

The Role of Prolactin Signaling in Epithelial Polarity and Lumen Formation

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1 Abstract

Cell polarity and lumenogenesis within epithelial cells are complex processes regulating normal tissue development and architecture. Deregulation in these processes lead to developmental defects and cancer. During pregnancy and lactation epithelial cells of the mammary gland undergo dramatic growth and morphogenesis, creating a fully differentiated lobuloalveolar network. These structures are characterized by polarized epithelial cells with tight junctions surrounding a central lumen crucial for the proper synthesis and directional secretion of milk proteins needed for breastfeeding. Extensive research has demonstrated that a major contributor of change seen during late pregnancy and lactation is the hormone prolactin (PRL). To investigate the role of PRL in this process, we have established a 3D culture assay using the mammary epithelial cell line HC11. For the first time, our lab reports PRL as a major contributor of epithelial polarity and lumen formation in mammary cells. HC11 cells cultured in the presence of PRL formed mammary acini with a single central lumen accompanied by the correct localization of the apical marker ZO-1 and the lateral/basal marker E-Cadherin. Treatment of HC11 cells with PRL also resulted in a small, yet reproducible inhibitory effect on cellular migration, but not proliferation. Using genetic manipulation, we found that the PRL-induced epithelial polarity was JAK2-dependent. Furthermore, we report that JAK2 is essential for proper PRLR localization, along with membrane localization of polarity proteins E-cadherin and ZO-1. Together, we report here that PRL is an important polarity signal in mammary epithelial cells, which gives new insight into the mechanisms governing its role in mammary gland development and tumour suppression.

2 Résumé

La polarité cellulaire, ainsi que la formation du lumen des cellules épithéliales, sont des procédés complexes qui régulent le développement et l'architecture des tissus normaux. La dérégulation de ces procédés mène à des déficiences du développement et au cancer. Durant la grossesse et l'allaitement, les cellules épithéliales de la glande mammaire subissent une croissance et un changement de morphologie dramatiques, créant ainsi un réseau lobulo-alvéolaire complètement différencié. Ces structures sont caractérisées par des cellules épithéliales polarisées, avec des jonctions serrées entourant un lumen central qui est crucial pour la synthèse et la sécrétion des protéines du lait nécessaires à l'allaitement. Des recherches approfondies ont démontré que l'hormone prolactine (PRL) est une contributrice majeure aux changements observés durant les derniers stades de la grossesse et l'allaitement. Pour déterminer le rôle de la PRL dans ce procédé, nous avons établi un test de culture 3-D en utilisant la lignée de cellules mammaires épithéliales HC11. Pour la première fois, notre laboratoire rapporte la prolactine comme étant une contributrice majeure à la polarité épithéliale et à la formation du lumen des cellules mammaires. Les HC11 cultivées en présence de la PRL forment des acini mammaires avec un seul lumen central accompagné d'une localisation appropriée du marqueur apical ZO-1 et du marqueur latéral/basal E-cadherin. Le traitement des HC11 avec la PRL résulte aussi à une faible mais consistante inhibition de la migration cellulaire, mais pas de la prolifération. En utilisant des manipulations génétiques, nous avons trouvé que la polarité épithéliale induite par la PRL était dépendante de JAK2. De plus, nous rapportons que JAK2 est essentielle à la localisation appropriée du PRLR, ainsi qu'à la localisation des protéines de polarité E-cadherin et ZO-1. Nous rapportons ici que la PRL est un important signal de polarité dans les cellules épithéliales mammaires, ce qui nous donne une meilleure compréhension du mécanisme gouvernant son rôle dans le développement de la glande mammaire et la suppression de tumeur.

3 Literature Review

3.1 Breast Cancer

3.1.1 Breast Cancer Risk

Breast cancer is a complex disease that affects more people than the individual diagnosed, which by itself is a staggering number when considering the statistics. According to the World Health Organization (WHO) 7.6 million people died worldwide from cancer in 2008 and it is estimated to rise to 12 million in 2030. Breast cancer is the leading cancer in women worldwide, in both developing and developed nations. In Canada, breast cancer is the most common cancer amongst women (excluding non-melanoma skin cancer). The chance of a female developing breast cancer in Canada, according to the Canadian Cancer Society, is one in nine and her chance of dying from the disease is one in twenty-nine. Although breast cancer incidences have risen since 1980, partially due to increased mammography screening and disease awareness, breast cancer death rates have declined since the mid 1980's. This highlights the importance for research into diagnostic and therapeutic targets.

3.1.2 Breast Cancer and Pregnancy

There are a number of risk factors that affect breast cancer risk, the most significant and well-studied being gender, age, family history and genetic background. There are a number of other factors that are not as well characterized, one of which being early pregnancy. It has been known for some time that one of the most consistent risk factors for women is parity and the age at first birth¹. This has logically been hypothesized to be in part due to a reduced total number of ovulations and therefore overall reduced exposure of estrogen. However, follow up studies have demonstrated a reduced risk of breast cancer associated with age of first full term

pregnancy and duration of breastfeeding, which highlights other factors other than estrogen exposure². These studies were amongst the first to link the physiological changes between pregnancy, lactation and breast cancer risk.

3.2 Prolactin Signaling

3.2.1 Origin and Overview

Pregnancy and lactation are closely linked by regulating factors that circulate in the blood including proteins, growth factors and hormones. In 1928 two French scientists Sticker and Grueter discovered that mammary development and milk secretion could be artificially initiated by injection of a pituitary extract in rabbits³. This discovery was later shown in other animals and later in 1933 by Riddles, Bates and Dykshorn it was purified for its active component and classified as an anterior hormone named Prolactin (PRL)⁴. Since its discovery, research has attributed PRL with over 300 separate actions, making it the most diverse hormone known. Due to this diversity, the biological functions have attempted to be classified into 6 major groups: 1) water and electrolyte balance, 2) growth and development, 3) endocrinology and metabolism, 4) brain and behavior, 5) reproduction, and 6) immunoregulation and protection⁵. PRL has also been studied for its association with a number of unique diseases ranging from Lupus Erythematosus to Parkinson's disease to a number of cancers. Though the pituitary hormone has a wide range of functions in health and disease, PRL is still most recognized for its originally discovered role as a major regulator of lactation and mammary gland development.

3.2.2 PRL/GH/PL Family

The hormone PRL very closely resembles two other polypeptide hormones, growth hormone (GH) and Placental Lactogen (PL). As indicated early on with structural similarities and later confirmed by genomic, binding, and functional features, PRL is widely accepted to belong to the PRL/GH/PL family^{6,7,8,9}. Early on similarities recorded between hPRL and hGH were so similar that there was controversy in the field as to whether or not they were even separate, unique hormones, which was eventually clarified in 1972 by the purification of hPRL by Freisen and colleagues^{10,11}. PRL and its other family members have further been associated with the family of proteins hematopoietic cytokines, due to its receptor similarities, predicted up-up-down-down four α -helix bundle fold and its effects on the immune system¹¹. Although there is a high level of similarities, these unique proteins were thought to have diverged from a common ancestral gene some 400 million years ago^{6,12,13,14}. However, it should be noted that other evolutionary studies using fish have suggested that this divergence may have occurred much earlier, even up to 820 million years ago¹⁵. Regardless of the exact time of divergence, there are many unique characteristics of PRL that makes it a unique hormone from its ancestral protein family members. Even within the PRL lineage itself there are many differences. Although PRL is highly conserved within classes (ex. Carp and salmon PRL have 77% similarity), there is a high level of difference the further you diverge between species (ex. Carp and human only have 36% similarity)¹⁶. This level of divergence within PRL proteins along with unique functions of PRL from GH and PL highlights not only the similarities and differences that natural selection has chosen, but also the importance and relevance of experimental models. For instance one could speculate that the more primitive an animal is, the more likely its PRL being studied would be to

its family member GH due to the smaller amount of divergence throughout time. None the less, PRL is a unique polypeptide hormone in humans and other animals that is encoded by a specific gene that has diverged from its evolutionary ancestors.

3.2.3 PRL Gene and Integrin Structure

The gene encoding PRL is unique and is found in all vertebrates. In humans, the *PRL* gene is located on chromosome 6 and consists of six exons and four introns for an overall length of approximately 10kb^{17,18}. The cDNA of hPRL consists of 914 nucleotides, containing 681 nucleotide open reading frames, resulting in a 227 amino acid (aa) prehormone with a putative 28 aa residue signal peptide¹². Thus, the major form of mature PRL found in humans is 199 aa's in length and is 23kD.

The polypeptide hormone is synthesized and secreted by numerous cells in the body. There are different variants of PRL caused by alternative splicing, proteolytic cleavage, dimerization or polymerization, and glycosylation¹⁹. Although the predominant PRL found in humans is 23kD and is secreted from specialized cells in the anterior pituitary called lactotrophs, different levels of PRL variants have been found in circulation. These variations in PRL affect the hormone's binding, metabolic clearance, immunologic reactivity and biological activity. The largest extrapituitary source of PRL in humans is the placenta; however PRL is also secreted in a wide range of tissue including deciduae, brain and mammary epithelia²⁰. It is widely accepted that the majority of biological effects recognized in humans are caused by the prevalent 23kD form of PRL by the anterior pituitary, however the level of significance that different variants and local secretion of extrapituitary PRL has on autocrine/paracrine signaling and therefore the overall biological effects is still to be determined in full.

3.2.4 PRL Receptor

The PRL receptor (PRLR) is a transmembrane protein consisting of three major domains: an extracellular domain (ECD) required for ligand binding, transmembrane domain (TM), and an intracellular domain (ICD). There are seven known isoforms of the PRLR that have unique signaling properties, the most common and well-studied in humans being the long form hPRLR (LF-hPRLR) (Figure 3.2.4). The ECD contains an S1 (which contains two highly conserved cysteine residues which make two disulfide bridges) and an S2 domain (which contains the WSXWS motif that is conserved throughout the cytokine receptor family)^{21,22}. The TM domain contains 24 aa's and aside from connecting the ECD and ICD its function is relatively unknown. Finally the ICD contains a juxtamembrane region that contains a Box1 and Box2 domain (which are also conserved throughout the cytokine family) and a unique C-terminal²³. The Box1 domain contains a proline-rich motif which is critical for folding of the molecule and both recognition and binding by transducing molecules, whereas Box2 contributes to activation of transducing molecules, is less conserved and is absent in the short form of PRLR (SF-PRLR)^{5,19}. The ICD of PRLR, regardless of isoform, does not have any domain with enzymatic capability, including kinase activity. PRLR therefore must rely on cytoplasmic kinases for signal transductions, the most important and investigated for breast development being Janus Kinase 2.

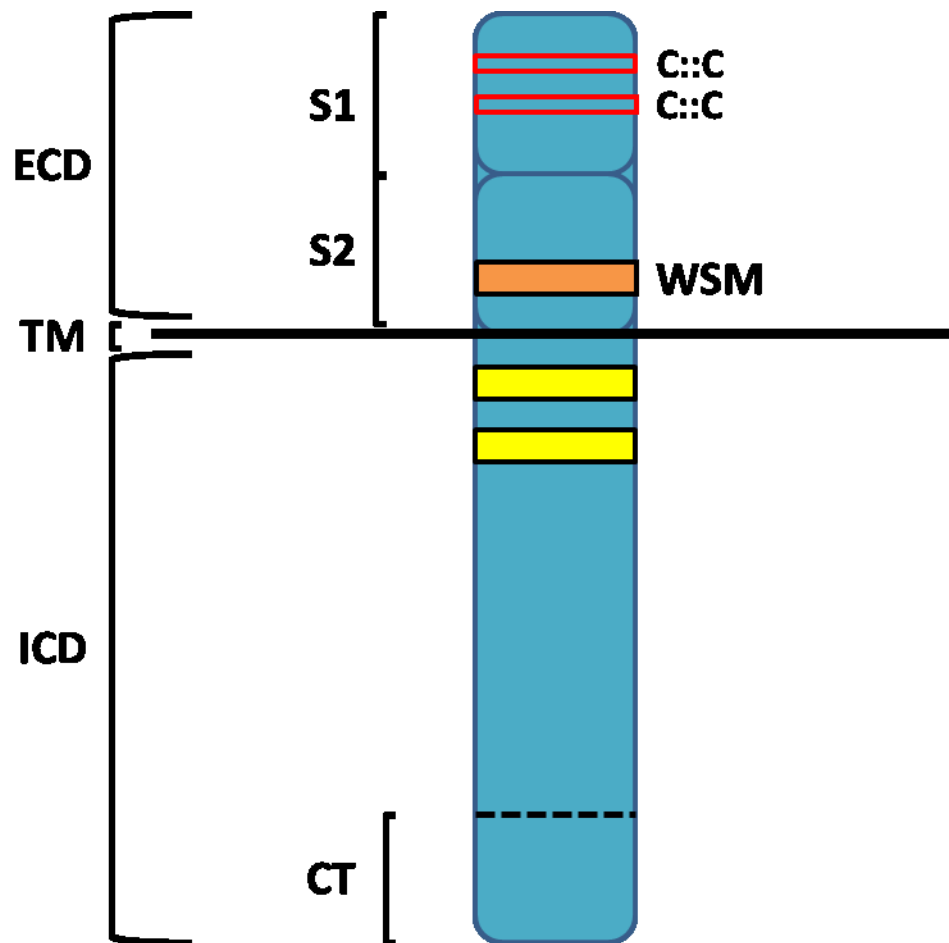


Figure 3.2.4. Protein Structure of the Long Form PRLR. ECD: Extracellular domain that contains the S1 and S2 domains. S1 domain contains two highly conserved cysteines that are cross-linked by disulfide bridges (C::C). The S2 contains a WSXWS motif (WSM) that is conserved throughout cytokine receptors. TM: Transmembrane domain. ICD: Intracellular domain that contains a unique C-terminal (CT). Each ICD is unique to the isoform.

3.2.5 Janus Kinase 2

Since their discovery as “just another kinase”, the family of protein tyrosine kinases was given the acronym JAK. They would eventually become known as Janus Kinases after the two-faced Roman god Janus for the two kinase-like domains in its primary structure^{24,25,26}. In mammals, there are four members of the JAK family: JAK1, JAK2, Jak3 and Tyrosine Kinase 2 (Tyk2)²⁷. JAK1 and JAK2 are expressed almost ubiquitously throughout the body, whereas Jak3 and Tyk2 expression is mainly limited to hematopoietic cells²⁸. There is a high level of homology amongst the four members of the JAK kinases, most of which is within the seven different JAK Homology Domains (JH1-JH7), which are numbered from carboxyl to the amino terminus (Figure 3.2.5). Each JAK member contains a Kinase domain (JH1), a catalytically inactive pseudokinase domain (JH2), a primitive Src Homology 2 (SH2) domain motif (JH3 and C-terminal half of JH4), and a band-four point one, extrin, radixin, moesin homology domain (FERM) protein-interaction domain (JH4-7)²⁹. Although each member has a high level of homology, they are all non-receptor tyrosine kinases and therefore one of the major factors in their signaling is the receptor in which they associate with.

It was determined in 1994 by three independent labs that JAK2 was the Janus kinase that associated with the PRLR^{30,31,32}. JAK2 interaction with PRLR is not dependent on ligand PRL binding, but instead this association has shown to be constitutive^{32,33}. How this interaction occurs is still not fully understood, however substitution experiments demonstrated that the most C-terminal Proline in Box1 of the PRLR is crucial for its association with JAK2³⁴. All of the functions of JAK2 and their mechanisms are still undetermined. The majority of functions reported are believed to occur through the catalytically active kinase domain (JH1) which

contains numerous tyrosine residues. The pseudokinase domain (JH2) has shown to suppress basal JAK2 activity and the FERM domain can down regulate the cytokine signaling through autophosphorylation^{35,36}. The function of JAK2 is likely to be ligand and tissue dependent. However, in pertains to mammary gland development, JAK2 plays a major role in signal transduction from the PRLR to Signal Transducers and Activators of Transcription 5 (Stat5).

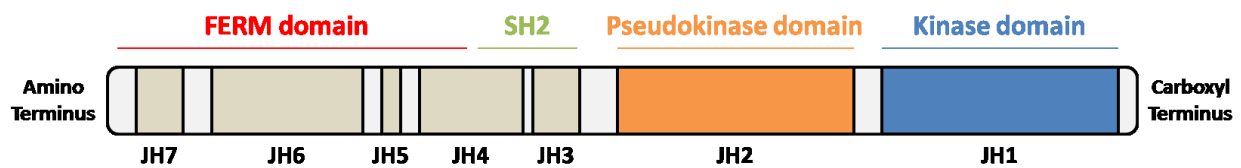


Figure 3.2.5. Common Janus Kinase Structure. JH: JAK Homologue domain. JH1: Catalytically active tyrosine kinase domain that contains conserved tyrosine residues. JH2: catalytically inactive pseudokinase domain, which distinguished JAK proteins from other tyrosine kinases. JH3-JH4 (partial): contains a Src Homology 2 (SH2) domain that can bind phosphorylated tyrosine residues and as such is important for protein-protein interactions. JH4 (partial)-JH7: contains a four-point one, exrin, radixin, moesin homology domain that is another protein-interaction domain responsible for interactions with cytokine receptors and/or other kinases.

