Short report

Prolactin/Jak2 directs apical/basal polarization and luminal lineage maturation of mammary epithelial cells through regulation of the Erk1/2 pathway

Fengming Liu a, Andrew Pawliwec a, Zhenqian Feng a, Zivart Yasrue la, Jean-Jacques Lebrun b, Suhad Ali a,⁎

a Division of Hematology, Department of Medicine, Cancer Research Program, McGill University Health Centre, Montreal, QC, H4A 3J1, Canada
b Division of Medical Oncology, Department of Medicine, Cancer Research Program, McGill University Health Centre, Montreal, QC, H4A 3J1, Canada

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ABSTRACT

Tissue development/remodeling requires modulations in both cellular architecture and phenotype. Aberration in these processes leads to tumorigenesis. During the pregnancy/lactation cycle the mammary epithelial cells undergo complex morphological and phenotypic programs resulting in the acquisition of apical/basal (A/B) polarization and cellular maturation necessary for proper lactation. Still the hormonal regulations and cellular mechanisms controlling these events are not entirely elucidated. Here we show that prolactin (PRL)/Jak2 pathway in mammary epithelial cells uniquely signals to establish A/B polarity as determined by the apical localization of the tight junction protein zona occludens 1 (ZO-1) and the basal/lateral localization of E-cadherin, and the apical trafficking of lipid droplets. As well, our results indicate that this pathway regulates mammary stem cell hierarchy by inducing the differentiation of luminal progenitor (EpCAMhi/CD49fhi) cells to mature luminal (EpCAMhi/CD49flow) cells. Moreover, our data indicate that PRL/Jak2 coordinates both of these cellular events through limiting the mitogen activated protein kinase (Erk1/2) pathway. Together our findings define a novel unifying mechanism coupling mammary epithelial cell A/B polarization and terminal differentiation.

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1. Introduction

The mammary tissue undergoes extensive morphological and phenotypic alterations during the pregnancy/lactation cycle forming a complex network of alveolar structures (Rosen et al., 1994; Macias and Hinck, 2012). This cellular architecture exhibits well defined A/B polarization established by the adherence and tight junction protein complexes. This polarized epithelial sheet with tight junction barrier consists of mature luminal epithelial cells functionally capable of synthesis and directional secretion of milk into the lumen of the mammary gland during lactation (Anderson et al., 2007).

While our understanding of the phenotypic differentiation of the mammary epithelial cells is still limited, however, signature molecules for various stem/progenitor cell populations have been described recently (Visvader and Stingl, 2014). Adult mammary glands maintain a population of stem cells which are capable of generating epithelial progenitor cells giving rise to terminally differentiated cells. Current knowledge indicates the complex nature of the mammary stem cell (MaSC) hierarchy (Fu et al., 2014; Shehata et al., 2012). Based on the expression pattern of cell surface markers EpCAM and CD49f, a differentiation model of MaSC was proposed: EpCAMlow/CD49fhi (bipotent progenitors)-EpCAMhi/CD49fhi (luminal progenitors)-EpCAMhi/CD49flow (mature luminal cells) (Fu et al., 2014; Shehata et al., 2012). The hormonal regulations and cellular mechanisms controlling these morphogenic/phenotypic events are not fully determined.

The hormone prolactin (PRL) is known to be indispensable in regulating the development of the mammary gland and promoting the terminal differentiation of mammary epithelial cells. PRL is known to mediate these effects through activation of the Jak2/Stat5 pathway. Indeed, genetically engineered knockout mice lacking either PRL, the PRL receptor, Jak2 or Stat5 showed limited mammary alveolar development and loss of lactation capacity, implicating a potential role for PRL in mammary alveolar development (Horseman et al., 1997; Ormandy et al., 1997; Wagner et al., 2004; Liu et al., 1997). Furthermore, we have previously shown that PRL signaling through Jak2 to induce epithelialization of mesenchymal breast cancer cells by suppressing the process of EMT further pointing to a potential role for PRL in regulating mammary epithelial morphogenesis/polarity (Nouhi et al., 2006).

