Prolactin-Jak2 Signaling in Mammary Epithelial Cellular Morphogenesis, Stemness and Genomic Integrity

Fengming Liu

Division of Experimental Medicine

Department of Medicine

McGill University

Montreal QC Canada

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Abstract

During the pregnancy/lactation cycle, the mammary epithelial cells undergo complex morphological and phenotypic transitions resulting in the acquisition of apical/basal (A/B) polarization and cellular maturation necessary for proper lactation. The hormonal regulations and cellular mechanisms controlling these events are not entirely elucidated to date. Here we show that prolactin(PRL)/Jak2 pathway in mammary epithelial cells uniquely signals to establish apical/basal 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. The functionality of terminally differentiated mammary epithelial cells is further demonstrated by the apical trafficking of lipid droplets and accumulation of β-casein in the lumen. Our results indicate that PRL/Jak2 signaling regulates mammary stem cell hierarchy by inducing the differentiation of luminal progenitor ($EpCAM^{hi}/CD49f^{hi}$) cells to mature luminal ($EpCAM^{hi}/CD49f^{low}$) cells. Moreover, our data indicate that PRL/Jak2 coordinates both of these cellular events through limiting the mitogen activated protein kinase (Erk1/2) pathway. Moreover, our data also revealed that PRL/Jak2 signaling is essential in regulation of centrosome duplication and maintenance of genomic stability. Indeed, loss of Jak2 expression in mammary epithelial cells resulted in centrosome amplification and accumulation of nuclear DNA damage. This abnormal phenotype is similar to that observed in aggressive human breast cancer cells. Together our findings define a novel PRL-Jak2 dependent mechanism coupling

mammary epithelial cell apical/basal polarization and terminal differentiation. In addition, these findings are in support of the potential tumor suppressive role of PRL in breast cancer and provide molecular insights into the protective effect of breastfeeding against breast cancer.

Résumé

Durant le cycle de la grossesse et de l'allaitement, les cellules épithéliales mammaires subissent des transitions morphologiques et phénotypiques complexes, entraînant l'acquisition d'une polarisation apicale/basale (A/B) ainsi qu'une maturation cellulaire nécessaire à un allaitement adéquat. À ce jour, la régulation hormonale et les mécanismes cellulaires qui contrôlent ces événements ne sont pas entièrement élucidés. Dans cette étude, nous démontrons que dans les cellules épithéliales mammaires, la voie de signalisation de la prolactine (PRL)/Jak2 est uniquement responsable de l'établissement de la polarité apicale/basale; tel que déterminée par la localisation apicale de la protéine zona occludens 1 (ZO-1), une protéine des jonctions serrées, ainsi que la localisation basale/latérale de la E-cadhérine. La fonctionnalité des cellules épithéliales mammaires, une fois différenciées, est également illustrée par le trafic de gouttelettes lipidiques vers la région apicale des cellules ainsi que par l'accumulation de β-caséine dans le lumen. Nos résultats indiquent que la voie PRL/Jak2 régule la hiérarchie des cellules souches mammaires en induisant la différenciation des cellules progénitrices luminales (EpCAMhi/CD49fhi) en cellules luminales matures (EpCAMhi/CD49flow). De plus, nos données indiquent que PRL/Jak2 coordonne ces deux événements cellulaires en limitant la voie de la protéine kinase activée par un mitogène (Erk1/2). Nos données révèlent également que la signalisation PRL/Jak2 est essentielle dans la régulation de la duplication des centrosomes et dans le maintien de la stabilité génomique. En effet, la perte de

l'expression de Jak2 dans les cellules épithéliales mammaires entraîne une amplification de centrosomes et une accumulation de dommages à l'ADN nucléaire. Ce phénotype anormal est similaire à celui observé dans les cellules agressives du cancer du sein. L'ensemble de nos résultats définissent un nouveau mécanisme dépendant de PRL/Jak2 couplant la polarisation des cellules épithéliales mammaires A/B et la différenciation terminale. En outre, ces résultats supportent un rôle suppresseur de tumeur de la prolactine dans le cancer du sein et fournissent une évidence moléculaire sur l'effet protecteur de l'allaitement sur le cancer du sein.

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This thesis is also dedicated to my late wife Mrs. Xiaoxia Zhang, I could have let go of my dream a long time ago without her inspiration.

Table of content

Preface

Rationale and Objective

The hormone prolactin (PRL) is indispensable in regulating the development of the mammary gland and promoting the terminal differentiation of the mammary epithelial cells through activation of the Jak2/STAT5 pathway. The detailed molecular mechanisms mediating PRL effects during this complex morphological program is not fully elucidated. Furthermore, the role of PRL in regulating the differentiation of the mammary stem cells is also not fully characterized. Previous work in this lab has demonstrated prolactin to play a suppressive role in breast tumorigenesis via EMT suppression. In addition, recent studies have highlighted that components of PRL/Jak2 signaling pathway are favourable prognostic markers in human breast cancer clinical cases. The objective of this thesis is to detail the mechanisms by which PRL induces mammary epithelial differentiation program, providing further insights into the protective role of PRL in breast cancer. The specific aims of this study include: (1) the role of PRL/Jak2 signaling in mammary epithelial cell morphogenesis, does it contribute to the establishment of apical/basal polarity and determination of cell fate of mammary epithelial cells? (2) the role of PRL/Jak2 signaling in planar cell division and tissue homeostasis of mammary epithelial cells, how the acini are developed in the presence of PRL? (3) the role of PRL/Jak2 signaling in maintenance of genomic integrity, we are to examine how PRL/Jak2 signaling is involved in the regulation of centrosome duplication and maintenance of genomic stability.

Author Contribution

This thesis is written by Fengming Liu and revised by Dr. Suhad Ali, the supervisor. It consists of four major chapters. Chapter 1 is the introduction, where an extensive review of related literature material is presented, chapter 2 includes materials and methods utilized in this study, chapter 3 is the results section and chapter 4 is the conclusion and general discussion. In chapter 3, I performed all the experiments described except (1) Figure 2.1A (left panel): the E-cadherin/ZO1 staining in HC11 cells cultured in 3D (conducted by Ms. Zhenqian Feng); (2)Figure 3.5: the β-casein staining in mouse primary epithelial cells cultured in 3D (conducted by collaborator Ms. Alaa Moamer), (3)Figure 3.18: Loss of Jak2 leads to EMT in HC11 cells (conducted by Ms. Anwar Shams) and (4) Figure 3.22: PTEN overexpression rescued junctional organization in Jak2 KD cells(conducted by Ms. Zhenqian Feng). Based on these results, I wrote the manuscript "PRL/Jak2 directs apical/basal polarization and luminal linage maturation of mammary epithelial cells through regulation of the Erk1/2 pathway" for publication in Stem Cell Research in 2015 (Liu F. Pawliwec A. Feng Z. Yasruel Z. Lebrun JJ. Ali S. Stem Cell Res. 2015 Sep; 15(2):376-83. Liu F. designed and performed experiments, analyzed data and wrote the manuscript, Pawliwec A. and Feng Z. performed experiments, Yasruel Z. provided technical support, Lebrun J-J. reviewed the manuscript and provided input and Ali S. supervised project and revised manuscript). In this publication, we demonstrated for the first time that PRL functions as a polarity cue in mediating junction organization and acini development of mammary epithelial cells. I also showed that PRL-Jak2

singaling promotes the maturation of mammary luminal progenitor cells. These findings contributed significantly to expanding the field of PRL functions and provided new evidence supporting the tumor suppressive role of PRL. A second manuscript entitled “PRL/Jak2 signaling mediates centrosome duplication and maintains genomic stability of mammary epithelial cells” described in the results section is in preparation, in which an essential role of PRL/Jak2 signaling in regulation of centrosome duplication and maintenance of genomic integrity is demonstrated.

Original Scholarship and Contribution to Knowledge

In this thesis, I demonstrated evidence for the first-time distinguishing PRL as a polarity cue in mediating apical/basal polarization and terminal differentiation of mammary epithelial cells. PRL/Jak2 signaling is required for mammary epithelial cells in cell/cell junction organization and proper localization of polarity complexes through inhibition of Erk1/2 activation. Secondly, the role of PRL in adult mammary stem cell is described. PRL promotes the maturation of luminal progenitor cells and depletion of the progenitor cell pool. This pro-differentiation effect implicates a potential tumor suppressive role of PRL in breast carcinogenesis. Thirdly, loss of Jak2 confers mammary epithelial cells basal/mesenchymal phenotypes including up-regulation of cytokeratin 5 and epithelial-mesenchymal transition markers such as Slug, Snail, Vimentin and Zeb1/2. In addition, I demonstrated PRL/Jak2 signaling is essential in regulation of centrosome duplication and maintenance of genomic stability. Loss of Jak2 leads to centrosome amplification and nuclear DNA damage accumulation in mammary epithelial cells. This finding adds to the pool of known regulators of controlled cell division. Jak2 is a newly identified guardian of genomic integrity in assuring proper centrosome duplication during cell proliferation.

Together these newly described roles of PRL/Jak2 increase our understanding and expand the spectrum of PRL functions in the mammary tissue. More importantly, these findings lend support and provide molecular insights into the protective role of breastfeeding against breast cancer, suggesting PRL as a valuable target for

therapeutic strategies against breast cancers. All those elements of the thesis are considered original scholarship and distinct contributions to knowledge.

List of Abbreviations

ALDH, aldehyde dehydrogenase;

BSA, bovine serum albumin;

CD, Cluster of differentiation;

CDC42, cell division control protein 42;

CDK, cyclin-dependent kinase;

CSC, cancer stem cells;

Dlg, Disc-Large;

DMEM, dulbecco's modified Eagle's medium;

DMSO, dimethyl sulfoxide;

ECM, extracellular matrix;

EGF, epidermal growth factor;

Elf5, E74-like factor 5;

EMT, epithelial to mesenchymal transition;

ER, estrogen receptor;

Erk1/2, extracellular signal-regulated kinase 1/2;

FACS, fluorescence-activated cell sorting;

FBS, fetal bovine serum;

Grb2, growth factor receptor binding protein 2;

HDAC, histone deacetylase;

HER2, human epidermal growth factor receptor 2;

IL6, interleukin 6;

MRAS, muscle Ras homolog;

Lgl2, lethal giant larvae 2;

Lgr5, Leucine-rich repeat containing G protein coupled receptor 5;

MAPK, mitogen-activated protein kinase;

MMP, matrix metalloproteinase;

NuMA, nuclear mitotic apparatus;

PAK1, p21-activated serine-threonine kinase 1;

Par3, partition-defective 3;

PI3K, phosphatidylinositol-3 kinase;

PR, progesterone receptor;

PRL, prolactin;

PRLR, prolactin receptor;

PTEN, phosphatase and tensin homolog deleted on chromosome ten;

Rb, retinoblastoma;

RANKL, Receptor activator of nuclear factor κ-b ligand;

SDS, sodium dodecyl sulfate;

STAT, signal transducer and activator of transcription;

TGFβ, transforming growth factor-beta;

TNBC, triple negative breast cancer;

WAP, whey acidic protein;

Wnt, wingless-type mammary tumor virus integration;

Chapter 1: Introduction

1. Mammary Gland Development and PRL Signaling

The extensive epithelial morphogenesis events occur dynamically at the mammary gland during the female reproductive cycle make it an ideal system for research in developmental mechanisms, gene regulation, tissue organization, hormonal action, and stem cell biology. Moreover, the mouse mammary gland is widely used as a model system for breast cancer research, adding greatly to our understanding of the molecular mechanisms for breast cancer development and progression. Experimental approaches utilized to study mammary gland development including genetically modified mouse models, transplantation experiments as well as *in vitro* recapitulation of mammary epithelial cell organization such as the 3D acinus formation assay. Here, I will provide a description of mammary gland morphological changes during different stages of development, followed by hormonal interplay controlling these events. The hormones involved in postnatal mammary gland development, primarily estrogen, progesterone and PRL, are further discussed in the coming sections.

1.1 Mammary gland development at a glance.

After embryonic development, postnatal development of the mammary gland includes stages of ductal morphogenesis where the rudimentary ductal tree at birth elongates and branches to fill the mammary fat pad. During pregnancy/lactation cycle the process of alveologenesis commences where cells at the terminal end bud proliferate and differentiate into milk-secreting unit. During lactation, terminal differentiation of

the mammary epithelial cells and milk secretion occur. Finally, involution post lactation where terminally differentiated mammary epithelial cells are eliminated by apoptosis and restoration of the mammary gland to pre-pregnant stage. These processes are regulated by interplay of various hormones and local growth factors (Figure 1.1).

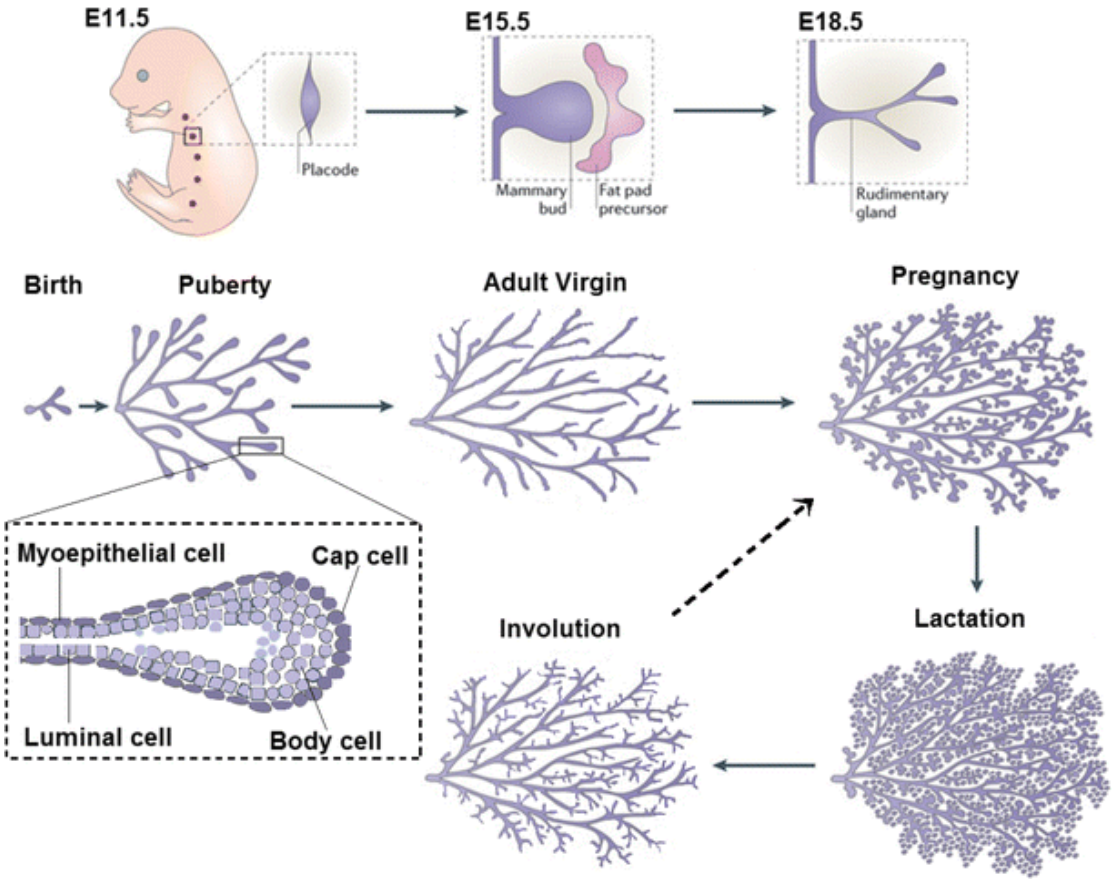

Figure 1.1 Distinct stages of mammary gland development. During embryogenesis, the ectoderm forms mammary line and grows into placodes which descend into the underlying mesenchyme and generate newborn rudimentary ductal structure. Pubertal growth, pregnancy, lactation and involution occur postnatally under the regulation of multiple hormones. Branching morphogenesis starts at puberty to create the ductal tree that fills the fat pad. Upon pregnancy, the combined actions of progesterone and PRL generate alveoli, which secrete milk during lactation. Lack of demand for milk at weaning initiates the process of involution and the gland is remodeled back to its pre-pregnancy state. A schematic structure of terminal end bud(TEB) is presented in the frame. Image adapted from S. McNally et.al. Methods in Mol. Bio. 1501 (2017).

1.1.1 Embryonic development of the mammary gland

Mammary glands are epidermal appendages evolved over 300 million years ago, most likely from apocrine sweat glands(Oftedal 2002). Early at E10 to E11 in the mouse, at the ventral-lateral boundary of the embryonic trunk, surface ectodermal cells coalesce to form primordia consisting of five pairs of placodes appearing along two milk lines(Daniel and Smith 1999, Parmar and Cunha 2004).These placodes increase in size and develop into mammary buds at E15 and then come to a temporary suspension in development(Sakakura, Kusano et al. 1987). Mesenchymal expression of androgen receptor in male embryos leads to irreversible condensation of the mesenchyme around the mammary bud and degeneration of the mammary bud epithelium during E13.5 to E15.5 (Kratochwil 1971). The mammary bud elongates and invades the fat pad precursor by E18.5. The other end of the mammary bud develops into the open lumen under the skin surface as future nipple. Slight branching of the mammary bud occurs in the fat pad, presenting the small gland at birth (Veltmaat, Mailleux et al. 2003). In human, the mammary bud develops in a similar pattern, except (1) no degeneration of mammary bud epithelium in male embryo and (2) ductal network merges at the nipple at birth whereas there is one single duct opening at the nipple in the mouse(Howard and Gusterson 2000). Mammary ducts are made up of epithelial cells surrounding a central lumen(Richert, Schwertfeger et al. 2000).

1.1.2 Pubertal development of the mammary gland

Between birth and puberty, the mammary gland exists in a relatively quiescent state,

which is composed mainly of stroma of fibroblast and connective tissue with the primary ductal network filling partially the mammary fat pad. Interestingly, these rudimentary ductal system is competent to produce milk under the influence of maternal hormones exposure in human infants.

