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Review

Involvement of the PRL-PAK1 Pathway in Cancer Cell Migration

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Abstract. *Prolactin (PRL) is a polypeptide hormone synthesized in the lactotrophs of the adenohypophysis and in extrahypophyseal glands (such as the prostate and breasts) where it promotes their development. PRL is also involved in cancer development in these glands. It has been shown to stimulate cancer cell migration, suggesting its possible involvement in metastasis, in which cell migration plays an essential role. However, the role of PRL in cell migration is still unclear. Moreover, the intracellular mechanisms activated by PRL to carry out cell migration are less well understood. PRL exerts its effects via the PRL receptor (PRLR), which leads intracellularly to phosphorylation of Janus protein kinase 2 (JAK2), which in turn phosphorylates p21-activated protein kinase (PAK1), leading to an increase in cell migration. Although several studies have described the involvement of the PRL-PAK1 pathway in breast cancer cell migration, the molecular mechanisms have not been fully elucidated and there is no integration of these into signaling pathways. This study was conducted based on literature search of review articles and original research in the PubMed database, using the following keywords: PRL, cell migration, PRL and cell migration, PAK1 and signaling pathways. The aim of this review article was to describe the major signaling pathways controlled by PRL-PAK1 and propose a comprehensive model of the signaling pathways associated with PRL-PAK1.*

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Prolactin (PRL) is a peptide hormone found in all vertebrates (1) and is synthesized and secreted mainly by lactotrophs in the adenohypophysis (2, 3). PRL is also produced in several extrahypophyseal organs with autocrine/paracrine effects (4). This hormone has several important biological functions, *e.g.*, stimulation of lactation and proliferation and differentiation of the mammary epithelium (5). Interestingly, PRL is also involved in tumorigenesis of the mammary gland (6-8), prostate (2, 9), and ovarian cancer (10), to name a few. In ovarian cancer, PRL receptor (PRLR) has been found to be highly expressed, and the biological relevance of this finding remains unknown (10). In breast cancer, high levels of PRL are thought to be associated with an increased risk of invasive

breast cancer. In addition, expression levels of PRL and its receptor are elevated in most estrogen receptor $(ER)^+$ tumors and ER^- tumors (5). Similarly, the development and progression of androgen-independent malignant neoplasms have been reported to be promoted by PRL. Therefore, treatments to advanced prostate cancer must take into account the down-regulated PRL plasma expression (11).

Although it is known that PRL is involved in cancer development, the exact mechanisms are not fully understood. One of the main problems in cancer is its ability to metastasize, constituting the main cause of mortality in cancer patients (12, 13). For cancer cells to metastasize, they must have the ability to migrate. Cell migration is a crucial event for the execution of numerous physiological and developmental processes. In addition, abnormal migration contributes to the development of metastases (14, 15). Therefore, understanding the mechanisms involved in cell migration is one of the most important goals of modern cancer research (15, 16).

Cell migration depends on ordered mechanisms involving hundreds of proteins and hormonal stimuli that trigger it. In this sense, PRL plays an important role in cancer cell migration (17-28). Therefore, it is important to know the mechanisms that control these actions, that are still not fully understood. One signaling pathway that has been studied in breast cancer cells is PRL-JAK-PAK1. Janus tyrosine kinase 2 (JAK2) activated by PRL has been shown to phosphorylate PAK1 at tyrosines (Tyr) 153, 201, and 285, thereby enhancing cell migration and invasion in breast cancer cells (24, 25). Although the PRL-JAK-PAK1 pathway has been studied as a mechanism for understanding cell migration, other mechanisms associated with this pathway have also been described. Therefore, it is important to describe and integrate the major signaling pathways associated with the PRL-PAK1 pathway to elucidate the mechanisms involved in cancer cell migration.

Prolactin and the Prolactin Receptor

PRL is a protein hormone that belongs to the cytokine family and is encoded by a single gene on chromosome 6 (29, 30). It was originally described as consisting of five exons and four introns, and a total length of 10 kb, then an additional exon (1a) was described (30, 31). This hormone is synthesized and secreted in the lactotropic cells of the adenohypophysis. However, several studies have shown that PRL is also synthesized and secreted in peripheral tissues and organs such as the decidua, mammary glands, ovaries, prostate, testis, endothelial cells, lymphocytes, adipose tissue, and cochlea (30). PRL secretion is mainly regulated by dopamine via dopamine D2 receptors on the surface of lactotrophs (29, 30).