3.2.6 Signal Transducers and Activators of Transcription 5

The Stat family is a group of latent cytoplasmic proteins that were identified for their association with cytokine signaling^{37,38}. The name Stat came from their functional role as signal transducers and activators of transcription, or Stat³⁹. There are eight members of the Stat gene family: Stat1 (α and β), Stat2, Stat3, Stat4, Stat5a, Stat5b, Stat6 (or IL-4 Stat), and dStat (a Stat5 homolog found in *Drosophila*). Translation of these genes results in a family of proteins, of ~90-100kDa, that contain five conserved structural features: C-terminal transactivating domain, a ubiquitous tyrosine, a SH2 Domain, DNA binding domain and an N-terminal domain (from carboxyl to amino-terminal, respectively)(Figure 3.2.6)^{40,41}. These proteins once activated by tyrosine-phosphorylation in the cytoplasm, dimerize and translocate to the nucleus to mediate gene transcription. Although the mechanism in which Stat-mediated gene transcription is not well known, Stats have been shown to recognize DNA sequences within the promoters of target genes, such as the gamma interferon activated sequence (GAS motif), which contains a palindromic consensus sequence TTCxxxGAA³⁰.

The Stat members that have been identified thus far as transducers of PRLR signaling are: Stat1, Stat3 and mainly Stat5. Cloning of Stat5 in mouse, rat and human identified two separate genes, Stat5a and Stat5b, which encode several isoforms with 90-95% similarity, the majority of difference being within the carboxyl-terminal^{42,43,44,45,46,47}. Stat 5, which was originally known as mammary gland factor (MGF), has been shown to be essential in PRL-induced milk protein gene activation^{48,33}. All isoforms have shown to possess tyrosine 694, which is the functionally essential site of Jak2 tyrosine phosphorylation, as determined by B-casein expression⁴⁹. Similarly, truncation of the C-terminal domain of Stat5, and ultimately loss of Tyrosine 694,

leads to a dominant-negative mutant^{50,51,52}. Likewise, PRLR mutants unable to activate JAKs are unable to activate Stat5^{53,54}. These studies have not only highlighted factors in Stat signaling, but have also identified mechanisms for PRL signaling and mammary gland development.

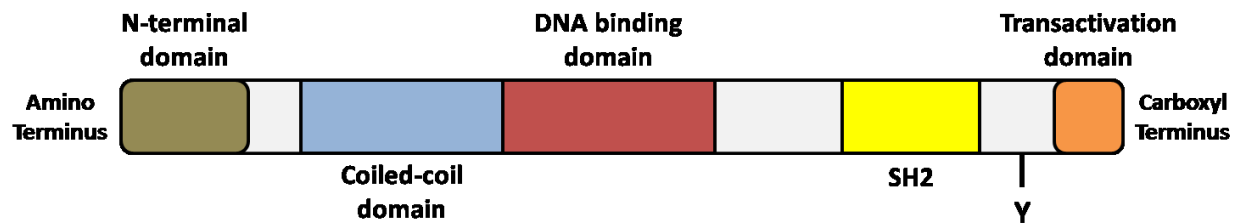


Figure 3.2.6. Common STAT Structure. Transactivation domain: essential for proper PRL-induced gene regulation. Y: a conserved tyrosine (Y) residue important for phosphorylation and activation of STAT proteins. SH2: a Src Homology 2 domain that binds with other STAT members during dimerization. DNA-binding domain: important for STAT's function in gene regulation. Coiled-coil domain: important in recruitment to receptors and cell membrane. N-terminal domain: also referred to as a dimerization or oligomerization domain for its role in protein-protein interactions, especially between STAT dimers.

3.2.7 Signal Transduction

PRL signaling is very complex with multiple PRLR isoforms that can potentially activate numerous signaling pathways. In the focus of mammary gland development however, transgenic mice models using functional knockouts of PRL, PRLR, Jak2 and Stat5 have demonstrated that the PRL/PRLR/Jak2/Stat5 pathway is the major and essential pathway in lactation and terminal lobuloalveolar development (Figure 3.2.7)^{55,56,57,58,59,60}. This pathway is initiated by the PRL ligand binding with its receptor, PRLR. Binding leads to homodimerization of the PRLR, which brings their associated Jak2s into close association. This leads to rapid activation of the Jak2s resulting in cross- and auto-phosphorylation and phosphorylation of the PRLR. Phosphorylation of the PRLR leads to the opening of tyrosine docking sites for SH2 domain-containing proteins like Stat5⁵³. After recruitment and binding with the PRLR, Stat5 is also phosphorylated on tyrosine residues by JAK2. Once activated, Stat5 proteins dissociates from the PRLR/Jak2 complex and homodimerize through their SH2 domains and translocate to the nucleus where they can bind to Stat5 response elements leading to subsequent mediation of gene translation⁶¹.

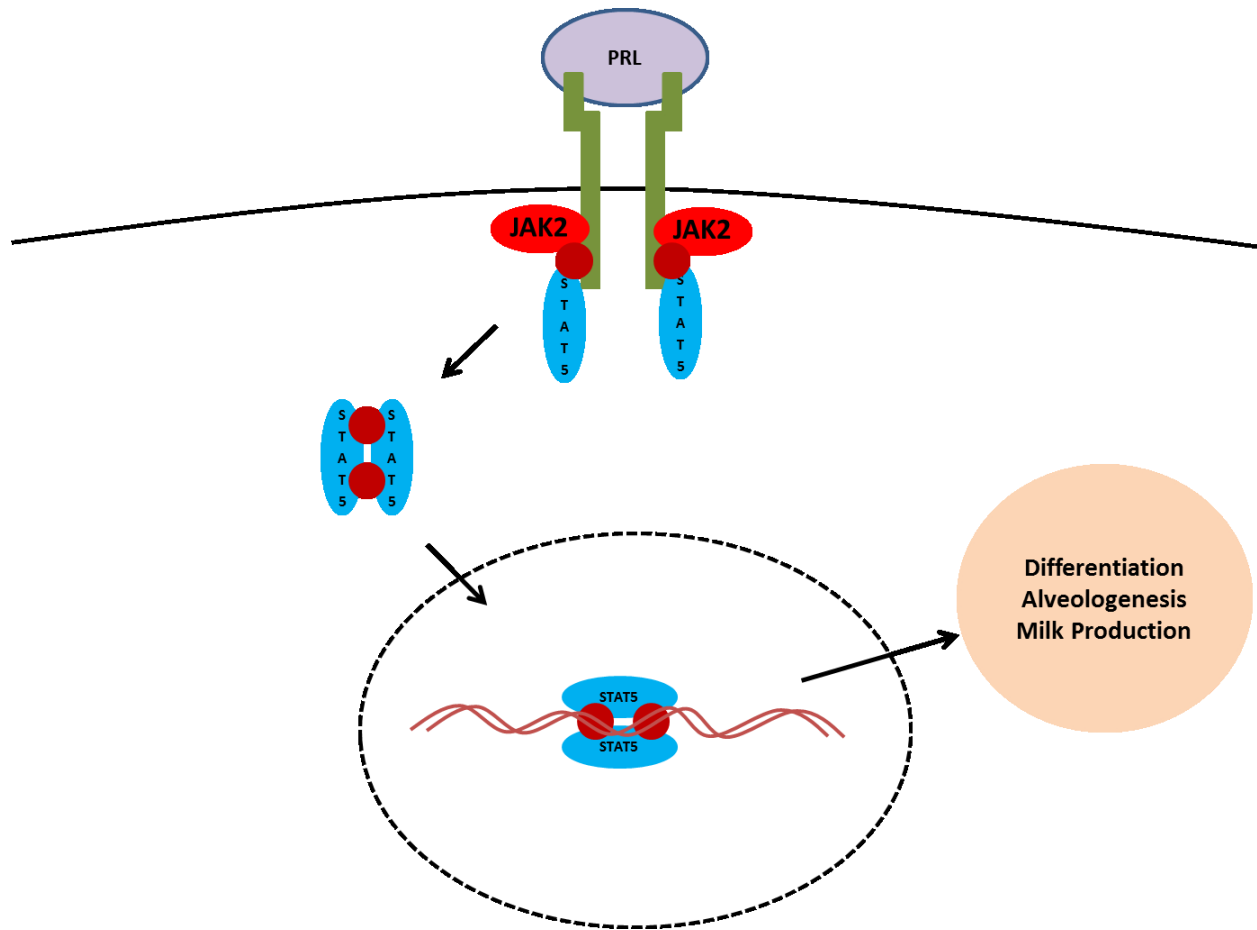


Figure 3.2.7. PRL/PRLR/JAK2/STAT5 Signaling Cascade. After PRL binds to its receptor, PRLR (green), PRLRs undergo homodimerization bringing the constitutively associated JAK2s into close proximity allowing them to auto- and cross-phosphorylate one another and the PRLRs. Phosphorylation results in the opening of docking sites for STAT5, which then interacts through its SH2 domain with tyrosine residues on the PRLR. STAT5 then is phosphorylated by JAK2, resulting in STAT5 activation and dissociation from the PRLR/JAK2/STAT5 complex. Activated STAT5s then form homodimers and translocate to the nucleus where they bind and activate transcription of target genes.

3.3 PRL's Role in Health and Disease

3.3.1 PRL Function

As previously described, PRL's functions are both numerous and diverse. The broad range of functions associated with PRL is likely due to the large number of potential variations throughout the signal cascade, which are not exclusive to, but include: tissue specificity, temporal specificity, specific form of PRL, isoform of PRLR being stimulated, isoform of JAK2 used in the signal transduction and the response element(s)/gene(s) being regulated. The most common cellular function associated with PRL acts through the signaling cascade previously described and is mainly associated with the gene regulation of milk proteins such as B-casein⁶². However, induction of milk protein expression is but one function of PRL during late pregnancy and lactation. PRL, along with Progesterone (Pg), plays a critical role in the process that leads to the formation of milk secretion structures, which has been referred to as the "Alveolar Switch"⁶³. The mammary gland development that occurs during pregnancy is unique compared to embryogenesis and is dependent on the synergy between PRL and Pg⁶⁴. Both hormones are thought to trigger an initial wave leading to the Alveolar Switch during days 2 to 6 of pregnancy⁶⁵. Furthermore, both hormones have been shown to affect the expression of each other, not only highlighting a synergistic role amongst hormones, but also a synergistic relationship between the anterior pituitary and the ovaries⁶⁶. Similar to the Pg Receptor (PgR), PRLR^{-/-} mice have demonstrated that PRLR in epithelial cells, but not stroma, is critical for normal lobuloalveolar differentiation⁶⁷. Early studies using ablative surgery demonstrated that post-puberty development, which includes side branching and terminal alveolar development, is dependent on the function of the anterior pituitary and ovaries⁶⁸. Similarly, the deletion of

the PRLR was further determined to not effect pubertal mammary development, but lead to a lack of secondary side-branching and alveolar buds in adults^{56,57,65}. Failure to side-branch in PRLR null mammary glands could be rescued by Pg, but could not recover the defect in terminal alveolar structures⁶⁹. These studies demonstrated PRL's role on mammary gland development to be both a direct regulator of terminal alveolar structures and an indirect regulator of side-branching during mammary gland development during pregnancy. The direct mechanism in which PRL acts on alveolar structures however, continues to be a major question in developmental biology and cancer research.

3.3.2 PRL and Cancer

The role of PRL in cancer and more specifically breast cancer, originally and continues to be controversial. This partially stemmed from early experiments that resulted in conflicting results when using hypophysectomy or bromocriptine treatment as a way to remove pituitary PRL to study its role in cancer⁷⁰. Similarly, epidemiological studies have either demonstrated moderately positive or null association with increased circulating levels of PRL and breast cancer^{71,72}. Undoubtedly, both healthy and cancerous epithelial cells and the surrounding stroma produce PRL demonstrating regulation by an autocrine/paracrine loop^{73,74,75}. This regulatory loop has been referred to as an oncogenic regulator by some, partially due to the finding that tumours express higher levels of PRL than normal or hyperplastic epithelium⁷⁶. Furthermore, this has been backed by transgenic mouse models that either demonstrates that autocrine PRL can lead to tumour formation or that PRL can have a permissive role in oncogene-induced breast tumours^{77,78,79}. However, these mouse models fail to contain convincing negative-control mice due to developmental abnormalities observed in the

mammary gland of these control mice. Therefore the question still remains whether PRL in these experimental models directly facilitates tumourigenesis or induces developmental changes that give rise to a cell population, which does not exist in the control mice, that is then vulnerable to cancer-initiating mechanisms and oncogenic events.

Although PRL's role in certain specific forms of breast cancer or oncogenic pathways is not yet fully determined, there is an increasing trend of acceptance within the PRL field that is backed by a growing body of evidence that PRL may act as a tumour suppressor. Investigation into PRL and PRLR antagonists has demonstrated that interference of the PRL autocrine/paracrine loop in breast cancer cells results in inhibition of proliferation and induction of apoptosis^{80,81,82,83}. Furthermore, PRL's downstream target STAT5a has been shown to induce cell adhesion and its activated form Phospho-STAT5a is correlated with a good prognosis and response to endocrine therapy^{84,85,86}. Similarly, surgery-induced rise in PRL levels was associated with an increase in disease-free survival in operable breast carcinoma in patients, regardless of axillary node involvement⁸⁷. More directly, it was recently demonstrated that PRL signaling can suppress the Epithelial-Mesenchymal Transition (EMT) and invasive potential in breast cancer cells, which was dependent on PRL's ability to suppress the oncogenic Mitogen-Activated Protein Kinase pathways (MAPK-ERK1/ERK2)⁸⁸. EMT is an evolutionary mechanism which is hijacked by cancer cells allowing them to undergo a transition from epithelial to mesenchymal phenotype. EMT allows for cancerous cells to adapt to microenvironments and is a major mechanism that partially accounts for tumour heterogeneity, drug resistance, migration, invasion and metastasis. The evidence suggests PRL

as a tumour suppressor and antagonist of EMT, which further helps to explain the observation that tumours that have been associated with PRL usually are low-grade, differentiated tumours.

3.4 Cellular Polarity

3.4.1 Overview

Cellular polarity classifies the asymmetry of a cell, which is defined by the segregation of cellular membranes into the apical and basal-lateral membranes and an asymmetric localization of particular proteins. These membranes are separated by tight junctions and help define the architecture and function of the cell by acting as binding sites leading to protein localization and cytoskeleton organization. Apical-basal polarity within epithelial cells allows for compartmentalization of tissues and transport of nutrients and metabolites across cell barriers⁸⁹. The fundamental tissue structure that makes up the branching and terminal alveolar architecture of the mammary gland, which is also conserved throughout Metazoa, consists of a layer of polarized epithelial cells that line and surround an internal hollow space called the lumen. This structure is essential for directional secretion of milk proteins during lactation and is often compromised in many diseases including breast cancer.

3.4.2 Lumen Formation

Lumen containing organs are common throughout the human body and the mechanism of lumen formation is of great interest to developmental biology and cancer. Research has unfolded a number of mechanisms by which lumens are formed, including: wrapping, folding, invagination or evagination of polarized cell sheets⁹⁰. There are three crucial design principles to this process: i) recognition of surrounding environment (cell-cell or cell-matrix), ii) apical-basal polarization and iii) the expansion of the luminal space (ex. Ion or fluid influx)⁸⁹. With

regards to the process that forms the terminal alveolar structures of the mammary gland, known as alveologenesis, earlier experiments using the spontaneously immortalized human mammary cells MCF10A pointed towards the need for apoptosis in lumen formation in a process known as cavitation^{91,92,93}. However, further extensive research in the area has demonstrated two unique de novo mechanisms for lumen formation in the mammary gland: cavitation (lumen space is cleared through apoptosis of cells) and hallowing (lumen is formed by exocytosis and membrane separation)^{94,95}. Furthermore, it has been demonstrated that there is an inverse relationship between ability to generate apical-basal polarity and apoptosis⁹⁶. This suggests that certain environmental cues, such as cellular spacing and polarity signals, control the mechanism of lumen formation. This is likely because it appears that polarization of epithelial cells is a two-step process: first an axis of polarization is selected through response to spatial or environmental cues, and second, cells generate molecular asymmetry along this axis⁹⁷. In a situation where an axis of polarization is not selected due to an absence of a polarity signal, a lumen may then be formed by apoptosis (also known as anoikis), somewhat as a default mechanism. It is therefore suspected that the early signals that cells receive both initiate and dictate the mechanism of lumen formation.

3.5 Cell-Matrix and Cell-Cell Recognition

3.5.1 Cell-Matrix

Aside from a cell's intrinsic genetic and molecular capabilities, the most important factor determining how a cell acts is the information it receives from the surrounding environment. During tissue formation, one of the first things a cell does is to take cues from its environment,

which may either be the surrounding matrix (also known as ECM for extra-cellular matrix) or neighboring cells.