Puberty initiates branching morphogenesis to create a ductal tree that fills the fat pad through proliferation of the terminal end buds(TEBs) of the growing ducts. The terminal end bud is described as bulbous, highly proliferative, hormone dependent structures penetrating the fat pad, which is driven by the proliferation of a single layer of cap cells at the tip. In human, TEBs share structural similarities with rodent(Howard and Gusterson 2000, Javed and Lteif 2013). Cap cells of the TEB differentiate into myoepithelial cells, which form the outer layer of the tubular duct that encircles inner luminal cells(Williams and Daniel 1983). The outlayer of myoepithelial cells can be distinguished by smooth muscle actin staining in addition to cytokeratin 5 and 14 in immunohistochemistry(Radice, Ferreira-Cornwell et al. 1997), whereas the inner epithelial luminal cells are distinguished by expression of cytokeratin 8, 11 and 22(Asch and Asch 1985). Besides ductal elongation, side branching occurs through lateral sprouting from the primary ducts, creating a tree like pattern of ducts occupying up to 60% of the available fatty stroma(Macias and Hinck 2012). Of note, the human architectural structure of the pubertal ductal tree differs from the mouse. In human, the lateral branches lead to terminal ducts that give rise to terminal ductal–lobular units comprising numerous blind-ended ducts, called acini(Howard and Gusterson 2000). These acini are embedded in fibroblastic,

intralobular stroma that is far more pronounced in the human breast than in the adipocyte-rich stroma surrounding the branches of the rodent mammary tree(Russo, Gusterson et al. 1990).

1.1.3 Mammary gland development during pregnancy and lactation

In the adult mammary gland, under the influence of ovarian hormones during each estrous cycle, the mammary epithelial cells undergo mild proliferation and differentiation that includes limited expression of milk proteins, followed by involution(Andres and Strange 1999). Upon pregnancy, extensive tissue remodeling occurs, the ductal tree undergoes tremendous secondary and tertiary ductal branching, providing ductal arbors for alveolar development under the combined actions of progesterone and PRL. Luminal epithelial cells proliferate to generate alveolar buds that cleave and differentiate into alveoli, which produce milk during lactation. The interstitial adipose tissue disappears and give room to proliferating epithelial cells till the alveoli encompass majority of the fat pad (McNally and Stein 2017). During lactation, infants suckling stimulates the release of pituitary PRL, activating Jak2 and STAT5 signaling for milk production. Release of oxytocin induces contraction of the myoepithelial cells around alveolus, promoting the outflow of milk into the ducts(McNally and Stein 2017).

1.1.4 The involuting (post-lactational) mammary gland

Lack of demand for milk at weaning initiates the process of involution whereby the gland is remodeled back to its pre-pregnancy state(Macias and Hinck

2012).Reduction in PRL levels in response to weaning leads to cessation of milk secretion and removal of unwanted epithelial cells in a controlled manner. A molecular process mediated by Stat3 and Akt signaling(Schwertfeger, Richert et al. 2001, Abell, Bilancio et al. 2005). Early involution is initiated by lysosome mediated programmed cell death of alveolar secretory epithelial cells, the alveoli collapse into epithelial clusters. Second phase of involution includes degradation of basement membrane and extracellular matrix proteins. After involution, the mammary gland resembles the virgin stage gland before pregnancy.

In addition to involution after weaning, the age-related lobular involution occurs within the human mammary gland with gradual loss of breast epithelial tissue, in which the terminal ductal lobular units (TDLUs) regress as a woman ages independent of prior lactational events. A completed age-related lobular involution reduces the mammary gland size and the complexity of both the ductal network and the terminal ductal lobular units, thereby reducing the incidence of breast cancer (Hutson, Cowen et al. 1985). Indeed, postmenopausal women with delayed lobular involution have a higher risk of developing breast cancer comparing to menopausal women with lobular involution(Milanese, Hartmann et al. 2006, Radisky, Visscher et al. 2016).

1.2 Hormones involved in mammary gland development.

Multiple hormones are involved in mammary gland development at different stages. I will discuss briefly the effects of growth hormone (GH), insulin-like growth factor 1

(IGF1), estrogen, progesterone and focus on PRL in the following sections.

1.2.1 Growth hormone and IGF1

Growth hormone is secreted from the pituitary gland and serves as a global regulator of mammary gland development, although recent evidence suggests its effects on mammary gland development is mediated largely through IGF1. The growth hormone receptor knock out mice, concomitant with a 90% decrease in serum IGF1 level, displayed delayed mammary gland development with eventual outgrowth of sparse ductal tree (Zhou, Xu et al. 1997). Later it was found that growth hormone signaling is not required in the mammary epithelium, instead, growth hormone signaling stimulates the IGF1 production in the stromal fibroblast cells (Ruan and Kleinberg 1999), which signals to the mammary epithelium for proliferation (Gallego, Binart et al. 2001). The *Igf1*$^{-/-}$ mice exhibit diminished ductal development like the growth hormone receptor knock out mice albeit normal growth hormone production, and the defects in mammary gland development can be rescued by IGF1 treatment(Gallego, Binart et al. 2001). On the contrary, overabundance of IGF1 drives increased mammary epithelial proliferation and enhanced mammary gland development(Cannata, Lann et al. 2010), and is associated with an increased risk of malignancy(Hankinson, Willett et al. 1998).

1.2.2 Ovarian hormone estrogen

Estrogen is responsible for the pubertal mammary gland development featuring surge of tubular-genesis via TEB formation and ductal branching. Initially, it was not clear

whether estrogen has direct effect on mammary gland development, given the broad expression of estrogen receptor in both epithelial and stromal compartments of the mammary gland or it functions indirectly through stimulating release of pituitary hormones like PRL(Lieberman, Maurer et al. 1978). Local delivery of estrogen directly to the mammary gland by the Elvax 40P implantation showed direct stimulatory effect on mammary ductal outgrowth(Silberstein and Daniel 1982), which was blocked by local administration of estrogen receptor antagonist, tamoxifen(Daniel, Silberstein et al. 1987, Silberstein, Van Horn et al. 1994).

1.2.3 Ovarian hormone progesterone

Progesterone is a soluble ovarian hormone responsible for the extensive side-branching and alveologenesis during pregnancy. In combination with PRL, progesterone promotes the differentiation and maturation of alveoli for milk production. Progesterone receptor knock out mice have simple epithelial tree, and there is no ductal proliferation and lobuloalveolar differentiation upon pregnancy (Lydon, DeMayo et al. 1995). Further studies indicated a paracrine role of the epithelial progesterone receptor in mammary gland development(Brisken, Park et al. 1998). RANKL (receptor activator of nuclear factor κ-B ligand) was recently identified as the key mediator of progesterone induced proliferation and alveologenesis. Like the progesterone receptor knock out mice, mice lacking RANKL fail to undergo alveologenesis during pregnancy(Fata, Kong et al. 2000). Progesterone induces RANKL expression for the pro-growth response of the mammary epithelial

cells(Fernandez-Valdivia, Mukherjee et al. 2008). In fact, RANKL inhibitors are currently being considered for breast cancer treatment(Gonzalez-Suarez, Jacob et al. 2010, Schramek, Leibbrandt et al. 2010). Clinically, removal of the ovaries, thereby eliminating mammary epithelium exposure to both estrogen and progesterone, reduces breast cancer risk by more than 50% in human (Hilton and Clarke 2015). In addition, RANKL is also a target of PRL, transgenic expression of RANKL in virgin mammary gland elicits characteristics of pregnancy including side-branching of ductal tree and alveolar budding (Fernandez-Valdivia, Mukherjee et al. 2009).

1.3 Role of PRL in mammary gland development

Numerous studies have demonstrated that PRL is the major driver of adult mammary gland development during pregnancy(Kelly, Bachelot et al. 2002). PRL collaborates with progesterone in generation of lactation-competent mammary gland through directly functioning on mammary epithelial cells and indirectly via modulation of the systemic hormone environment like inhibiting ovarian progesterone production(Brisken, Kaur et al. 1999, Binart, Helloco et al. 2000). In PRL and PRL receptor (PrlR) knock out mice, embryonic and postnatal mammary gland development appear to be normal, whereas side-branching and alveolar budding were absent, TEB like structures persisted at the ductal termini well into maturity (Horseman, Zhao et al. 1997, Ormandy, Camus et al. 1997). However, when *PrlR*$^{-/-}$ mammary epithelium was grafted into precleared wild type fat pads, these *PrlR*$^{-/-}$ mammary epithelium developed into normal mammary gland, suggesting PRL/PrlR

signaling is required in tissues other than the mammary epithelium for normal development, and endocrine PRL functions in paracrine manner to direct the development of PRL null mammary epithelium (Brisken, Kaur et al. 1999, Naylor, Lockefeer et al. 2003). In ovariectomized *Prl*$^{-/-}$ mice, exogenous supplementation of progesterone restored ductal side branching and partially rescued the infertility of *Prl*$^{-/-}$ mice, suggesting progesterone and PRL work together during lobuloalveolar outgrowth(Kelly, Bachelot et al. 2002). PRL receptor heterozygous animals showed normal ductal and alveolar development up to mid-pregnancy, but alveolar development stalled during late pregnancy preventing successful lactation, demonstrating an essential epithelial intrinsic role for PRL signaling in lobular alveolar development and maturation for milk production(Oakes, Rogers et al. 2008).

Downstream of PRL/PRL receptor signaling is the Jak2/STAT5 signaling pathway. Similarly, conditional Jak2 knock out mice and STAT5 knock out mice demonstrate failure of alveolar development(Han, Watling et al. 1997, Cui, Riedlinger et al. 2004, Wagner, Krempler et al. 2004). Alveolar development in the Jak2 null transplant was disrupted, whereas tubular genesis was not affected, as the Na-K-Cl co-transporter, a ductal marker, was still maintained but not the sodium phosphate co-transporter type IIb, a secretory cell marker(Shillingford, Miyoshi et al. 2002).

The laminin rich extracellular matrix surrounding the mammary alveoli is also involved in differentiation of mammary epithelial cells elicited by PRL. Laminin binds to its membrane receptor β1 integrin, and β1 integrin mediated cell adhesion plays a permissive role in PRL signaling through targeting STAT5(Streuli, Edwards et

al. 1995). Loss of β1 integrin in vivo results in impaired alveologenesis and lactation, and cultured β1 integrin null mammary epithelial cells no longer respond to PRL stimulation due to defective STAT5 signaling(Naylor, Li et al. 2005). β1 integrin may maintain STAT5 activation through activation of Rho GTPase Rac1. Dominant negative Rac1 inhibits PRL signaling in normal mammary epithelial cells and activation of Rac1restored β-casein production in β1 integrin null mammary epithelial cells (Akhtar and Streuli 2006). STAT5 regulates expression of target genes like β-casein and whey acidic protein (WAP) via binding to consensus DNA binding sites within their promoter region. In Jak2 conditional knock mouse model, disrupted nuclear STAT5 accumulation abolished expression of milk proteins. In addition to β1 integrin, the receptor tyrosine kinase ErbB4 acts to maintain STAT5 activation in mammary epithelium(Long, Wagner et al. 2003).

PRL signaling drives the specification of alveolar cell fate during pregnancy via Elf5, the epithelial-specific Ets transcription factor and GATA3, a zinc-finger transcription factor (Asselin-Labat, Sutherland et al. 2007), a determinant of luminal mammary cell fate(Kouros-Mehr, Slorach et al. 2006). Elf5 drive the differentiation of $CD61^{+}$ luminal progenitor cells(Oakes, Naylor et al. 2008). Other than these hormones, growth factors and transcription factors described previously, microRNAs such as miR-137 was also involved in the regulation of mammary gland development. Over-expression of miR-137 inhibited placode invagination during embryonic mammary gland development(Lee, Cho et al. 2015).

In summary, PRL initiates a genomic transcriptional network in controlling gene

expression within the mammary epithelial cells, which controls both epithelial cell proliferation and differentiation necessary for proper function of mammary gland during pregnancy and lactation.

2. PRL Hormone and Its Signal Transduction Pathways

2.1 Prolactin

PRL was initially discovered as a protein hormone of the anterior pituitary gland functions to promote lactation and thereby named after this role more than 90 years ago. More than 300 separate biological activities varying from mediating immune responses, osmotic balance to angiogenesis and regulation of citrate production in prostate have been ascribed to this hormone(Costello and Franklin 1994, Bole-Feysot, Goffin et al. 1998, Chilton and Hewetson 2005). Recent studies have expanded its functions to central nervous system regeneration and pathogenesis of the cardiovascular system(Ignacak, Kasztelnik et al. 2012). However, a thorough description of its roles is yet at large to date as new roles are continuing to be characterized, such as a recent studies just expanded PRL function to regulation of MicroRNAs(Yan, Zhao et al. 2016) and promotion of immune cell migration into mammary gland during lactation(Dill and Walker 2017).

PRL is a single chain hormone produced in both endocrine and autocrine/paracrine systems thereby functioning as either a circulating hormone or as a cytokine in a variety of physiological events in vertebrates from fish to mammals. In mammals, it is mainly secreted by lactotroph cells of the anterior pituitary gland.

Autocrine/paracrine sources of PRL include brain, prostate, immune system, the female reproductive organs and the mammary gland(Freeman, Kanyicska et al. 2000). In human, PRL is first synthesized as a proto-peptide. After cleavage of the signal peptide, a mature 23kDa protein consisting of 199 amino acids with three pairs of intra-molecular disulfide bonds is released to the blood circulation (endocrine PRL) or the cellular niche (autocrine PRL)(Sinha 1995). Structurally, PRL resembles growth hormone and placental lactogen, which evolved from a common ancestor gene by duplication. All these three hormones belong to the large haematopoietic cytokine family/group I of the helix bundle protein family(Cooke and Liebhaber 1995), sharing a common tertiary structure(Goffin and Kelly 1997, Forsyth and Wallis 2002). These hormones exert their biological effects through binding to their target receptors with a single membrane transmembrane domain and a non-intrinsic tyrosine kinase activity. However, these ligands can substitute one for another in receptor binding and induce subsequent signal transduction. As consequence of ligand promiscuity, concomitant or overlapping biological effects are elicited after ligand engagement, concealing the individual effect of these hormones. Target tissues of PRL include the mammary gland, prostate, ovary, immune cells, adipocytes and liver(Freeman, Kanyicska et al. 2000).

PRL variants result from alternative splicing of the primary transcript, proteolytic cleavage, and other post-translational modifications including phosphorylation, N-linked glycosylation(Sinha 1995), sulphation and deamindation(Sun, Lou et al. 1996). The 23kDa PRL is proteolytically cleaved into 14 kDa, 16 kDa and 22 kDa

isoforms(Bernard, Young et al. 2015).Importantly, phosphorylated PRL has much lower biological activities than non-phosphorylated form, and may act as an antagonist to PRL signaling pathway(Wang and Walker 1993, Coss, Kuo et al. 1999). One extensively studied mono-phosphorylated PRL mimic is the S179D human PRL(Walker 2007), which inhibits secretory activation and attenuates PRL induced gene transcription(Naylor, Oakes et al. 2005), albeit it was initially reported as an agonist, not an antagonist(Bernichtein, Kinet et al. 2001). Opposing effect of the N-terminal fragmented 16KDa PRL in angiogenesis was reported. The regular 23KDa PRL promotes angiogenesis(Yang and Friedl 2015), whereas the short form inhibits angiogenesis, impairing tumour vascularization and growth in tumorigenesis(Struman, Bentzien et al. 1999). In addition, the 16KDa PRL triggers apoptosis in endothelial cells(Tabruyn, Sorlet et al. 2003). These functions rendered the fragmented PRL as a potential therapeutic agent in cancer. Additionally, PRL may undergo dimerization, polymerization or association with immunoglobins, which reduced its biological activity(Sinha 1995).

A notable difference between human and rodent PRL genes is that human and other primates contain an alternative promoter, 5.8kbp upstream of their pituitary transcription start site, driving the expression of PRL outside the pituitary gland(Gerlo, Davis et al. 2006). However, rodent PRL gene does not have the alternative promoter(Berwaer, Martial et al. 1994). Accordingly, extra-pituitary PRL expression in human mammary epithelium is detectable in both non-pregnant and pregnant states, whereas in rodent, PRL expression in the mammary epithelium is only detected after

pregnancy and during lactation(Fields, Kulig et al. 1993, Kurtz, Bristol et al. 1993, Steinmetz, Grant et al. 1993). Expression of human PRL in mouse PRL knockout mice was achieved through first introduction of bacterial artificial chromosome harboring the entire human PRL gene into mice by oocytes microinjection and subsequent crossing with PRL knockout mice, conferring additional human PRL expression in several extra-pituitary sites including spleen, thymus, kidney, and reproductive tissues of both males and females, including mammary gland, uterus, ovary, prostate, and testis(Christensen, Murawsky et al. 2013), which restored the defective embryonic mouse mammary gland development of PRL knock out mice and generated fertile progenies(Horseman, Zhao et al. 1997).

Interestingly, non-receptor binding PRL can be detected in the milk itself(Grosvenor, Picciano et al. 1993). Radio-labelling and tracing of circulating PRL showed part of milk PRL originated from circulation(Grosvenor and Whitworth 1976, Mulloy and Malven 1979). Two potential mechanisms account for the internalization of circulating PRL after reaching the basal membrane of the mammary epithelial cell: (1) PRL binds to its receptor, ligand engaged receptor is internalized to endosomes, where the acidic environment facilitates ligand/receptor dissociation. It is not yet documented whether maternal PRL is released from the early endosome or late endosome, neither dissociated PRLR returns to membrane via recycling endosome or remains in later endosome/lysosome for degradation, and thirdly, internalized PRL and PRLR might be sorted to the trans-Golgi network by retrograde transport. Maternal PRL is re-packaged into these intraluminal vesicles and released to milk

during lactation. This could serve as a regulatory mechanism for PRL signaling input; and (2) PRL crosses the basal membrane of mammary epithelial cell through endocytosis. It was not clear whether a specific PRL binding protein at the basal membrane mediated this process. Exocytosis of PRL through the apical membrane of mammary epithelial cell into the alveolar lumen is not elucidated, and the physiological relevance of this finding is not yet fully appreciated. In rat, the milk PRL potentially went directly into the circulation system of the neonate(Grosvenor and Whitworth 1983).

