burden was measured from 14 days after dosing to 4.5 months. Doses exceeding 0.3 mg/kg displayed antitumor efficacy by day 3 post-injection. Moreover, LFA102 treated mice (doses exceeding more than 0.3 mg/kg) showed significantly higher survival compared to controls, with 50% of animals surviving 2-4 fold longer than IgG1 treated mice (88).

In addition to xenograft tumor models, a carcinogen induced model was utilized to assess LFA102 efficacy. Briefly, 7,12-Dimethylbenz[α]anthracene (DMBA) was administered to induce rat mammary tumors. LFA102 treatment (300 mg/kg) significantly reduced PRLR signaling and tumor growth in this rat mammary cancer model as a monotherapy and combination with letrozole (aromatase inhibitor, 10 μg/kg). LFA102 treatment reduced tumor volume to 809±279 mm^3 from 1964 ±243 mm^3 in case of control, while combination of LFA102 and Letrozole further reduced tumor volume to 436±144 mm^3, suggesting synergistic/additive anticancer activity (88). These data demonstrated that LFA102 has the potential to be the first effective antibody-based therapeutic agent for the treatment of PRL-responsive malignancies.

Following these studies, the clinical efficacy of LFA102 was assessed in patients with stage IV breast and castration-resistant prostate cancer. In this Phase I clinical trial, patients (n=73, female=34, male=39) received 3-60 mg/kg of LFA102 intravenously once every 4 weeks and the maximum tolerated dose (MTD) and/or recommended dose for expansion was determined to study the safety and antitumor efficacy of LFA102 (89). Drug-related toxicity was not observed during the dose escalation study, hence a MTD was not obtained during this study. The highest tested dose of 60 mg/kg was established as the recommended dose for expansion. The most common side effects recorded were fatigue (44%), nausea (33%), vomiting, constipation, and reduced appetite (21%), while 3 patients had adverse effects, which included decreased blood phosphorus, increased levels of serum lipase, and reduced blood lymphocyte count (89). The mean half-life of LFA102 ranged from 6-9 days. At the dose of 60 mg/kg, the C_{max} of LFA102 was found to be 1,495 ± 589 μg/ml, and mean area under the curve (AUC_{last}) was 230,991 ± 102,673 hour × μg/ml (89). There was no response noted in the patients with breast cancer after LFA102 treatment. Similarly, in prostate cancer patients, there was no PSA response. Overall, LFA102 treatment contributed to stable disease in 13 patients out of 73 (18%), while all other patients 67 out of 73 (92%) discontinued the study due to the cancer progression (89). This poor response of LFA102 was thought to

be because of insufficient exposure. The authors retrospectively hypothesized that a more frequent dosing of LFA102, such as once every 2 weeks, would have resulted in durable PRLR inhibition and superior antitumor efficacy

In another Phase 1 Trial study in East Asian patients of Japanese ancestry with breast (n=7) and prostate (n=7), similar results were obtained for MTD and anti-tumor activity of LFA102. Here, the antibody was administered at a dose of 3-40 mg/kg intravenously every 4 weeks (90). There were 14 patients enrolled in the study and grade 1 or 2 toxicities were reported in 9 patients out of 14 (64%), while the most frequent toxicity reported was nausea in 3 patients (21%) (90). The mean AUC_{last} of LFA102 (40 mg/kg) was found to be 5674 ± 507 μg/ml×day, C_{max} was found to be 1089 ± 227 μg/ml, while median $t_{1/2}$ was found to be 12.1 days (90). As with the previous Phase I trial, LFA102 did not display antitumor activity.

2.2.1.2 Anti-prolactin receptor (PRLR) antibody, F56

Cui et al. sought to design a new PRLR antagonist using a hybridoma technique to develop a series of monoclonal antibodies (91). After screening these antibodies, F56 was selected that specifically antagonized PRLR as assessed by enzyme-linked immunosorbent assay (ELISA) and western blot. The authors performed epitome mapping which identified a common binding epitope between F56 and PRL. In subsequent experiments, the authors determined that F56 inhibited PRL binding to PRLR in a dose-dependent manner suggesting that the F56 epitope overlapped with the PRL-binding site. Furthermore, F56 treatment (0.1-5 μg/ml) inhibited PRL-induced STAT3/5, AKT, and ERK phosphorylation in CHO cells expressing PRLR and Nb2 cells in a dose-dependent manner, confirming the antagonistic activity of F56 (91). Moreover, F56 inhibited PRL-induced proliferation of Nb2 cells, corroborating molecular data. These preclinical studies identified F56 as the first PRLR antagonist that has an overlapping epitope as PRL, which has potential to treat PRL-dependent diseases. However, early phase clinical trials will be required to assess toxicity, and preliminary efficacy.