Numerous experiments using manipulation of ECM components have demonstrated the importance of ECM composition to epithelial polarity. For example, MDCK cells grown on collagen were dependent on apoptosis for lumen formation, whereas when grown on Matrigel (an ECM mixture with laminin as its major component) cells were able to quickly polarize and form lumens independent of apoptosis⁹. In further studies laminin, through a Rac-1 dependent mechanism, has been shown to be crucial to the formation of a polarity axis⁹⁸. The importance of laminin and other ECM components such as collagen IV is also highlighted by the discovery that growing epithelial cysts secrete these components to create their own basal laminae and spatial cues, which is essential for proper localization of zonula occludens (ZO-1) and thus tight junction formation⁹⁸. These environmental cues need to be recognized before signaling can lead to a cellular response. The ECM- and cell-recognizing receptors known as integrins, and most notably β 1-integrin, have been shown to be essential for apical pole orientation during the Rac1/laminin-dependent alveologenesis⁹⁹. Integrins also detect spatial cues by playing an important role in cell-cell adhesions.

3.5.2 Cell-Cell Adhesion

Cell-cell recognition and adhesion is an important step in polarity and tissue formation. Furthermore adhesion can act as a tumour suppressor either by physically joining neighboring cells and preventing migration, or by facilitating other signaling events. Epithelial cells strongly adhere to each other through an intercellular junctional complex composed of two unique junctions: zonula adherens (ZA) and zonula occludens (ZO).

3.5.2.1 Zonula Adhesion

The primary adhesion structure between epithelial cells is the zonula adherens, or more commonly, adherence junctions (AJ). In mammalian cells these are adhesive belts that encircle cells basolaterally, or just below the apical surface. AJs are primarily composed of Cadherins, whose Ca^{2+} -dependent *trans*-dimerization accounts for the major mechanism allowing for adhesion between neighboring cells¹⁰⁰. E-cadherin is a commonly studied member of the Cadherin Superfamily and is the primary AJs component, which accounts for the protein's importance in adherence and polarity. E-cadherin mediated adhesion has been demonstrated to be enough to induce segregation of apical proteins to the non-contacting membrane from the contacting basolateral membrane in epithelial cells, an early process in establishing polarity^{101,102}. Similarly, it was experimentally shown that ectopic E-cadherin in non-polarized fibroblasts was able to induce Ca^{2+} -dependent cell-cell adhesion and site-specific protein localization¹⁰³. Cadherin-mediated cell adhesions also influence numerous other cellular processes. A family of cytoplasmic proteins, which include β -catenin, plakoglobin, and p120, bind Cadherins at their cytoplasmic domains, which then bind α -catenin who is thought to then interact with the cytoskeleton through actins⁹⁸. Furthermore, subsequent loss of E-cadherin and therefore loss of E-cadherin- β -catenin interaction can lead to nuclear accumulation of β -catenin and an increase in β -catenin/Wnt signaling resulting in tumorigenic events^{104,105}. This further highlights a link between cell-cell adhesion, polarity and cell signaling.

3.5.2.2 Zonula Occludens

At the apical most part of the intercellular junction are structures that reach across the intercellular space and connect the membranes of adjacent epithelial cells known as zonula

occludens, or what will be referred to from this point on as tight junctions (TJ). TJs are composed of three families of transmembrane proteins: occludins, claudins, and junctional adhesion molecules¹⁰⁶. These cellular structures have two important functions: first being its barrier functions, which refers to its ability to form tight seals between epithelial cells and create a selectively permeable barrier to diffusion through the intercellular space, and secondly its fence function, which physically separates the apical and basolateral membranes, preventing components of the two membrane domains and their interacting proteins from mixing^{107,108}. Both of which are crucial for the proper function of the mammary gland during lactation. In the mammary gland the barrier function, which requires fully closed tight junctions, has shown to be dependent upon prolactin stimulation and is required during lactations for proper directional secretion of milk proteins^{109, 110}. The later fence function is critical for establishing and maintaining epithelial polarity by separating distinct membranes and interacting with polarity complexes, which as previously mentioned, is needed for lumen formation and subsequently required for proper lactation.

3.6 Molecular Regulation

3.6.1 Molecular Control of Epithelial Polarity

Our current understanding of epithelial polarity and lumen formation in epithelial cysts, a large part of which comes from the 3-dimensional (3D) cellular models that reflect the *de novo* development of terminal mammary alveoli, is that it is a step-wise process. A model using MDCK cells demonstrated that Rac1-mediated-laminin assembly was essential for apical pole orientation¹¹¹. It appears, at least in these models, that epithelial polarity is a step-wise process where cells first interact with the ECM or neighboring cells, leading to basal organization

followed by apical orientation and polarity. Each process in this polarity mechanism is essential for the next, highlighting the importance of early polarity mechanisms and environmental cues. Although the exact step-wise mechanism, the timing of events and the level of redundancy in pathways is undetermined, experimental results have given us insight into important complexes and molecular events involved in establishing and maintaining epithelial polarity. To date, there are three central protein complexes that are widely accepted as polarity complexes important in the process of establishing and maintaining apicobasal polarity.

3.6.2 Scribble-Dlg-Lgl Complex

The use of genetic studies in *Drosophila* has uncovered three important proteins for the establishment of intercellular junctions and membrane polarity: Scribble (Scrib), Discs large (Dlg), and Lethal giant larvae (Lgl) (Fig.3.6.4.1)^{112,113}. These proteins colocalize at the basolateral membrane and although the exact mechanism is not known, nor is it completely determined if these proteins interact in a complex or a signaling cascade, they have all been associated with common signaling pathways, vesicle trafficking and Myosin II-actin cytoskeleton organization¹¹⁴. Furthermore, Scrib, Dlg, and Lgl have all been established as tumour suppressors in *Drosophila*, a common characteristic with polarity proteins, which is thought to be, attributed to their function in common pathways regulating apicobasal polarity, cell proliferation, survival, differentiation and migration/invasion¹¹². Although little is known about the mechanism or early events leading to their localization and association, the Scrib/Dlg/Lgl basolateral association is important in epithelial polarity and has been shown to act antagonistically with the apical CRUMBS and PAR complexes^{115,116}.

3.6.3 Crumbs-PALS1-PATJ Complex

Another protein complex important in epithelial polarity and whose subsequent lack of has been linked to tumorigenesis is the Crumbs-PALS1 (Stardust)-PATJ complex, or more commonly referred to as the Crumbs complex. The protein Crumbs Homologue 1 (CRB1), or more commonly referred to by its *Drosophila* name Crumbs under which it was first discovered, is an apical transmembrane protein. It has been shown to act as a positive regulator of apical membrane size and overexpression of Crumbs leads to expansion of the apical membrane^{117,118}. Crumbs contains an intracellular domain that can bind ezrin-radixin-moesin (ERM) proteins, although this domain's role in polarity is undetermined, and a binding domain in the C-terminal tail which directly interacts with a PDZ domain in PALS1^{119,120}. PALS1, or Stardust in *Drosophila*, is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins, which is known for their functions in receptor clustering and tumour suppression¹²¹. PALS1 acts as an adapter protein linking Crumbs and PATJ, formerly known as Discs Lost in *Drosophila*¹²². It can do this by interacting with Crumbs with one L27 domain, while interacting with PATJ through a second L27 domain¹²³. PATJ (PALS1-associated TJ protein) is a scaffold protein with multiple PDZ-domains which binds to a number of TJ proteins including claudins and zonula occludens 3 (ZO-3)¹²¹. The interactions between these three proteins which results in the Crumbs Complex that localize at the ZA are important in polarity, tight junction integrity and a number of other cellular functions (Fig. 3.6.4.1). In pertains to epithelial polarity, it was more recently demonstrated in *Drosophila* embryo that while the Scribble and PAR (Bazooka) complexes were required for the establishment of polarity in an epithelial tubular system, the Crumbs complex was only required to maintain polarity and TJ integrity during morphogenesis and tissue

remodeling¹²⁴. This unique role of the Crumbs Complex has yet to be determined in mammalian systems; however its role in polarity is widely accepted.

3.6.4 Par3-Par6-aPKC Complex

The last polarity complex to be discussed, and possibly the best understood and studied, is the Par complex. Par (Partitioning defect) proteins were first discovered in genetic screens in *C. elegans* for regulators of cytoplasmic partitioning and since have been heavily studied for their ability to form physical complexes which gives them the physical characteristics that allow them to act as fundamental mechanisms for cellular polarity in numerous systems and life forms¹²⁵. The polarity protein complex that is referred to as the Par complex is composed of three members: Par3 (Bazooka), Par6 and the atypical Protein Kinase C (aPKC) (Fig. 3.6.4.1). However, a more detailed mechanism between two unique complexes with two overlapping players has taken shape. Studies have demonstrated that Par6 and aPKC form a complex with Lethal giant larvae (Lgl) independent of Par3, which also contributes to regulation of epithelial polarity¹²⁶. Lgl appears to compete with Par3 for the Par6-aPKC complex. Par3 and Par6, like PALS1 and PATJ, are PDZ domain scaffold proteins involved in numerous protein-protein interactions and can bind with each other through this PDZ domain¹²⁷. The central effector in this mechanism involving a balance between two complexes is aPKC, which through its kinase activity can phosphorylate proteins leading to localization along a polarity gradient¹²⁸. During early stages of epithelial polarity, Lgl binds with Par6-aPKC creating an Lgl-Par6-aPKC complex at the basolateral membrane. Lgl phosphorylation by aPKC then leads to Par6-aPKC dissociation from Lgl and apical translocalization leading to interactions with Par3 creating a Par3-Par6-aPKC complex which localizes and regulates TJ formation and other protein interactions¹²⁹.

Overexpression experiments have further demonstrated this because increased Lgl-Par6-aPKC interaction led to a lack of Par-3-Par6-aPKC interaction and suppression of epithelial junctions¹²⁶. Although early events leading to protein localization, protein interactions, or signals that initiate aPKC phosphorylation are unknown, it is clear that the Par complex plays a crucial part in the establishment and maintenance of epithelial polarity throughout evolution.

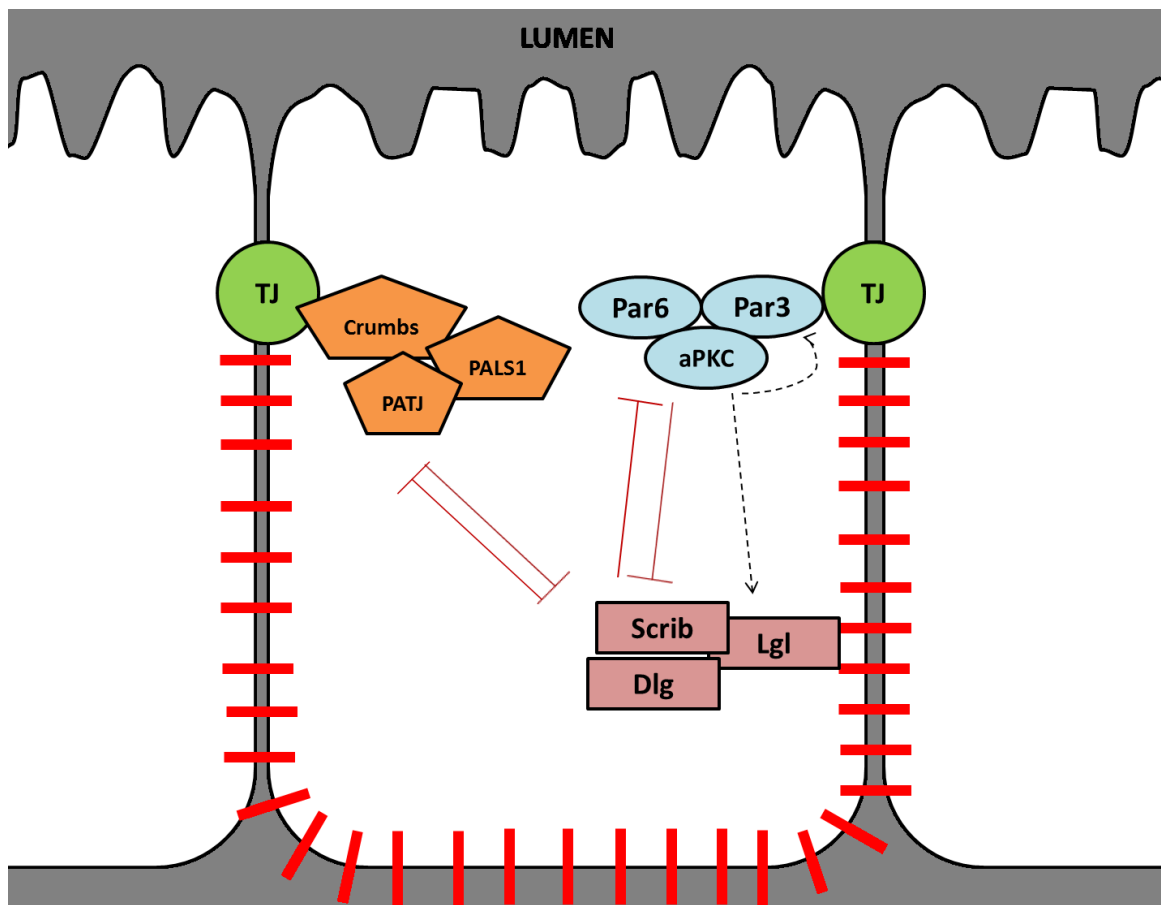


Figure 3.6.4.1. Polarity Protein Complexes. Lumen: marks apical side of the epithelial cells. TJ: Tight Junctions mark the beginning of the apical membrane. Red Dashes: resemble the adherence junctions which mark the basolateral membrane. Dashed arrow: represents the phosphorylation by aPKC which regulates the two polarity complexes: Par complex and Scribble Complex. Red T's: represent the inhibitory relationships between protein complexes.

4 Rational and Hypothesis

The objective of this study was to investigate the role of PRL and its signaling pathway in morphogenesis and epithelial polarity of the mammary gland. It has long been established that PRL plays a critical role in development of the mammary gland during late pregnancy and lactation. Furthermore, PRL acts as a differentiation factor on mammary epithelial cells¹³⁰. However, its role in processes that are associated with cell proliferation and tumourigenesis continue to be debated, further fuelling the need for new assays and experimental approaches. Our lab, along with other studies that have increasingly pointed towards PRL as a tumour suppressor, previously found that PRL inhibits EMT and the invasive ability of breast cancer cells⁹⁰. EMT is a process that results in loss of epithelial polarity and gain of stemness, which has been shown to increase motility and invasiveness in breast cancer cells. This has led to our current theory that EMT is a major driving mechanism for tumour progression and metastasis. It is therefore conceivable that PRL may inhibit breast cancer progression by inhibiting EMT through its promotion of epithelial polarity and tight junction integrity. We therefore hypothesize that PRL, through its downstream PRLR/JAK2/STAT5 pathway, acts as a polarity signal inducing epithelial polarity and tight junction formation. Furthermore, since JAK2 is a major tyrosine kinase and due to our understating that phosphorylation is the regulating mechanism that controls the PAR complex's ability to regulate tight junctions and cellular polarity, we further hypothesize that PRL regulates epithelial polarity and tight junction formation through regulation of the PAR complex.

5 Materials and Methods

5.1 Reagents and Antibodies

Ovine PRL (Sigma-Aldrich), Mouse EGF (Sigma-Aldrich), Bovine Insulin (Wisent Inc.), hydrocortisone (Sigma-Aldrich), Matrigel (BD Biosciences). Monoclonal antibodies: conjugated Zo1-Alexa Fluor®488 (Invitrogen), E-cadherin (BD Transduction Laboratories). Polyclonal antibody: JAK2 (Millipore), phospho-STAT5 (Invitrogen), PRLR (Santa Cruz Biotechnology), aPKC ζ (Santa Cruz Biotechnology), PAR3 (Millipore), β -catenin (Cell Signaling Technology).

5.2 Cell Culture

HC11 mouse mammary epithelial cells were provided by Dr. C. Shemanko (University of Calgary). These cells were cultured in growth media containing: RPMI-1640 media (Wisent) containing 10% fetal bovine serum (FBS), L-glutamine (0.1M), penicillin (50units/mL), streptomycin (50units/mL), mouse EGF (10ng/mL) and insulin (5 μ g/mL). They were induced for two days in induction media: RPMI-1640 media (Wisent) containing 10% fetal bovine serum (FBS), L-glutamine (0.1M), penicillin (50units/mL), streptomycin (50units/mL), insulin (5 μ g/mL), and hydrocortisone (1 μ M). Cells were starved using starvation media: RPMI-1640 media (Wisent) containing L-glutamine (0.1M), penicillin (50units/mL), streptomycin (50units/mL), insulin (5 μ g/mL), and hydrocortisone (1 μ M), transferrin (10 μ g/mL), Fetuin (0.5 μ g/mL) or for 3D cultures: RPMI-1640 media (Wisent) containing 3% fetal bovine serum (FBS), L-glutamine (0.1M), penicillin (50units/mL), streptomycin (50units/mL), transferrin (10 μ g/mL), Fetuin (0.5 μ g/mL). HC11-JAK2-KD cells were grown in the same media as previously described for HC11, with the addition of the antibiotic puromycin (3 μ g/mL).