PRL is involved in biological processes, such as reproduction, growth and development, metabolism, immune modulation, electrolyte transport, integument regulation, behavior, and carcinogenesis. However, one of its major roles is during pregnancy and lactation, where it promotes mammary gland development and milk synthesis and excretion (29, 32). The major isoform of PRL has a molecular weight of 23 kDa and is composed of 199 amino acids that exert their effects by binding to their membrane receptor, the PRLR (30).

PRLR belongs to class 1 of the cytokine receptor superfamily, and its structure consists of an extracellular domain, a transmembrane domain, and an intracellular domain that transduces signals (30). In humans, the gene encoding PRLR is located on chromosome 5 (p13→14) and contains at least 10 exons that give it a length of ~100 kb (1, 33); in mice, it is located on chromosome 15, and in rats, it is located on chromosome 2 (1). Alternative splicing has resulted in 6 isoforms in humans: one long (PRLR-L), one intermediate (PRLR-I), two short (PRLR-S), one variant lacking the D1 domain (DS1), and one soluble form (PRL-binding protein) containing only the extracellular domain. The long, medium, and short forms have identical extracellular and transmembrane domains; they differ in their intracellular domain, which has a different size and sequence (29, 30). This difference in the intracellular domain of the receptor appears to modulate intracellular signaling systems in different ways, *i.e.*, each isoform may exert different functions by activating different signaling pathways (34).

Since the extracellular domain is exactly the same for all PRLR isoforms, PRL binds to each of them equally, so the effect depends on the isoform to which PRL binds. In this sense, the PRL receptor complex can activate different signaling pathways. Through PRLR-L, PRL activates the JAK signal transducer and activator of transcription (STAT) signaling pathway (33); this group of proteins consists of 5 isoforms known as STAT1, STAT3, STAT5a/b, and STAT6 (35). When PRL binds to PRLR-S, it activates the mitogen-activated protein kinase (MAPK) pathway (33), also known as ERK (35). There are two isoforms, ERK1 (44 kDa) and ERK2 (42 kDa), which have different functions. While ERK2 is involved in cell proliferation, ERK1 negatively regulates ERK2 (35). Other intracellular kinases such as Src family kinases, focal adhesion kinase (FAK), and phosphoinositol 3-kinase (PI3K) are also activated (36).

PRLR does not have intrinsic tyrosine kinase activity, and therefore transmits signals via other associated cytoplasmic proteins such as JAK2, Fyn, Raf-1, and others (30, 34). Signal transduction begins when PRL binds to D1 and D2 extracellular interaction sites of predimerized PRLRs, causing a conformational change in the receptor dimer. These conformational changes in the extracellular domain induce structural changes in the intracellular domain that promote various processes that enable receptor activation. The intracellular domain, in turn, activates various signaling systems to modulate different cellular processes (30, 34).

Although PRL can activate STAT and MAPK signaling pathways to carry out, *e.g.* breast epithelial cell differentiation to promote milk protein gene expression or prostate epithelial cell proliferation, it is known to phosphorylate other cytoplasmic proteins such as PAK1 (30), which is involved in cell migration.

PRL and its Involvement in Cancer Cell Migration

Cell migration is the coordinated movement of a cell (individual migration) or a group of cells (collective migration) in response to mechanical or chemical stimuli. It is a fundamental process for creating and maintaining adequate organization of multicellular organisms (37, 38). Thanks to cell migration, physiological processes, such as gastrulation (in which cells migrate as sheets to form the endoderm, mesoderm, and ectoderm), organ formation (germ layer cells migrate to different destinations, specialize to different cell populations, and form tissues or organs in the embryo), adequate immune response (in infection, neutrophils migrate into infected tissues to destroy pathogens), wound renewal or healing (replacing old or damaged cells with newly formed cells that migrate from underlying tissue layers), tissue homeostasis, *etc*., can be carried out (16, 37-40). In contrast, defects in cell migration are associated with pathological conditions, such as metastasis in cancer (38).