2.2.2 Antibody-drug conjugates

2.2.2.1 ABBV-176

Antibody-drug conjugates (ADCs) have become a popular therapeutic design concept that combines the specificity of antibodies and potency of payload/cytotoxic drugs. Currently, 5 antibody-drug conjugates have been approved for the treatment of four hematological malignancies and one for solid tumors. For the purpose of targeting PRLR, Anderson et al. designed a novel pyrrolobenzodiazepine antibody-drug conjugate, ABBV-176 (92). To generate the PRLR-specific antibody used to produce the ABBV-176 ABC, a standard hybridoma technique following immunization with the PRLR extracellular domain was employed. The lead antibody selected was h16f (PR-1594804) based on affinity, epitope binding and activity (92). Initial screening was performed on antibodies conjugated to monomethyl-auristatin payload and studied based on ability to inhibit proliferation of the BT474 cell line. Based on this, the lead candidate ABBV-1776 was selected from the panel for further analysis. Surface plasmon resonance was performed with ABBV-176 and the extracellular domain of human PRLR, showing a strong affinity with a K_D value of 1 nM (92). In bioassays of anti-tumor activity, ABBV-176 was found to inhibit the growth of various cancer cell lines including breast cancer (IC50 value = 0.0055-0.77 nM), prostate cancer (IC50 value = 0.01 nM), endometrial cancer (IC50 value = 0.6 nM), ovarian cancer (IC50 value = 0.16 nM), colorectal cancer (IC50 value = 0.11 nM) and liver cancer (IC50 value = 5.2-8.6 nM) (92). These IC50 values were highly dependent on PRLR expression (i.e. more PRLR receptors was associated with higher IC50 for ABBV-176). Moreover, ABBV-176 was found to be nontoxic to normal/immortalized cell lines in kidney, breast, liver, lung, prostate, and vascular endothelium. Furthermore, the antitumor activity of ABBV-176 was evaluated in the BT-474 FP2 human xenograft breast cancer model. The single dose of 0.5 mg/kg was effective in significantly reducing tumor growth (92). A higher dose of 3 mg/kg produced the highest tumor reduction without affecting body weight as compared to control. Furthermore, there were no apparent physiological changes suggesting no impact on normal tissues. Similar results were obtained in a patient-derived xenograft (PDX) model. In these studies, the authors established the effect of ABBV-176 (0.1 mg/kg) in combination with the PARP inhibitor Valiparib (200 mg/kg) in CTG-0670 triple-negative, BRCA1 deficient, BRCA2 mutant PDX tumor models (92). It was determined that ABBV-176, both as a monotherapy and in combination with Valiparib, significantly inhibited PDX growth. This data suggests that ABBV-176 may be an effective therapy either alone or in combination with PARP inhibitors for the treatment of breast cancers (92).

Recently, Lemech et al. conducted a first-in-human Phase 1 dose-escalation study of ABBV-176 in patients with advanced solid tumors for evaluating safety, pharmacokinetics, and preliminary anticancer activity (93). Patients were given ABBV-176 once every three weeks with dose escalation based on level of exposure which was continually assessed. Drug-related toxicities were studied following each dose escalation to determine MTD. A group of 19 patients were enrolled, of which 11 had colorectal cancer, 6 had breast cancer and 2 had adrenocortical carcinoma. The patients were administered 2.7-109.36 μg/kg ABBV-176 (93). Dose-limiting toxicities occurred in four patients, which included two cases of thrombocytopenia, two cases of neutropenia, and one case of pancytopenia (93). The common adverse effects of ABBV-176 reported were thrombocytopenia, neutropenia, nausea, fatigue, increased aspartate aminotransferase, and pleural effusions. PRLR expression in tumors among these patients was varied, but no patient had an objective response. Unfortunately, there was

considerable toxicity associated with ABBV-176 in this Phase 1 dose-escalation study. One caveat is that the study analysis relied on a small patient cohort with differential PRLR expression. This may be the reason why no response was observed. This study was terminated following administration of the drug to 19 patients. Therefore, further evaluation may be necessary with a larger cohort of patients with high PRLR expression.