5.3 3D Cell Culture

HC11 cells were plated at 5,000 cells per well (100 μ L of 50,000 cells/mL solution) on Poly-D-Lysine 8-well Culture Slides (BD BioCoatTM) with Matrigel coating. An “On-top” method was used by then adding 100 μ L growth media containing 4% Matrigel to each well, creating a final media concentration of 2% Matrigel. After 2 days of growth, media was changed to induction media containing 2% Matrigel. Finally after 2 days of induction, media was replaced by starvation media containing 3% FBS, 2% Matrigel and one or a combination of stimulating factors: Prolactin, Insulin, Hydrocortisone and Progesterone. After growth and treatments colonies were fixed, immunostained, and analyzed using confocal microscopy.

5.4 Cell Lysate

Following ligand stimulation, cells were washed using cold phosphate buffer saline (PBS) on ice to halt ligand stimulation. Continuing on ice, cells were lysed using lysis buffer (10 mM Tris-HCl pH7.5, 5mM EDTA pH8.0, 150 mM NaCl, 30 mM sodium pyrophosphate, 30 mM sodium fluoride, 1 mM activated sodium orthovanadate, 2 mM leupeptin, 5 mM aprotinin, 0.4 mM Pefabloc and 0.5% Triton X-100). Cell lysates were then collected and centrifuged at 13,000 rpm at 4°C for 10 minutes to remove cellular debris. Protein levels were then quantified and equalized using Bradford assay.

5.5 Immunoprecipitation and Western Analysis

For immunoprecipitation experiments, after total protein levels were equalized, cell lysates (800 μ L) were incubated at 4°C on a rotor with Protein-A-Sepharose (PAS) beads (10 μ L) and antibody (1 μ L) for 3 hours. Following incubation, PAS beads containing the immunoprecipitates

were washed 3 times in HNTG (20 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol).

PAS beads containing immunoprecipitates and whole lysates, equalized for protein levels as previously described, were denatured with loading buffer (0.25M Tris-HCl pH6.8, 5% SDS, 0.1M DTT, 50% Glycerol) at 100°C for 5 minutes. Denatured proteins were separated using an acrylamide SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes (Whatman) and examined using western analysis.

Nitrocellulose membranes containing proteins were blocked overnight at 4°C on a rocker in blocking buffer containing either 0.25% gelatin, for analysis of milk or phosphorylated proteins, or 7.5% milk in TBST (100 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.5% Tween-20). After membranes were equilibrated to room temperature, they were incubated for 1 hour in primary antibody at a concentration of 1:1000 to 1:10000 in 0.25% gelatin. Membranes were washed 3 times for 10minutes in TBST, and then incubated in a secondary antibody (HRP-conjugated) at a concentration 1:10000 in either 0.25% gelatin or 7.5% milk. Membranes were washed 4 times for 20 minutes in TBST solution and then subjected to ECL luminescence substrate for 1 minute in the dark and luminescence was then detected on film.

5.6 Immunofluorescence

Cells were fixed using 3.2% paraformaldehyde (PFA) in PBS for 10 minutes, permeabilized with 0.1 % Triton X-100 in PBS for 10 min, and blocked using 2 % BSA in PBS for 1 hour at room temperature. Following blocking, cells were incubated for 1 hour at room temperature in primary antibody diluted in 2% BSA in PBS at the following concentrations: E-cadherin (1:100), Par 3 (1:100), aPKC ζ (1:300), ZO-1 conjugated with Alexa 488 (1:100), β -catenin (1:100), Lgl

(1:100) and Par 6 (1:100). Cells were washed 3 times in PBS and then incubated for 1 hour in the dark at room temperature with secondary antibody (1:400). Finally the nuclei of cells were counterstained with DAPI for 10 minutes, mounted with antifade mounting media overnights and analyzed using Carl Zeiss LSM 510 Confocal imaging.

5.7 Proliferation and Migration Assay

Proliferation assays were done using the IncuCyte HD live-cell imaging system as previously described in “Kinetic Proliferation Assays Using IncuCyte™”¹³¹. In short, cells were plated at 5000 or 10000 cells per well (100µL of 50,000 or 100,000 cells/mL respectively) and incubated in the IncuCyte machine at 37°C. Cell growth was determined using monolayer cellular confluence data, which was collected every 4 hours. All data and images were collected and analyzed using IncuCyte 2011 software.

Migration assays were again done using the IncuCyte HD live-cell imaging system as previously described in “CellPlayer™ 96-Well Cell Migration Assay”¹³². Briefly, cells were plated at 40,000 cells per well (100µL of 400,000 cells/mL) and incubated at 37°C for 24 hours until they reach total confluence. Scratches were made using the 96-pin WoundMaker (Essen Bioscience) ensuring equal wound width. Cells were then washed 2 times with RPMI and then treated with ligand at a concentration of 1µL/mL in 100µL of RPMI containing Fetuin and transferrin. Cells were then incubated in the IncuCyte machine at 37°C and analyzed using the “Wound Healing” program. All data and images were collected and analyzed using IncuCyte 2011 software.

6 Results

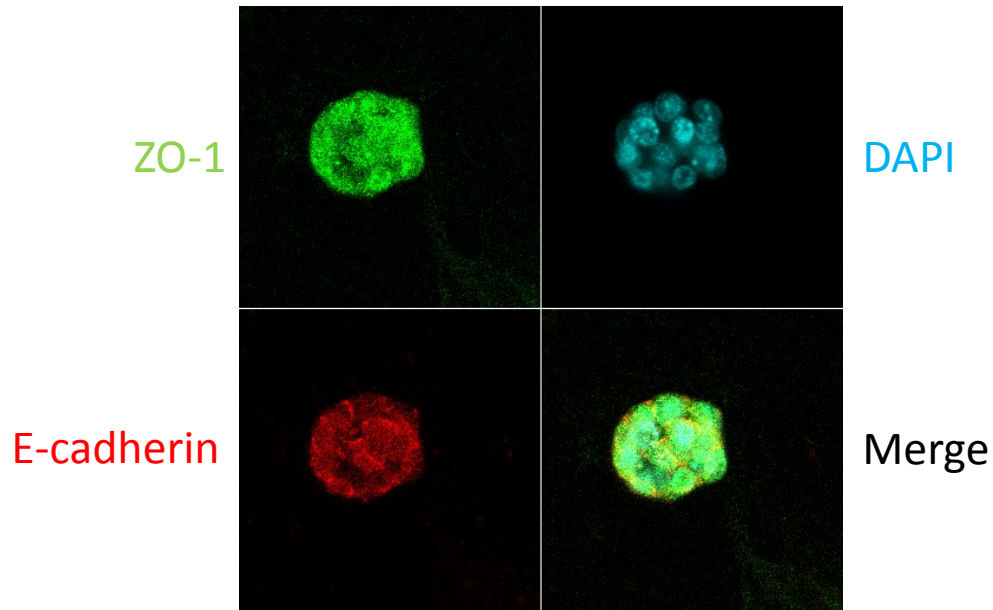
6.1 Ligand dependent epithelial polarity and lumen formation of HC11 cells.

The development of the mammary gland during late pregnancy and lactation relies greatly on the controlled morphogenesis and polarization of epithelial cells. *In vitro* techniques have been critical to underlying the cellular events that take place in the growth and development of epithelial cells and their subsequent deregulation during tumourigenesis. However, a vast majority of these studies were done in 2-dimensional (2D) monolayer cultures. Although monolayer cell cultures are novel and still greatly useful, it is becoming increasingly known that the function of epithelial cells and their regulating signaling pathways can act very different in 2D monolayer cultures compared to 3D cultures^{133,134}. In the field of mammary gland development, 3D cultures have been essential to understanding conditions and events necessary for lumen formation and epithelial polarity. 3D cultures are a powerful tool for investigating the mammary gland because the tubular and cyst structures that are able to be cultured, greatly reflect the tubular branches and terminal acini, respectively, that occur *in vivo*. However, to date the major focus has been on composition of the ECM and the intrinsic genetic and molecular activities of the cell^{96,135}. There have been no studies to our knowledge that investigate the effects of ligand stimulation on epithelial polarity or lumen formation such as the events observed *in vivo* during late pregnancy and lactation.

To investigate the effects of lactogenic ligands, namely PRL, on epithelial polarity we used a 3D basement membrane model using HC11 mammary epithelial cells and Matrigel. Cells were grown using the “on-top” technique for 2days in growth media, followed by a treatment of either EGF or HIP for 3-5 days. Cysts were then immunostained for the tight-junction protein

marker ZO-1 (green), the basolateral protein marker E-cadherin (red) and the nuclei were stained with DAPI. Confocal images were taken at different planes from top to bottom. Central plane images clearly demonstrate a difference in cyst morphology and protein localization between EGF and HIP conditions (Fig. 6.1.1). It was qualitatively observed that cysts treated with EGF tended to be larger with more cells on average. However, even when considering cysts of equal size, there was a clear difference. HC11 cells treated with EGF developed into unorganized cysts with no lumen and a low level of protein localization (Fig. 6.1.1 A). Any localization of proteins, ZO-1 or E-cadherin, appeared between cells at intercellular-junctions and not surrounding a hollow cavity or lumen (Data not shown). In contrast, HC11 cysts that were treated with HIP demonstrated a high level of organization with localization of the adherence junction protein E-cadherin to the basolateral membrane and the tight junction marker ZO-1 to the apical most region of the cell surrounding a hollow central lumen (Fig. 6.1.1 B). In this model using HC11 cells, it was determined epithelial polarity and lumen formation was ligand dependent.

A)



B)

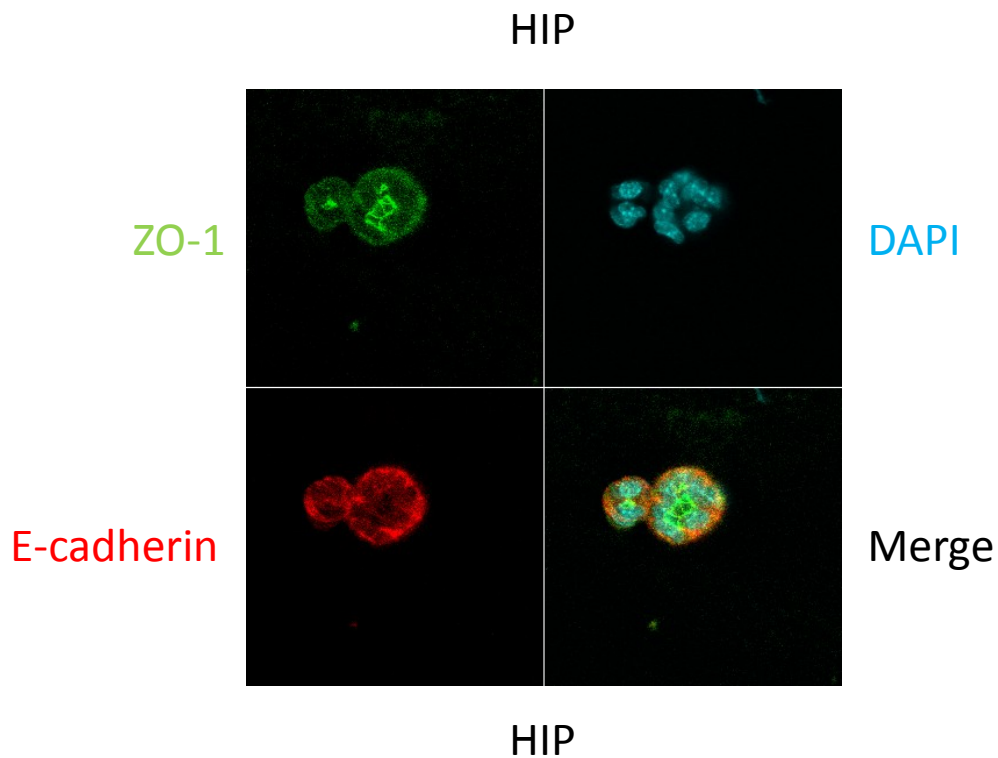


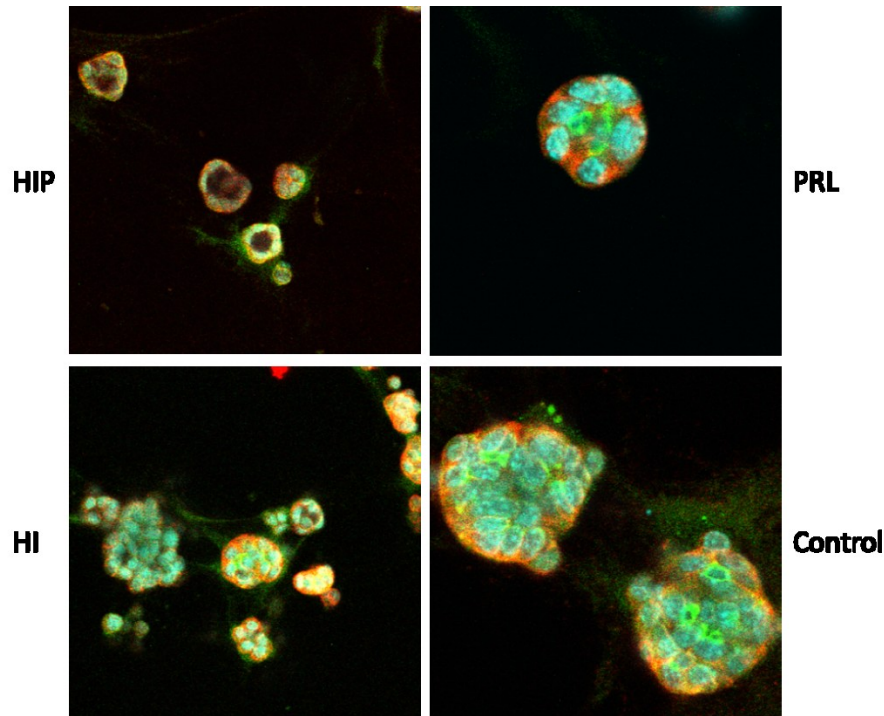
Figure 1. Epithelial polarity and lumen formation in an HC11 3D model is dependent on specific ligand stimulation. HC11 cells were grown using the 3D method with Matrigel for 2 days in growth media, then treated for 3 days with either a) EGF or b) HIP (see Materials & Methods). Colonies were immunostained for E-cadherin (red) and ZO-1 (green), while nuclei were stained with DAPI. A central plane Z-section photo demonstrates an average cyst with either the (B) presence or (A) absence of a central lumen.

6.2 Prolactin-induced epithelial polarity and lumen formation in HC11 cysts.

We further studied the effects of the lactogenic treatment on HC11 cells by investigating whether the induction of epithelial polarity was an effect of the treatment HIP as a whole or an effect of individual ligands. Due to its role in terminal acini development and EMT inhibition, we hypothesized that PRL was the major inducer of epithelial polarity and lumen formation. To explore this hypothesis the HC11 3D culture model was again used. HC11 cells were grown with Matrigel for 2 days in growth media, induced for 2 days in induction media, and then treated with either: serum only (control), PRL, HI, or HIP. Cysts were then immunostained for ZO-1 (green), E-cadherin (red), and nuclei using DAPI. Images were taken at different planes from top to bottom using a confocal microscope. Cross-sectional images at a central plane of an average cyst for each condition demonstrates the presence or absence of a central lumen (Fig. 6.2.1 A). Both treatments, HIP and PRL, resulted in similar results of organized cysts with localized basolateral E-cadherin and apical ZO-1 surrounding a central lumen. Similarly, both HI and serum treatments yielded a majority of cysts that were unorganized with no lumen. Occasionally, localized ZO-1 was observed surrounding what has been previously referred to as multi-vacuoles or multi-lumen in similar amounts in all conditions. More research is needed to determine the true meaning of this phenomenon, however to date it is thought to either be due to disorganization of a central lumen or a pre-cursor event leading to an organized central lumen. Regardless, this phenomenon represents either a pre- or post- event to an organized central lumen and since it was found at equal levels in all conditions, all cysts containing such were considered as unorganized cysts for statistics. The relative number of organized cysts, as determined by organized central lumens, were counted for each condition for three separate

independent experiments and compared statistically using ANOVA(Fig. 6.2.1 B). Here we experimentally demonstrated that PRL (either as HIP or PRL alone treatments) significantly induced polarized epithelial cysts with central lumens more than 3-4 times that of treatments without PRL.