Here we set to explore the possible role of PRL as a regulator of A/B polarity establishment and cell fate determination of mammary epithelial cells. Using 3-dimensional (3D) cellular culture systems of mammary epithelial cells we show that PRL specifically is a key factor in inducing A/B cell polarization and generation of mature luminal cells.

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Interestingly these complex functions of PRL/Jak2 were linked to the ability of this signaling cascade to limit the activity of the Erk1/2 pathway. Together our results describe novel functions for PRL, in addition to its well-recognized lactogenic role, as an A/B polarity inducer and a cell fate determinant factor.

2. Methods

2.1. Antibodies, plasmids and other reagents

Antibodies and reagents are listed in supplemental table 1. Small hairpin shRNAs in the pLK0.1 lentiviral vector against mouse Jak2 were obtained from The RNAi Consortium (TRC-Mm1.0) lentiviral shRNA library (Open Biosystems, GE Healthcare). Mature antisense shRNA and their efficacy in Jak2 suppression were listed in supplemental table 2. The scramble shRNA in pLK0.1 vector was obtained from Addgene (Addgene plasmid #1864). Human Jak2 cDNA in pcDNA vector was a generous gift from Dr. Olli Silvennoinen (University of Tampere Finland). Other reagents were used as described.

2.2. Cell lines and cell culture

Mouse mammary epithelial cell line HC11 was routinely maintained in growth media (Haines et al., 2009). Jak2 suppressed HC11 cell populations were generated by transfecting shRNAs with Lipofectamine™ 2000 (Invitrogen) and cells were screened with 1 μg/ml puromycin (InvivoGen) selection. Jak2 suppressed HC11 cells were rescued with expression of human Jak2/PCIneo selected in 250 μg/ml G418.

2.3. 3D cell culture

The Poly-o-Lysine coated 8-well culture slides (BD Biosciences) were utilized for 3D culture. Briefly, each well of the culture slide was first coated with 100 μl growth factor reduced Matrigel® (BD Biosciences), after polymerization, 5000 cells in 100 μl growth medium were plated and allow 1.5 h for cells to attach. 100 μl growth medium containing 10% Matrigel® was added on top, creating a final concentration of 5% Matrigel® in full growth medium. Cells were maintained in growth medium with 5% Matrigel® for two days for cell colonies outgrowth. The morphology of the colonies were evaluated after 3 days of 5 different treatments: (1) control (CTL): 2% FBS, (2) EGF: EGF 10 ng/ml and 2% FBS or (3) HIP, 1 μM hydrocortisone, 5 μg/ml insulin, 2 μg/ml ovine PRL and 2% FBS, (4) HI, 1 μM hydrocortisone, 5 μg/ml insulin and 2% FBS and (5) PRL, 2 μg/ml ovine PRL and 2% FBS. Mouse primary mammary epithelial cells were prepared from mid-pregnant C57BL/6 (Jackson Mice) females in DMEM/F12 media with 5% FBS using a kit, STEMCELL Technologies Inc. (Canada). Isolated MECs (5000 cells/well) were plated for 3D culture.