Model systems utilized to study PRL function evolved from pigeon crop-sac to human mammary epithelium and other tissues. Conflicting conclusions have been reached from different model systems utilized. It is now appreciated that PRL exerts diverse biological effects in a context dependent and tissue specific manner. For example, the Nb2 rat lymphoma cells depend on PRL for proliferation(Campbell, Argetsinger et al. 1994, Rui, Lebrun et al. 1994), however, it is not applicable to generalize PRL as a mitogen for other types of cells. PRL maintains hair follicle stem cells in quiescence(Goldstein, Fletcher et al. 2014), which is in consistency with the previously observed hair growth cycle delay in PRLR knockout mice(Craven, Ormandy et al. 2001).On the other hand, PRL promotes the differentiation of mammary epithelial cells, as my work will demonstrate in this thesis (see chapter 3, results).

2.2 The PRL receptor (PRLR)

2.2.1 PRLR gene expression

PRLR belongs to the class I cytokine receptor superfamily, featuring an extracellular ligand binding domain as the signal receiver, a single transmembrane domain and a signal transducing non-catalytic intracellular domain. Class I cytokine receptors fall into three major families, IL-2R, IL-3R and IL-6R, as determined by usage of shared receptor chains(Leonard and Lin 2000). In the extracellular ligand binding domain, two pairs of disulfide-bridged cysteines are crucial for ligand binding and the WS motif (W-S-X-W-S) undergoes conformational changes upon PRL engagement which is important for receptor activation(Dagil, Knudsen et al. 2012). The intracellular domain is relatively conserved among species, with a conserved proline-rich Box 1 domain necessary for docking and activation of Jak2 kinase, and a Box 2 domain features 11 amino acids in the succession of hydrophobic, negatively charged and then positively charged residues and it is less conserved among species(Bernard, Young et al. 2015). Additionally, there are three lipid-interacting domain (LID) in the intracellular portion of PRLR, which might confer specificity of signal transduction(Haxholm, Nikolajsen et al. 2015). There is currently not many known genetic diseases linked to PRLR in humans. Bogorad et al. reported in 2008 a heterozygous single nucleotide polymorphism in exon 6 of the PRLR gene, encoding Ile146Leu substitution in its extracellular domain from multiple fibroadenomas (MFA) patients. This single amino acid substitution confers constitutive activation of PRLR(Bogorad, Courtillot et al. 2008). Other than this gain-of-function mutation,

later in 2014, a His188Arg PRLR loss-of-function mutation is reported to cause familial hyperprolactinaemia(Newey, Gorvin et al. 2014). PRLR shares significant structural homology to other members of this family including the receptors for growth hormone, erythropoietin, leptin, and interleukins(Bazan 1990). The PRLR primary transcript originates from one single gene and undergoes alternative splicing and translates into multiple isoforms(Kelly, Djiane et al. 1991, Brooks 2012). In human short, intermediate and long form PRLRs exist with varying cytoplasmic domains(Bole-Feysot, Goffin et al. 1998). In mice, one long form and three short forms, slightly varying in their C-terminal part of the cytoplasmic tail, have been identified with identical extracellular ligand binding domain(Davis and Linzer 1989). Regulation mechanisms controlling PRLR isoform expression in human and mice are different from each other(Shao, Nutu et al. 2008). However both forms were needed for reproduction(Devi and Halperin 2014) as corpus luteum needs both isoforms for normal function(Stocco 2012).In addition to membrane bound PRLR, soluble PRLR isoforms were identified potentially originating from either alternative splicing of the primary mRNA or proteolytic cleavage of membrane bound PRLR(Bernard, Young et al. 2015). It is not clear how the signaling mechanisms differ from each other when different isoforms were utilized. It is believed other than activating signaling cascades like MAPK and Src, the short PRLR blocks regular PRL signaling through dominant negative ligand competition and its inability for intracellular signal transduction to the Jak/STAT cascade(Berlanga, Garcia-Ruiz et al. 1997). These non-redundant isoforms might be involved in fine-tuning the PRL signaling output.

For example, low ratio of short/long PRLR isoform is observed in some breast tumor patients and some but not all breast cancer cell lines, although it is not clear how the reduced short isoform expression contributes to mammary tumor development/progression(Meng, Tsai-Morris et al. 2004). Also, multiple isoforms of PRLR account for the diversity of PRL functions in different tissues and organs, mediating alternative signaling cascades unique to each individual target (Figure 1.2).

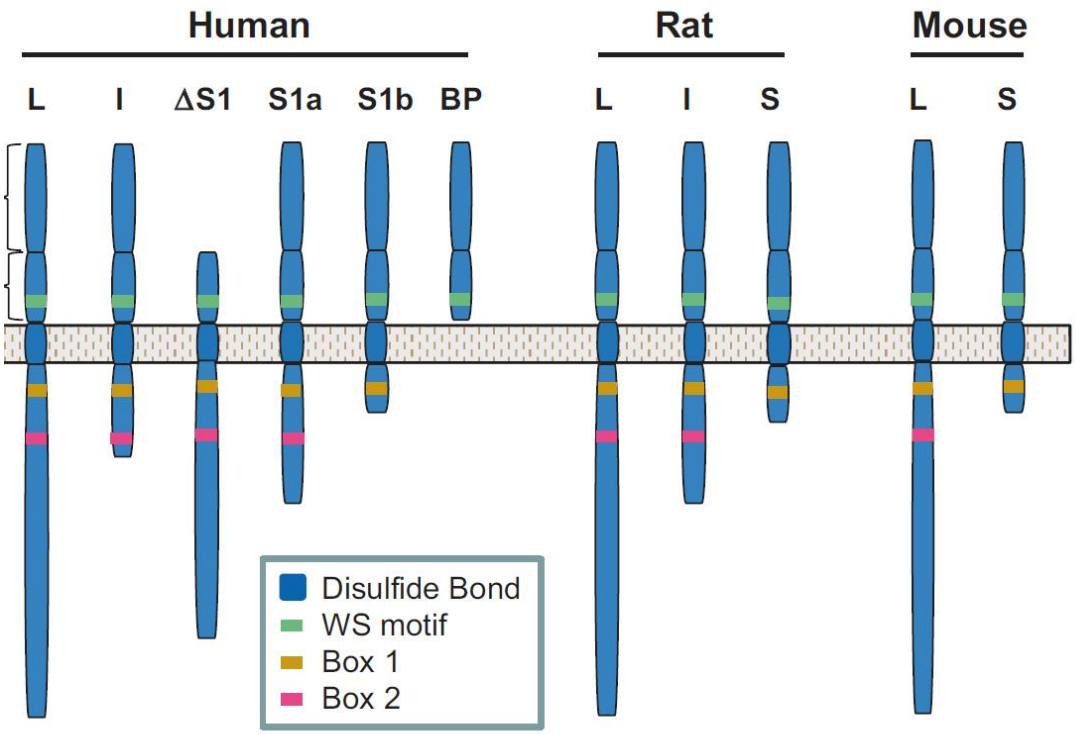

Figure 1.2 PRLR isoforms. The extracellular domain contains two pairs of disulfide-bridged cysteines which are crucial for ligand binding. The WS motif (W-S-X-W-S) undergoes conformational changes upon ligand engagement, which is important for receptor activation. Cytoplasmic domains are relatively conservative among species, with a conserved Proline-rich Box 1 domain necessary for docking and activation of Jak2 kinase. Box 2 domain features 11 amino acids in the succession of hydrophobic, negatively charged and then positively charged residues and it is less conservative among species. I: intermediate form. Image adapted from Goffin et.al LeRoith D Advances in Mol Cell Endocrinol. 2: 1-33 (1998)

Results from in situ hybridization and immunohistochemistry pointed to a broad spectrum of PRLR expression. Besides primarily expressed in mammary tissues, PRLR was also identified in non-mammary tissues including kidney, liver(Davis and Linzer 1989), prostate(Nevalainen, Valve et al. 1997), lung and pancreas(Freemark 2001). Intra-cellularly, PRLR was reported to localize to cell membrane, cytosol, however, nuclear localization of PRLR is not clear.

2.2.2 PRL/PRLR binding

There are two binding sites of PRL on its receptor(Goffin, Struman et al. 1994, Kinet, Goffin et al. 1996). Binding of a single PRL molecule to the predimerized PRLR induces conformational changes of the receptor, bringing the intracellular domains of the receptors together with its constitutively associated tyrosine kinase Jak2(Lebrun, Ali et al. 1994) into close proximity for auto-phosphorylation or trans-phosphorylation(Finidori and Kelly 1995). The subsequent phosphorylation/activation event is strictly contingent upon homo-dimerization of PRLR and its associated Jak2 kinase(Chang, Ye et al. 1998). Of note, human PRLR is less sensitive to non-human PRL(Utama, LeBaron et al. 2006, Utama, Tran et al. 2009), this need to take into consideration when interpreting data from xeno-transplant modeling of human breast cancer cells in mice, as these xenografts proliferate in a relatively PRL free environment regardless of circulating mouse PRL.

A mutant human PRL G129R(Chen, Ramamoorthy et al. 1999, Chen 2015), which harbors the specific single amino acid substitution, is drawing a lot of attention as

PRL antagonist in seeking treatment of breast, ovary and prostate cancer through blockage of PRL signaling. Indeed, decreased cell migration and survival, as well as cell apoptosis were observed after ectopic expression of PRL antagonists in several cancer cell lines(Rouet, Bogorad et al. 2010, Tan, Chen et al. 2011, Wen, Zand et al. 2014),whereas these achievements were far from reaching clinical trial due to short half life and low affinity plus limited patients eligible for such therapy given the high heterogeneity of these cancers. Also, further study is requested to identify the direct mediator of the observed phenotype after PRL antagonist administration.

2.3 The Jak/STAT pathway

Engagement of PRL with its receptor at the membrane activates varying signaling cascades, which is dependent upon the cellular microenvironment. A key factor, whereas not often investigated, is the stiffness of collagen matrice, laminin enriched compliant collagen matrix promoted STAT5 activation, while collagen I enriched stiff collagen matrix activated the PRL-SRC-FAK-MAPK signaling(Barcus, Keely et al. 2013, Barcus, Keely et al. 2016). The Jak (Janus Kinases)/STAT (Signal Transducer and Activation of Transcription) pathway relays the membrane to cytosol and nucleus signaling. Extracellular messengers like PRL (binding to type I family of cytokine receptor) and interferons (IFNs), which binds to the type II cytokine receptor family, exerted their biological effect through the activation of STAT transcription factors(Hynes, Cella et al. 1997).

2.3.1 The Janus Kinases

Four members of the mammalian Janus Kinase family exist: Jak1, Jak2, Jak3 and

tyrosine kinase 2(Tyk2). All Jaks contain a conserved C terminus kinase domain plus a catalytically inactive, pseudo-kinase domain. There are two tyrosine residues in the kinase domain, which can be phosphorylated after ligand stimulation. The divergent N terminus domain mediates the binding to respective receptors(Shuai and Liu 2003). (Figure 1.2) Jak1 and Tyk2 are ubiquitously expressed kinases mainly involved in interferon signaling(Ihle 1994, Ihle 1994, Ihle, Witthuhn et al. 1994). Jak2 is widely expressed and involved in many signaling by single chain hormone receptors like PRLR(Campbell, Argetsinger et al. 1994), growth hormone receptor(Argetsinger, Campbell et al. 1993) and certain members of the class II receptor cytokine family(Figure 1.3)(O'Sullivan, Liongue et al. 2007). Jak3 expression is restricted to cells of hematopoietic origin and functions mainly in immunity(Zhu, Berry et al. 1998).

Jak2 activation depends on localizing in proximity with its associated receptors near the membrane via its N-terminal Four-point-one/Ezrin/Radixin/Moesin(FERM) domain, the conformational changes elicited by ligand/receptor engagement bring the Jak2 kinase close to each other and trigger auto or trans-phosphorylation of the tyrosine residues in its kinase domain. Using Fluorescence Resonance Energy Transfer (FRET) to monitor the conformational change of receptor associated Jak2 dimers, Waters et al. demonstrated activation of the receptor dimer induced a separation of its Jak2 binding motifs which leads to removal of the pseudo-kinase domain from the kinase domain of the partner Jak2 and pairing of the two kinase domains, facilitating trans-activation of Jak2(Brooks, Dai et al. 2014). Other receptor

tyrosine kinases and members of the Src family kinases can also activate Jak2(Rane and Reddy 2002). Activated Jak2 phosphorylates the tyrosine residues of the receptor, which serves as the docking sites for SH2-domian containing scaffold proteins or signaling molecules like the STATs. Genetically, Jak2 knock out in mice is embryonically lethal at day 12.5 due to failure of erythropoiesis(Parganas, Wang et al. 1998). A tissue specific Jak2 conditional knock out mice was established to evaluate its function in mammary gland development, which was elaborated in the previous sections(Wagner, Krempler et al. 2004).

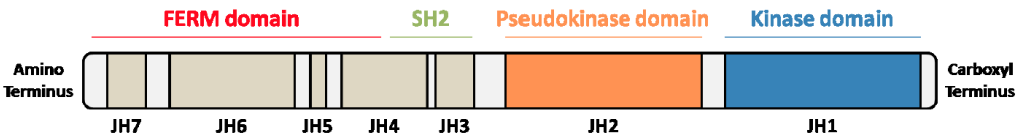

Figure 1.3 Schematic structure of Jak2 kinase. The tyrosine kinase domain localizes at C-terminus followed by a unique pseudo-kinase domain JH2 domain which is catalytically inactive (JH: Jak Homologue domain). A Src homology 2 (SH2) domain localizes next to the N-terminus FERM domain (4.1, ezrin, radixin and moesin domain which is responsible for the PIP2 regulated binding of ERM (Ezrin/Radixi/Moesin) proteins to the membrane. Image adapted from Yamaoka et al. Genome Biol. 5(12):253 (2004).

2.3.2 The STATs

STATs are a family of latent cytoplasmic transcription factors respond quickly to cytokine stimulation. One of its member STAT5 was once called the mammary gland factor (MGF) named after its functions during lactation(Tourkine, Schindler et al. 1995). They are one of the well described Jak kinases substrates in signal transduction associated with cytokine receptors(Pellegrini and Dusanter-Fourt 1997). In mammals, seven members of this family have been identified (STAT1, STAT2, STAT3, STAT4,STAT5a,STAT5b and STAT6), all expressed ubiquitously except STAT4, which is expressed mainly in the thymus and the testes(Duncan, Zhong et al. 1997). Structurally, all STATs contain (1) a relatively conserved N terminal domain responsible for major protein/protein interactions like homo-dimerization or interacting with other nuclear transcriptional co-activators or regulatory proteins like the protein inhibitor of activated STAT (PIAS)(Shuai 2000), which interact with nuclear STATs, inhibiting the transcriptional activity of STATs (Shuai and Liu 2003), (2) in the middle a DNA binding domain resembling DNA binding domains of NF-κB and p53(Chen, Vinkemeier et al. 1998), and (3) the SH2/tyrosine activation domain for recognition of highly specific phosphorylated tyrosine residues of cytokine receptors and subsequent recruitment to this site, whereas the (4) divergent C terminal transcriptional activation domain (TAD) confers the specificity of different members(O'Shea, Gadina et al. 2002) (Figure 1.4).

Figure 1.4 schematic structure of STAT: The N-terminus domain is the dimerization domain upon tyrosine phosphorylation. The coiled-coil domain links a DNA binding domain and Src homology 2(SH2) domain to the C-terminus transactivation domain, which is subject to multiple post-translational modifications including sumoylation and phosphorylation on Tyrosine and Serine residues.

Post-translational modifications of STATs include methylation, acetylation, ubiquitylation and sumoylation and most importantly, tyrosine phosphorylation have been documented(Shuai, Schindler et al. 1992). Tyrosine phosphorylation is required for STATs dimerization and subsequent nuclear transportation and DNA binding(Shuai, Stark et al. 1993). Dimerization of STATs is achieved through interactions of the SH2 domain of one monomer with the tyrosine phosphorylated tail of the other monomer(Strehlow and Schindler 1998). In general, STATs form homodimers of the same family member. Heterodimerization of STATs also occurs in the same reciprocal SH2-C terminal phosphor-tyrosine binding manner, adding functional diversity in cytokine signaling(Schindler and Darnell 1995). Tyrosine phosphorylation is also required for the nuclear retention of activated STATs(McBride, Banninger et al. 2002). Besides, serine residues on STATs are targets for phosphorylation by MAPK/ERK1/2 activation, adding another layer of regulation in cross-talking of multiple signaling cascades(Decker and Kovarik 2000, Levy and Darnell 2002). Interestingly, PRL could modulate the serine phosphorylation of STAT5a and STAT5b in COS7 and Nb2 cells independent of MAPK(Yamashita, Xu et al. 1998). However, these observations are not yet reported in the mammary epithelium.

Besides their canonical functions as transcription factors after tyrosine-phosphorylation and translocation into the nucleus, the functions of unphsophorylated STATs (U-STATs) are being increasingly acknowledged recently. U-STAT3 regulates expression of genes distinct from those induced by

tyrosine-phsophorylated STAT3 dimer, including oncoproteins like MRAS (muscle RAS oncogene homolog) and receptor tyrosine kinase MET, which might contribute to the major role of STAT3 in cancer(Yang, Chatterjee-Kishore et al. 2005). Outside the nucleus, U-STAT5 constitutively associate with Golgi apparatus and rough endoplasmic reticulum in vascular cells to preserve their structure and function, loss of STAT5 led to dramatic phenotypes including fragmentation of Golgi cisternae and mitochondria(Lee, Yang et al. 2012).