A)



B)

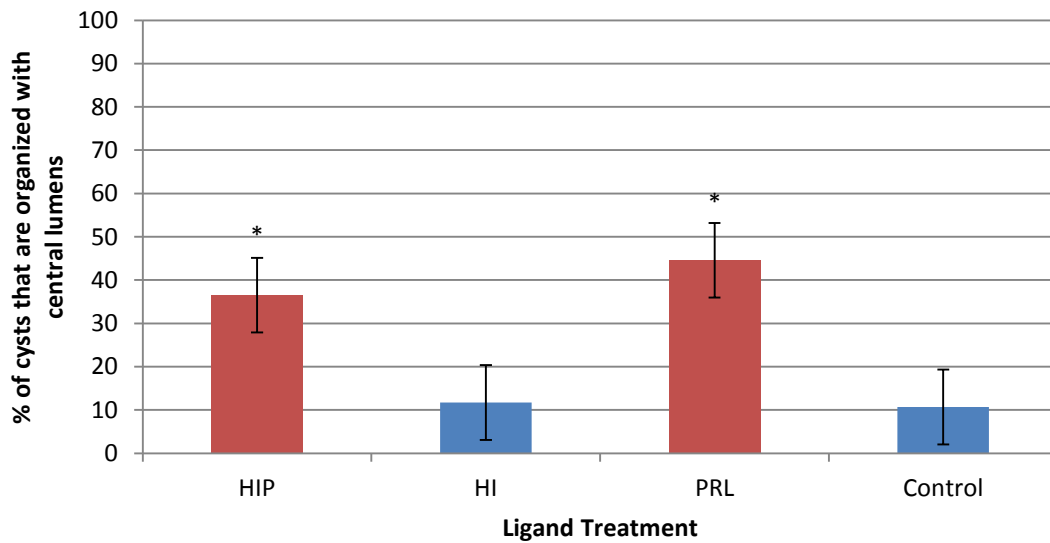


Figure 2. Prolactin-induced epithelial polarity and lumen formation in mammary acini. HC11 cells were grown using 3D method for 2 days in growth media, 2 days in induction media, then treated for 3 days in either: Control (Serum only), PRL, HI, or HIP. (A) Colonies were immunostained for E-cadherin (red), ZO-1 (green) and nuclei using DAPI. (B) Statistics were conducted using three separate experiments where approximately 50 colonies per condition, per experiment were analyzed. Error bars represent standard error and stars (*) represents a significant change ($P \leq 0.05$) compared to control as determined by ANOVA.

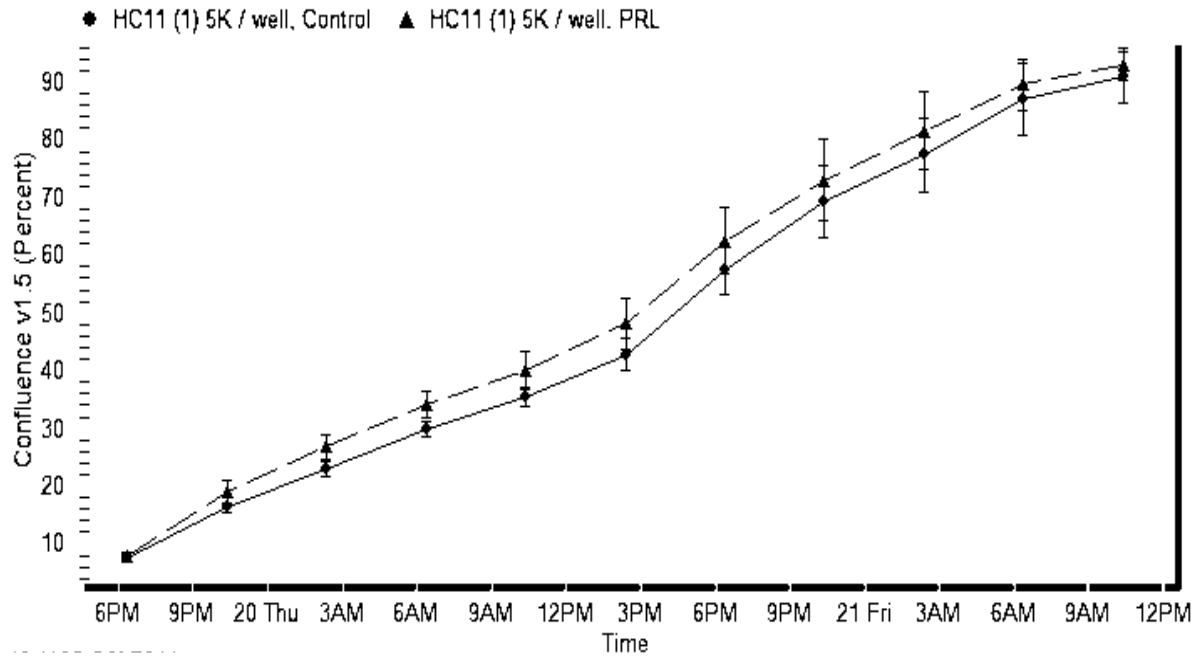
6.3 PRL's small inhibitory effect on migration, but not proliferation

In many other 3D culture models using other cell lines, such as MCF-10A, polarity and lumen formation in cysts is usually accompanied with growth arrest. Furthermore, tumour progression and EMT leads to an increase in cell growth, migration and a loss of apicobasal polarity. With our new found role of PRL as an inducer of epithelial polarity and previous findings demonstrating PRL as an inhibitor of EMT and invasion, we would expect PRL in our model to inhibit proliferation and invasion⁸⁸. However, PRL's role in cellular growth, similarly to its role in cancer, has continuously been a topic of debate^{136,137}. We therefore investigated the effects of PRL on proliferation and migration in HC11 cells using IncuCyte HD live-cell imaging system. HC11 cells were plated at 5,000 cells/ well in media containing either: serum, PRL, EGF or EGF +PRL. Cells were then incubated and allowed to grow at 37°C. Cells treated with EGF reached confluency and demonstrated a stereotypical growth curve, while cells grown without EGF only underwent linear growth as they never reached full confluency (Fig. 6.3.1 A & B). Due to these differences, we analyzed the effects of PRL on its own and in the presence of EGF. In all conditions, PRL did not have any significant effect on proliferation regardless if confluency was reached or the presence of EGF (Fig. 6.3.1 A&B).

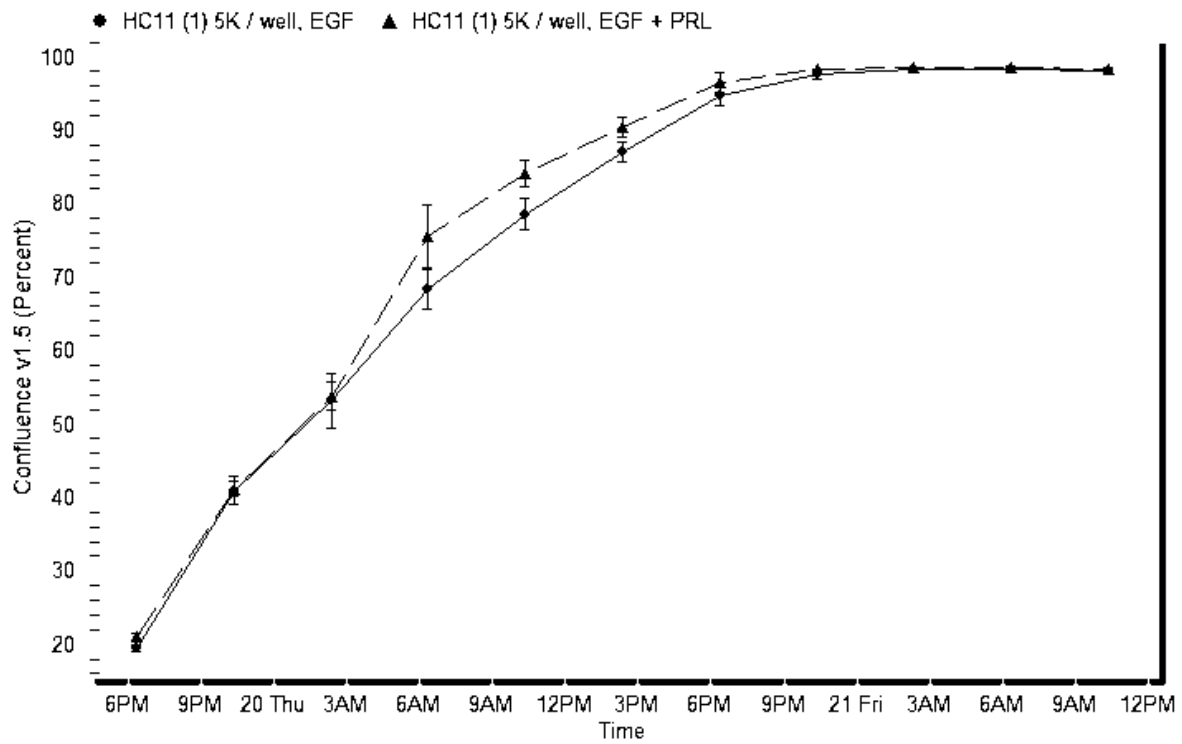
Similarly to that of proliferation, migration often in connection with EMT or tumour progression is a topic of debate in the field of PRL. We therefore investigated the effects of PRL on migration in HC11 cells using a standard scratch-wound assay with an IncuCyte HD live-cell imaging system. HC11 cells were plated at 30,000cells/well and incubated at 37°C and allowed to grow until full confluency. After a wound was made, cells were treated with either: serum only (control), PRL, EGF, or EGF + PRL. It should be noted that in all treatments the wound was

never fully closed and the migration differences observed were determined based on change in confluency over time compared to that of the original wound (Fig. 6.3.1 D). Regardless, a small inhibitory effect was reproducibly observed in HC11 cells with the treatment of PRL, regardless of the presence of EGF (Fig. 6.3.1 C). With our findings with the proliferation assay, these findings can only be attributed to migration and not differences in proliferation. Therefore in HC11 cells, we found that PRL has a small inhibitory effect on migration, but no effect on proliferation.

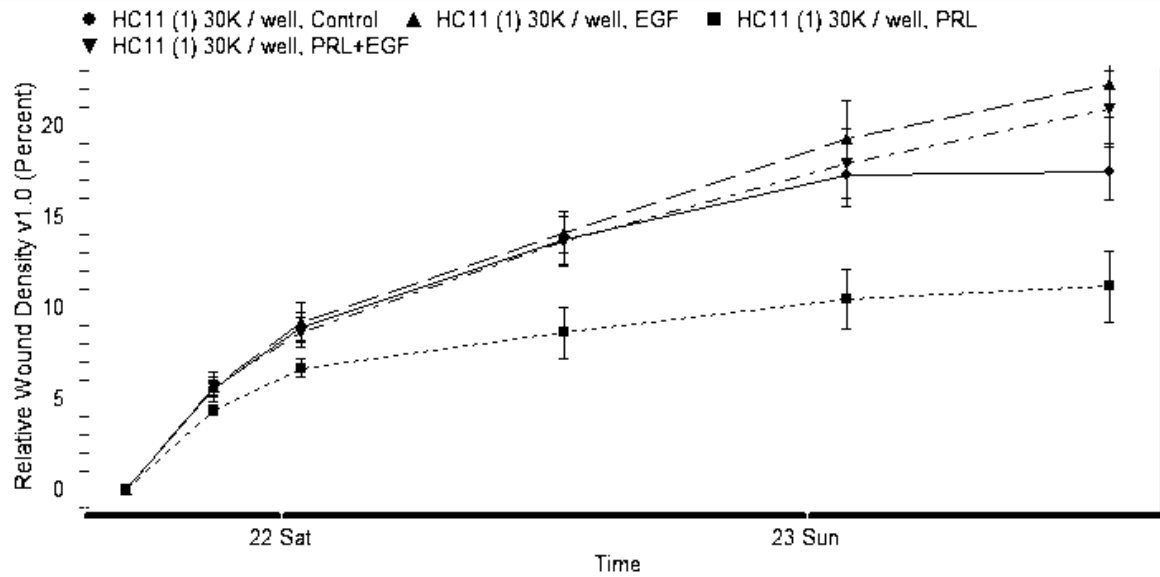
A)



B)

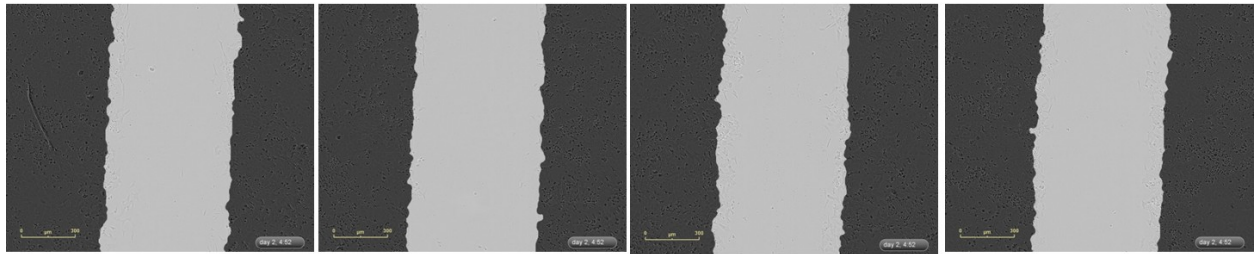


C)



D)

i. Phase-contrast of migration assay



ii. Scratch wound mask of migration assay

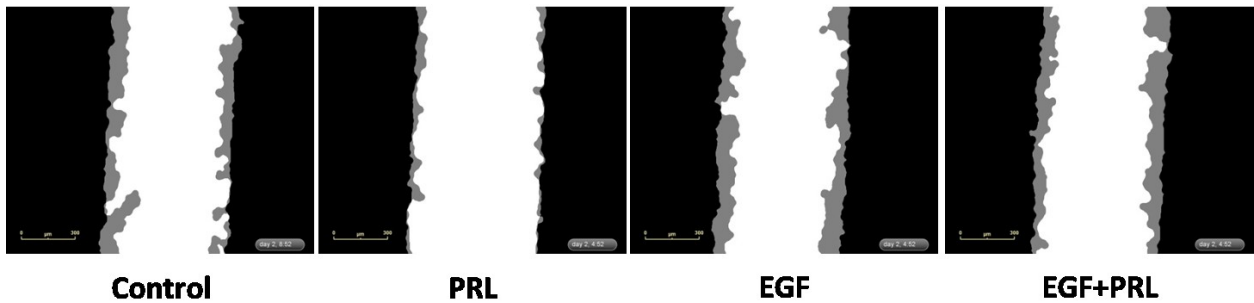
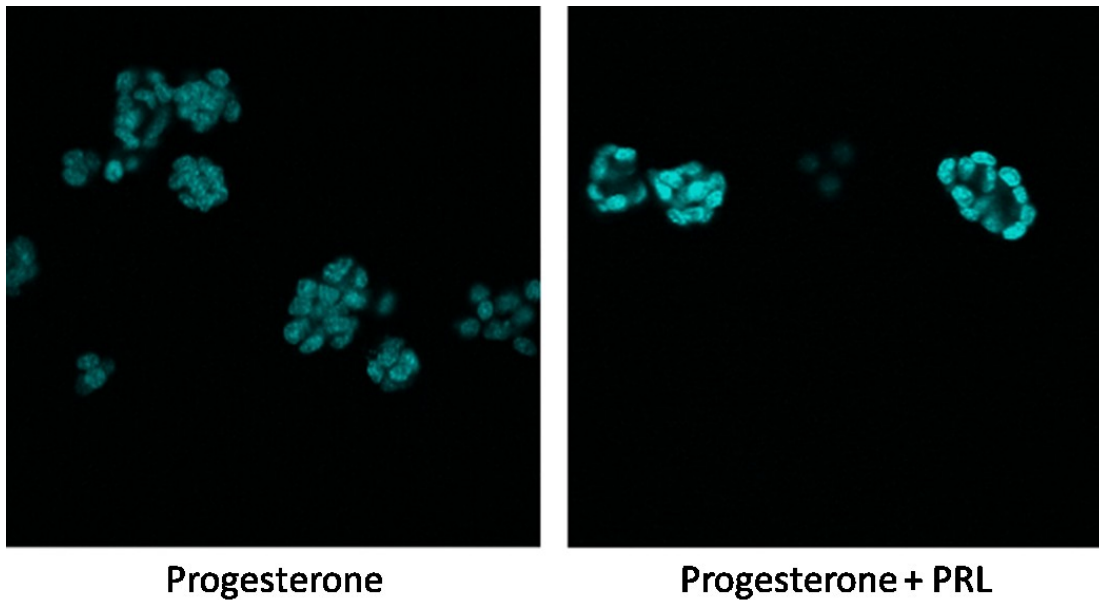


Figure 3. No effect on proliferation and a small, yet reproducible inhibitory effect on migration by prolactin stimulation on HC11 cells. Cells for proliferation assays (A&B) were plated at 5,000 cells/mL. Cells were treated as following: A) serum only (control) or PRL, and B) EGF, or EGF + PRL. Migration Assays (C&D) were conducting by plated cells at 30,000cells/mL and grown until fully confluent. A wound was then made and cells were treated with one of the following conditions: serum only (control), PRL, EGF, or EGF + PRL. Proliferation and Migration assays were both conducted and analyzed using IncuCyte HD live-cell imaging system (see Materials & Methods). Dii) Scratch wound masks demonstrate initial scratch (black) and migration area (grey) as determined by monolayer confluence.

6.4 The null effect of Pg on polarity and lumen formation in HC11 cells

Due to the synergistic roles of PRL and Pg during mammary gland development, we investigated the possibility of Pg having either a similar polarity effect on HC11 cells or a complementary role with PRL. The 3D culture model was conducted as previously described with other ligands. Following a 2 day growth and 2 day induction period, cells were treated with either Pg or a combination of Pg and PRL. Treatment of Pg alone had no noticeable effect on cyst organization or lumen formation in comparison to the control (Fig. 6.4.1). There was a reproducible increase of cyst organization and lumen formation with Pg and PRL in comparison to control and PG alone, an increase that was comparable to our previously reported HIP treatments (Fig. 6.4.1 B). Therefore in our model with HC11 cells, Pg had no effect on epithelial polarity or lumen formation, nor did Pg act synergistically with PRL.