3. Results

3.1. Prolactin functions as a polarity signal in mammary epithelial cells

The use of 3D culture of mammary organoids, primary mammary epithelial cells as well as mammary cell line was instrumental in deciphering the contributions of hormonal/growth factors, extracellular matrix proteins and intracellular signaling pathways in mammary epithelial morphogenesis, gland (Lo et al., 2012; Shaw et al., 2004). To investigate the possible role of PRL in establishing A/B polarity in mammary epithelial cells, we used HC11 mouse mammary epithelial cells in 3D Matrigel culture system. HC11 cells recapitulate many aspects of mammary luminal epithelial cells. They require EGF for cell proliferation and undergo differentiation following PRL stimulation (Taverna et al., 1991; Ball et al., 1988). We found that HC11 cells grown in the presence of EGF to form large disorganized mammary colonies that are characterized by the lack of lumen and A/B polarization as determined by ZO-1 and E-cad localization. In contrast, colonies treated with the lactogenic hormone combination of hydrocortisone (H), insulin (I) and PRL (HIP) formed mammary acini in which the cells are polarized and organized around a single lumen resembling the inner lobular core of alveoli in lactating mammary glands (Fig. 1A, left panel). To distinguish the specific effector hormone mediating these structural organizations, cells were grown in the presence of PRL alone, HI or serum (CTRL). Interestingly, only PRL treated cells showed organized mammary acini. In contrast, no organized acini were observed in HI or serum treated cells (Fig. 1A, left panel, Fig. S1A). This data together establish the role of PRL as a mammary epithelial morphogen inducing A/B polarization. To further validate and extend our findings that PRL initiates alveolar morphogenesis, we performed similar experiments using primary mammary epithelial cells (MECs) dissociated from mid-pregnant C57BL/6 mice. Importantly, similarly organized alveolar acini were observed only in cells treated with either a mixture of HIP or PRL alone (Fig. 1A right panel, Fig. S1B). To enumerate the observed effects of PRL on cellular polarization, we quantified the organized acini within the various treatments. As can be seen in Fig. 1B & C, cells treated with HIP or PRL showed significant increase in the development of organized acini in comparison to cells treated with EGF, HI or serum. Occasionally, we also observed the generation of multi-lumen acini in colonies grown in the presence of EGF (Fig. S1C). Moreover, the size of the colonies grown in PRL treated cells were the smallest of all (Fig. S1D). This data highlight PRL as a polarity cue in mammary epithelial cells.

During lactation, directional secretion is an important functional property of alveolar cells and is determined by the establishment of A/B polarity. Therefore, we next examined the functionality of these acini by staining with Nile Red, a fluorescent dye labeling cytoplasmic lipid droplets (Greenspan et al., 1985). Indeed, formation/accumulation of lipid droplets is contingent upon alveolar cell secretory differentiation (Russell et al., 2011). Notably, only HIP treated acini were found to accumulate large granular lipid droplets localized at the apical/luminal side of the acini (Fig. 1D). No lipid droplets were observed under all other conditions tested. Moreover, no lipid accumulation was observed in EGF treated colonies showing multilumen acini or structurally normal acini in HI treated colonies (Fig. 1E). This data together indicate that PRL is required for lipid droplet apical trafficking/accumulation, signature of alveolar cell secretory differentiation.

3.2. Prolactin/Jak2 signaling mediates mammary epithelial polarity establishment and junction organization through regulating Erk1/2 activity

The Jak2 kinase is known to be the major kinase mediating PRL signaling in mammary gland development and lactation (Wagner and Rui, 2008). To determine the downstream signaling mechanism mediating PRL morphogenic effects, we used multiple short hairpin RNAs (shRNAs) targeting mouse Jak2 to block PRL signaling. We generated 6 independent stable populations of HC11 cells with Jak2 knockdown. After screening, two of the stable cell lines J5 and J6 showed Jak2 knockdown (63.4% and 63.6% suppression, respectively) and were used for our analyses (Fig. S2A–C). Moreover, we verified Jak2 knockdown in J6 cells using qRT-PCR (Fig. S2D). We then generated J6Rh stable cell line overexpressing human Jak2 cDNA in J6 cells for rescue experiments.
We compared the Erk1/2 activation levels between the parental HC11 cells and J6 cells, showing diffused cytoplasmic staining, while rescuing Jak2 expression was able to restore membrane localization of both markers in J6Rh cells (Fig. 2B, Fig. S2G, top panel). These results emphasize the critical role of Jak2 in cellular junctional organization. To further investigate whether other polarity protein complexes were affected in J6 cells, we considered the Par protein complex which has previously been described as part of an evolutionarily conserved complex that plays an important role in establishing cellular polarity (Goldstein and Macara, 2007). In polarized cells, the protein Par3 is known to be localized in association with apical junction complexes (Nelson et al., 2013). Therefore, we next examined the localization of Par3 protein in J5 and J6 cells. We found Par3 to be mislocalized in Jak2 suppressed cells (Fig. 2C, Fig. S2G, bottom panel). Although western blotting results suggested no differences in expression levels of Par3 as well as other par-complex proteins including aPKCz and Igl (Fig. S2H). Importantly, similar to ZO-1 and E-cad, the membrane localization of this juncional protein marker Par3 was restored in J6Rh cells (Fig. 2H). Together, this data highlights Jak2 as a critical mediator of PRL signaling in acinar morphogenesis, A/B polarization and junctional organization.