2.3.3 The PRL/Jak2/STAT5 signaling cascade.

The Jak-STAT pathway is paradigm signaling transduction cascade initiated by protein/protein interactions (ligand/receptor engagement) at the cell surface and message is conveyed directly to genes in the nucleus(Stark and Darnell 2012). The Jak2/STAT5 pathway is the primary pathway activated upon PRL/PRLR binding(Campbell, Argetsinger et al. 1994, Rui, Kirken et al. 1994, Liu, Robinson et al. 1995). Unlike the ligand binding dependent association with growth hormone receptor(Argetsinger, Campbell et al. 1993), Jak2 pre-associates with PRLR and phosphorylates itself for activation and subsequently phosphorylates tyrosine residues on the intracellular domain of PRLR, providing docking sites for STAT5 recruitment. Jak2 phosphorylates the recruited STAT5, leading to dimerization and nuclear transportation of these transcription factors(Goffin, Binart et al. 2002). Additionally, many other kinases, including Fyn kinase(Clevenger and Kline 2001), proto-oncogene-tyrosine-protein kinase Src(Garcia-Martinez, Calcabrini et al. 2010),

Mitogen-Activated Protein Kinase (MAPK)(Bole-Feysot, Goffin et al. 1998) and the phosphoinositide-3-kinase (PI3K)(Berlanga, Gualillo et al. 1997, Bishop, Nien et al. 2006) were reported to be activated upon PRL/PRLR binding. In the liver, PRL mimics action of growth hormone in tyrosine phosphorylation of Insulin Receptor Substrates (IRSs) for P85 docking during PI3K activation in a Jak2 dependent manner(Yamauchi, Kaburagi et al. 1998). Interestingly, Jak2 also phosphorylates the tyrosine residues (153,201 and 285) in the p21-activated serine-threonine kinase (PAK1) in Nb2 cells in response to PRL(Rider, Shatrova et al. 2007), although it is not clear the temporal order of these phosphorylation events and Rac1/cdc42 mediated PAK1 activation from its trans-autoinhibitory conformation(Parrini, Lei et al. 2002). Tyrosine phosphorylated PAK1 was reported to mediate cyclin D1 activation(Tao, Oladimeji et al. 2011), focal adhesion kinase de-phosphorylation via activation of tyrosine phosphatase(Hammer and Diakonova 2016), and adhesion turnover(Hammer, Oladimeji et al. 2015) in T47D cells. Activation of FAK1 is inhibited by PRL induced de-phosphorylation. PRL induced PAK1 tyrosine de-phosphorylation and breast cancer metastasis(Hammer and Diakonova 2016). However, these observations should be interpreted in a context dependent scenario since altered PRL signaling transduction occur in breast cancer cells. For example, in breast cancer cell lines like MCF7 and SKBR3, but not in in the COMMA-D-derived murine mammary epithelial cell line HC11(Ball, Friis et al. 1988) or PRLR deficient breast cancer cell lines like MDA-MB-231, PRL/PRLR binding leads to co-activation of JAK1 in a JAK2 dependent manner, and subsequent activation of ERK and STAT3

following Jak1 activation(Neilson, Zhu et al. 2007). Namely, in addition to Jak2 kinase, PRL/PRLR signaling activates different kinases in a tissue specific manner. To date, little is known about PRL effects mediated by the other PRLR isoforms and the assumption other isoforms inhibit PRL signaling through ligand competition prevails.

In addition, recent advances in microarray technology has allowed the identification of transcriptional targets induced by PRL signaling(Oakes, Rogers et al. 2008). The effects of these transcriptional targets including cyclin D1(Brockman, Schroeder et al. 2002), receptor activator of NFκB ligand (RANKL)(Ormandy, Naylor et al. 2003), GATA3(Naylor, Oakes et al. 2005), IGF2(Brisken, Ayyannan et al. 2002) and Elf5(Harris, Stanford et al. 2006) will be discussed in the coming sections. A schematic PRL/Jak2 signaling pathway before and after ligand engagement is summarized in figure 1.5.

During mammary gland development, the PRL/Jak2/STAT5 signaling is critical for growth and differentiation of alveolar progenitor cells. In breast cancer initiation and metastatic progression, contradicting effects of Jak2/STAT5 signaling was reported(Wagner and Rui 2008). The role of PRL/Jak2 signaling in breast cancer is discussed in the coming section.

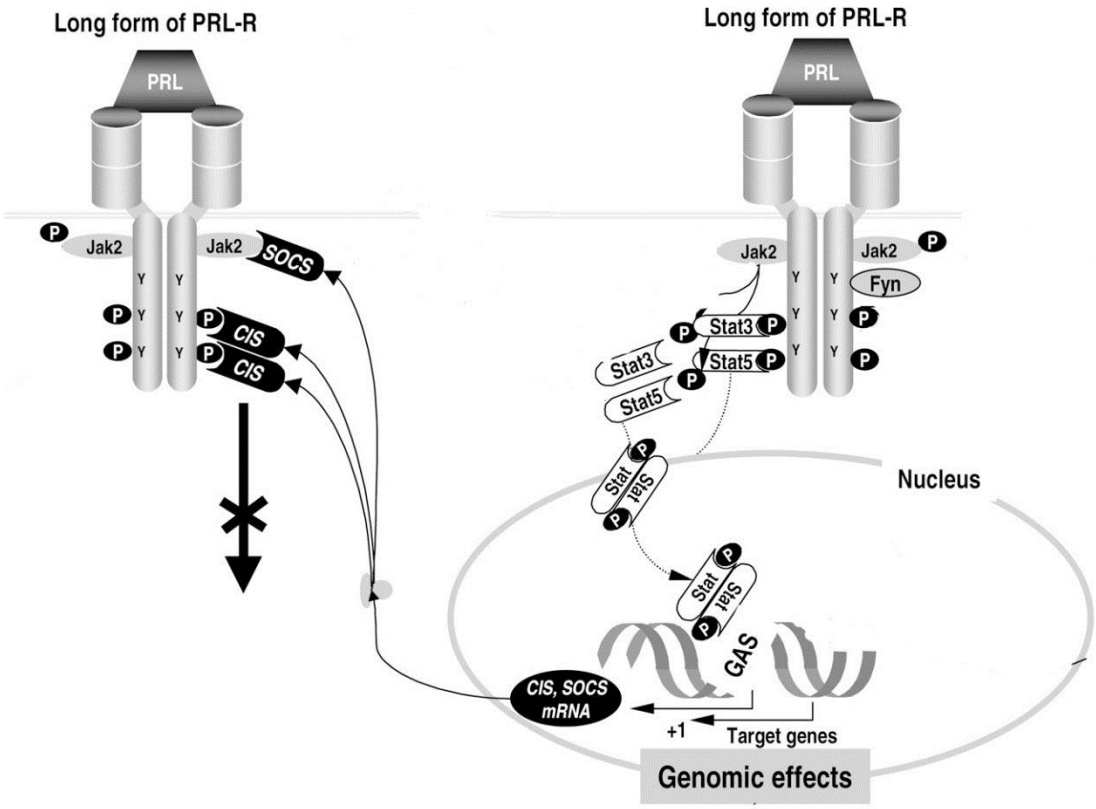

Figure 1.5 PRL signaling pathway. Cytoplasmic domain of PRLR is required for signal transduction, before ligand binding, Jak2 constitutively associates with pre-dimerized PRLR, and non-tyrosine phosphorylated STATs localizes to the cytosol. Upon PRL binding, formation of ligand/receptor complex leads to receptor activation, and Jak2 is trans-phosphorylated (P). This induce Jak2 activation and tyrosine phosphorylation of PRLR cytoplasmic domain, which serves as docking sites for STATs recruitment and phosphorylation by Jak2. Phosphorylated STATs dimerize and translocate into the nucleus, inducing the expression of various genes like β-Casein and whey acidic protein. Tyrosine phosphorylated PRLR is also docking sites for adaptor proteins inducing activation of C-Src and Fyn kinases. Negative regulators of PRL/Jak2 signaling is indicated on the left.

2.4 Negative regulators of Jak2/STAT5 signaling pathway.

Negative regulators of Jak2/STAT5 pathway includes (1) suppressor of cytokine signaling (SOCS) proteins, a group of proteins induced by Jak/STAT signaling and act as a negative feed back loop to switch off Jak2 activation(Tomic, Chughtai et al. 1999), (2) protein tyrosine phosphatases (PTPs) like Src homology2 (SH2) -domain-containing PTP1/2 (SHP1,SHP2), and CD45 dephosphorylate tyrosine residues and restore target proteins including Jak2, PRLR and STAT5 to inactive stage. (3) Tyrosine phosphorylated Jak2 in its active form may undergo polyubiquitination and destined for proteasomal degradation (Ungureanu, Saharinen et al. 2002). (4) The PRL bound PRLRs undergo ubiquitination, internalization and degradation after Jak2 activation for signaling termination(Swaminathan, Varghese et al. 2008). Furthermore, the protein termed cytokine-inducible SH2-containing proteins (CIS) attenuate PRL signaling through either phosphatase recruitment or phosphor-tyrosine docking sites blocking(Helman, Sandowski et al. 1998).

3.Mammary Epithelial Morphogenesis, From Stem Cells to Polarization

3.1Mammary epithelial cell stemness and hierarchy

Adult stem cells were defined as a small group of cells capable of self-renewal and tissue/organ regeneration. The existence of locally resided mammary stem cells were postulated and demonstrated by *in vivo* serial transplantation assay in which the mammary gland can be regenerated by transplantation of epithelial fragments to

de-epithelialized recipient syngeneic mouse fat pad back in the 1960s(Deome, Faulkin et al. 1959, Daniel, De Ome et al. 1968, Kordon and Smith 1998). Subsequent work showed successful engraftments from any segment of the mammary epithelial tree at any developmental stages(Daniel 1975, Smith and Medina 1988). Microscopically visualized mammary stem cells rely heavily on the development of fluorescent activated cell sorting (FACS) and expanding pool of cell surface markers identified to distinguish these cells(Smalley, Kendrick et al. 2012). It took almost 40 years before these self-renewing multipotent stem cells were isolated from the mouse mammary gland in 2006 by FACS. A subpopulation of mammary cells bearing either $Lin^{-}CD45^{-}CD29^{hi}CD24^{+}$ or $CD31^{-}EpCAM^{low}CD49f^{high}$ were isolated and found to contribute to both the luminal and myo-epithelial lineages and the generation of functional lobuloalveolar units during pregnancy. In both studies, TER119 and CD45 were used to exclude the haematopoietic cells and CD31 was used for the endothelial cell exclusion. These basally positioned stem cells distributed throughout the mammary epithelium in low frequencies ranging from 1 mammary repopulating units (MRU) in 100 to 4,900 total cells with the variation arose from different procedures used to dissociate and transplant cells(Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006). A recent quantitative analysis of the cellular composition of the mammary epithelium demonstrated the ventral-most large ducts contain the reservoir of stem cells(Fernandez-Gonzalez, Illa-Bochaca et al. 2009). In human, similar stem cells were also described with different cell surface markers(Stingl, Eaves et al. 2001, Clarke 2005). A recent addition to the expanding surface markers is

CD10(Bachelard-Cascales, Chapellier et al. 2010), a Zinc dependent metalloprotease responsible for the cleavage of signaling peptides in the niche(Turner and Tanzawa 1997). Unlike the almost unlimited self-renewal potential of cancer stem cells, mammary stem cells undergo senescence after 5 to 10 transplant generations(Visvader and Smith 2011).

Extensive studies have established that adult mammary gland harbours more than one type of stem cell populations featuring slow-cycling(dos Santos, Rebbeck et al. 2013) or putative quiescent(Boras-Granic, Dann et al. 2014), or quick expansion during pregnancy(Asselin-Labat, Vaillant et al. 2010, Visvader and Clevers 2016). These stem cell subtypes organized in hierarchy with a unipotent progenitor resides at the apex, giving rise to two primary epithelial cell lineages including myoepithelial progenitors which differentiate into myoepithelial cells and luminal progenitors comprising ductal and alveolar subtypes for generation of ductal luminal cell and alveolar cell (Visvader and Stingl 2014). Myoepithelial progenitors contain overlapping cell surface markers with the constituent cells in basal compartment, which make them more difficult to distinguish due to lack of exclusive markers(Stingl, Eirew et al. 2006). Better understanding has been achieved for luminal progenitor cells comparing to the relatively mysterious myoepithelial progenitors(Asselin-Labat, Sutherland et al. 2007, Booth, Boulanger et al. 2007). Recent work from the laboratory of Dr. J. Visvader identified a subpopulation localizing to the proximal ductal tree with signature of $Lgr5^{+}Tspan8^{high}$ may originate from the embryonic mammary primordia and may lie at the apex of the mammary stem cell hierarchy,

which switch to quiescent state postnatally and are responsive to ovarian hormones oestrogen and medroxyprogesterone acetate (MPA)(Fu, Rios et al. 2017). This is consistent with the previous results that mammary stem cells and progenitors are highly responsive to steroid hormones (Asselin-Labat, Vaillant et al. 2010), albeit these cells do not express estrogen or progesterone receptors(Asselin-Labat, Shackleton et al. 2006). Meanwhile, lineage tracing studies, which relies on expression of lineage specific DNA recombinase to activate a reporter gene driven by a strong ubiquitous promoter, renders permanently marking and tracking the fate of specific cell population *in vivo*(van de Moosdijk, Fu et al. 2017), have also yielded discrepant data. A. Van Keymeulen et.al. showed both luminal and myoepithelial lineages contain long-lived unipotent stem cells capable of tissue regeneration, suggesting distinct stem cells contribute to mammary gland development and maintenance(Van Keymeulen, Rocha et al. 2011). Notably, the transplantation studies mimic the regenerative state in which stem cells might be forced to de-differentiate or develop into other lineages which they usually do not contribute to under physiological conditions. An earlier study showed medium switch can force the luminal cells to differentiate into myoepithelial cells, arguing a interconversion between progenitor cells(Pechoux, Gudjonsson et al. 1999). Moreover, a recent study from J. Stingl challenged a hierarchically organized stem cell subtypes by characterizing the stem cell division kinetics during postnatal mouse mammary gland development. Their data indicate each cell type is largely maintained by its own restricted progenitors. Secondly, rather than the previously established hormone

receptor positive cells do not proliferate, most detected cell division in the adult virgin gland is restricted to oestrogen receptor positive luminal cell lineage. Thirdly, as it would be anticipated in accordance with the stem cell hierarchy theory (Figure 1.6), stem cells at the apex have longer telomere length than their progenies, whereas J. Stingl reported disparate telomere lengths in different mammary epithelial subpopulations, with no correlation between cell subtypes and telomere length(Giraddi, Shehata et al. 2015). Furthermore, stem cell fate determination is subject to the microenvironment cells are exposed to. For example, epithelial cells inside terminal end bud function as highly proliferative, multiple lineage-committed stem cells follows a stochastic growth pattern leading to heterogeneity in the ductal network during pubertal mammary development(Scheele, Hannezo et al. 2017), however, these very same cells undergo terminal differentiation for milk production during pregnancy, articulating another layer of temporal hormonal regulation on stem cell fate.

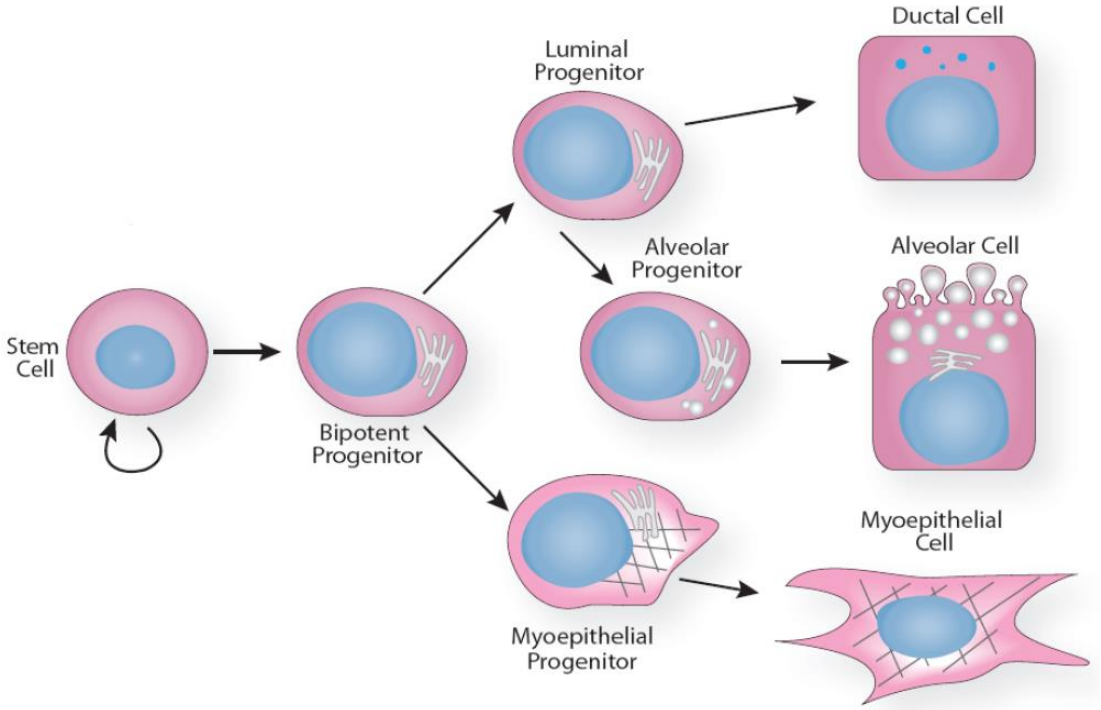

Figure 1.6 Schematic representation of a commonly agreed mammary cell hierarchy. Stem cell at the apex give rise to bipotent progenitor cells which differentiate into both luminal progenitor and myoepithelial progenitors. Myoepithelial progenitors differentiated into myoepithelial cells, and it is not clear how the luminal progenitor further differentiated into progenitors dedicated to either alveolar cell or luminal cells, a postulated intermediate progenitor is yet to be identified. Discrepancies arise from molecular signatures of progenitor cells and reversibility of progenitor to reconstitute the whole mammary gland in transplantation assay. Images adapted from H. Macias Wiley Interdiscip Rev Dev Biol.1 (4):533-557 (2012)

Mammary stem cells exist in a reversible state of quiescence or dormancy, presumably this is evolved to protect the genomic integrity of long-lived cells, indeed dormant stem cells demonstrate higher repopulating potential than cycling stem cells in transplantation assay (Orford and Scadden 2008). In response to physiological stimulus like hormonal regulation during estrous cycle and pregnancy, quiescent cells are activated to proliferate and return to quiescence after establishing new homeostasis. Dysregulation of dormant state can lead to impaired tissue function like neoplasia and eventually breast tumorigenesis. To date, exhaustion and replenishment of stem cell pool remains a poorly understood field. Data from DNA nucleotide analogue-retaining studies suggest that mother stem cells undergo asymmetrical cell division, generating a differentiated progeny and an identical replica for stem cell pool maintenance(Smith 2005). Exhaustion of stem cell pool is speculated given the limited transplant generations, although it is not clear senescence of mammary stem cells is induced by repeated artificial manipulation in these transplant assays. Furthermore, exhaustion of stem cell pool might account for the epidemiological statistics that multiple parous /breast-feeding women have lower incidence of breast cancer based on the assumption that these women bear less error prone stem cells after multiple pregnancies than those with single or no pregnancy(Russo, Moral et al. 2005). In particular, breastfeeding women benefit further from extended periodical PRL exposure during lactation against breast tumorigenesis, suggesting a potential protective role of PRL in breast cancer (Giudici, Scaggiante et al. 2016) , a point will be discussed in the following section. In our

study, we will focus on the ductal/alveolar progenitor cells giving rise to ductal luminal cell and alveolar cell, which bear a surface marker signature of $EpCAM^{+}CD49f^{low}$.