A)



B)

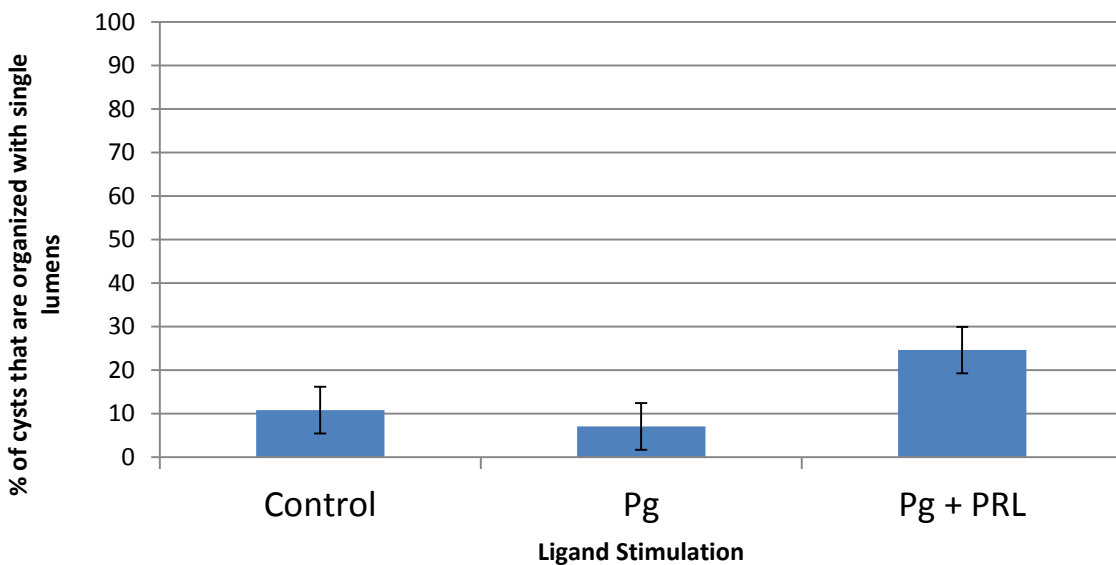


Figure 4. Progesterone has a null effect on epithelial polarity or lumen formation on HC11 acini. Cells were grown using 3D protocol with Matrigel for 2 days in growth media, 2days in induction media, and then treated with either Pg alone or Pg and PRL. A) Nuclei were stained with DAPI as seen with the central cross-sectional photo demonstrating an average cyst with the presence or absence of a central lumen. B) Average number of acini with organized central lumens were calculated from over 100 cysts analyzed from two separate experiments, error bars represent standard error.

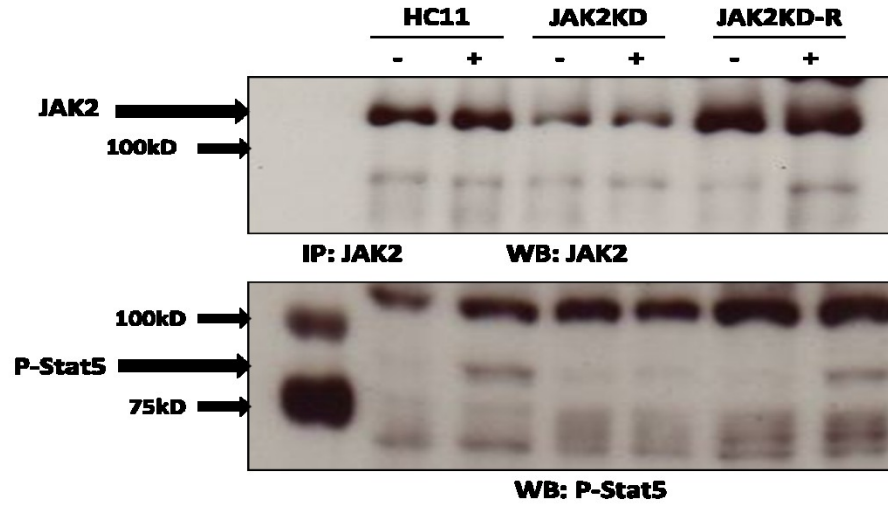
6.5 Loss of JAK2 in HC11 cells results in a phenotypic change resembling an EMT process

To further our study into the role of PRL and its downstream signaling cascade, our lab previously generated an HC11-JAK2-knock down (HC11-JAK2KD) cell line and its subsequent recovery cell line, HC11-JAK2KD-R. However, due to antibody issues, these cell lines' genetic variations were only able to be analyzed indirectly using anti-PY (data not shown). Therefore to further the investigation into the molecular signaling responsible for the regulation of epithelial polarity and lumen formation we needed to characterize these cells line. As expected, using an IP-JAK2 technique to pool JAK2 and then using western analysis to probe JAK2, we determined that in comparison to the parental HC11 cell line, we had a loss of and subsequent recovery of JAK2 in the HC11-JAK2KD and HC11-JAK2KD-R cell lines respectively (Fig. 6.5.1 A). Due to the importance of the downstream PRLR/JAK2/Stat5 pathway, western analysis of Phosphorylated-Stat5 (P-Stat5) confirmed that the transfections were functional knock downs and recoveries (Fig.6.5.1 A).

During EMT when apicobasal polarity is lost, a phenotypic change occurs in which epithelial cells lose their epithelial characteristics and gain mesenchymal ones. These new stem-like cells tend to appear more elongated and fibroblastic in culture^{138,139}. Using phase-contrast microscopy, it was evident that loss of JAK2 had similar phenotypic effects. Loss of JAK2 in HC11 appeared to cause cells to be more fibroblastic in 2D culture, with an overall longer soma and longer protrusions in comparison to parental HC11 cells which appear more symmetric with numerous small finger-like projections (Fig. 6.5.1 Bii). Furthermore, at high confluence HC11-JAK2KD cells appeared to overlap compared to HC11 cells which have the expected epithelial

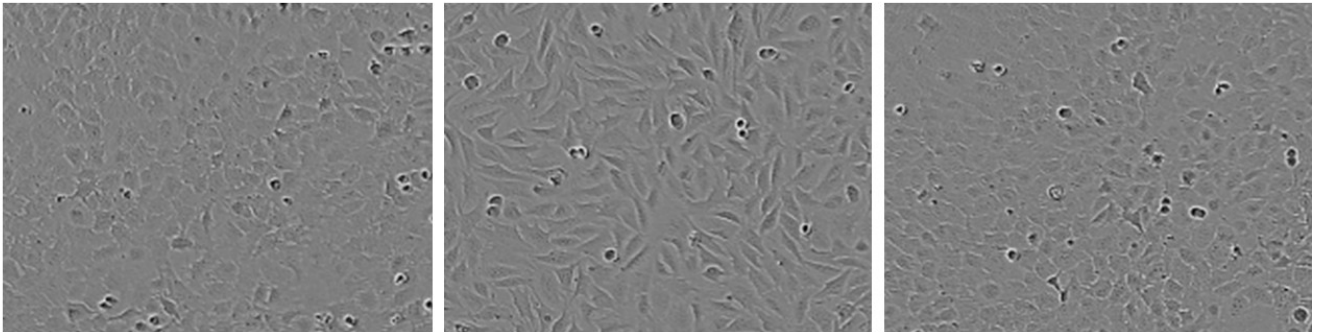
“cobble-stone” phenotype with rigid cell boundaries (Fig. 6.5.1 Bi). Finally, in 3D culture loss of JAK2 resulted in a less compact and organized cyst with more protrusions that project into the ECM (Fig. 6.5.1 C). All three of these likely-connected phenotypes were recovered by reintroducing JAK2 in HC11-JAK2KD-R cells (Fig. 6.5.1 A-C).

A)

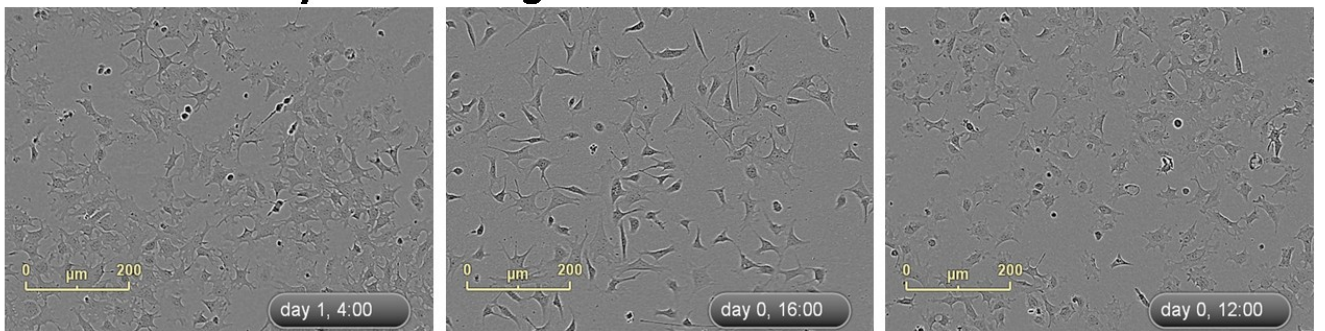


B)

i. High Confluency High Magnification



ii. Low Confluency Medium Magnification



c)

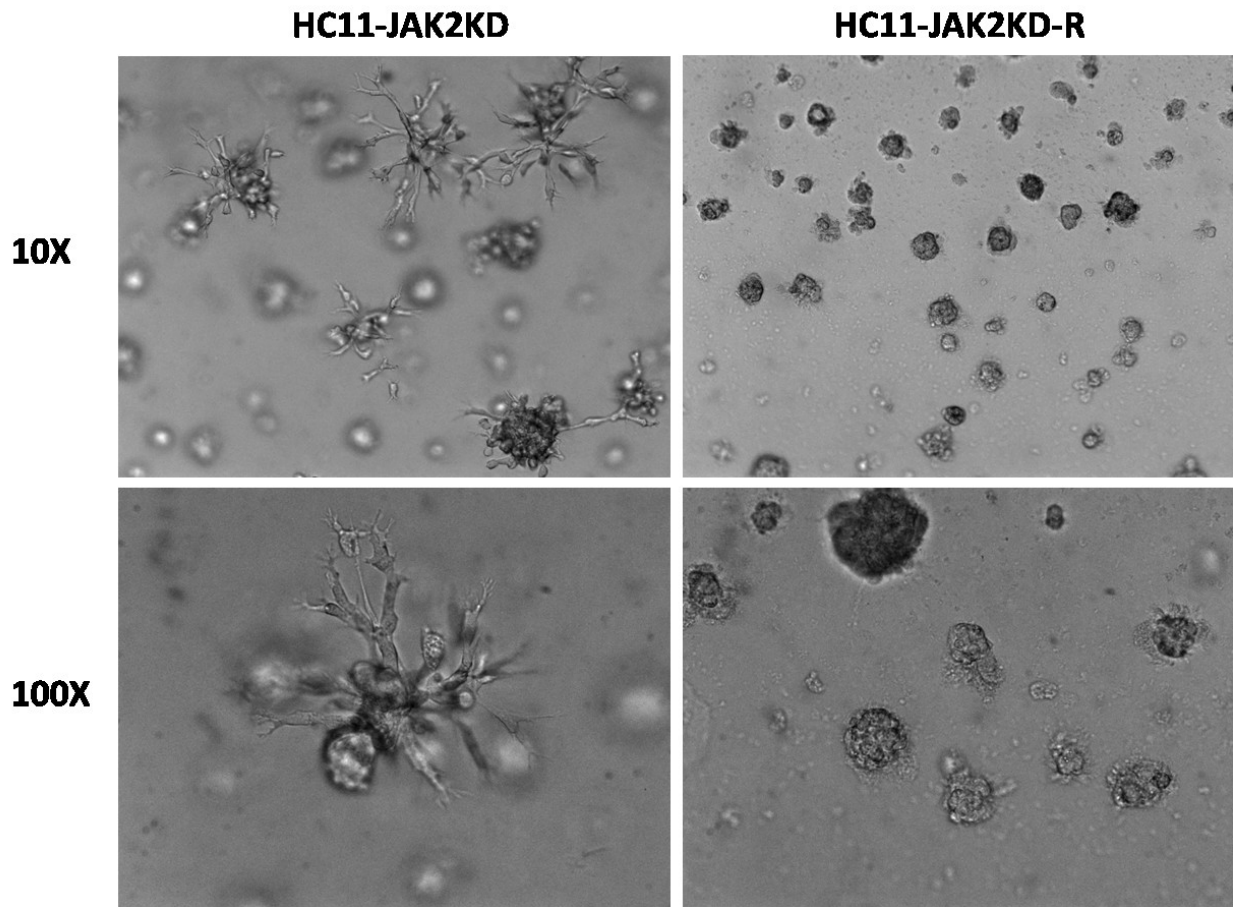


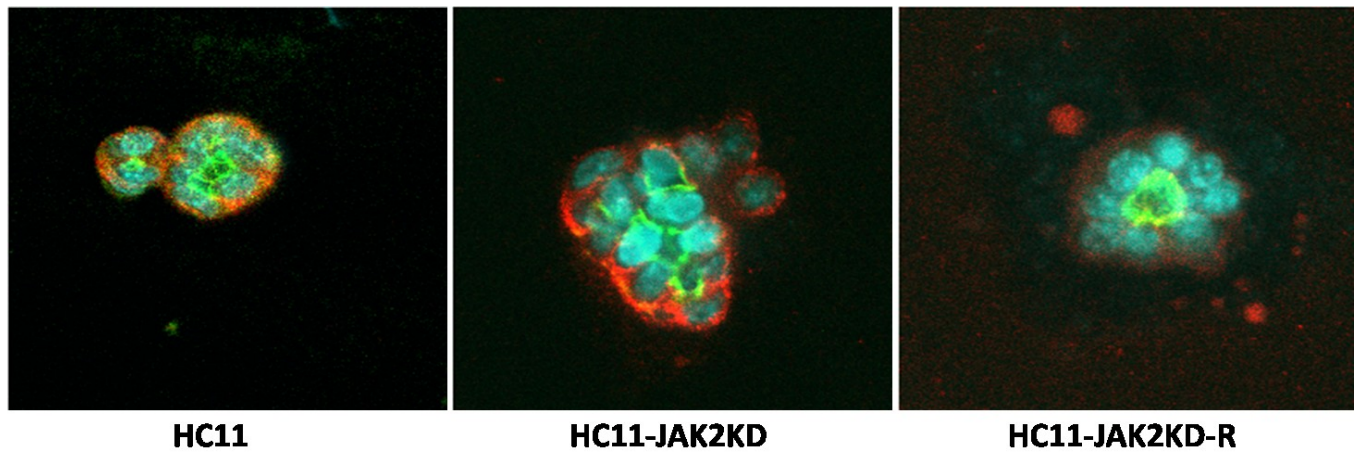
Figure 5. Characterization of JAK2 knockdown and JAK2-Recovery cell lines. A) HC11, HC11-JAK2KD and HC11-JAK2KD-R cells were grown for 2 days in growth media, 2days in induction media, and 24 hours in starvation media and either treated (+) or not (-) with PRL and then lysed for Western analysis (see Materials & Methods). Lysates were either: IP with JAK2 and then probed for JAK2 (top panel), or used as total lysates and probed for P-stat5 (lower panel). B) Cells were grown in 2D monolayer culture in growth media and photographs were taken at varying confluency by the IncuCyte HD live-cell imaging system. C) Cells were grown using the 3D “on top” method and after 5 days (2days EGF, 3days HIP) photographs were taken with an inverted phase-contrast microscope.