2.2.2.2 REGN2878-DM1

Another antibody-drug conjugate REGN2878-DM1 that is reported to target PRLR was developed by Kelly et al. to target PRLR positive breast cancer (31). This antibody-drug conjugate is composed of a high-affinity anti-PRLR IgG1 antibody conjugated to a cytotoxic maytansine derivative DM1, *via* a noncleavable Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate linker. The antibody was generated in VelocImmune mice, which contain genes encoding human immunoglobulin heavy and kappa light chain variable regions. The mice were immunized with recombinant protein of the extracellular domain of human PRLR. Hybridomas were generated and joined to the human IgG1 constant region. REGN2878 was selected as the lead antibody after screening more than 300 antigen-binding clones. This antibody was further conjugated to DM1 *via* a non-cleavable SMCC (Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) linker and purified by size exclusion chromatography. The concentration of antibody-drug conjugate was confirmed by UV spectroscopy and MALDI-TOF mass spectrometry analysis. Both REGN2878 and REGN2878-DM1 were determined to have high-affinity binding to hPRLR with a K_D value of 1.05 and 1.24 nM respectively, and blocked prolactin binding to PRLR as measured by ELISA with an IC50 value of 5.0 and 4.4 nM, respectively (31). REGN2878-DM1 also inhibited prolactin-induced STAT5 activity in the HEK293/PRLR/STAT5-Luc reporter cell line, demonstrating inhibition of PRLR signaling. Moreover, REGN2878-DM1 treatment induced cell death in breast cancer cells with IC50 values between 0.06 nM and 0.97 nM (31). Proof of principle *in vivo* studies were performed using MCF7 and MCF7-PRLR over-expressing breast cancer xenograft mouse models in NCr nude mice. The xenografts were established and treated with a single dose, or thrice single-weekly doses of REGN2878-DM1 (5, 10, 15 mg/kg). Single dosing of 15 mg/kg significantly impaired tumor growth, which was also observed with 10 and 15 mg/kg repeated injections compared to control in both MCF7 and MCF7-PRLR overexpressing tumors (31). REGN2878-DM1 was further tested in breast cancer mouse xenografts of T47Dv11, which exhibit high levels of endogenous PRLR. As seen with previous studies, REGN2878-DM1 inhibited tumor growth in this model even at 2.5 and 5 mg/kg doses, while complete regression was observed with the highest 15 mg/kg dose (31). REGN2878-DM1 (2.5 mg/kg) was also test in combination with Fulvestrant (150 or 250 mg/kg), standard-of-care for ER+ breast cancer, showed greater inhibition of tumor growth in T47Dv11 xenografts in mice compared to monotherapy, suggesting synergistic or additive effects (31). Follow-up pharmacokinetic studies, in which 5 mg/kg of REGN2878-DM1 was delivered, resulted in serum levels above 16 µg/mL for at least 10 days, suggesting a long-lasting concentration sufficient to produce anti-tumor effects in the conducted mouse models (31). Collectively, these data suggest that the REGN2878-DM1 antibody-drug conjugate has potential to target PRLR and may have implications in the treatment of breast cancer with high expression of PRLR. Further early phase clinical trials will be required to assess toxicity, and preliminary efficacy in patients.

2.2.3 Bispecific antibodies targeting PRLR

Bispecific antibody (BsAb) is a novel technology that contains two binding sites towards two different epitopes. This provides significant clinical advantages compared to monoclonal antibodies, due to an increased range of applications. Currently, more than 110 BsAbs are being evaluated in clinical trials (94), demonstrating the functionality, and excitement of this technology in targeting applications for human diseases and conditions. Two different BsAbs have been developed targeting PRLR.

2.2.3.1 PRLR-DbsAb targeting CD3 and PRLR

Zhou et al. have recently developed a bispecific antibody, PRLR-DbsAb, that targets both PRLR and T-cell surface antigen, CD3 using the "Bispecific Antibody by Protein Trans-splicing" (BAPTS) system (95, 96). Briefly, Fragment A (CD3 antibody fusion protein) and Fragment B (PRLR antibody fusion protein) were expressed in CHO and 293E cell lines, respectively, and purified using protein L affinity chromatography. The authors identified that the PRLR-DbsAb-mediated cytotoxicity of immune effector cells is dependent on the ratio of effector to target cells; PRLR-DbsAb showed dramatic T-cell toxicity at the ratio of 5:1. Further, PRLR-DbsAb mediated cell killing of T47D (PRLR high) cells was 60% at a dose of 100 ng/ml at a ratio of 10:1 (97). It was shown that PRLR-DbsAb induced cytotoxicity *via* the synergistic effect of immune cell recruitment and not solely on the combined effect of PRLR and CD3 antibody. When T47D cells were treated with single targeting antibodies towards PRLR or CD3 alone, they produced less cytotoxic activity compared to PRLR-DbsAb. The EC50 values of PRLR-DbsAb against breast cancer cell lines MDA-MB-231, MCF-7, SKBR-3, and T47D were found to be 5.053, 1.78, 46.68, and 7.63 ng/ml, respectively (97). Mechanistically, PRLR-DbsAb was found to recruit T cells to PRLR expressing T47D breast cancer cells, which further induced cytotoxicity. Moreover, PRLR-DbsAb was found to activate T-cells *in vitro* as shown by increased CD69 levels in peripheral blood mononuclear cells (PBMCs) without target cells, while $CD8^+CD69^+$ T-cells had more activity than $CD4^+CD69^+$ T-cells when cultured with target cells (97). Rather, PRLR-DbsAb was found to activate $CD4^+CD69^+$ T-cells,