Elucidating the fate determination of the mammary epithelium is not only of interest from a developmental perspective, but also for breast cancer research, as it is widely appreciated the signaling cascades maintaining normal homeostasis are often dysregulated during tumorigenesis. Cancer stem cells, defined from their self-renewal capacity and tumor initiating ability, are a small group of cells in the heterogeneous tumor driving tumor growth and progression(Siddique and Saleem 2012),share a similar molecular signature with stem cell. It was also demonstrated that only a small proportion of the solid tumor cells are capable of forming colonies(Wicha, Liu et al. 2006). Similar with the identification of mammary stem cells, breast cancer stem cells were first screened by flow cytometry using various cell surface markers, and then validated by *in vitro* soft agar colony formation assay and the *in vivo* tumor generating capacity through xeno-transplanting into immune-deficient mice. In search of tumorigenic breast cancer cells out of the phenotypically diverse/heterogeneous population, a small portion of cancer cells bearing the $Lineage^{-}ESA^{+}CD44^{+}CD24^{-/low}$ surface marker signature were identified, as few as 200 $ESA^{+}CD44^{+}CD24^{-/low}$ cells gave rise to tumors, comparing to 50,000 non-sorted cells required to generate a tumor in NOD/SCID mice(Al-Hajj, Wicha et al. 2003). Later work demonstrated breast tumor initiating cells positively express aldehyde dehydrogenases(ALDH)(Ginestier, Hur et al. 2007). Notably, these

individual markers label distinct group of cancer stem cells, a recent study demonstrated ALDH positive cancer stem cells are more active in proliferation comparing to the $CD44^{+}CD24^{-/low}$ labelled ones(Liu, Cong et al. 2014). Expression of cancer stem cell markers showed big variation among primary tumors, suggesting multiple subtypes of breast cancer stem cells exist (Hwang-Verslues, Kuo et al. 2009). No matter whether these stem-like breast cancer cells originate from dysregulation of normal stem cell self-renewal and differentiation(Al-Hajj, Wicha et al. 2003) or develop from epithelial-mesenchymal transition (EMT)(Mani, Guo et al. 2008), it is implicated that distinct breast cancer subtypes originate from different subpopulations of mammary cells(Velasco-Velazquez, Homsi et al. 2012). Targeting these cancer stem cells is of interest in seeking therapeutic strategies against breast cancer, as accumulating evidence point to the small portion of cancer stem cells accounting for tumor resistance to conventional chemo/radio-therapy and tumor recurrence or metastasis. Progress achieved in the field of mammary stem cell research will interactively direct the breast cancer stem cell study in search of drug targets and treatment paradigms.

3.2 Cell polarity in mammary epithelial cells

Quiescent epithelial cells organize as mono-layered or pseudo-stratified sheets to create boundaries between different environments. A defining feature of epithelial cell is polarization, i.e. asymmetric distribution of cellular and membrane contents. Epithelial cells are polarized along their apical-basal (A/B) axis, with the apical side

facing the exterior or the lumen of the epithelial tube and the basal side facing the extracellular matrix where the epithelial cells are embedded in. In the mammary gland, luminal epithelial cells exhibit apical/basal polarity, with the apical domain facing the central lumen of the mammary duct or the terminal end bud. The basal-lateral domain contacting neighboring luminal cells or myoepithelial cells and the basement membrane. The A/B polarity is established and maintained by the asymmetric segregation of evolutionarily conserved proteins that assemble into dynamic protein complexes. Proper formation of apical/basal cell polarity combined with planar cell polarity (PCP), the cellular organization within the plane, is essential for normal epithelium physiology and tissue homeostasis. On the other hand, loss or dysregulation of cell polarity can cause misoriented cell divisions and tissue disorganization, increased self-renewal of adult epithelial stem cells, which are the hallmarks of cancer development.

3.2.1 Junctional proteins in epithelial cells

A distinctive feature of polarized epithelium is the presence of adhesive structures like adherent junctions, desmosomes, gap junctions and tight junctions(Niessen, Leckband et al. 2011). Adhenrens junction is mediated through E-cadherin, a transmembrane calcium-dependent cell adhesion protein, which forms extracellular contacts with cadherin on opposing cells through their N-terminal extracellular domain(Shapiro and Weis 2009). The cytoplasmic domain of E-cadherin links the intracellular actin

cytoskeleton through α-catenin and β-catenin, which regulate cadherin turn over(Weis and Nelson 2006). E-Cadherin regulates localization and activity of Rho family GTPases to modulate actin organization and function(Kaibuchi, Kuroda et al. 1999, Fukata and Kaibuchi 2001). Desmosomes are localized patches that hold two cells together and links to the intermediate filaments intracellularly, providing mechanical integrity of the epithelium(Cheng and Koch 2004). Gap junctions are intercellular cylinder channels made by a variety of connexion species and modulate direct exchange of ions and small molecules between neighboring cells. Recent studies have demonstrated signaling events at gap junctions contribute to the bio-synthesis and release of secretory products in exocrine and endocrine glands(Meda 2017). Apical tight junctions link neighboring cells through transmembrane proteins including claudins, occludins and junction adhesion molecule A. In practice , the adaptor protein zonula occludens 1 (ZO1), which links the tight junction to the actin cytoskeleton, is usually used as a marker of tight junctions(Bazzoun, Lelievre et al. 2013). Epithelia form barriers with functions essential for life like controlled exchange of nutrients solutes and waste between biological compartments, and tight junctions are the primary determinant of epithelial barrier function(Marchiando, Graham et al. 2010). Sometimes, the adhenrens junctions and tight junctions are referred to as apical junctional complexes (AJC).

Besides the cell/cell adhesion, cell/ECM adhesion at the basal side is also essential for proper function of polarized epithelium. ECM provides signaling cues for the initiation of cell polarization predominantly through integrin, the transmembrane

ECM receptor mediated signaling (Manninen 2015). In a recent study, rotational motion of polarized mammary epithelial acini cultured in 3D culture system was documented, whereas none of the non-polarized human cancer derived cell spheres undergo such rotation. Rotation of cancer derived cell spheres was restored when the basement membrane was dissolved, demonstrating the involvement of cell/ECM interactions for proper cell polarization (Wang, Lacoche et al. 2013). Integrins and cadherins join forces to form the adhesive networks, which respond to and integrate signal inputs from ECM, neighboring cells and soluble factors, guiding each individual cell to make right decisions when to differentiate, migrate, divide or die during normal tissue morphogenesis and homeostasis (Weber, Bjerke et al. 2011).

Of note, organization of the cytoskeleton network, including actin, microtubule and intermediate filaments, in polarized epithelial cells is different from other cell types. Actin filaments are organized in three forms: (1) as bundles within apical microvilli, (2) as filament bundles in association with the apical junctional complexes, and (3) as dense networks lining the lateral and basal membranes (also referred to as cortical cytoskeleton). Microtubules are generally organized in bundles parallel to the lateral membrane, with their minus-end uniformly oriented toward the apical membrane, or as networks of mixed orientation underneath the apical and basal membranes. Intermediate filaments link desmosomes across the cell(Nelson 2003).

Core components of the cadherin-catenin cell adhesion complex are E-cadherin and β-catenin. E-cadherin is a tumor suppressor gene and renowned for its potent malignancy suppressing activity(Berx, Becker et al. 1998). Conversely, catenin is an

oncogene(Fodde and Brabletz 2007), functioning as a Wnt pathway coactivator of genes that induce cell cycle progression(Nelson and Nusse 2004). Cadherin mediated cell/cell adhesion is involved in contact inhibition of the epithelial sheet(Eagle, Levine et al. 1965, Fagotto and Gumbiner 1996). Disrupted E-cadherin junction or loss of E-cadherin leads to increased cell proliferation, migration and aberrant epithelial homeostasis(Berx and van Roy 2009).

3.2.2 Polarity proteins in epithelial cells

Polarized epithelium feature a distinctive spatial distribution of polarity protein complexes around the apical junctional complexes(St Johnston and Ahringer 2010). Genetic studies in *Drosophila* and *C. elegans* have identified three major evolutionarily conserved polarity networks in the formation and maintenance of apical/basal polarity(Tepass, Tanentzapf et al. 2001). The Crumbs complex (Crb3, PALS1, and PATJ) localizes to the apical side of apical junctional complexes, and is required for tight junction formation(Fogg, Liu et al. 2005) and subsequent establishment of the apical membrane domain(Tanentzapf and Tepass 2003). The PAR complex, which includes a multi-domain scaffolding protein Par3, the adaptor protein Par6, the serine/threonine kinase atypical protein kinase C (aPKC), localizes close to the apical junctional complexes(Izumi, Hirose et al. 1998). Par6 contains a semi-CRIB domain, which interacts with small GTPases like cell division control protein 42 (CDC42) or Rac only in their activated GTP-bound form(Bose and Wrana

2006) (Figure 1.7).The Scribble complex (including Scrib, Dlg, and Lgl2) localizes to the lateral membrane below adhenrens junctions and tight junctions, and defines the basolateral plasma membrane domain(Bilder, Schober et al. 2003). It is necessary to maintain E-cadherin mediated cell/cell adhesions (Qin, Capaldo et al. 2005), *vice versa*, junctional scribble recruitment is dependent on E-cadherin engagement (Navarro, Nola et al. 2005). These three complexes act interactively and spatiotemporally to regulate epithelial polarization(Tepass 2012). For example, both the Crumbs complex and PAR complexes are implicated in tight junction assembly, the transmembrane protein Crb3 competes with Par3 for binding Par6 and recruit Par6 to the apical cell surface (Hurd, Gao et al. 2003). The distribution of apical and basal/lateral polarity proteins complexes are mutually exclusive (Bilder, Schober et al. 2003). The apical membrane localization of the PAR complex is restricted by the basolaterally localized Par1b, which phosphorylates Par3 and promotes its dissociation from the cell cortex, excluding it from the basolateral membrane domain(Suzuki and Ohno 2006), Conversely, aPKC phosphorylates Par1b to expel it from apical membrane(Suzuki, Hirata et al. 2004). Moreover, Par3 competes with the adaptor protein lethal giant larvae (Lgl2) for binding to Par6/aPKC. Phosphorylated Lgl2 is expelled from Par6/aPKC upon aPKC activation (Chalmers, Pambos et al. 2005). In addition, the tumor suppressor phosphatase and tensin homolog (PTEN) functions at the apical membrane by dephosphorylating the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and producing phosphatidylinositol (4,5)-bisphosphate (PIP2), and the PIP2 enriched apical membrane domain recruits annexin 2 (Anx2),

which elicits apical localization of CDC42 and PAR complex(Gassama-Diagne, Yu et al. 2006).

Of note, the small GTPase CDC42 plays fundamental role in promoting cell polarization. It promotes the formation of apical junctions(Rojas, Ruiz et al. 2001, Wallace, Durgan et al. 2010). CDC42-GTP induces conformational change of Par6 for aPKC activation (Schonegg and Hyman 2006, Chen and Zhang 2013). It also directs the basolateral protein trafficking(Kroschewski, Hall et al. 1999) and apical surface formation(Martin-Belmonte, Gassama et al. 2007). In non-epithelial cells, directed astrocytes migration is modulated through integrin mediated CDC42 activation(Etienne-Manneville and Hall 2001). On the other hand, aPKC activation is not necessarily associated with polarization under all circumstances, for example, aPKC phosphorylates Par6 and facilitates transforming growth factor beta (TGFβ) induced epithelial-mesenchymal transition in non-small-cell lung cancer (NSCLC), which is a process of de-polarization(Gunaratne, Thai et al. 2013).

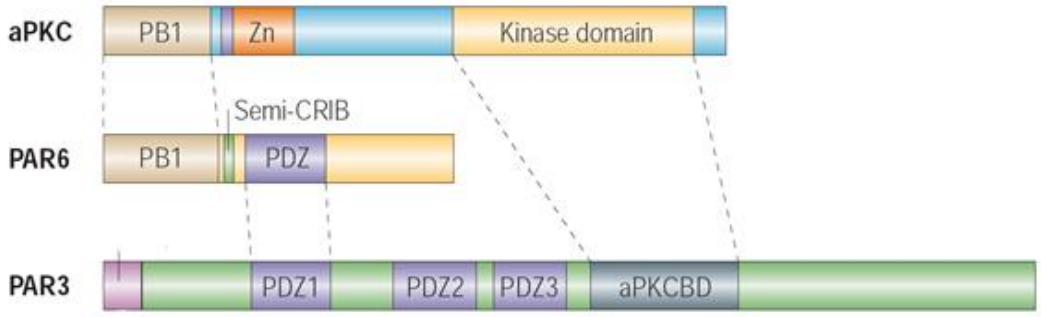

Figure 1.7 Schematic structure of PAR complex: aPKC contains a Zinc-finger (Zn) motif as regulatory domain, its kinase domain phosphorylates the aPKC-binding domain (aPKCBD) of Par3. Connecting lines indicate regions of proteins interacting with one another. Image adapted from Macara IG Nat.Rev. MCB 5,220-231(2004).

3.2.3 Establishment and maintenance of cell polarity

The mechanism and the sequence of events leading to cell polarization are not completely elucidated to date. Recent advances implicated that polarity complexes collaborate with junctional proteins to establish epithelial cell polarity(Coopman and Djiane 2016). Studies showed that cadherin/catenin mediated cell/cell adhesion is required for initiation of epithelial polarization(Cox, Kirkpatrick et al. 1996), and maturation of junctional complexes needs aPKC kinase activation(Suzuki, Ishiyama et al. 2002). A model is postulated regarding the initiating events of cell polarization. First, adhenrens junction starts with the contact of actin based protrusions between two neighboring cells triggered by E-cadherin molecules(Mandai, Rikitake et al. 2013), and this activates Rac signaling and promotes early adhenrens junction maturation(Collinet and Lecuit 2013, Ivanov and Naydenov 2013). Once adhenrens junction matured, Par3 and α-catenin are recruited to inhibit Rac activation and concomitant activation of Rho signaling, leading to formation of stable actin bundles that stabilizes the junctions. Par3 functions as a localization clue for adhenrens junctions and it is actively involved in the formation and stabilization of tight junctions which are in proximity with adhenrens junctions in mammalian cells(Chen and Macara 2005, Harder and Margolis 2008). In addition, cell polarization also relies on correct sorting of proteins to each plasma membrane domain at the Trans-Golgi Network (TGN)(Folsch 2008, Mellman and Nelson 2008). In summary, a basic set of evolutionarily conserved core mechanisms is adapted by both single-cell organisms

and multi-cell tissues for initiation and maintenance of polarization, which includes localized signaling complex assembly, cytoskeleton remodeling and targeted marker protein delivery from intracellular pools. It is initiated by a cell-surface landmark or spatial cue and defines a point on the cell surface to which the cell orients. Once the landmark/axis of polarity is defined, cytoskeleton reorganizes to facilitate the propagation of polarization by correct delivery of asymmetrically distributed polarity proteins to their right destination(Nelson 2003). Established polarity is subsequently maintained by interplays between polarity complexes and junctional proteins. Figure 1.8 showed a representative polarized epithelial cell with its general distribution of polarity proteins and junctional proteins.