6.6 PRL-induced epithelial polarity and lumen formation is JAK2-dependant

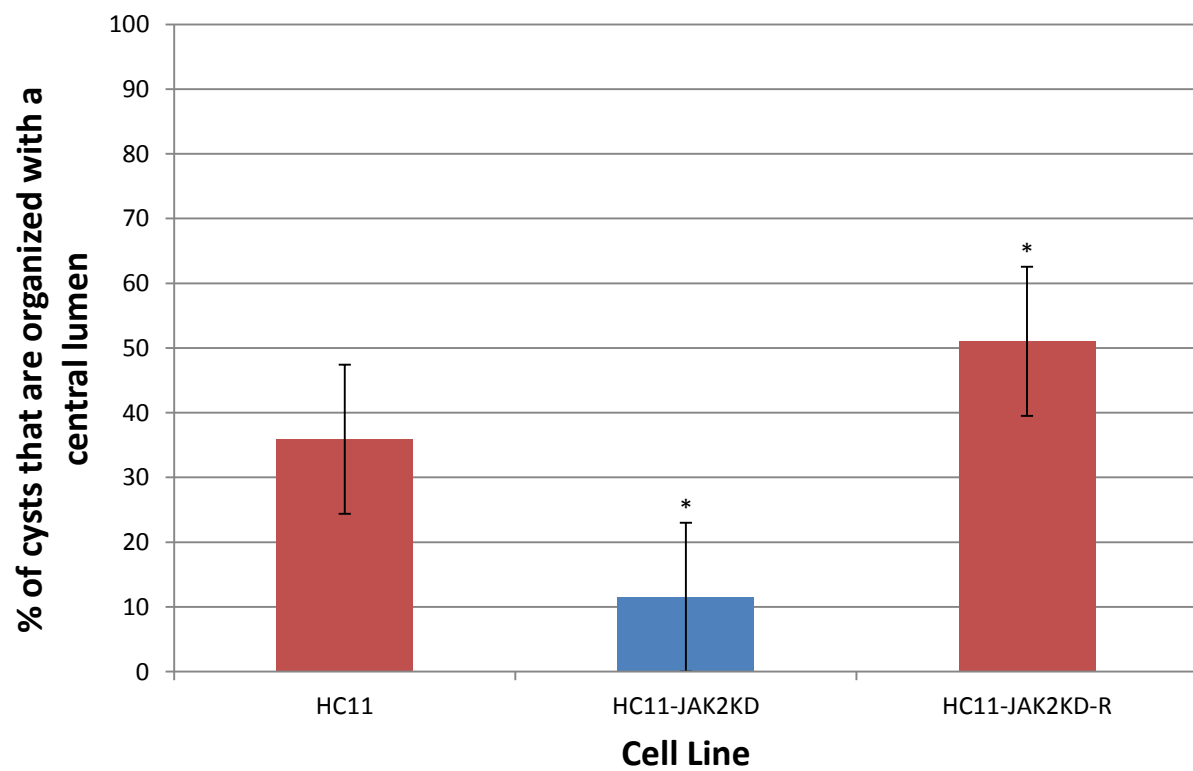
To continue our study into the role of PRL in mammary epithelial polarity and lumen formation we utilized our 3D-culture assay. With this model we used our HC11-JAK2KD and HC11-JAK2KD-R cell lines to determine if the PRL-induced polarity was JAK2-dependant. We grew HC11, HC11-JAK2KD and HC11-JAK2KD-R cells and treated them all with HIP for 3 days. Cysts were stained for ZO-1 (green), E-cadherin (red), and nuclei using DAPI. From top to bottom images were taken at different planes using a confocal microscope. Cross-sectional images at a central plane of an average cyst for each condition demonstrate the presence or absence of a central lumen surrounded by the localized tight junction protein ZO-1 (Fig. 6.6.1 A). The knock down of JAK2 in HC11 cells resulted in a significant reduction of approximately half the number of organized polar cysts with central lumen (Fig. 6.6.1 B). Recovery of JAK2, HC11-JAK2KD-R, lead to a significant increase in approximately 3 times the number of organized polar cysts (Fig. 6.6.1 B). A level of significance in JAK2-dependent polarity was statistically determined using ANOVA with a $P\text{-value} \leq 0.05$. Furthermore, the knock down of JAK2 in HC11 cells resulted in the loss of cell-cell junction protein localization. HC11 cells had well developed cell-cell junctions in 2D monolayer culture as demonstrated by membrane localized E-cadherin and ZO-1 (Fig. 6.6.1 C). These junction proteins lost membrane localization with the subsequent genetic knock down of JAK2 and then recovered with the JAK2-recovery cell line, HC11-JAK2KD-R (Fig. 6.6.1 C). In both 2D and 3D culture, epithelial polarity was determined to be JAK2-dependant.

A)

ZO-1/E-cadherin/DAPI



B)



c)

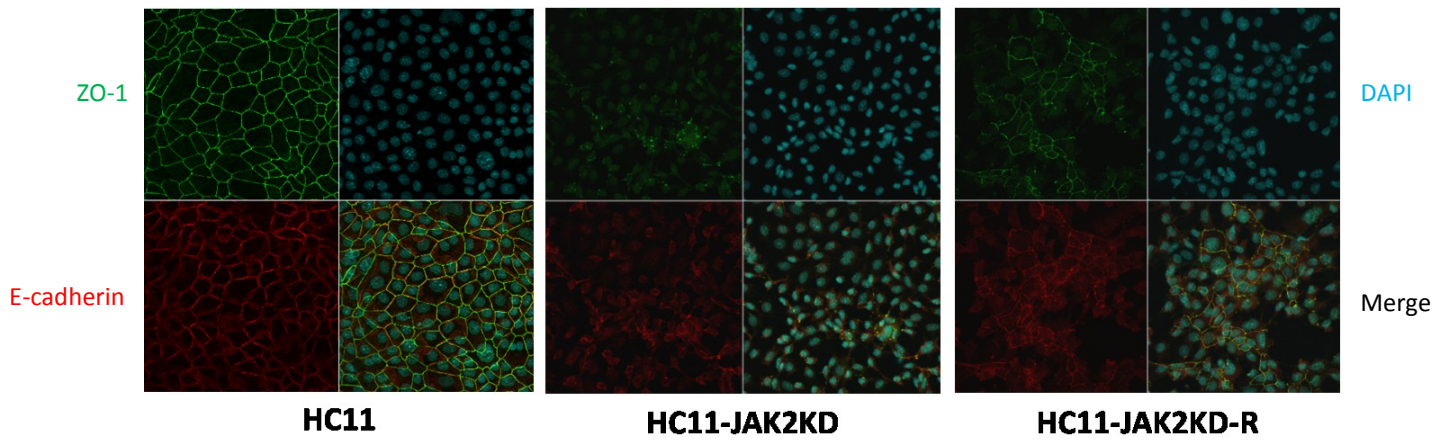
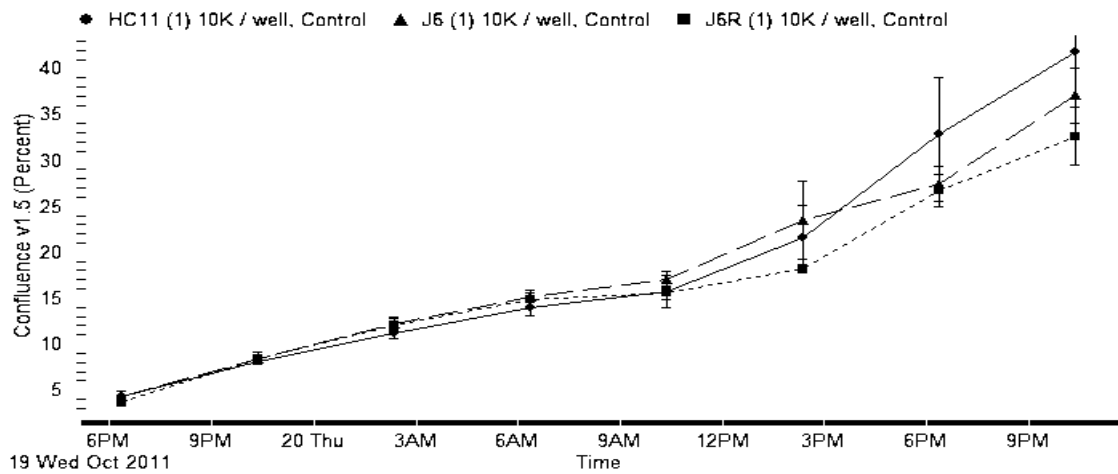


Figure 6. JAK-dependent establishment of epithelial polarity and lumen formation. HC11, HC11-JAK2KD and HC11-JAK2KD-R cells were grown in (A) 3D and (B) 2D monoculture culture and stained for ZO-1 (green), E-cadherin (red) and nuclei were stained with DAPI (blue). A) Z-sections were taken and a central cross-section was chosen to display the presence or absence of a central lumen. B) Colonies from three separate experiments were analyzed and averaged for the percent of cysts that were organized with central lumens. Error bars represent standard error and stars (*) represents a significant change ($P \leq 0.05$) compared to control as determined by ANOVA.

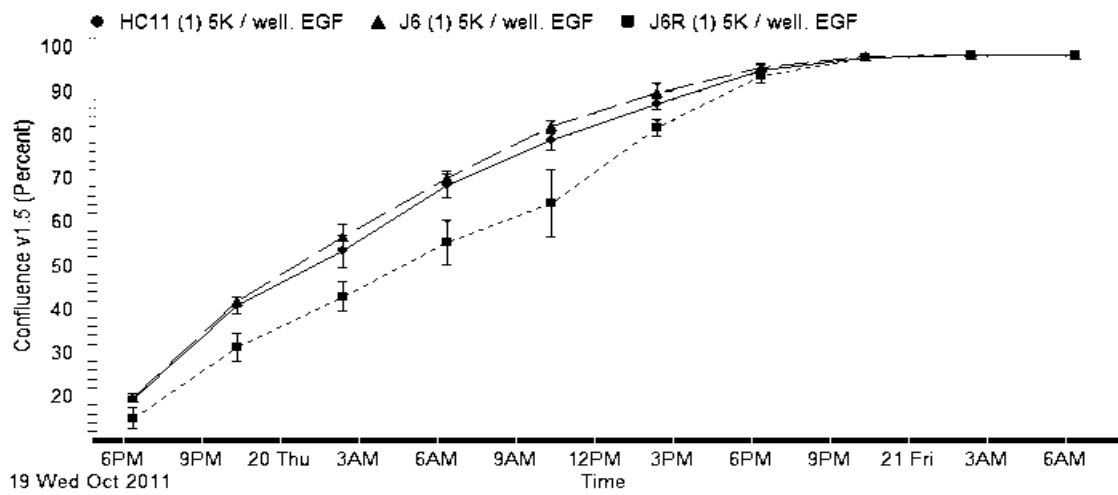
6.7 JAK2-KD in HC11 has no effect on proliferation nor migration

Considering our results for PRL and our findings that epithelial polarity and lumen formation was JAK2 dependent, we investigated the role of JAK2 in proliferation and migration using our genetically modified and parental HC11 cells. As previously described we used the IncuCyte HD live-cell imaging system to conduct proliferation assays comparing HC11, HC11-JAK2KD, and HC11-JAK2KD-R cells. Cells were grown in either serum only (control), EGF or EGF + PRL, to determine the role of JAK2 in HC11 cells during different phases: basal level, normal EGF-induced growth, and EGF-induced growth with the presence of PRL, respectively. Similar to the effects of PRL, genetic manipulation of JAK2 had no significant effect on proliferation under any condition (Fig. 6.7.1 A-C). Continuing into our investigation of the role of JAK2 we used the wound healing assay with the IncuCyte HD live-cell imaging system to assess any differences in the migratory capabilities of the cell lines HC11, HC11-JAK2KD, and HC11-JAK2KD-R. Surprisingly, there were no comparable or significant differences between cell lines regardless of the presence of PRL as seen with our two conditions used: EGF and EGF + PRL (Fig. 6.7.1 D-F). Analysis of the migration assay videos (not shown) and the scratch wound masks demonstrates that the changes recorded as migration were likely a combination of small cellular mobility and proliferation due to the inability of the cells to close the wound and the extremely small changes in the cell boarder(Fig. 6.7.1 F). With the data collected, JAK2 did not play a significant role in proliferation or migration in our HC11 model.

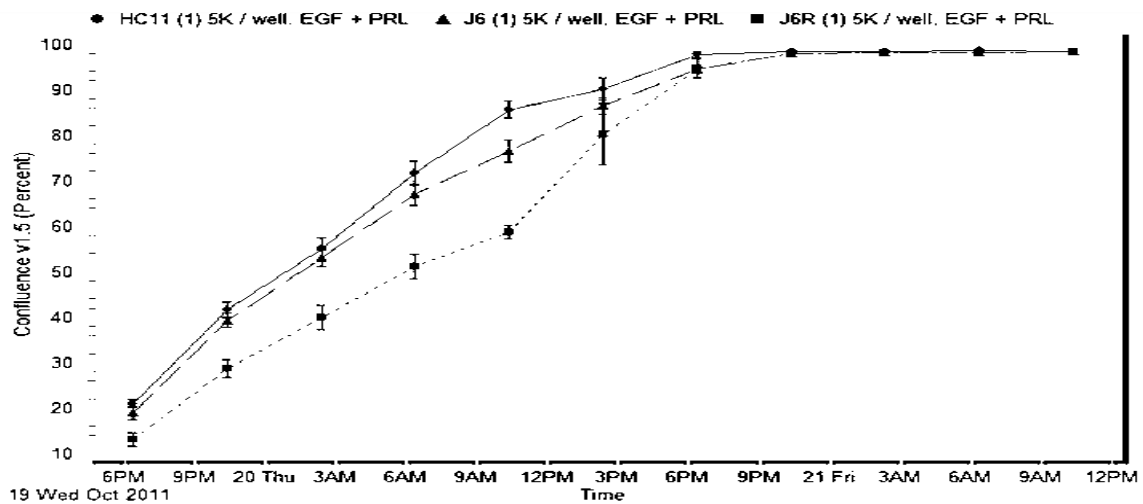
A)



B)



C)



D)

Treatment: EGF + PRL



Treatment: EGF

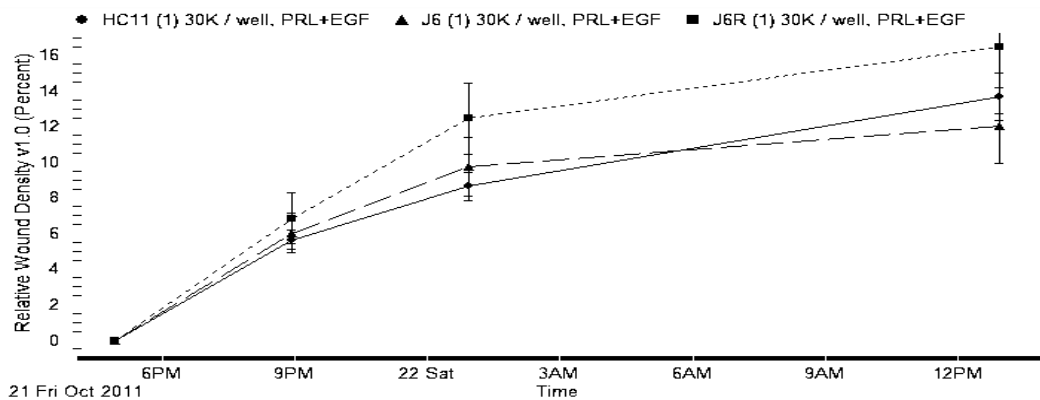


HC11

HC11-JAK2KD

HC11-JAK2KD-R

E)



F)

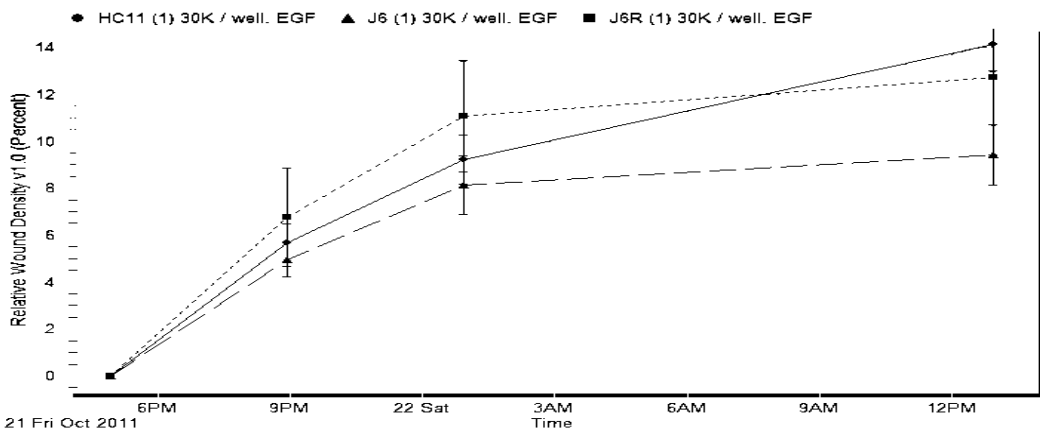


Figure 7. Null effect on proliferation or migration with the knock down of JAK2 in HC11 cells. Proliferation assays using IncuCyte HD live-cell imaging system were done using HC11, HC11-JAK2KD and HC11-JAK2KD cells grown in either: Serum (A), EGF (B) or EGF + PRL (C). Migration assays comparing cell lines used EGF as the migratory stimuli and were treated with either PRL (E) or not (F). Scratch Wound masks (D) were determined using the initial scratch (black) and migration (grey) areas by relative monolayer confluency via IncuCyte HD live-cell imaging system.