while $CD8^+CD69^+$ T cell activation is dependent on combination with the target cells engagement. Moreover, cytokine release (IL10 and TNF-alpha) was significantly increased after PRLR-DbsAb treatment, supporting the T-cell activation mechanism (97). The *in vivo* activity of PRLR-DbsAb was evaluated in the NOD/SCID mice where T47D cells together with healthy human PBMCs were co-injected subcutaneously. PRLR-DbsAb was delivered once weekly at 0.33, 1, and 3 mg/kg intraperitoneally and compared with a 3 mg/kg PRLR monoclonal antibody (97). PRLR-DbsAb treatment significantly inhibited tumor growth at 0.33 mg/kg, which was comparable to PRLR monoclonal antibody alone. At higher doses of 3 mg/kg, PRLR-DbsAb substantially suppressed tumor growth, as both tumor volume and weight were impaired compared to control, and further increase survival of mice (97). Moreover, PRLR-DbsAb stimulated T-cell infiltration and expression of PD-L1 in these tumor tissues. Lastly, when PRLR-DbsAb was delivered in combination with a PD-1 antibody, anti-tumor activity was enhanced against MDA-MB-231 cells supporting the rationale of targeting PRLR with the novel BsAbs technology for PRLR-expressing breast cancers (97).

2.2.3.2 Growth hormone receptor/Prolactin receptor BsAbs (H53)

As PRLR and growth hormone receptor (GHR) are closely involved in the incidence and development of breast cancer (98) which typically express PRLR, GHR, and GHR-PRLR heterodimers (99), the use of a combination PRLR and GHR antagonists may be a better strategy for breast cancer treatment. As such, Chen et al. have used a hybridoma technology to design a dual GHR-PRLR targeting antibody called H53 (100). Using competitive ELISA, receptor binding analysis, and immunofluorescence assays, the authors identified that H53 behaved like a typical anti-idiotypic antibody (Ab2β) (100). Further testing revealed that H53 treatment (0.05-1 μg/ml) inhibited not only the growth of CHO cells expressing PRLR and GHR but also PRLR-induced JAK2-STAT5 signaling (100). H53 also inhibited the PRL-induced phosphorylation of both STAT3 and STAT5, and AKT at a dose of 5-10 g/ml in T47D and MCF7 breast cancer cell lines, and further attenuated PRL-induced proliferation (100). Moreover, H53 also inhibited clonogenic potential, and migration that was accompanied by decreased expression of PRLR and GHR (100). The H53 BsAbs also displayed robust antitumor activity in proof-of-principle T47D and MCF-7 tumor xenografts models. When delivered at 15 and 30 mg/kg twice a week the expression p-STAT3/5 and p-AKT were downregulated in tumor tissue (100). H53-treated tumors also displayed a reduction in Ki67 that was accompanied by increased tunnel staining, indicating that H53 induced apoptosis in tumor cells. This preclinical study demonstrates the application of dual GHR/PRLR antibodies as a useful strategy for the treatment of breast cancer by impeding the PRLR signaling axis.

2.3 Small molecular inhibitors of PRLR

Extensive research has been conducted on developing antibody-based targeting and competitive antagonists of PRLR, but to date have unfortunately failed to produce sufficient anticancer activity in clinical trials. While these strategies have shown antagonization of PRLR in pre-clinical studies, poor bioavailability and stability can result in less durable responses, leading to tumor progression. While these technologies may still produce effective therapies, and certainly warrant further studies, an alternative solution to the noted clinical challenges may be resolved through the development of small molecule inhibitors. Advantages of small molecule inhibitors include oral delivery, low/no immunogenic properties, ability to cross the blood-brain barrier, easy to synthesize and optimize, and lower cost due to ease in manufacturing, transport, and storage compared to antibody based strategies (101). We have summarized below several studies focused on developing small molecule inhibitors for targeting PRLR, which have largely been conducted in the context of cancer.