Metastasis is a process in which tumor cells detach from the primary tumor and colonize distant organs; it accounts for more than 90% of all cancer-related deaths (40). In metastasis, abnormal cells that exhibit genomic heterogeneity undergo epithelial-mesenchymal transition (EMT) and phenotypically exhibit increased cell migration and invasiveness compared to normal cells. Moreover, increased cell migration is an essential requirement for cancer cell invasion and metastasis during cancer development (41). It appears that although normal and cancer cells use similar mechanisms for migration, cancer cells often lose the ability to correctly interpret chemical and mechanical signals from their environment and have lost the signaling mechanisms that tell the cell where to stop, which is important for their anchorage and adhesion (42).

Cell migration is regulated by many hormones, cytokines, and growth factors and requires protrusive and contractile forces provided by the actin cytoskeleton through a combination of actin polymerization and depolymerization, cross-linking of actin filaments, and the interaction of myosin-based motors with actin filaments. The complexity of these processes suggests that there are multiple signaling mechanisms (43). In this sense, the mechanisms underlying cell migration processes induced by PRL are poorly understood, although it has been documented that this hormone induces migration in breast cancer cell lines (T47-D, MDA-231, MCF-7, ZR75-1) (17, 18, 23-26, 44), ovarian cancer cell lines (TOV112D) (28), human endometrial cancer cell lines (Ishikawa and RL95-2) (20), teratocarcinoma cell lines (P19 and NTera 2) (21), pancreatic ductal adenocarcinoma cell lines (MiaPaCa-2 and Panc-1) (45) and in glioblastoma multiforme cell lines (C6 and LN229) (22).

One mechanism by which PRL triggers cell migration is the PRL-JAK-PAK1 pathway, which has been demonstrated in T-47D cells. To confirm this, Hammer et al. used T-47D cells stably overexpressing a) wild-type (WT) PAK1, b) the PAK1 mutant Y3F in which the three PAK1 sites phosphorylated by JAK2 [Tyr(s) 153, 201, and 285] were mutated by phenylalanine, and c) green fluorescent protein (GFP) as a control vector. Thus, T-47D PAK1 WT cells stimulated with PRL (200 ng/ml) for 18 h migrated significantly faster (31-34% reduction in wound closure) compared to T-47D GFP cells (15% wound closure). In contrast, T-47D PAK1 Y3F cells migrated at the same level as control cells without PRL (5-6% wound closure) during the same period. To support the above, Boyden chamber assays demonstrated that after 48 h of stimulation with 500 ng/ml PRL, T-47D PAK1 WT cells (>50 cells migrated) and T-47D GFP (30 cells migrated) migrated significantly faster than the controls. There were no significant differences in T-47D PAK1 Y3F cells (<20 cells migrated). When PAK1 expression was abrogated by siRNA targeting PAK1, cells maintained basal migration in the presence of PRL; the same effect was observed in MCF -7 cells (24). This underscores the importance of PAK1 activated by PRL for cell migration.

PAK1 and its Involvement in Cancer Cell Migration

p21-activated kinases, better known as PAKs, are effector proteins for the Rho GTPases Cdc42 and Rac (46). PAKs are divided into two groups: Group I (PAKs 1-3), which are activated in response to intracellular signals in a GTPase-dependent or -independent manner, and Group II (PAKs 4-6), which are likely regulated by intracellular mechanisms and not by GTPases (43). PAKs are involved in cellular processes such as cell morphogenesis, cell development, growth, mitosis, survival, cytoskeletal organization, EMT, motility, and invasion. However, they are also involved in malignant transformation and cancer (46, 47). This functional diversity depends on the intracellular localization of PAK1. In this context, PAK1 moves between the plasma membrane, adhesion sites, cell-cell junctions, and the nucleus. Approximately 20% of nuclei in interphase contain endogenous PAK1, where it phosphorylates histone H3. Under serum-free conditions, overexpressed PAK1 WT is localized exclusively in the cytoplasm; however, treatment with EGF results in translocation to the nucleus in 40% of cells (47).