Previous work suggested that PRL/Jak2 signaling exerts inhibitory effects on Erk1/2 activation (Nouhi et al., 2006; Haines et al., 2009). We compared the Erk1/2 activation levels between the parental HC11 cells and J6 cells. Interestingly, as can be seen in Fig. 2D, we found sustained Erk1/2 activation in J6 cells. Indeed, the extent of Erk1/2 activity was found to be similar to that induced by EGF, a well-known inducer of Erk1/2 activation. Similarly, activation of Erk1/2 was also observed in HC11 cells treated with a Jak2 specific kinase inhibitor (Jak2i) (Fig. 2D). We also examined the activation of PI3K/Akt pathway under these conditions and found no difference in Akt activation in J6 cells in comparison to HC11 cells (Fig. S2I). Together, these results suggest a potential role for sustained Erk1/2 activation in disruption of cellular polarization and junctional organization following loss of Jak2 expression. To test this hypothesis, J6 cells were treated with the Mek inhibitor PD0325901 for a 24 hour time period and cell/junction formation was examined. Notably, treatment of J6 cells with PD0325901 fully restored the membrane localization of ZO-1, E-cad and Par3 (Fig. 2E & F). Together, this data underscore Jak2 regulation of cellular junctional organization through modulation of Erk1/2 activities.

3.3. Prolactin promotes the maturation of mammary luminal progenitor cells

During alveogenesis, alveolar cells also undergo coordinate and irreversible phenotypic maturation besides acquiring A/B polarization. Therefore, we next examined whether PRL plays a role in alveolar cell fate determination. We hypothesized that PRL regulates MaSC hierarchy to enrich for mature luminal cells. For this reason, we assessed the expression profile of EpCAM and CD49f using flow cytometry in HC11 cells following stimulation with EGF, HH, HIP or PRL (Fig. 3A). In control EGF treated cells, we identified two distinct cellular sub-populations, one featured a surface marker signature with EpCAMhi/CD49fl defining the luminal progenitor cells and a second population with EpCAMhi/CD49flew defining mature luminal cells. Interestingly, treatment of HC11 cells with HIP or PRL resulted in the differentiation of luminal progenitor (EpCAMhi/CD49fl) cells into mature luminal (EpCAMhi/CD49flew) cells (Fig. 3A). Indeed, we observed a significant – 30% of luminal progenitor cells differentiated into mature luminal cells in these samples (Fig. 3B). In HI treated cells, on the other hand, we observed no change in pools of progenitor or mature luminal cells, suggesting that HI plays no role in the differentiation of luminal cell hierarchy (Fig. 3A & B). Moreover, histogram display of individual markers confirmed the decrease in expression of CD49f (Fig. 3C, C1) and the increase in expression of EpCAM (Fig. 3C, C2) in HIP treated cells, in comparison to EGF treated cells. Similar results were obtained in PRL treated cells in comparison to EGF treated cells (Fig. 3C, C3 & C4). To further validate the role of PRL in inducing the differentiation of mammary luminal progenitor cells into mature luminal cells we also examined the effects of PRL using primary mouse mammary epithelial cells isolated from mid pregnant mice (Fig. 3D). Importantly, our data show that treatment of primary cells with either PRL or HH induced the maturation of progenitor (EpCAMhi/CD49fl) cells into mature luminal (EpCAMhi/CD49flew) cells. Altogether, our data reveal a central role for PRL in promoting the maturation of the mammary luminal lineage.

We next evaluated the role of Jak2 in promoting the transition of luminal progenitor cells to mature luminal cells. Interestingly, we found that loss of Jak2 significantly abrogated the maturation of luminal progenitor cells to mature luminal cells (Fig. 3E). Indeed, the ratio of mature luminal to luminal progenitor cell populations was found to be higher in HC11 in comparison to J6 cells most notably at the 48 hour time point (Fig. 3F). Histogram display of CD49f further showed that there was sustained expression of CD49f in J6 cells (Fig. 3G) in comparison to HC11 cells whereas no significant change in EpCAM expression was found in the two different cell lines (Fig. 3H). This data demonstrates the critical role of Jak2 in mediating PRL-induced luminal maturation of mammary epithelial cells.