2.3.1 Small molecule inhibitors that target the ECD of PRLR

Borcherding et al. sought to identify a small molecular inhibitor targeting the extracellular domain (ECD) of PRLR (102). First, they performed *in silico* docking of a virtual library of 340,000 small molecules and evaluated their binding to the ECD of PRLR, of which 1000 compounds were predicted to affect PRL binding (102). Moreover, 50,000 diverse compounds were selected in addition to the predicted 1,000 compounds through virtual screening. For high-throughput screening, three sequential assays were performed on selected compounds. All three assays were designed under conditions where cells were incubated with PRL alone, compound alone, and PRL and compound in combination. Compounds that displayed significant cytotoxicity in the absence of PRL were eliminated. In the first assay, Nb2 cells, which are sensitive to PRL stimulation, were treated with selected compounds to determine the effect on proliferation at a concentration of 10 μM. The authors identified 120 potential compounds for further screening. In the second assay, a stably transfected PRLR cell line Ba/F3 was utilized for calculating IC50 values. Verification was performed in a third assay of T47D breast cancer cells stably transfected with luciferase reporter driven by a PRL-responsive

promoter. Seven compounds were selected based on the IC50 values of 0.09 to 2.07 μM in the Ba/F3 assay. These were further analyzed for PRLR ECD binding using Microscale Thermophoresis (MST) technique. Three compounds, SMI-1, -6, and -7 bound to PRLR-ECD with K_D values of 1.26, 3.31, and 2.69 μM, respectively (102). Interestingly, SMI-1 was predicted by virtual screening and by molecular docking. Receptor binding was further confirmed by isothermal titration calorimetry. The ~40X ratio of antagonist/PRL binding affinities was found to be 1.26 μM vs. 29.9 nM (102). The incubation of SMI-1 and -6 at 1 μM concentration inhibited PRL-induced migration of MDA-MB-468 cells in the Boyden chamber transmigration assays. Moreover, both compounds also inhibited PRL-induced proliferation of Jurkat lymphocytes, as well as PRL-induced phosphorylation of JAK2 in Ba/F3 cells (102). SMI-6 was selected for further testing based on the absence of *in vitro* off-target toxicity. Further evaluation of SMI-6 identified that it inhibited PRL-induced phosphorylation of STAT5 in MDA-MB-468 cells without affecting the ability of growth hormone to phosphorylate STAT5 in PRLR deficient-T47D cells. To study the selectivity of SMI-6, the DiscoverX platform was used and tested against 168 G-protein-coupled receptors (GPCRs). In addition to PRLR, SMI-6 inhibited the serotonin receptors 2C, 2A, and hypocretin receptor 1 with IC50 values of 3.476, 2.395, and 6.712 μM, respectively. Moreover, SMI-6 was also tested against 468 kinases and failed to display inhibitory activity towards the tested kinases, including JAK2 (102). Subsequently, the authors evaluated the anti-proliferative activity in six breast cancer cell lines (BT474, MCF7, T47D, MDA-MB-231, ZR75-1, and MDA-MB-468). SMI-6 produced dose-dependent antiproliferative activity with IC50 values ranging from 0.29-1.68 μM (102). In non-malignant cells (fibroblasts, keratinocytes, and mammary epithelial cells) IC50 values were determined between 4.5-20.4 μM, suggesting low toxicity and a plausible therapeutic window (102). To confirm these findings, the authors assessed SMI-6 antitumor efficacy in proof-of-concept *in vivo* models utilizing athymic nude mice implanted orthotopically with control MDA-MB-468 cells or with Doxycycline regulated PRL producing cells (MDA-PRL). MDA-PRL produced larger tumors compared to control, while delivery of SMI-6 significantly inhibited tumor growth of MDA-PRL tumors. Moreover, SMI-6 did not show any apparent signs of toxicity or discomfort in mice. These data demonstrate that SMI-6 serves as a potent small molecular inhibitor targeting PRLR that may have implications for the treatment of breast cancer.