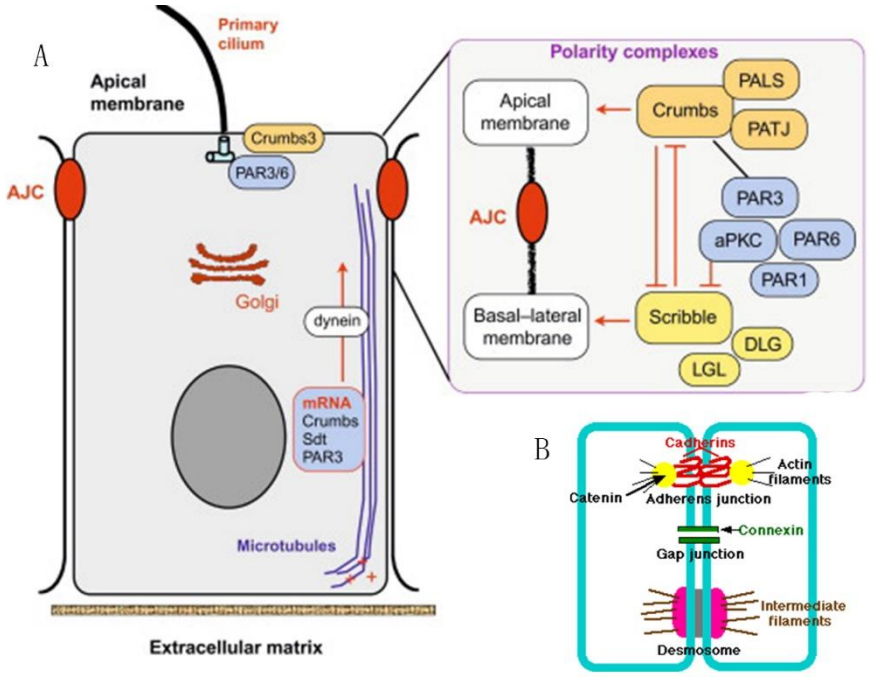

Figure 1.8 A representative cartoon of polarized epithelial cell showing (A) the distribution polarity complexes of and (B) the junctional proteins. AJC, (orange) localizes apically at the interface between the apical and basal membrane domains characterized by antagonizing protein complexes: the apical Par6/aPKC (light blue), and the basal-lateral Dlg/Lgl/Scrib (yellow). Par3 accumulates apically due to a double restriction by the apical kinase aPKC and by the basal kinase Par1 (see text). Polarity was maintained through both correct sorting at the Golgi apparatus and active transportation via the cytoplasmic dynein/microtubule meshwork. The cell/ECM interactions via integrin mediated focal adhesion and the actin cytoskeleton is skipped in (A). The centrosome showed in blue function as basal body for primary cilium in somatic cells. Panel A was adapted from Nelson WJ et.al Prog Mol Biol Transl Sci. 116: 3-23 (2013)

Establishment and maintenance of cell polarity is regulated through various conserved cell signalling pathways including TGFβ, Integrin and WNT signalling(Khursheed and Bashyam 2014). Loss of cell polarity is a hallmark for cell malignancy. Most solid tumors are derived from epithelial tissues. Malignant tumors are characterized by the loss of epithelial characters such as cell-cell adhesion and apical-basal cell polarity, appearance of mesenchymal characters including increased cell motility and expression of mesenchymal genes such as N-cadherin and vimentin, a process designated as epithelial-mesenchymal transition (EMT), which is regulated by multiple transcription factors including Snail, Slug, Twist and Zeb1/2 to suppress epithelial genes and activate mesenchymal expression program(Thiery, Acloque et al. 2009). EMT occurs during both normal tissue remodeling and pathological conditions like tumor metastasis. EMT and its reverse process mesenchymal-epithelial transition (MET) mediate normal development events like neural crest formation and wound healing, meanwhile, the motile and invasive phenotype after EMT endows epithelial cell with more proliferative and motile characteristics during tumorigenesis (Yang and Weinberg 2008). Several signaling pathways like TGFβ, WNT Notch, EGF and FGF pathways have been implicated in regulating EMT or MET(Polyak and Weinberg 2009). For example, TGFβ signaling promotes EMT by phosphorylation of Par6 and consequent disassembly of tight junction (Ozdamar, Bose et al. 2005). ErbB2 activation disrupts cell polarity by competing with Par3 for Par6/aPKC binding (Aranda, Haire et al. 2006). In mammary epithelial cells, loss of apical/basal polarity de-regulates proliferation and apoptosis, promoting invasion and metastasis in breast

cancer(Chatterjee and McCaffrey 2014). Other EMT regulators include the micro ribonucleic acid (miRNA) miR-200 and Elf5. miR-200 maintains apical/basal polarity in mammary epithelium by suppressing expression Zeb1(Gregory, Bert et al. 2008), and the latter inhibits EMT through Snail2 repression in mammary gland development and breast cancer metastasis(Chakrabarti, Hwang et al. 2012).

Notably, physiological de-polarization occurs at the mammary gland, luminal cells lose polarity temporarily and rearrange within the epithelium for multiple-layering during branching morphogenesis(Ewald, Brenot et al. 2008, Ewald, Huebner et al. 2012). Polarity is also partially lost during terminal differentiation of mammary epithelial cells, where the usually apically localized protein NHERF1(Na/H exchanger regulatory factor 1) forms complexes with PRL receptor, as well as β-Catenin, E-Cadherin and ezrin for correct localization of the PRL receptor to the basal membrane(Morales, Hayashi et al. 2012), suggesting a potential crosstalk between apical/basal polarity and hormone signaling.

3.2.4 Planar cell division, the orientation of cell division

During adult life, tissue homeostasis and regeneration of damaged tissues require fine-tuned regulation of cell division and growth rate. Cell division is controlled both in timing and orientation. Oriented cell division is a key regulator of cell fate determination and tissue architecture, which is crucial for tissue morphogenesis and homeostasis. In polarized epithelium, mitotic division initiates with actomyosin

contraction at the cytokinesis furrow after karyokinesis(Tanaka 2010) and followed by planar alignment of the mitotic spindle which is generally orthogonal to the plane of epithelial sheet(Morin and Bellaiche 2011). This uniform cellular organization within the epithelial plan, typically orthogonal to the apical/basal polarity axis, is referred to as planar cell polarity (PCP) (Sebbagh and Borg 2014). Both polarity proteins and epithelial junctions are involved in regulation of mitotic spindle orientation during epithelial morphogenesis (Durgan, Kaji et al. 2011, Nakajima, Meyer et al. 2013, Vorhagen and Niessen 2014). Polarity proteins might orient axis of cell division through regulation of spindle orientation by positioning adaptor protein Leu-Gly-Asn repeat-enriched (LGN) to the cortical landmark at the lateral membrane. LGN binds to the membrane anchored myristoylated heterotrimeric G-protein $G_{\alpha i}$ to the membrane(Du and Macara 2004), and it also binds to Dlg for lateral membrane positioning. Eventually, LGN binds to the astral microtubules of the centrosome, which have minus ends embedded in the spindle poles and plus ends extending toward the cell cortex, through adaptor protein nuclear mitotic apparatus (NuMA) and determines the relative positioning of the mitotic spindle in the dividing cell(Johnston, Hirono et al. 2009, Bergstralh, Lovegrove et al. 2013). Synergistically, apically restricted aPKC phosphorylates LGN and expel it from the apical membrane (Hao, Du et al. 2010). Another study from cyst formation assay of 3D cultured Caco-2 cells demonstrated Par6 and aPKC regulate mitotic spindle orientation in a CDC42 dependent manner (Jaffe, Kaji et al. 2008). In addition, the cell/ECM interactions are also involved in regulation of spindle orientation during mitosis. Loss of β1-integrin

in the basal layer of the mammary epithelium causes spindle orientation defects and resulted in the mixing of the basal and luminal layers (Taddei, Deugnier et al. 2008) and blocked apical/basal polarity(Akhtar and Streuli 2013).

In glandular epithelial tissues, the axis of cell division determines expansion/regenerative repair of epithelial sheet or stratification, with the latter in general is detrimental to the integrity of the epithelium. Oncogenic transformation such as Ras hyper-activation in the epithelium leads to neoplasia characterized with multiple layering in epidermis and lumen filling in glandular structures in kidney and breast tissues. An interesting question arises regarding which strategy epithelial cells are utilizing in lumen formation. In both the cyst formation assay of Madin-Darby-Canine Kidney (MDCK) cells(Bryant and Mostov 2008) and mammary acini formation assay of MCF10A cells(Debnath, Muthuswamy et al. 2003, Underwood, Imbalzano et al. 2006), where a single cell was seeded into Matrigel and grown into polarized cysts that inherit a single lumen or mammary acini in the latter. It was proposed cavitation and subsequent lumen clearing is induced by apoptosis of inner cells due to lack of contact with extracellular matrix. Other than the reported MCF10A cells, mammary epithelial cells also form functional acini when cultured in 3D(Akhtar and Streuli 2006, Mroue and Bissell 2013). Are they utilizing similar mechanism? Is it possible that the lumen is developed through strictly controlled planar cell division from a single cell and the cell/ECM contact is maintained for all daughter cells? I will go back to this question in the discussion chapter.

3.2.5 Role of PRL in mammary epithelial cell polarity

Epithelial cells of the lactating acini are highly polarized, with the apical domain facing the lumen for milk secretion and matured tight junctions between neighboring cells to prevent the backflow of secreted milk(Anderson, Rudolph et al. 2007). Tight junctions between alveolar epithelial cells undergo complete closure around parturition(Nguyen and Neville 1998). PRL plays an important role in tight junction formation and maintenance, PRL alone or in combination with glucocorticoids increases the expression of tight junction proteins including occludin and ZO-1, promoting maturation of tight junction formation exemplified by increased trans-epithelial resistance (Stelwagen, McFadden et al. 1999). Another study demonstrated PRL regulates tight junction structure possibly by modulating the localization, stability or assembly of tight junction proteins to the membrane(Peixoto and Collares-Buzato 2006).

A lot of questions remain with respect to the detailed role of PRL in mammary epithelial cell polarization and terminal differentiation. I set to elucidate whether PRL plays alternative roles during mammary gland development other than the conventionally recognized lactogen hormone. Our attempts and discoveries are presented in the result section.

3.3 Regulation of centrosome amplification and maintenance of genomic stability

3.3.1 Centrosomes in cell division

Centrosomes are subcellular organelles composed of two orthogonally positioned

centrioles surrounded by pericentriolar material (PCM)(Bettencourt-Dias and Glover 2007).They function as microtubule organization center (MTOC) for construction of the mitotic spindle during cell mitosis and serve as basal body for primary cilium assembly in somatic epithelial cells(Kim and Dynlacht 2013). Centrosome duplication is tightly controlled during cell cycle in human and most mammalian species. Throughout development and adult life, this single centrosome of zygote originated from the sperm needs to be duplicated once and only once during every single cell cycle, which is initiated in early G1 phase and is completed at the end of S phase(Nigg 2007). In addition, proper mitotic spindle assembly ensures accurate segregation of chromosomes into daughter cells during cell division, maintaining genome integrity(Prosser and Pelletier 2017). During embryogenesis, proper spindle positioning is crucial for segregation of developmental determinants into different daughter blastomeres(Moorhouse and Burgess 2014). In adult tissue, maintenance of epithelial homeostasis in either monolayer or glandular morphology requires proper mitotic spindle orientation to establish the right axis of cell division for both self-renewal and regenerative repair. Spindle positioning determines the location of contractile ring assembly, and centrally placed mitotic spindle results in daughter cells of equal size(Green, Paluch et al. 2012). The planar cell division is determined by intrinsic factors like distribution of polarity determinants like the PAR complex and extrinsic factors like the niche of mother cell, which is ultimately executed by directional centrosome segregation after duplication through proper mitotic spindle assembly.

During mitosis, the two centrosomes nucleate and organize microtubules at the spindle pole, although bipolar spindles can assemble and mitosis is completed in *Drosophila melanogaster* cells lacking centrioles or mammalian cells after laser ablation of centrosomes(Khodjakov, Cole et al. 2000). Indeed, it turned out that chromatin can mediate microtubule nucleation in parallel with centrosome for spindle assembly via Aurora A kinase(Sardon, Peset et al. 2008, Pinyol, Scrofani et al. 2013). A critical regulator of centrosome duplication is polo-like kinase 4 (PLK 4), which triggers the assembly of daughter centrioles during G1/S phase(Habedanck, Stierhof et al. 2005, Rosario, Ko et al. 2010, Holland, Fachinetti et al. 2012). Overexpression of PLK4 by disruption of its transcription repressor Kruppel-like factor 14 (KLF14) induces centrosome over-duplication (Fan, Sun et al. 2015). Centrinone is a reversible PLK4 inhibitor derived from pan-Aurora kinase inhibitor VX-680, which can induce centrosome depletion in vertebrate cells. Centrinone treated normal cell can complete mitosis, whereas progenies are arrested in a senescence like G1 state after centrinone withdrawal because of failure in the assembly of new centrioles(Wong, Anzola et al. 2015). P53 recruits histone deacetylases (HDAC) repressor to the PLK4 promoter and inhibits PLK4 transcription(Li, Tan et al. 2005), and loss of p53 is associated with increased centrosome numbers in mouse fibroblast(Fukasawa, Choi et al. 1996). In MCF10 A cells, p53 induces expression of γ-Tubulin associated N-myc downregulated gene 1 (NDRG1) in mediating centrosome homeostasis and consequent genomic stability(Croessmann, Wong et al. 2015). Although normal centrosome number is observed in the $p53^{-/-}$ mouse(Marthiens, Rujano et al. 2013),

indicating that p53 loss alone is not sufficient to induce centrosome amplification. Cells lack a functional p53 pathway are proposed to acquire multiple centrosomes through failure of a G1 phase checkpoint that should eliminate cells after aborted division. Other key regulators of centrosome duplication include Polo-like kinase 1 (PLK1), Mps-1 and Cyclin-dependent kinase2-cycline E (Cdk2-E)(Hinchcliffe 2014). Despite studies questioning the absolute requirement for centrosomes in spindle assembly(Kwon, Godinho et al. 2008), centrosomes act prevailingly to dictate spindle polarity when present.

3.3.2 Centrosome abnormalities and genomic stability

Alternations in centrosomes number and structure lead to defective mitosis and consequently in chromosome instability, leading to aneuploidy in tumorigenesis. The presence of supernumerary centrosomes typically leads to multipolar spindle which promotes chromosome mis-segregation(Ganem, Godinho et al. 2009, Silkworth, Nardi et al. 2009). Altered centrosome structure in breast epithelial cells disrupts tissue integrity and favors proliferation(Schnerch and Nigg 2016). However, most multiple polar divisions cause severe chromosome mis-segregation and therefore constitute lethal events. Occasionally, they might give rise to cells with chromosomal compositions that favor survival in the microenvironment of the tumor. In tumor cells, genes that are involved in alternative mechanisms for spindle formation might be up-regulated or re-expressed. This might cause several centrosomes to coalesce and allow the formation of bipolar spindles despite of excessive centrosome numbers. On

the other hand, loss of centrosome leads to assembly of mono-polar spindle or delayed bipolar spindle assembly, chromosome instability and aneuploidy(Sir, Putz et al. 2013)(Figure 1.9).

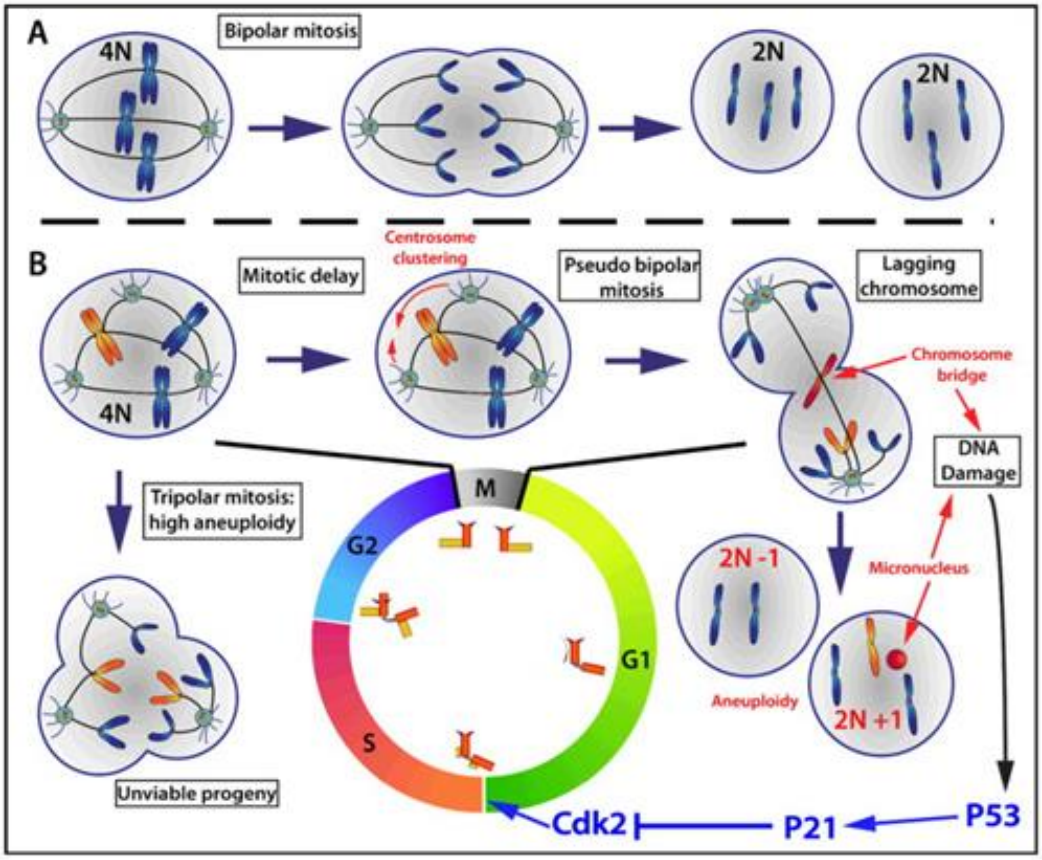

Figure 1.9 The centrosome cycle and consequence of centrosome aberrations. (A) centrosome duplication was tightly controlled to only once during cell division for bipolar spindle formation for equal segregation of chromosomes. (B) multiple centrosomes due to de-regulation of centrosome duplication leads to unequal segregation of chromosomes during mitosis, inducing DNA damage and aneuploidy. In the events of multi-polar mitosis, either unviable or transformed progeny with growth advantage is generated. Key regulators of centrosome duplication are indicated in blue. Image adapted from Cosenza MR et.al. Chromosome Res. 24(1): 105-26 (2016)

Centrosome aberration and chromosome instability are expected to enhance each other. Although under some circumstances, cells harboring defective centrosome duplication regulators exhibit slowed cell proliferation, a disadvantage in tumor progression, tumor cells do show high frequency of centrosome aberrations particularly centrosome amplification and consequent genomic instability, indicating an underlying deregulation of centrosome structure, duplication or segregation. In human breast cancer, centrosome amplification is associated with high grade features(Denu, Zasadil et al. 2016). However, cancer cells seem to have adapted alternative strategies like centrosome clustering to cope with supernumerary centrosomes. On the other hand, depletion of centrosomes by PLK4 inhibition does not inhibit cancer cell proliferation like it does to normal cells. Cancer cells return to its intrinsic centrosome number "set point" after PLK4 inhibition withdrawal, indicating a fundamental difference from normal cells in response to centrosome loss(Wong, Anzola et al. 2015).

3.3.3 Centrosomes in DNA damage response

In response to DNA damage, which occur naturally during DNA duplication cycle and homeostasis, DNA damage response (DDR) is initiated to repair the lesions at all cost and communicate the presence of damaged DNA at the same time. Expression of genes involved in DNA repair is up-regulated and the cell-cycle is arrested by the DNA damage checkpoint. Cell cycle arrest is not released till DNA damage is repaired, otherwise, cells harboring damaged DNA undergo apoptosis or senescence(Jackson

and Bartek 2009, Ciccia and Elledge 2010). Once the DNA damage is recognized, ATM (Ataxia Telangiectasia Mutated), ATR (ATM and Rad3 related) and members of PARP (poly ADP-ribose polymerase) family will be recruited and activated for DNA damage repair and cell cycle arrest will take place via p53 dependent p21 inhibition of CDK (Cyclin D kinase)(Mullee and Morrison 2016).