PAK1 is the best-studied group I kinase, expressed in a variety of tissues, and involved in functions such as cell

proliferation, survival, motility, and invasion (43). Because PAK1 is associated with cancer cell migration, it is important to understand its structure and activation mechanisms. PAK1 is a 545-amino acid protein consisting of an N-terminal regulatory region with a GTPase-binding domain (GBD) that partially overlaps with an autoinhibitory domain (AID) and a C-terminal serine/threonine kinase domain from which its enzymatic activity is derived (43, 48). The catalytic domain has a single activation site (Thr423) within the activation loop. At the N-terminus there are several sequence motifs responsible for interaction with associated proteins. Residues 75-90 are part of the CDC42/RAC1 interactive binding domain (CRIB), in addition, it contains three proline-rich N-terminal motifs (Pro) that interact with the SH3 domain containing the adapter proteins: GRB2, NCK, and the exchange factor that interacts with p21 PIX (48). Similarly, PAK1 has three nuclear localization signals (NLS) and several phosphorylation sites that allow it to stabilize its active form. Seven of these are autophosphorylation sites: Serine (Ser) 21, 57, 144, 149, 199, 204 and Threonine (Thr) 423 (43).

PAK1 has been found in various tissues such as mammary glands, muscle, spleen, prostate, and nervous system, but PAK1 is overexpressed in tumors such as breast, kidney, and colon. Although PAK1 activity is high in some tumors, this kinase is generally found in its wild-type form, without mutations that activate it. Moreover, PAK1 is elevated in invasive prostate cancer cells compared to non-invasive cells. It has been shown to play a predominant role in microinvasion and is required for prostate tumor growth and micrometastasis. Therefore, PAK1 has been associated with metastatic potential (46) and thus with cell migration. Indeed, one of the first described functions of PAK1 was its role in regulating the actin cytoskeleton and cell migration (43). In this sense, the work of Diakonova and coworkers investigated the involvement of PAK1 in cancer cell migration depending on the phosphorylation of this kinase by PRL. Using mass spectrometry and two-dimensional peptide mapping, they showed that PAK1 is phosphorylated by PRL-activated JAK2 at tyrosines (Tyr) 153, 201, and 285, whereas similar mutation of these phosphorylation sites by phenylalanine (Phe) has no effect on PAK1 activity (49).

The PRL-JAK2-PAK1 Pathway

The PRL-JAK2-PAK1 pathway has been shown to be one of the mechanisms by which PRL can trigger cell migration. However, this pathway is even more complex, and the mechanisms are not fully understood. Therefore, it is important to describe the signaling pathways associated with PRL-JAK2-PAK1 to try and understand the mechanisms involved in cancer cell migration.

The signaling pathway begins with the binding of PRL to its receptor (Figure 1), which dimerizes and causes the activation and autophosphorylation of JAK2 at Tyr1007 and 1008. As a result, focal adhesion kinase (FAK) is autophosphorylated at Tyr397. On the other hand, PAK1 is phosphorylated at Tyr153, 201, and 285, resulting in tyrosine-phosphorylated PAK1 (pTyr-PAK1), which promotes breast cancer cell migration and invasion (25). FAK is a non-receptor protein tyrosine kinase located in focal adhesions of cells and has the ability to make multiple connections to affect the cytoskeleton, cell adhesion site structures, and membrane protrusions to regulate cell migration (50-52). In addition, it is overexpressed in metastatic tumors of the breast, prostate, colon, and brain (51).

Once FAK autophosphorylates itself at Tyr397, it can phosphorylate other proteins, including paxillin, which is an important regulator of adhesion turnover. Paxillin, along with talin and actin, among others, forms small adhesion complexes known as nascent adhesions at the distal edge of the lamellipodium. However, for these new adhesions to mature into focal complexes, FAK Tyr397 must phosphorylate paxillin at Tyr31 and 118, which increases the affinity between the two proteins and favors the binding of other scaffolding proteins, ultimately promoting the maturation of the new adhesion in the focal complex. The latter can take two paths: they can mature into larger focal adhesions, or they can dissolve by a process known as turnover (43, 51). The protein pTyr-PAK1 is able to phosphorylate paxillin at Ser273, thereby increasing the turnover of adhesions by decreasing the affinity of FAK for paxillin, initiating the breakdown of adhesions, and releasing FAK to facilitate the formation of new nascent adhesions. However, these adhesions cannot form mature focal complexes because the involvement of paxillin is required, increasing the rates of adhesion formation and degradation (43).