2.3.2 Repurposing antipsychotic drugs for targeting the JAK-2 binding site of PRLR.

Several attempts were made to target the extracellular domain of PRLR using competitive antagonists, neutralizing antibodies, antibody-drug conjugates, and small molecule inhibitors but none have produced a clinically effective and acceptable antitumor response to date. In our own studies, we sought to identify novel targets involved in pancreatic ductal adenocarcinoma (PDAC) progression, and came across a pilot clinical trial that studied serum prolactin levels in women with different cancers (27). The authors observed 3-4 times greater prolactin levels in women with PDAC, which led us to investigate the role of PRL and PRLR in pancreatic cancer. In initial studies, we determined that PRLR is overexpressed in PDAC patient tissues by immunohistochemistry (25). The expression of PRLR in the normal pancreas was limited to the islet cells, while high cytoplasmic expression was observed in PDAC tissues. Moreover, we observed PRL released by PDAC tissues and cell lines using IHC and ELISA techniques, suggesting the role of both autocrine and paracrine PRL in PDAC progression. Furthermore, when we treated PDAC cells (MiaPaCa-2 and Panc-1) with PRL, it induced phosphorylation of canonical JAK2, STAT3, and ERK in a time- and dose-dependent manner, suggesting the functionality of the PRLR in PDAC cell lines (25). Interestingly, while PRL treatment failed to increase proliferation of MiaPaCa-2 and Panc-1 cells, we observed a significant increase in spheroid formation and migration (25). Furthermore, when we knocked down PRLR (PRLR KD) from PDAC cell lines (MiaPaCa-2 and mouse UKNC-6141) using CRISPR-Cas9 and shRNA approaches. PRLR KD resulted in significant inhibition of proliferation, colony formation, migration, and spheroid formation, suggesting that PRLR regulated multiple hallmarks of cancer progression (25). Moreover, when we treated PRLR knockdown cells with PRL, PRL failed to induce phosphorylation of JAK2, STAT3, and ERK suggesting the inhibition of PRL : PRLR regulated signaling pathways. In proof-of-concept studies, we injected PRLR knockdown UNKC-6141 cells in the pancreas of C57BL/6 mice to generate syngeneic orthotopic tumors. PRLR KD significantly impaired growth of orthotopic tumors compared to controls (25). These data suggested that PRLR affected PDAC progression and can be an attractive target for therapeutic interventions. These studies further demonstrate the feasibility and druggability of PRLR.

Due to limited the success achieved by targeting the PRLR ECD, we decided to approach targeting PRLR from a different perspective. In initial studies, we observed the presence of multiple isoforms of PRLR in PDAC cell lines. Structurally, all PRLR isoforms retain a conserved JAK2 binding domain. Following PRL binding to PRLR, JAK2 binding is the first downstream event that occurs in PRL-PRLR signaling. Hence, we sought to target the JAK2 binding domain of PRLR. We performed *in silico* virtual screening of small molecular inhibitors using a homology model of the intracellular domain (ICD) of PRLR, due to the lack of a published crystal structure

for the ICD. We utilized I-TASSER software to predict inhibitors followed by virtual screening of small molecules using the IDOCK program. We selected two classes of compounds based on these predictions. We decided to use a fragment-based drug design approach to select commercially available small molecules. Since previous attempts achieved limited success in producing anticancer activity in clinical trials, we first screened these compounds for antiproliferative activity against PDAC cell lines. We found a single compound Penfluridol produced antiproliferative activity against MiaPaCa-2 and Panc-1 cells in a dose- and time-dependent manner, with an IC50 value of 3-4 μM concentration (25). Penfluridol is a first-generation antipsychotic drug used for the treatment of schizophrenia. We further performed multiple assays to study Penfluridol : PRLR binding and inhibition of PRL-induced signaling. First, we pretreated PDAC cells with Penfluridol at 4 μM concentration and subsequently stimulated with PRL. We determined that pretreatment of Penfluridol inhibited PRL-induced phosphorylation of STAT3 and ERK in both MiaPaCa-2 and Panc-1 cells (25). Moreover, we performed cell-based and cell-free drug-protein binding assays. Surface plasmon resonance and magnetic relaxometry using a peptide encoding the JAK-2 binding site of PRLR confirmed a dose-dependent response in Penfluridol binding (25). We further validated these results using cell-based binding assays. We performed a cellular thermal shift assay (CETSA), in which MiaPaCa-2 cells were treated with Penfluridol (5-20 μM) and subjected to a thermal gradient to assess PRLR denaturation in the presence or absence of drug. We observed that PRLR denatured at ~58°C, while denaturation occurred at 66°C in Penfluridol treated cells, suggesting that Penfluridol bound to PRLR and provided stabilization to thermal denaturation (25). These results were validated with the Drug Affinity Responsive Target Assay (DARTS). Similarly, Penfluridol provided stability to PRLR against pronase-induced proteolysis demonstrating Penfluridol binding. Collectively, these data confirmed that Penfluridol binds to PRLR.

We further tested the anticancer activity of Penfluridol in a variety of PDAC animal models. Penfluridol was delivered at 5 mg/kg intraperitoneally for 21 days in all models. We used UNKC6141 and KPC cell lines to generate orthotopic tumors in C57BL/6 mice. In a second model, we used Panc-1 cells to generate subcutaneous xenografts in Nude mice. In the third model, we generated a PDX in NSG mice. Penfluridol produced significant antitumor activity in all three animal models, and further induced LC3B and p62 mediated autophagy in PDAC cells as well as in orthotopic tumors (25). Our study is the first to target the JAK2 binding domain of PRLR. We demonstrate that Penfluridol binds to the PRLR ICD, and potently inhibits PRL : PRLR signaling that results in the inhibition of PDAC growth.