To decipher the mechanism by which PRL/Jak2 mediates mammary cell luminal maturation we focused on the Erk1/2 pathway as J6 cells show constitutive Erk1/2 activation. We hypothesized that this increased Erk1/2 activity is impeding J6 cells to undergo luminal maturation. Most importantly, treatment of J6 cells with the Mek1 inhibitor resulted in the transition of EpCAMhi/CD49fl to mature EpCAMhi/CD49flew cells (Fig. 3I). This transition process was not further enhanced by the addition of PRL (Fig. 3I). Together, this data indicate that mammary luminal cell maturation requires tight control of the Erk1/2 pathway exerted by PRL/Jak2 signaling. Collectively, our findings show that PRL coordinates both polarity cues and cell fate determination for mammary epithelial cells. These newly defined functions for PRL are of high significance in understanding the normal development of the mammary gland and its carcinogenesis where these mechanisms are deregulated.

4. Discussion

Understanding mammary gland biology is of critical significance given the prevalence of breast cancer worldwide. To characterize mechanisms involved in regulating mammary morphogenesis, extensive studies have used ex vivo culture model of mammary epithelial cells on extracellular matrices in the presence of various hormonal and growth factors. These original studies showed that mammary epithelial cells to organize into functional acinar architecture resembling mammary alveoli. Information generated using these cellular model systems have highlighted the role of the ECM component like laminin (Streuli et al., 1991, 1995) and integrin (Lee and Streuli, 2014) as important regulators of mammary acini morphogenesis. Furthermore, Xian et.al has reported the development of mammary acini using HC11 cells cultured in Matrigel in the presence of EGF (Xian et al., 2005). However, there have been no studies examining explicitly the role of PRL hormone in regulating the various aspects of acini morphogenesis. Here we describe...
a new role for PRL as a crucial regulator of mammary epithelial A/B polarization and luminal cell fate determination.

While there is limited information with respect to physiological ligands inducing mammary acini morphogenesis, the literature presents several growth factors, oncogenes and signaling pathways that are involved in disrupting mammary cell polarity and acini formation. Indeed, it was shown that FGF (Xian et al., 2005); TGF-β (Ozdamar et al., 2005); Erbb2 (Aranda et al., 2006) and Ephrin B1 (Lee et al., 2008) as well as NFκB (Becker-Weimann et al., 2013) to interfere with mammary acini formation/organization. Thus, our results

Fig. 2. PRL/Jak2 signaling regulates cellular junction organization through inhibition of Erk1/2 activity. (A) Western blot showing Stat5 phosphorylation in HC11, vector transfected (CTL), J5, J6 and J6Rh cells following PRL stimulation for 10 min. Membranes were reprobed for total Stat5 and β-tubulin. (B) HC11, J6 and J6Rh cells grown in 2D culture were stained for ZO-1 (green) and E-cad (red). (C) HC11, J6 and J6Rh cells grown in 2D culture were stained for Par3 (green) and E-cad (red). Scale bar 10 μm. (D) Western blot analysis using antibody to phospho-Erk1/2 (Thr202/Tyr204) in HC11 and J6 cells following the indicated treatments. EGF was used at 50 ng/ml for 15 min and Jak2 kinase inhibitor (Jak2I) was used at 20 μM for an overnight period. Membranes were probed for Erk1/2 and β-tubulin. (E) & (F) HC11 and J6 cells were subjected to 200 nM (L) or 500 nM (H) PD0325901 or DMSO mock treatment for 24 h before staining for ZO1 (green) and E-cad (red) (E) or Par3 (green) and E-cad (red) (F). Scale bar 10 μm. Merged images show nucleus counter-stained with DAPI (blue).
demonstrating an organizational role for PRL in mammary acini morphogenesis is highly significant. Indeed, our results demonstrate a novel regulatory PRL-dependent mechanism coordinating mammary acini organization.