Outside the nucleus, centrosomes are sensitive to DNA damage as well. Multiple components of DNA damage response apparatus have been identified at centrosomes, these include ATM, ATR, PARPs(Kanai, Uchida et al. 2000), BRCA1(Hsu and White 1998), and p53(Tarapore and Fukasawa 2002). PARPs localize to centrosomes throughout the cell cycle(Kanai, Tong et al. 2003) and their loss leads to dysregulation of centrosome number(Kim, Dudognon et al. 2012). BRCA1 contributes to genome stability by mediating homologous recombination DNA repair(Savage and Harkin 2015), together with BARD1, BRCA1 function as a ubiquitin E3 ligase complex targeting γ-tubulin(Hsu, Doan et al. 2001) to regulate centrosome activity in microtubule nucleation and centrosome number(Starita, Machida et al. 2004). BRCA1 mutation leads to centrosome amplification in mouse embryonic fibroblasts (Xu, Weaver et al. 1999). P53 is activated in response to cellular stresses to suspend cell cycle progression and direct cells for apoptosis in the event of fatal un-repairable errors(Batchelor, Loewer et al. 2009). It also localizes to centrosomes, and p53 deficiency leads to centrosome amplification as well(Fukasawa, Choi et al. 1996). DNA-damaging treatment causes structural alterations of centrosome like splitting of centrosome into individual centrioles(Inanc, Dodson et al. 2010) or disruption of

PCM(Antonczak, Mullee et al. 2016) and allows centrosome duplication outside S phase, resulting in abnormal numbers of centrosomes within the cell(Godinho and Pellman 2014), implicating a commonly occurred centrosome amplification in events of un-repaired DNA damage.

Centrosomes were postulated as a promising anti-cancer target, as factors controlling the centrosome duplication may be potential targets for cancer therapy. Over 100 years ago, Theodor Boveri hypothesized that aneuploidy induced by centrosome amplification promotes tumorigenesis. Either numerical or structural defects in centrosomes are observed in most human solid tumors, with the numerical aberrations being the most frequently described centrosome defect in cancer. Centrosome duplication could result from prolonged S phase in response to activation of DNA damage checkpoint. BRCA plays a key role in the control of genomic stability through regulation of DNA repair and centrosome duplication(Venkitaraman 2002, Kais, Chiba et al. 2012). Impaired p53 in MCF7 cells led to centrosome amplification and distant metastasis of tumor cells(D'Assoro, Busby et al. 2008). Overexpression of PCM components, such as pericentrin, also leads to centrosome overduplication(Loncarek, Hergert et al. 2008). Similarly, overexpression of γ-tubulin is observed in BRCA1 loss induced centrosome amplification(Starita, Machida et al. 2004). In addition, prolonged G2 phase during cell cycle leads to de-coupling of centrosome duplication and cell cycle progression, resulting in re-duplication of centrosomes mediated by polo-like kinase 1 (PLK1)(Li, Tan et al. 2005, Loncarek, Hergert et al. 2010).

In summary, extra copies of centrosomes could arise from over duplication within one single cell cycle, aborted cell division, cell fusion or de novo genesis. Accumulating evidence points to aborted division as an important cause of excessive centrosome numbers. Although it is not clear whether centrosome de-regulation is cause or consequence of tumorigenesis, neither how the viable aneuploidy generated from centrosome malfunction affects tissue integrity and function(Nano and Basto 2016).

4: PRL and Its Signaling Pathway in Breast Cancer

Breast cancer is a heterogeneous disease comprising multiple distinct subtypes. It is the most frequent cancer among women, one in every seven women could potentially develop breast cancer during lifespan (Ferlay, Soerjomataram et al. 2015), and it is the leading cause of cancer death in women(Mahoney, Bevers et al. 2008). As expected, risk of developing breast cancer and mortality rate increase with age(Senior 2012). The role of PRL in breast tumorigenesis has been a topic of debate for decades. Here we review the controversial findings about PRL and the PRL/Jak2 signaling pathway in breast cancer. We speculate the reproductive hormonal exposure during pregnancy and lactation deplete or passivate the mammary stem cells to account for the well documented protective role of pregnancy and lactation in breast cancer. This leads to interesting questions like the role of PRL in mammary stem cell differentiation, which will be addressed in this thesis.

4.1 PRL promotes breast tumor progression, the prevailed notion

Breast cancer takes a step-wise progression development, it begins with benign epithelial atypia and atypical ductal hyperplasia (ADH) to malignant ductal carcinoma in situ (DICS) or lobular carcinoma in situ (LCIS), and eventually develops into invasive ductal carcinoma (IDC) or invasive luminal carcinoma (ILC), which breaches the basement membrane and metastasizes(Bombonati and Sgroi 2011). Invasive ductal carcinomas are categorized into subtypes like Luminal A/B, HER2 positive and triple negative breast cancer (TNBC) based upon histological features, molecular expression signatures, and clinical outcomes(Malhotra, Zhao et al. 2010). Triple negative breast cancers are further subdivided into basal like and claudin-low groups, with the former expressing basal myoepithelial and mesenchymal markers and the latter enriched in stem cell markers, lacking differentiation markers and junction gene expression(Metzger-Filho, Sun et al. 2013). Recently, Lehmann et al. categorized triple negative breast cancers into 6 subtypes based on gene expression profiles and ontologies, which includes two basal like (BL1 and BL2), immunolodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal adndrogen receptor (LAR) subtype(Lehmann, Bauer et al. 2011). Given the gene expression signatures of different breast cancer subtypes resembling different populations along the mammary stem cell differentiation hierarchy, breast cancer subtypes may derive from specific stem cell populations(Visvader 2009).

Epidemiological evidence on plasma PRL as a risk marker for breast cancer development indicated positive association between high plasma PRL and increased

breast cancer risk (Tworoger, Eliassen et al. 2004, Tworoger, Eliassen et al. 2007, Tworoger, Eliassen et al. 2013). However, using plasma prolactin concentration as a marker did not represent the real efficacy of prolactin, as the PRL association with immunoglobins in circulation system is not evaluated, and the locally produced extra-pituitary prolactin cells are exposed to is not considered either in these studies. Studies from various groups concluded PRL a tumor promoting hormone(Bernard, Young et al. 2015). Locally produced PRL in breast and prostate cancer is proposed to function as a growth factor for cancer cells via autocrine or paracrine pathways(Goffin, Touraine et al. 2006, Sethi, Chanukya et al. 2012). Indeed, overexpression of PRL in mammary epithelial cells led to tumorigenesis in rodents (Wennbo, Gebre-Medhin et al. 1997, Rose-Hellekant, Arendt et al. 2003). PRL signaling plays a permissive role in polyoma middle-T antigen and SV40-induced mammary tumors (Vomachka, Pratt et al. 2000, Oakes, Robertson et al. 2007). During breast cancer bone metastasis, PRL/PRLR signaling escalate this process through inducing lytic osteoclast formation(Sutherland, Forsyth et al. 2016). PRL induces claudin-low mammary carcinomas when p53 is lost (O'Leary, Rugowski et al. 2013). Elevated PRLR expression is detected in a rare lung disease pulmonary lymphangioleiomyomatosis (LAM) after loss of tumor suppressor gene encoding Tuberous Sclerosis Complex (TSC)1 and TSC2, therefore, up-regulation of PRL signaling in these cells stimulated cell proliferation(Alkharusi, Lesma et al. 2016). Therapeutic approaches to block PRL signaling for breast cancer treatment have been postulated based on the proposed pro-oncogenic effects of PRL signaling(Jacobson,

Hugo et al. 2010, Damiano, Rendahl et al. 2013, Damiano and Wasserman 2013). Recently, blockage of PRL receptor signaling with G129R, an antagonist peptide of PRL, promotes autophagy-related cell death in human ovarian cancer cells(Wen, Zand et al. 2014). As a downstream mediator of PRL signaling, overexpression of wild-type STAT5 or constitutively active STAT5 promoted sporadic mammary cancers occurrence in mice, highly differentiated micropapillary and papillary adenocarcinoma developed after a latency of 8 to 12 months(Iavnilovitch, Cardiff et al. 2004). Meanwhile, breast tumor suppressive role of STAT5 is proposed based on the observation that many high grade breast tumors lack phosphorylated STAT5(Wagner and Rui 2008). More recently, PRL/STAT5 signaling interferes the function of BRCA1, implicating a tumor promoting role of PRL/Jak2 signaling(Chen and Walker 2016).

However, the PRL signaling effect on tumor initiation is not clear, animal models over-expressing PRL induced higher tumor occurrence only at late stage of the animal life span, when various cell types are prone to carcinogenesis due to aging. It is not clear neither at which step of tumor progression, nor which subpopulation of tumor initiating cells PRL exerts its growth promoting effect in these reported studies.

Generation of PRL receptor antagonists for treatment of advanced breast or prostate cancer has long been proposed(Jacobson, Hugo et al. 2010, Damiano and Wasserman 2013). However, previous developments of therapies aimed at reducing tumor growth by suppressing PRL production or by blocking its receptor failed unanimously, expelling PRL from the active participants in tumorigenesis. Recently, in a phase I

clinical trial of PRL receptor antagonist LFA102, a humanized PRL receptor binding monoclonal antibody targeting its extracellular domain, did not produce clinical efficacy in treatment of PRL receptor positive metastatic breast cancer or metastatic castration resistant prostate cancer despite its previously reported potent tumor suppressive potentials (Damiano, Rendahl et al. 2013, Agarwal, Machiels et al. 2016). The aforementioned molecular mimic of phosphorylated PRL S179D and G129R also possess strong preclinical data regarding treatment of various tumors, they were developed as clinical candidates(O'Sullivan and Bates 2016). G129R, when used in combination with other tumor suppressive agents like trastuzumab, demonstrated potent growth inhibition on Her2 positive breast cancer cells(Scotti, Langenheim et al. 2008). Another clinical trial of Jak2 inhibitor AZD1480 aimed to supress the STAT5 activation in prostate cancer(Plimack, Lorusso et al. 2013) was abandoned due to lack of efficacy albeit its strong precedent pre-clinical data(Gu, Liao et al. 2013).

4.2 A compelling different voice

First, the proposed autocrine proliferative function of PRL in breast cancer was subject to extensive interrogation. The well studied dopamine exerts the main inhibitory effect on pituitary PRL secretion(Freeman, Kanyicska et al. 2000). Hyperprolactinemia is a frequent adverse effect in patients treated with antipsychotic drugs due to their competitive binding to dopamine receptor D2 on the lactotroph cells. Concerns arise whether use of these drugs positively correlates with risk of breast cancer given the reported tumor promoting effect of elevated PRL, whereas no

causal link between either typical(Sabbe, Detraux et al. 2016) or atypical(Azoulay, Yin et al. 2011) antipsychotic drugs and development of breast cancer was found. Fond et al. concluded antipsychotic drugs as a class cannot be considered as a risk factor for breast cancer in humans(Fond, Macgregor et al. 2012). A recent population based cohort study found no increased risk of breast cancer among patients with hyperprolactinemia(Dekkers, Ehrenstein et al. 2015). In fact, the initial efforts to utilize bromocriptine, antagonist to dopamine, for treatment of breast cancer failed(Freeman, Kanyicska et al. 2000), although it was later argued bromocriptine does not alter the presence of locally produced PRL, which accounts for tumor growth and progression despite decreased serum PRL. Discrepancy recurred from a recent study in MCF7 cells lacking endogenous PRL expression, in which bromocriptine inhibited cancer cell proliferation independent of PRL involvement (Pornour, Ahangari et al. 2015). In a qPCR analysis of autocrine PRL expression in group of breast cancer cell lines, results showed non-detectable autocrine PRL in majority of cells lines tested. And T47D cells ectopically expressing PRL exhibited decreased PRL receptor expression and lower proliferation rate, suggesting locally synthesized PRL is unlikely to be a general mechanism promoting breast cancer progression(Nitze, Galsgaard et al. 2013).

On the contrary, accumulating evidence points to a protective effect of PRL exposure against breast cancer. An epidemiological study in patients with pre-existing breast cancer showed these patients benefit from pregnancy and lactation for prolonged survival despite elevated plasma PRL(Bercovich and Goodman 2009). In animal

studies, a previous pregnancy alone, and thus a PRL-driven lobuloalveolar development process in the mammary gland, or in combination with lactation after parturition confers protective effect against carcinogen exposure in rodents (Stoker, Robinette et al. 1999, Yang, Yoshizawa et al. 1999).

Secondly, results from laboratory studies also indicate a tumor suppressive effect of PRL and its signaling cascade. First line of evidence comes from its inhibition on proto-oncogene B-cell CLL/lymphoma 6 (BCL6), BCL6 is a transcriptional repressor(Chang, Ye et al. 1996) upregulated in poorly differentiated breast cancer, which competitively binds to DNA target sequences recognized by STAT5(Meyer, Laz et al. 2009). It seems the mutually exclusive STAT5 or BCL6 dominancy, i.e. the loss of PRL/STAT5 signaling and concomitant up-regulation of BCL6 expression represents a molecular switch for differentiation (STAT5 dominant) or transformation (BCL6 dominant) of breast epithelial cells (Tran, Utama et al. 2010). A further molecular dissection of these interactions expanded the role of PRL to regulation of MicroRNA-339-5p, PRL upregulates the MicroRNA-339-5p, which targets BCL6 for gene suppression(Yan, Zhao et al. 2016). Indeed, activation of STAT5 in human breast cancer cells like T-47D, ZR-75-1, and BT-20 following PRL stimulation increased E-cadherin expression in these cells and alleviated cell invasiveness (Sultan, Xie et al. 2005), accounting for the favorable prognosis associated with active STAT5 in human breast cancer(Nevalainen, Xie et al. 2004). In T47D cells, PRL stimulation inhibited the expression of TGF-β and matrix metalloproteinases (MMPs), counter-striking the tumor progression(Philips and McFadden 2004). On the other hand, restoring the

Jak2/STAT5 signaling cascade in mammary cancer cells reverses epithelial-mesenchymal transition (Sultan, Brim et al. 2008). In line with the pro-differentiating effect of active PRL/Jak2 signaling, suppression of Jak2 in immortalized HC11 mouse mammary epithelial cells leads to abrupted differentiation, hyper-proliferation and reduced rate of apoptosis correlated with inhibition of PRL induced STAT5 tyrosine phosphorylation and constative activation of oncoprotein STAT3(Xie, LeBaron et al. 2002). Canonical activation of STAT3 is through cytokines like IL-6 and non-IL6 cytokine family and receptor tyrosine kinases with intrinsic kinase activity like human epidermal growth factor receptor (EGFR/HER/ErbB), vascular endothelial growth factor receptor (VEGFR) and non-receptor tyrosine kinases like Src, as well as serine kinase PKCε. STAT3 is tightly regulated as a latent transcription factor in normal cells, however, it is constitutively activated in more than 40% of breast cancers and functions as a central linking point for a multitude of signaling processes in tumorigenesis(Banerjee and Resat 2016).

Another line of laboratory evidence comes from characterization of down-stream gene products following PRL/Jak2 signaling activation. A global profiling of PRL modulated transcripts in xeno-transplanted T47D cells following 48 hours of human PRL stimulation revealed PRL up-regulated genes were enriched in pathways involved in differentiation(Sato, Tran et al. 2013). Moreover, expression of Whey Acidic Protein (WAP) has been found in the milk of rodents and other species excluding human, its expression in mammary epithelial cells during late pregnancy and throughout lactation is regulated by PRL. Other than its initially assumed

functions as proteinase inhibitor and milk nutrient, WAP was found to inhibit lobulo-alveolar development *in vivo* and cell cycle progression of HC1 cells *in vitro* (Nukumi, Ikeda et al. 2004). And WAP reduced human breast cancer progression via reduction of tumorigenesis and invasion (Nukumi, Iwamori et al. 2007). Likewise, another PRL regulated gene, the milk protein α-casein, also functions as tumor suppressor in preventing breast tumor growth and metastasis (Bonuccelli, Castello-Cros et al. 2012).

The third line of evidence originates from previous work in the current lab. Restoring PRL/Jak2 signaling by transient co-expression of PRL receptor (the rat long isoform) and Jak2 in the triple negative MD-MBA-231 breast cancer cells suppressed its invasion at the presence of human PRL, and suppression of Jak2 with a kinase inhibitor in T47D cells sufficiently blocked PRL induced STAT5 phosphorylation, inducing ERK1/2 and SMAD2 activation, the latter was required for EMT and cancer metastasis(Nouhi, Chughtai et al. 2006). Later a negative cross talk of PRL signaling with Epidermal Growth Factor (EGF) was described, in which PRL induces tyrosine phosphorylation of growth factor receptor-bound protein 2 (Grb2) to attenuate EGF induced MAPK activation (Haines, Minoo et al. 2009). A tissue microarray analysis of PRL receptor gene expression in human breast cancer specimens revealed a significant down-regulation in invasive subtypes compared to benign cases (Hachim, Hachim et al. 2016). Recently, reconstitution of PRL/STAT5 signaling cascade by inducible exogenous PRL receptor expression in MDA-MB-231 cells led to reduced tumorigenesis in a xeno-transplant model (Lopez-Ozuna, Hachim

et al. 2016). These findings suggested the pro-differentiation roles of PRL/Jak2 signaling exert tumor-suppressive effect in breast cancer development.

4.3 What accounts for the protective role of pregnancy and lactation in breast cancer?

Reproductive factors like late age at menarche, parity and breastfeeding have been shown to protect against the development of breast cancer. It was proposed that reduced exposure to ovarian hormones and differentiation of breast lobules might account for the protective effect. Previous studies from multiple breast cancer cell lines and various mouse tumor models demonstrate PRL in breast cancer is not as a classic oncogene, neither an autocrine/paracrine growth factor.

A recent report suggested inflammatory breast cancer patients could benefit from combined treatment of conventional chemo/radio-therapies with reagents like PARP inhibitor or CDK2 inhibitor which sensitizing breast cancer stem cells(Curtin and Szabo 2013). Recently, PRL was proposed to be used as an adjuvant agent for breast cancer, not as tumor suppressor, but rather a mitogen to sensitize the dominant cancer stem cells for conventional chemo/radio therapy. Again, this is grounded on the prevailing notion that PRL functions as an autocrine/paracrine growth factor produced locally within the mammary glands, which sensitizes the cancer stem cells to conventional chemotherapy, as it induces proliferation of mitotic quiescent cancer stem cells.