3 Summary and conclusions

It is becoming clear that PRLR mediated signaling plays a critical role in multiple human diseases and malignancies, and therefore is an attractive target for developing therapies. Since the early 1990s, researchers have attempted to generate PRLR antagonists and inhibitors with mixed success in pre-clinical and clinical applications. While none of these studies have resulted in FDA approval to date, they have provided a foundation for future discoveries that may yet be exploited. At the very least, our understanding of PRLR biology has expanded, and the studies to date have provided tools to interrogate this to greater depths.

First generation human PRL analogs exhibited weak agonistic activity towards PRLR despite numerous studies demonstrating antagonism, leading to reluctance for use in clinic (55). This contributed to the development of second generation analogs, such as Δ1–9-G129R-hPRL, which exhibits pure antagonism across multiple bioassays. Unfortunately, there remain challenges for clinical applications. Since these antagonists are small peptides (~23 kDa), these are quickly filtered by the kidneys, leading to suboptimal half-lives to maintain a potent and durable response. Nevertheless, the high selectivity of these analogs remains attractive with clinical prospects. With recent technological advancements, hormone based analogs may yet have therapeutic use. As shown in the recent studies by Yu et al., the fusion of second generation analogs with stabilizing proteins/peptides, such as albumin binding domain, can extend analog half-life substantially (71). As such, hormone analogs should not be discounted, and certainly further investigation is warranted to determine therapeutic implications.

Due to the clinical challenges innate to current hormone based analogs, and the significant advancement in antibody and protein engineering and recombinant DNA technology, antibody-based strategies have become of interest for antagonizing PRLR activity. Generally speaking, these strategies are attractive due to their success for the treatment of multiple human conditions, including cancer. Structurally, PRLR is an attractive target for antibody-based technologies due to the presence of a defined extracellular domain. Monoclonal antibodies and antibody-drug conjugates targeting PRLR have shown promising results in pre-clinical applications, antagonizing PRL induced signaling and cellular growth and migration (88–90, 92). Unfortunately, these have failed in Phase I clinical trials assessing toxicity and preliminary anti-tumor efficacy due to disease progression or development of dose-limiting adverse effects. This potentially could be improved by adjusting dosing frequency in therapies with minimal toxicities, though that is highly speculative. In such cases, antibody design

is essential to preclinical and clinical success, and requires stringent study in regard to specificity, biodistribution, toxicity and efficacy. Moreover, antibody-drug conjugates are an exciting and novel technology. Though current designs have shown substantial toxicity in Phase I trials, there are significant advantages in concept design compared to monoclonal antibodies, combining the high specificity of antibodies and potency of cytotoxic drugs. Overall, there have been few antibody-based strategies that have been evaluated in clinical trials to date for targeting PRLR, largely due to the recency of technologic developments supporting their generation. As such, antibody based therapies may have significant potential for use in the future, though further study and designs are required to fully assess their prospective use.

The use of small molecule inhibitors may also provide alternatives to inhibiting PRLR signaling, and improving upon the challenges related to antibody and hormone based approaches. There are significant advantages to the use of small molecules; of particular note, oral delivery, ease of synthesis and optimization, and cost effectiveness make small molecule inhibitors a highly attractive approach (103). *In silico* screening tools combined with bioassays can provide a high-throughput screening pipeline for thousands of compounds. Prospective leads can then be validated for selectivity, and serve as scaffold platforms for additional analogs/derivatives to improve target binding, potency, bioavailability and stability.

To date, a handful of small inhibitors have been designed for targeting PRLR. SMI-6, developed by Borcherding et al., has been well characterized, demonstrating high selectivity for PRLR extracellular domain (102). Efficacy was also validated in both *in vitro* and *in vivo* models of breast cancer. Though SMI-6 is at the level of experimental investigation, the potential for therapeutic applications remains open, and certainly will be of interest to follow. In our own studies, we identified that Penfluridol, which has been approved for the treatment of schizophrenia, binds to PRLR at the JAK2 binding site within the intracellular domain (25). Penfluridol effectively inhibited PRLR signaling and maintained potent anti-tumor activity in multiple mouse models of PDAC (25). Penfluridol is a first generation antipsychotic with a long half-life, which may confer advantages in maintaining potent and durable responses in clinic. Unfortunately, Penfluridol is no longer licensed in the United States based on current FDA drug database information. Nevertheless, these studies demonstrate the feasibility of small molecule inhibitors targeting the intracellular domain of PRLR.