Similarly, autophosphorylation of FAK at Tyr397 enhances its kinase activity and provides an important binding site for Src family kinases (50). This FAK/Src binding is essential for FAK phosphorylation in the kinase domain at Tyr576/577, fully activating it (51). However, other FAK phosphorylation sites have been identified in the C-terminal domain at Tyr861 and Tyr925, which also contribute to its activation (50). The FAK-Src complex controls cell shape and rotation of focal contacts during cell migration and mediates phosphorylation of several adhesion components involved in dynamic regulation of cell migration, including p130Cas (50, 51). The latter is a scaffold protein that is recruited to focal adhesion sites thanks to the contact of its SH3 domain with FAK. Phosphorylation of p130Cas promotes binding of the adaptor protein Crk, which in turn recruits the DOCK180 protein, ultimately leading to activation of the Rac1 protein of the Rho GTPase family. Activation of Rac1 promotes membrane maturation, lamellipodia formation, and actin reorganization by acting on the Arp 2/3 complex, which stimulates actin polymerization and forms a network of branched filaments as this complex nucleates new actin filaments alongside the pre-existing ones (43, 53).

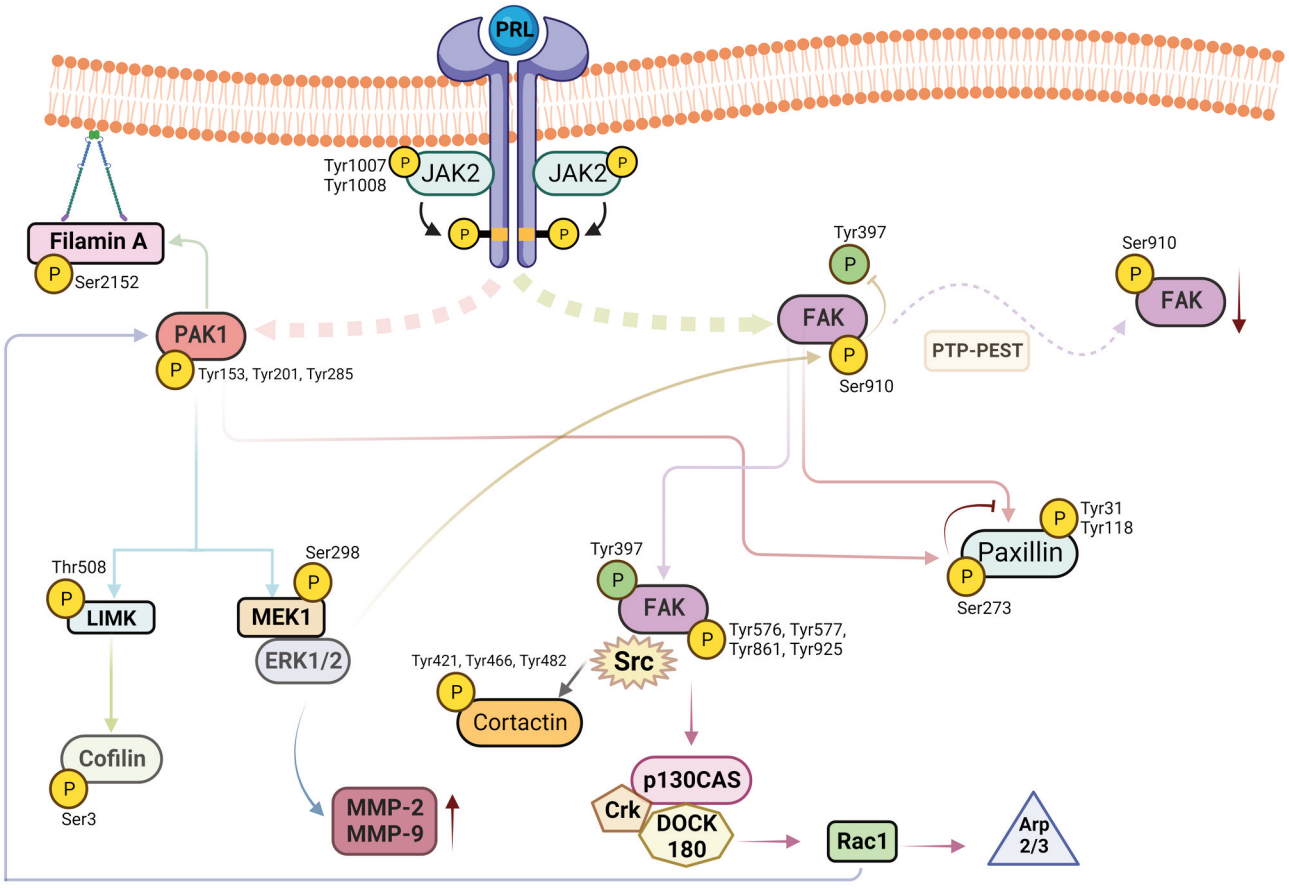

Figure 1. *Signaling pathways involved in cell migration in response to PRL and interaction with PAK1. PRL binds to its receptor, PRLR, which dimerizes, leading to activation and autophosphorylation of JAK2 at Tyr1007 and 1008, whereupon FAK is autophosphorylated at Tyr397. Phosphorylated FAK induces phosphorylation of paxillin at Tyr 31 and 118; it also provides a binding site for Src family kinases. The FAK/Src complex is required for the phosphorylation of FAK at Tyr576, 577, 861, and 925. This causes binding of other proteins such as p130CAS, which, when phosphorylated, promotes binding of the adaptor protein Crk, which in turn recruits the protein DOCK180, leading to activation of the Rac1 protein. Rac1 acts on the Arp 2/3 complex to stimulate actin polymerization, likewise Rac1 can phosphorylate PAK1. Similarly, PAK1 protein is phosphorylated after phosphorylation of JAK2 at Tyr 153, 201, and 285. Phosphorylated PAK1 has the ability to phosphorylate paxillin at Ser273, which reduces the affinity of this protein for FAK. Another protein phosphorylated by PAK1 is LIMK at Thr508, which in turn phosphorylates cofilin