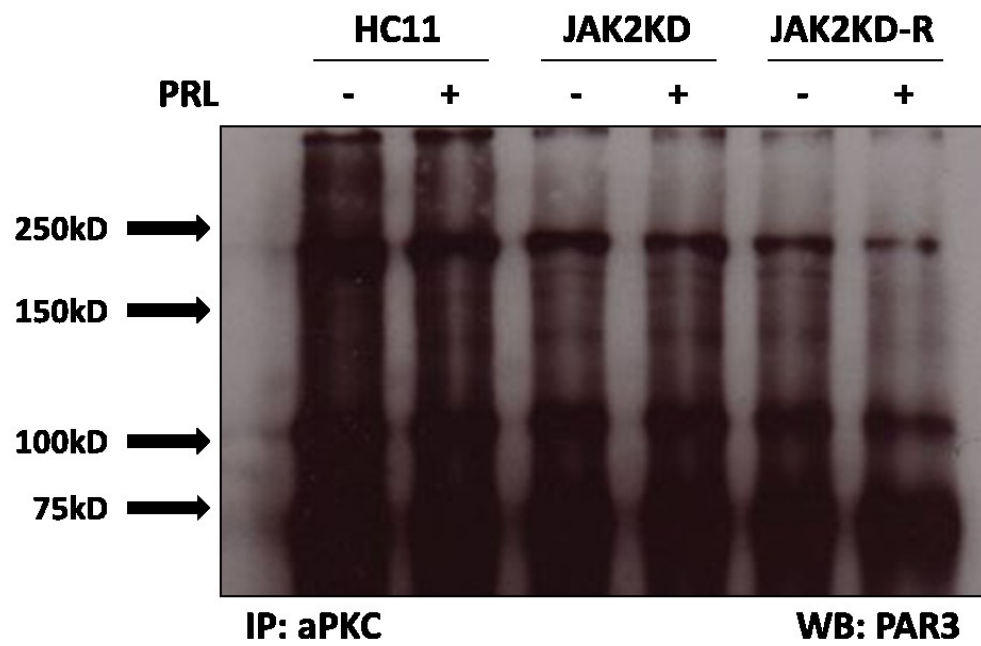
6.8 JAK2-dependant regulation of polarity protein interactions

After the confirmation of the critical role by PRL and its downstream transducer JAK2 in epithelial polarity and the characterization of their peripheral effects, it was important to next investigate possible mechanisms. With our current understanding of PRL's role in tight junction regulation during lactation, our findings that PRL can induce epithelial polarization with localized ZO-1 in both 2D and 3D culture in a JAK2-dependant manner, and due to preliminary data previously gathered by our lab, we investigated the possibility that PRL played a role in regulation of the aPKC/PR3 interaction that regulates the PAR complex and tight junctions. Although there was some preliminary data suggesting this hypothesis (data not shown), all protein analyses using Co-IP techniques demonstrated that the aPKC/PR3 interaction was neither PRL nor JAK2 dependent (Fig. 6.8.1 A). Along with this rationale we investigated the potential regulation of the Lgl/aPKC interaction, which we would have expected to act in an inverse relationship to the aPKC/PR3 interaction. However, we did not see any JAK2-dependance with respect to Lgl/aPKC interaction or Lgl protein level (Fig. 6.8.1 B).

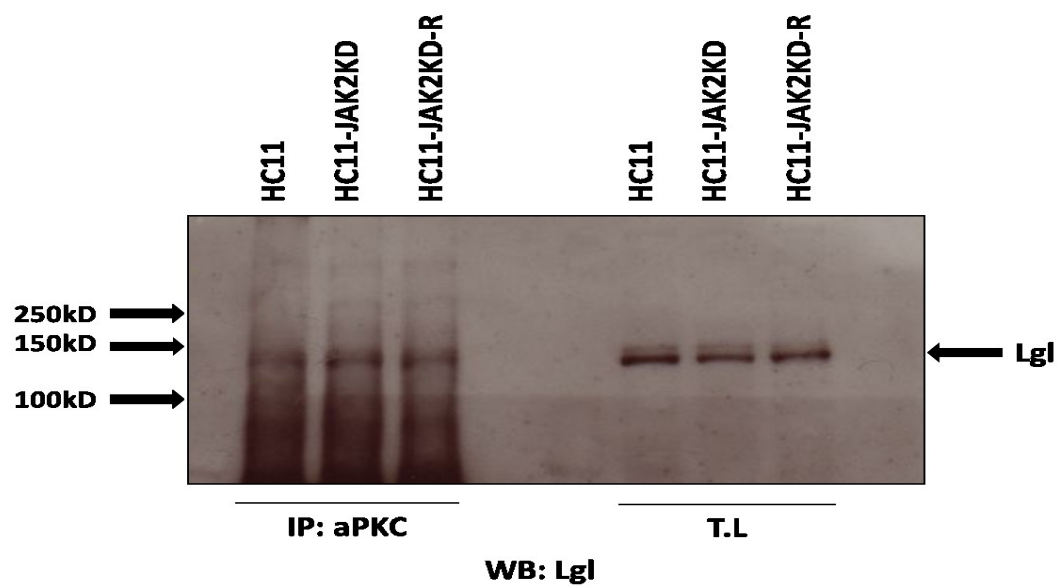
Since epithelial polarity depends on tight junction and adherence junction integrity, as well as our current understanding that basal polarity is established first during establishment of apicobasal polarity, we investigated the potential role of the PRL/JAK2 pathway in regulation of adherence junctions. Using 2D monolayer culture, we studied the localization of adherence junction proteins by immunostaining: E-cadherin (green), β -catenin (red) and nuclei using DAPI. Interestingly, we found that E-cadherin and β -catenin colocalized to cell-cell junctions at the cellular boarder of cells in a JAK2-dependant manner (Fig. 6.8.1 C upper panel). However, in

using Co-IP protein analysis we found no significant difference in E-cadherin/ β -catenin interaction regardless of PRL stimulation or presence of JAK2 (Fig. 6.8.1 C lower panel).

A)



B)



c)

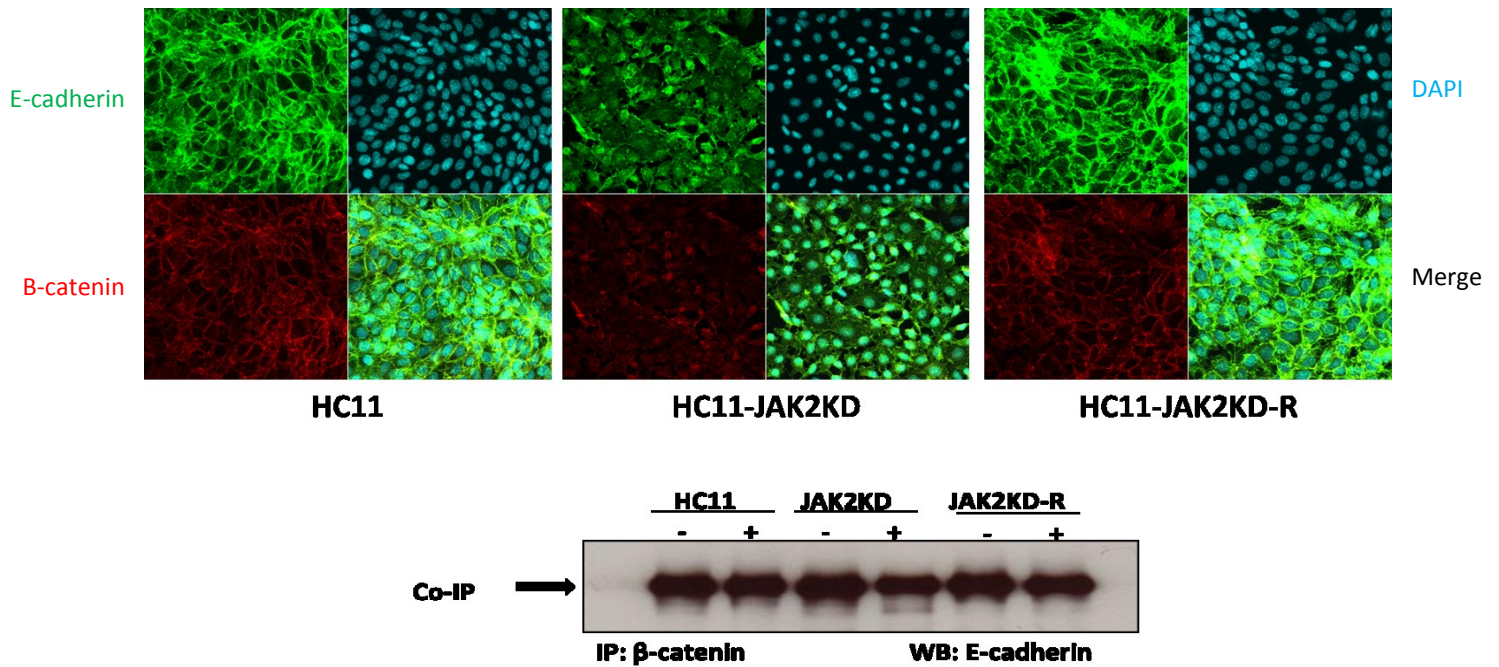


Figure 8. JAK2-dependent regulation of interactions between polarity proteins. Western analyses were done by growing HC11, HC11-JAK2KD, and HC11-JAK2KD-R cells in 2D monolayer culture (see Materials & Methods). A) Cells treated for 24hrs with (+) or without (-) PRL, were lysed and incubated with 1μL of aPKC 1° antibody and PAS beads, and then probed for Par3 by western analysis. B) All cells were treated with PRL overnight then lysed. Lysis was then used either for total lysate (TL) or incubated with 1μL of aPKC AB and PAS beads (IP). The IP and TL lysates were then probed for Lgl using western analysis. C) Co-immunoprecipitate (Co-IP) was done similar to (A) with the exception of lysates being incubated with 1μL of β-catenin 1° antibody and PAS beads, then probed for E-cadherin (Bottom panel). Cell were also grown in 2D and immunostained for E-cadherin (green), β-catenin (red) and nuclei were stained with DAPI (Top panel).

6.9 JAK2-dependant regulation of PRLR

During our study of polarity proteins we wanted to determine where the PRLR is localized especially since there is very limited information on this in the literature. To do this we grew HC11, HC11-JAK2KD and HC11-JAK2KD-R cells in 2D monolayer cultures and immunostained with antibodies for the PRLR (red), as well as ZO-1 (green) and DAPI for reference of the membrane and nuclei respectively. We found that in HC11 cells, PRLR appears to be homogenously distributed throughout the x-plane (Fig. 6.9.1). Interestingly, loss of JAK2 led to the distinct localization of the PRLR just above the nuclei (Fig. 6.9.1). The change in receptor localization caused by the loss of JAK2 in HC11 cells is similar to that of the association of other members of Janus Kinases, including JAK2, with paired receptors such as the thrombopoietin receptor, common γ chain receptor, and the erythropoietin receptor^{140,141,142}. This change in phenotype was partially recovered with the rescue of JAK2 (Fig. 6.9.1). These findings demonstrate a positive feedback loop within the PRLR/JAK2/STAT5 pathway in which JAK2 is needed for proper PRLR localization, as well as a new Janus Kinase-Receptor chaperone pairing.

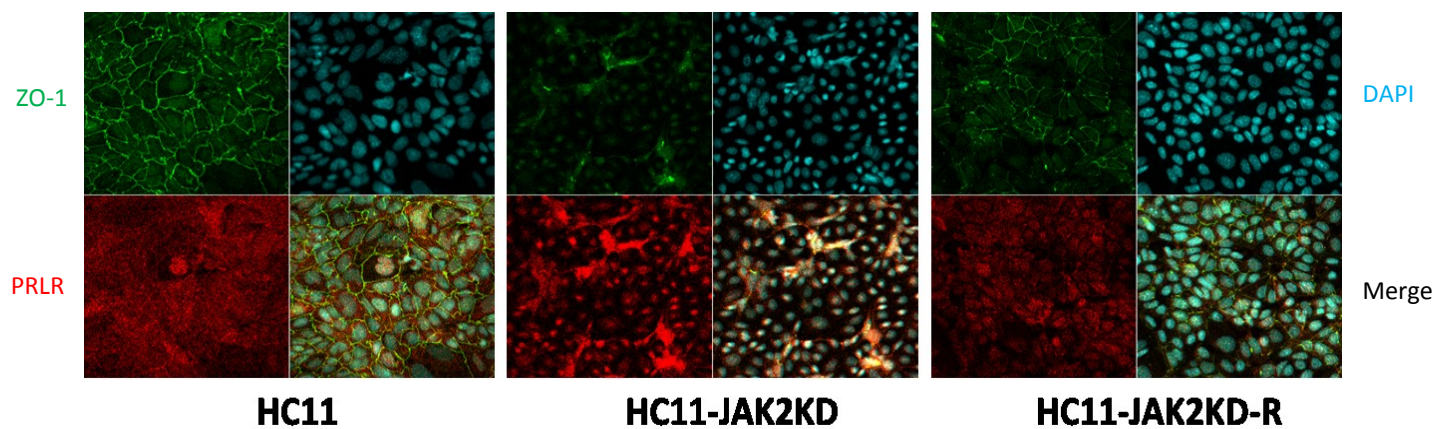


Figure 9. Jak2-dependent localization of PRLR. Cells were grown in 2D monolayer culture until full confluency was reached, fixed and immunostained for ZO-1 (green), PRLR (red), and nuclei were stained with DAPI.

7 Discussion

In our attempt to understand breast cancer, one of our best tools is to look at the development of breast tissues like the mammary gland. If we hope to one day find effective diagnostic tools and therapeutic treatments for the disease, it is essential to first understand the mechanisms that regulate the mammary gland as these are the same mechanisms which will either be compromised or hijacked by the disease. Extensive work has revealed that PRL and its downstream pathway, PRLR/JAK2/STAT5, is one of the major key regulators of mammary gland development. This pathway is essential for terminal differentiation of end buds leading to alveolar structures. However, the full mechanism controlling this is not well understood. These structures are composed of polar epithelial cells surrounding a central lumen, which is essential for directional secretion of milk proteins during breastfeeding. The creation of 3D *in vitro* assays has given us insight into the events and molecules that are critical to these alveolar structures and have also contributed to the increasing popularity of the emerging polarity field. However, the field of epithelial polarity has exclusively focused on ECM composition, integrins, and polarity proteins. Our results are the first to make the connection between the hormones that initiate and regulate morphogenesis of the mammary gland and the polar epithelial structures that result.

Our findings show that PRL is a polarity signal that acts through its downstream transduction molecule JAK2 to induce cellular polarity and lumen formation in mammary epithelial cells. Our findings not only add to the long list of functions for the hormone, but are the first to demonstrate a ligand as a polarity signal. Furthermore, our results show that PRL is not a major inducer of growth or migration as has been suggested by some groups. Instead, we

found that PRL and its downstream pathway had no effect on growth and a very small to null effect on migration. PRL's effects on migration, as suggested by our labs previous findings on EMT suppression, are potentially much greater in cancerous cells than in our HC11 cells⁹⁰. This is because although we used genetic manipulation and ligand stimulation, our cell lines were still epithelial in nature and therefore relatively immobile, as demonstrated by the results showing that no wound fully closed in our migration assays. Further investigation into PRL's role in migration or invasion should be done using more mobile or aggressive cells line.

It should also be noted, that although our results did not show Pg to have an effect on epithelial polarity and lumen formation, this possibility should not be ruled out. Our preliminary findings were conducted using our 3D culture assay, which was optimized for PRL signaling. It is then possible that these conditions are not ideal for Pg signaling and therefore we were unable to capture these Pg effects. Research has implicated Pg as an important regulator of side branching during mammary development, a process that results in structures with polar epithelial cells surrounding a central lumen in a tubular structure rather than a terminal alveolar structure as observed with PRL^{143,144}. Although our results showed that Pg does not play any complementary role in PRL-induced epithelial polarity and lumen formation in mammary acini, further research is needed to understand Pg's effects on morphogenesis and mammary epithelia.

Although our study has yet to reveal the full mechanism that regulates the PRL-induced epithelial polarity and lumenogenesis, we did uncover that JAK2 is essential for proper PRLR localization. Our observations have led us to suspect that loss of JAK2 leads to an inability for the PRLR to be released from the Golgi apparatus due to its localization above the nucleus,

although this has yet to be confirmed. Our finding, although preliminary, is in line with previous reports of the role Janus Kinases play with receptor chaperoning¹⁴⁴. Further research into the mechanisms regulating this and other receptor localizations is needed, as loss of membrane receptors is a major step in tumour progression. Understanding the early events that regulate localization of receptors to the membrane may lead to effective diagnostic markers that could help us treat or predict the state and future progression of tumours before receptors are lost. This would be highly useful because current diagnostic tools only tell us what receptors are present or absent, which is often very limiting on a physician's options for treatments or plans of actions. Finally, our finding of PRL as a polarity signal and inducer of epithelial polarity and lumen formation, along with our labs previous reports of its role in EMT suppression, highlight the potential for PRL or its signaling pathway as a treatment for breast cancer. Although PRL is not likely to kill or eliminate tumours, which is the classic goal of cancer treatments, it may act to keep tumours in a low grade, stable state. PRL therefore could be used in combination with other treatments, preventing a tumour from metastasizing while a second or combination of treatments eliminates it, or used to keep tumours at a low grade and manageable state. The latter suggestion being an increasingly intriguing and academically-accepted idea for treatment; where the goal of the treatment becomes making cancer a chronic disease rather than completely defeating it. Our understanding of the effects differentiation and polarity signals have on epithelial and cancer cells is essential to clarifying the debate about their roles in cancer. Our findings and future direction aim to clarify the role of PRL, a differentiation and now polarity signal, in mammary gland development and tumour progression.

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