With the development of more accurate *in silico* screening tools, repurposing FDA approved drugs may serve as a means for rapid approvals for treating PRLR dependent conditions outside the original scope. FDA-approved drugs have been extensively screened for toxicity, safety, and pharmacokinetic and pharmacodynamic properties, and hence, may potentially decrease overall development timelines and costs for new applications. In this regard, It is important to address that the majority of PRLR targeting approaches have been designed against the extracellular domain, and it would be wise to expand these approaches to include the intracellular domain. Ultimately, the goal is to develop therapeutic strategies that can modulate PRLR signaling to promote positive clinical responses, either through agonistic or antagonistic mechanisms. For the purpose of studying PRLR biology, we already have numerous tools available that have been extensively characterized, and have been outlined in this review and Table 1, yet there remain challenges with the development of PRLR targeting therapeutics. As such, expanding our developmental strategies to include additional sites within PRLR may yield promising candidates for future clinical applications.

TABLE 1 Summary of PRLR inhibitors in preclinical and clinical stages of development.

Inhibitor	Class	Development Stage	Cancers tested	Effects	Reference
G129R-hPRL	hPRL protein analog	Preclinical	Ovarian, Breast	Inhibited cancer growth	(6, 21, 36–39, 41–46)
A1-9-G129R-hPRL	hPRL protein analog	Preclinical	Breast, Glioblastoma, Prostate	Inhibited cancer cell growth, chemoresistance	(47, 48, 50, 54–60)
ABD-PrIRA	Neutralizing antibody	Preclinical	Glioblastoma	Inhibited PRL induced signaling, extended serum half-life	(63)
LFA102	Neutralizing antibody	Clinical	Breast, Prostate	Inhibited cancer cell growth in preclinical studies, failed to inhibit tumor growth in clinical trials	(80–82)
F56	Neutralizing antibody	Preclinical	N/A	Inhibited PRL induced signaling	(83)

(Continued)

TABLE 1 Continued

Inhibitor	Class	Development Stage	Cancers tested	Effects	Reference
ABBV-176	Antibody-drug conjugate	Clinical	Breast, Prostate, Endometrial, Ovarian, Colorectal, Liver	Inhibited growth of cancer cells in preclinical studies, Clinical trial was stopped due to severe adverse toxicities	(84, 85)
REGN2878-DM1	Antibody-drug conjugate	Preclinical	Breast	Inhibited cancer growth	(22)
PRLR-DbsAb	Bispecific antibody	Preclinical	Breast	Activated T-cells, Inhibited cancer growth	(89)
H53	Bispecific antibody	Preclinical	Breast	Inhibited cancer growth, migration	(92)
SMI-6	Small molecule	Preclinical	Breast	Inhibited cancer growth, invasion	(94)
Penfluridol	Small molecule	Preclinical	Pancreas	Inhibited cancer growth, invasion, stemness	(18)

In summary, PRLR has become an attractive target for therapeutic development due to the broad expression of PRL and PRLR within biological tissues and human diseases. Hormone based approaches have yielded the development of specific antagonists, though their potential for clinical use is limited due to rapid filtration from blood and excretion. Antibody-based strategies have shown promise in preclinical applications, though they have failed in clinical trials due to toxicities and poor response. Nevertheless, there remains potential with antibody-based approaches due to the defined extracellular domain of PRLR. Similarly, the development of small molecule inhibitors has also shown potential in preclinical applications. The challenge now is to further assess lead candidates in clinical trials, as well as design new candidates with increased potency, with limited adverse toxicities.

Author contributions

DS produced and edited the final manuscript. PD wrote the first draft of the manuscript. SA edited the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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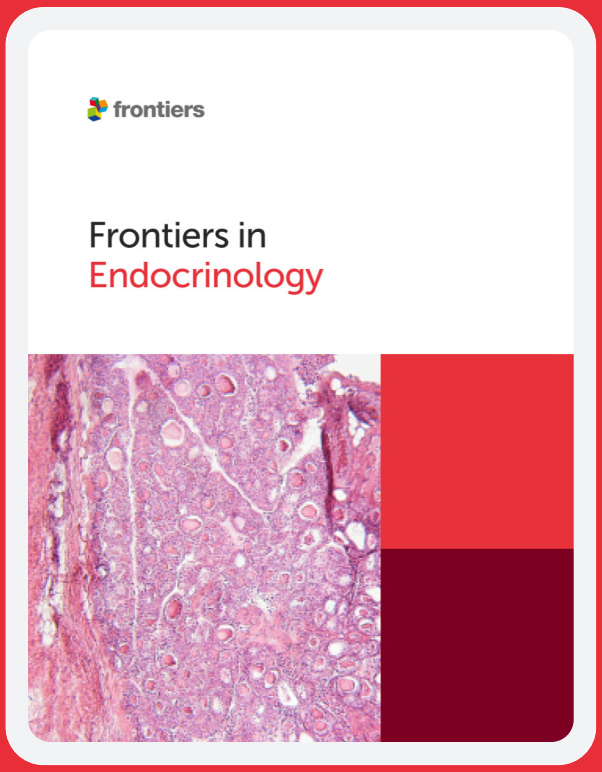

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