at Ser3. In addition, PAK1 phosphorylates MEK1 at Ser298, which causes activation of ERK1/2. The latter phosphorylates FAK at Ser910, which causes dephosphorylation of FAK at Tyr397 and also promotes recruitment of protein tyrosine phosphatase PTP-PEST. ERK1/2 is involved in modulating the expression of MMP-2 and MMP-9. Cortactin can be phosphorylated by Src protein kinase at Tyr 421, 466, and 482. Filamin A can also be phosphorylated by PAK1 at Ser2152, inducing both autophosphorylation and kinase activity of PAK1 to regulate adhesion turnover during cell migration. PRL: Prolactin; JAK2: Janus kinase 2; Tyr: tyrosine; FAK: focal adhesion kinase; PAK1: p21-activated kinase-1; Ser: Serine; MEK1: MAPK/ERK kinase 1; ERK1/2: extracellular-signal-regulated kinase 1/2; PTP-PEST: protein tyrosine phosphatase with residues rich in proline, glutamic acid, serine and threonine; MMP-2: matrix metalloproteinase 2; MMP-9: matrix metalloproteinase 9; LIMK: LIM-kinase; Src: stored response chain; p130CAS: p130 Crk-associated substrate; DOCK180: dedicator of cytokinesis 180; Rac1: RAS-related C3 botulinum toxin substrate 1; Arp2/3: actin-related proteins 2/3; Crk: CT10 regulator of kinase. Created in BioRender.com.*

It is known that continuous activation of FAK-Src resulting from increased expression of focal adhesion components (FAK, Src, p130Cas, or Crk) in tumor cells can limit the maturation of focal complexes into stable focal adhesions or cause rapid turnover that promotes cancer cell motility (51, 54-59).

Once activated, Rac can phosphorylate PAK1, which in turn can phosphorylate the protein LIM-kinase (LIMK) at Thr508 (60). This protein is involved in cytoskeletal reorganization by regulating actin polymerization through phosphorylation of the protein cofilin at Ser3, causing its inactivation (61). Cofilin is a protein that promotes actin depolymerization. However, this ability is inhibited by phosphorylation of the Ser3 residue, so that Rac is able to inhibit the depolymerization induced by cofilin and allow

greater accumulation of polymerized actin at the cell leading edge (59). Similarly, there is evidence that cofilin is necessary to promote lamellipodia expansion, which has the purpose of promoting cell migration, either by releasing actin monomers that can bind to growing actin filaments in the plasma membrane and/or by shortening actin filaments, making more ends available for actin polymerization (61).