The current view of mammary acini organization and lumen formation implicate apoptosis in shaping acini lumens (Humphreys et al., 1996; Debnath et al., 2002; Maillieux et al., 2008). Indeed, studies utilizing the mammary epithelial cell line MCF10A have indicated that within the mammosphere the inner cell population undergoes anoikis due to lack of matrix attachment and growth factor exposure resulting in lumen formation and have implicated the pro-apoptotic protein Bim in this process. Our results highlight two potentially coordinated mechanisms regulating lumen formation. Indeed, while PRL treated acini show well established polarity we noticed that the lumen of these colonies are small in comparison to acini grown under HIP treatment conditions. This data implicates insulin and/or hydrocortisone in apical membrane generation/expansion. Indeed, studies performed with MDCK cells do implicate lipid metabolism in apical membrane generation (Bryant et al., 2010). Therefore, we propose that lipid metabolism downstream of insulin is potentially leading to the expanded lumen seen in HIP treated acini.

Notably, our stem cell profiling analyses using EpCAM and CD49f stem cell markers in HC11 and in primary mammary epithelial cells isolated from mid-pregnant mice showed similar profile, confirming the luminal origin of HC11 cells. Furthermore, these profiles identified only two subpopulations comprising the luminal progenitors and mature luminal cells. No basal or stromal cells could be detected (EpCAMlow/CD49fluc or EpCAMlow/CD49fpro) as has been documented using primary mammary epithelial cells isolated from virgin mice. Together, these results indicate the enrichment of the luminal lineage during pregnancy (Vissader and Stingl, 2014; Shehata et al., 2012). Moreover, using these cellular model systems we show that PRL through Jak2 plays a critical role as a mammary cell fate determinant inducing the differentiation of mammary progenitor cells into mature luminal cells capable of acinar morphogenesis. The role of PRL/Jak2 pathway in regulating mammary luminal maturation is also supported by the observations that Stat5alpha/b knockout mice show loss of mammary luminal cell population (Yamaji et al., 2009). Together our results demonstrate that PRL hormone through Jak2 kinase couples both cellular A/B polarization and mammary stem cell hierarchy.

The Erk1/2 signaling cascade regulates a variety of cellular processes by phosphorylating multiple target proteins (Lefloch et al., 2009; Yoon and Seger, 2006). Our results highlighted the negative cross-talk between PRL/Jak2 and the Erk1/2 pathway to be critical in regulating both mammary epithelial A/B polarity and stem/progenitor cell differentiation. We have reported previously that this negative cross-talk was found to be important in PRL’s ability to block EGF-induced mammary epithelial cell proliferation as well as in PRL’s ability to block EMT process in breast cancer cells. Although the detailed network of crosstalk between PRL/Jak2 and Erk1/2 pathway is yet to be established, it is likely to involve multiple mechanisms that needs to be further elaborated. Interestingly, it was reported that EpCAM expression itself may regulate cadherin mediated cell adhesion through suppression of the MAPK signaling cascade (Maghzal et al., 2013). Therefore, it is possible to postulate that PRL-mediated increase in EpCAM expression observed may ultimately lead to suppression of the Erk1/Erk2 pathway in mammary epithelial cells allowing acinar morphogenesis.

Finally the results described here have important implications in expanding our understanding of the role of PRL in breast tumorigenesis. The role of PRL in breast tumorigenesis is not fully clarified. There have been reports indicating a pro-oncogenic role for PRL functioning as a local autocrine growth factor in mammary cells. Moreover, data generated from in vivo studies have highlighted PRL to play a permissive role in oncogene-induced mammary tumorigenesis (Fernandez et al., 2010; Wennbo et al., 1997; Rose-Hellekant et al., 2003). Indeed, as lumen filling/regeneration of the luminal space is a hallmark of early breast tumors, we expect restoration of PRL/Jak2 signaling in breast tumor cells to induce cell polarization and promote lumen clearance. On the other hand it is known that lumen filling/regeneration of the luminal space is a hallmark of early breast tumors, therefore, we propose that restoration of PRL/Jak2 signaling in breast tumor cells to induce cell polarization and promote lumen clearance. Moreover, our findings that PRL promotes the maturation of luminal progenitor cells and induces mammary morphogenesis combined with our previous study showing PRL as a suppressor of EMT process in breast cancer cells (Nouhi et al., 2006) underscore PRL as a potential tumor suppressor and therapeutic modality against breast cancer.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.08.001.

Conflict of interest

The authors declare no conflict of interest.

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