Cortactin is another protein that can be the target of PRL signaling. It is an actin-binding protein found in the cortical actin cytoskeleton, where it regulates actin nucleation, endocytosis, and polymerization during cell motility and adhesion (62). However, it has also been observed to play an important role in actin assembly for invadopodia formation, where it promotes protease secretion (63). Full activation of cortactin requires that some protein kinases such as Src phosphorylate it at Tyr421, 466, and 482. Subsequently, the level of phosphorylated cortactin decreases and favors migration and invasion, both through the formation of lamellipodia and through degradation of the extracellular matrix (ECM) by matrix metalloproteases (MMPs) that may be present in the invadopodia. Finally, it has been suggested that cortactin may regulate the stability or assembly of branched actin thanks to its direct interaction with the Arp 2/3 complex and actin filaments (64).

Similarly, pTyr-PAK1 phosphorylates MEK1 at Ser298, leading to activation of extracellular signal-regulated kinase 1 and 2 (ERK 1/2). The active ERK is able to phosphorylate FAK at Ser910, which promotes the recruitment of protein tyrosine phosphatase with proline-, glutamic acid-, serine-, and threonine-rich residues (PTP-PEST) in the lamellipodia of migratory cells and leads to the dephosphorylation of FAK at Tyr397. This process causes a decrease in FAK kinase activity and promotes renewal of cancer cell adhesion and migration (25). Downregulation of FAK activity by PTP-PEST promotes degradation and renewal of focal contact and thus cancer cell migration, invasion, and metastasis (65).

Some studies have suggested a possible role of ERK in the modulation of MMPs, zinc-dependent endopeptidases whose main function is the degradation of the ECM. In particular, it was mentioned that the ERK signaling pathway could affect the expression of MMP-2 and MMP-9, because in cell cultures where the action of this protein is inhibited, this leads to a decrease in the activity of both MMPs. This suggests that specific blockade of the ERK pathway inhibits tumor cell invasiveness by negatively regulating MMPs, whereas activation of this pathway increases the activity of the above MMPs (66).

On the other hand, pTyr-PAK1 can also phosphorylate the protein filamin A (FLNa) directly at Ser2152, which promotes actin cytoskeleton reorganization and cell ruffling. FLNa is an actin-binding protein that binds actin filaments to the plasma membrane and can intervene with other proteins in cytoskeletal restructuring to mediate cell adhesion, migration, and spreading. Once FLNa is phosphorylated, it interacts and colocalizes with endogenous PAK1 in membrane ruffling, necessary for cell migration. Thus, FLNa is important for stabilizing orthogonal actin networks suitable for locomotion, and decreased activity is thought to promote cell migration, invasion, and metastasis (24, 67).

Conclusion

The main function of PRL is synthesis of milk in the mammary glands. However, although this hormone is not carcinogenic, it is also involved in the development of diseases such as cancer. The main problem with cancer is its ability to metastasize, and for cells to invade and metastasize, they must migrate. In this sense, PRL is also shown to be involved in cell migration as it triggers migration in breast, prostate, and ovarian cancer cell lines. However, the mechanisms that trigger this migration have not been fully elucidated. The mechanism that has been studied in more detail is the PRL-JAK2-PAK1 pathway, which has been described primarily in breast cancer cell lines. Other works have described this pathway and evaluated the proteins activated by this pathway, such as FAK, paxillin, MMPs, *etc*., involved in cell migration. Since the role of PRL in cell migration is relevant, we set out to integrate and describe the signaling pathways associated with PRL-JAK2-PAK1 to better understand the role of PRL in cancer cell migration. However, further studies need to be performed, for example, in prostate cancer cell lines (LNCaP and DU145) known to express PAK1, although the involvement of the PRL-JAK2-PAK1 pathway has not yet been investigated. Because PRL induces migration in cancer cell lines, it is likely that this hormone is involved in metastasis. Therefore, PRL should be considered as a therapeutic target in the fight against metastatic cancer.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

The Authors contributed equally to all aspects of the article.

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