Going back to the old question about what accounts for the protective role of breastfeeding and lactation against breast cancer, the answer might simply be PRL.

The elevated PRL production during pregnancy and lactation maintained the genomic integrity of mammary epithelial cells (see results and discussion), and its pro-differentiation role is exhausting the pool of error prone mammary stem cells, a major source of breast tumorigenesis.

Chapter 2: Materials and Methods

1. **Antibodies, plasmids and other reagents**

Antibodies and reagents are listed in supplemental table 1. Small hairpin shRNAs in the pLKO.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 pLKO.1 vector was obtained from Addgene (Addgene plasmid #1864). Human Jak2 cDNA in pCIneo vector was a generous gift from Dr. Olli Silvennoinen (University of Tampere Finland). Other reagents were used as described.

2.Cell lines and cell culture

Mouse mammary epithelial cell line HC11 (obtained from Dr. Nancy Hynes, Friedrich Miescher Institute, Basel, Switzerland) was routinely maintained in RPMI1640 medium (Wisent) containing 100mM L-Glutamine 10% fetal bovine serum (Gibco), 1% 50 units/mL Penicillin/50 units/mL Streptomycin (Wisent), 5 μg/ml bovine insulin (Sigma-Aldrich) and 10 ng/ml mouse epidermal growth factor (Sigma-Aldrich). (referred as growth media hereafter) Jak2-suppressed HC11 cell populations were generated by transfecting anti-Jak2 shRNAs with lipofectamin^{TM} 2000 (Invitrogen) and cells were screened with 1μg/ml puromycin (InviroGen) selection. Jak2-suppressed HC11 cells were rescued with expression of human Jak2/PCIneo

selected in 250μg/ml G418. PD032059 was used at 200nM (low) or 1μM (high) for 2D and 1μM for FACS.

3. Isolation and transfection of mouse mammary epithelial cells.

Mouse primary mammary epithelial cells were dissociated from mid-pregnant (E14-16) C57BL/6 (Jackson Mice) females using a kit from Stemcell Technologies following manufacture's instruction. 4,000 isolated mammary epithelial cells per well (MEC) were plated for 3D culture. Transfection of primary mouse mammary epithelial cells with Premo FUCCI Cell Cycle Sensor (ThermoFisher) is performed per manufacture's instruction. Transfected cells were synchronized with double thymidine block and 4,000 single suspended cells were plated in Matrigel.

4.3D culture

The Poly-D-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, 5,000 cells in 100 μL growth medium were plated and allow 1.5 hours for cells to attach. 100μL growth media 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 mammosphere outgrowth. The morphology of mammospheres were evaluated after 3 days of 5 different treatments:

(1) control (CTL): 2% FBS, (2) EGF: EGF 10ng/ml and 2% FBS or (3) HIP, 1μM hydrocortisone, 5μg/ml insulin, 2μg/ml ovine PRL and 2% FBS, (4) HI, 1μM hydrocortisone, 5μg/ml insulin and 2% FBS (5) PRL, 2μg/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 (5,000 cells/well) were plated for 3D culture.

5.RNA extraction and Quantitative PCR

Total RNA was extracted from cells with Trizol (Invitrogen) kit according manufacturer's instructions. Total RNA (1 μ g) was used as template for reverse-transcription using iScript (BioRad). The resulting cDNA was diluted 1:10 and subject to qRT-PCR analysis with SsoFast Sybr Green super mix (BioRad) on Rotorgene PCR cycler (Corbett) using primers specific for mouse Jak2, human Jak2, mouse E cadherin, mouse Slug, mouse Snail, mouse zeb1, mouse zeb2, mouse Vimentin and mouse GAPDH respectively in a 20 μl final reaction volume. Primer sequence was listed in supplemental table 3. Each sample was run in triplicates and normalized to GAPDH. Expression difference was assessed by $2^{-\Delta\Delta Ct}$ relative quantitative analysis.

6.STAT5 phosphorylation assay

Cells were grown to confluence then allowed to undergo differentiation for 1 day in RPMI1640 medium supplemented with 10% FBS, 5μg/ml insulin and 1 μM hydrocortisone. Cells were then starved or treated with ovine PRL for 10 minutes. Cells were lysed in 1% SDS lysis buffer (1% SDS, 50 mM Tris-HCl, pH8.0, 5mM EDTA pH8.0, 2.5mM EGTA pH8.0) and subject to SDS-PAGE and Western Blotting.

7.Western Blotting

Unless indicated otherwise, cell lysates were prepared from confluent cells grown for 2 days in 2D. Cells were washed and lysed in 1% SDS lysis buffer described above. Protein concentrations were determined by a bicinchoninic acid assay (Pierce Biotechnology). Equal amounts of protein were diluted in 4x reducing Laemmli buffer, loaded onto SDS-PAA gels, and transferred onto PVDF membrane (Millipore). Secondary antibodies for Western blots comprised HRP-conjugated donkey anti rabbit IgG and mouse IgG (Santa Cruz Biotechnology Inc.) and blots were visualized by enhanced chemiluminescence (ECL).

8.Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) analysis was performed. Cells were collected by calcium depletion with 10mM EDTA and 2.5mM EGTA in 1xPBS$^-$ and

filtered through 40-micron cell strainer (Becton Dickinson) to prepare single cell suspension after the indicated treatments. The cell surface markers CD49f and EpCAM were stained and analyzed on a FACSAria II cell sorter (Becton Dickinson). Gates were set in reference to negative controls stained with isotype antibodies conjugated to individual fluoro-chromes. For DNA content analysis, cells were collected in single suspension following the same procedure and stained directly with 0.5μg/ml DAPI for 5 minutes. Data were analyzed with Flowjo® software. (Tree Star Inc.)

9.Immunofluorescence staining and confocal microscopy

For all 2D staining, cells plated confluently on poly-D-Lysine (Sigma Aldrich) coated coverslips were fixed in 4% PFA for 10 minutes at room temperature, Samples were permeabilized in 0.1% Triton X-100/PBS (PBST) and blocked with 5% normal donkey serum in PBST for 1 hour; incubations with primary and secondary antibodies were done in the same buffer. Samples were mounted in FluorSave™ (Calbiochem) supplemented with 10 μg/ml DAPI to stain nuclei. Cells in 3D culture were fixed in 4% PFA with 5% Sucrose for 20 minutes at room temperature and stained as described above. Samples were imaged on a Zeiss 510 or 780 LSM confocal microscope with an Axiovert 200M microscope and a C-Apochromat 63x/1.2W Corr lens. Images showing single confocal slices were adjusted for brightness with Adobe Photoshop CS6 and composite images with scale bars were made with Adobe Illustrator CS6.

10.Statistical Analysis

Statistical significance was determined by a paired t test. P values less than 0.05 were considered significant.

11. list of supplemental tables

Supplemental Table 1: list of antibodies and reagents

Antibodies/Reagents	**Vendor Cat#**	**Application**
Rat ZO1 Ab	Invitrogen (339188)	1:100 IF
Rat E Cadherin Ab	Sigma(U3254)	1:200 IF
Mouse E Cadherin Ab	BD (611082)	1:2,000 WB
Rabbit Par3 Ab	Millipore (07-330)	1:100 IF,1:1000 WB
Rabbit aPKCζ Ab	Santa Cruz(sc216)	1:1,000 WB
Rat EpCAM Ab	eBioscience 17-5791-82	1:500 FACS
Rat CD49f Ab	eBioscience 12-0495-82	1:400 FACS
Mouse β-Tubulin Ab	Santa Cruz (sc53140)	1:2,500 WB
Rabbit Jak2 Ab	Millipore (06-255)	1μg for 1mg cell extract, IP
Mouse Jak2 Ab	Abcam (Ab37226)	1:1,000 WB
Mouse STAT5a Ab	Invitrogen (13-3600)	1:2,000 WB
Rabbit p-STAT5 Ab	Invitrogen (71-6900)	1:1,000 WB
Rabbit p-ERK1/2 Ab	Cell Signaling (9101)	1:1,000 WB
Rabbit ERK1/2 Ab	Cell Signaling (9102)	1:1,000 WB
Rabbit p-AKT Ab	Cell Signaling (9271)	1:1,000 WB

Rabbit AKT Ab	Cell Signaling (9272)	1:1,000 WB
Anti rabbit IgG HRP	Santa Cruz (sc2004)	1:5,000 WB secondary
Anti mouse IgG HRP	Santa Cruz (sc2005)	1:5,000 WB secondary
Anti rat 488	Invitogen (A21208)	1:200 IF
Anti mouse 546	Invitrogen (A10036)	1:200 IF
Anti rabbit 488	Invitrogen (A21206)	1:200 IF
Jak2 Inhibitor II	Calbiochem (420132)	20uM to inhibit Jak2 activity
Nile Red	Sigma (N3013)	100ng/ml stains lipid droplets
DAPI	Sigma (D9542)	1μg/ml Nucleus counter staining
Rabbit Pericentrin Ab	Abcam (Ab4448)	1:300 IF
Mouse γ-Tubulin Ab	Sigma (T6557)	1:500 IF
Rabbit γH2AX Ab	Abcam (Ab11174)	1:300 IF
Mouse β-Casein Ab	Santa Cruz (sc-166684)	1:100 IF
Rabbit BRCA-1 Ab	Abcam (ab16780)	1:100 IF 1:1000 WB
Mouse BRCA1 Ab	Santa Cruz (sc6954)	1:100 IF 1:1000 WB
Mouse CDC42 Ab	BD (610929)	IF not work, 1:1000 WB
PD0325901	Abcam (Ab120639)	200nM, 500nM, 1uM for FACS

Rabbit CDC42 Ab	Santa Cruz (sc-87)	IF not work, 1;1000 WB

Supplemental Table 2: list of shRNAs targeting mouse Jak2

Alias in this manuscript	**Products #**	**Mature antisense (5'to 3')**	**Efficacy**
J2	RMM3981-9591057	AATATGTTCCTTGTTGCCAGG	YES
J3	RMM3981-9591058	AACAGGCTGTTAAGATCACGG	NO
J4	RMM3981-9591059	AAATCCATTGATATTGGGCCG	NO
J5	RMM3981-9591060	ATCATTCGCATAAATTCCACG	YES
J6	RMM3981-9591061	ATTATGCCTCTGTAATGTTGG	YES

Supplemental Table 3**:** list of primers for qRT-PCR

Primers	**Sequence (5’ to 3’)**
Mouse Jak2 forward	GACATGATGAGAATAGCTAAGGAG
Mouse Jak2 reverse	TGGTAAGAATGTCTTGTAGCTG
Human Jak2 forward	GACAGATGATCGTGTTCCA
Human Jak2 reverse	TCATATAGATCTCATCTGGGCA
Mouse GAPDH forward	GAGAAACCTGCCAAGTATG
Mouse GAPDH reverse	TCAGTGTAGCCCAAGATG
Mouse Ecadherin forward	TGCCCAGAAAATGAAAAAGG
Mouse Ecadherin reverse	GTGTAYGTGGCAATGCGTTC
Mouse Vimentin forward	GAGAACTTTGCCGTTGAAGC
Mouse Vimentin reverse	GCTTCCTGTAGGTGGCAATC
Mouse Slug forward	CTCACCTCGGGAGCATACAG
Mouse Slug reverse	GACTTACACGCCCCAAGGATG
Mouse Snail forward	AAGATGCACATCCGAAGCCA
Mouse Snail reverse	CTCTTGGTGCTTGTGGAGCA
Mouse Zeb1 forward	GTTCTGCCAACAGTTGGTTT
Mouse Zeb1 reverse	GCTCAAGACTGTAGTTGATG
Mouse Zeb2 forward	TCTGAAGATGAAGAAGGCTG
Mouse Zeb2 reverse	AGTGAATGAGCCTCAGGTAA

Chapter 3: Results

To detail the mechanisms by which PRL induces mammary epithelial differentiation and examine the potential alternative roles of PRL other than the conventionally recognized lactogen hormone, we first established a mammary epithelial cell 3D culture model system and examined the role of PRL/Jak2 signaling in mammary epithelial cell morphogenesis. We evaluated the role of PRL during establishment of apical/basal polarity and cell fate determination of mammary epithelial cells. We next examined the role of PRL/Jak2 signaling in regulation of positioning mitotic spindle during mitosis and tissue homeostasis of mammary epithelial cells, postulating a model on mammary acini development in the presence of PRL. A surprising finding led us to examine how PRL/Jak2 signaling is involved in the regulation of centrosome duplication and maintenance of genomic stability. Besides, I examined the effect of PRL on CDC42 activity in HC11 cells to answer whether PRL promotes mammary epithelial cell polarization via CDC42 activation. I also explored the potential role of PRL in mediating distribution of polarity complexes and regulation of planar cell polarity in mammary epithelial cells. Naturally, not all attempts were successful, no conclusive data was achieved after tremendous efforts. These trials are reported in the results section of this thesis to spur further discussion and investigation.

1.PRL 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 lines was instrumental in deciphering the contributions of hormonal/growth factors, extracellular matrix proteins and intracellular signaling pathways in mammary epithelial morphogenesis, gland (Shaw, Wrobel et al. 2004, Lo, Mori et al. 2012). 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 (Figure 3.1). They require EGF for cell proliferation and undergo differentiation following PRL stimulation (Ball, Friis et al. 1988, Taverna, Groner et al. 1991). We found that HC11 cells grown in the presence of EGF to form large disorganized mammospheres 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 mammospheres in which the cells are polarized and organized around a single lumen resembling the inner lobular core of alveoli in lactating mammary glands (Figure 3.2A, left panel). To distinguish the specific effector hormone mediating these structural organizations, cells were grown in the presence of PRL alone, HI or serum (CTL). Interestingly, only PRL treated cells showed organized mammary acini. In contrast, no organized acini were observed in HI or serum treated cells (Figure 3.2A, left panel). 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 (MEC)

dissociated from mid-pregnant C57BL/6 mice. Importantly, similar organized alveolar acini were observed only in cells treated with either a mixture of HIP or PRL alone (Figure 3.2A right panel). To enumerate the observed effects of PRL on cellular polarization, we quantified the organized acini within the various treatments. As can be seen in Figure 3.2B & Figure2.2C, 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 (Figure 3.3C). Moreover, the size of the mammospheres grown in PRL treated cells were the smallest of all (Figure 3.3D). To verify the specificity of PRL in inducing the acini development, a kinase inhibitor specific to Jak2(Jak2I) was added to primary mammary epithelial cells cultured in 3D at presence of HIP, acini development was completed abolished with Jak2I treatment (Figure 3.4). 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 the triglyceride-rich cytoplasmic lipid droplets(Greenspan, Mayer et al. 1985). Only fully differentiated alveolar mammary epithelial cells are capable of synthesis and secretion of milk lipids(Russell, Palmer et al. 2007). Indeed, formation/accumulation of lipid droplets is contingent upon alveolar cell secretory differentiation (Russell, Schaack 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 (Figure 3.2D). 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 (Figure 3.3E). To further demonstrate the functionality of these acini, we stained these acini with antibody against β-Casein, and our results showed positive β-Casein staining in HIP and P treated primary mammary epithelial cells (Figure 3.5). This data together indicates that PRL is required for lipid droplet apical trafficking/accumulation, signature of alveolar cell secretory differentiation.

2.PRL/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 (Figure 3.6A-C). Moreover, we verified Jak2 knockdown in J6 cells using qRT-PCR (Figure 3.6D). We then generated J6Rh stable cell line overexpressing human Jak2 cDNA in J6 cells for rescue experiments (Figure 3.6E). To further evaluate the extent of loss of Jak2 in J5

and J6 cells, we used Stat5 phosphorylation as readout of PRL/Jak2 signaling. In contrast to parental and vector transfected cells, PRL was unable to induce Stat5 phosphorylation in J5 and J6 cells (Figure 3.7A). Whereas PRL induced Stat5 phosphorylation was restored in J6Rh cells (Figure 3.7A). We next examined the ability of J5 and J6 cells to form acini in 3D culture. Importantly, both cell lines failed to form organized acini in the presence of HIP (Figure 3.6F) or PRL (data not shown). Of note, J5 and J6 cells grown in EGF showed striking morphological features. These colonies formed disorganized spreading cell clumps with diffused ZO-1 and E-cad staining (Figure 3.6F). To get a better understanding of the role of Jak2 in cellular junction formation, we next examined the localization of cellular junction marker proteins ZO-1 and E-cad in 2D culture model. Interestingly, we found a complete mislocalization of ZO-1 and E-cad proteins in both J6 and J5 cells, showing diffused cytoplasmic staining, while rescuing Jak2 expression was able to restore membrane localization of both markers in J6Rh cells (Figure 3.6G top panel, Figure 3.7B). These results emphasize the critical role of Jak2 in cellular junction 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, Dickinson 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 (Figure 3.6G bottom panel,

Figure 3.7C). Although western blotting results suggested no differences in expression levels of Par3 as well as other par-complex proteins including aPKCζ and lgl (Figure 3.6H). Importantly, similar to ZO-1 and E-cad, the membrane localization of this junctional protein marker Par3 was restored in J6Rh cells (Figure2.7H). Together, this data highlight 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, Chughtai et al. 2006, Haines, Minoo et al. 2009). We compared the Erk1/2 activation levels between the parental HC11 cells and J6 cells. Interestingly, as can be seen in Figure 3.7D, 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) (Figure 3.7D). 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 (Figure 3.6I). 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 Erk1/2 inhibitor PD0325901 for 24hr and cell/cell junction formation was examined. Notably, treatment of J6 cells with PD0325901 fully restored the membrane localization of ZO-1, E-cad and Par3 (Figure 3.7E and F). Together, this data underscore Jak2 regulation of cellular junctional organization through modulation of Erk1/2 activities.

3.PRL promotes the maturation of mammary luminal progenitor cells

During alveologenesis, 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 mammary stem cell 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, HI, HIP or PRL (Figure 3.8A). In control EGF treated cells, we identified two distinct cellular sub-populations, one featured a surface marker signature with $EpCAM^{hi}/CD49f^{hi}$ defining the luminal progenitor cells and a second population with $EpCAM^{hi}/CD49f^{low}$ defining mature luminal cells. Interestingly, treatment of HC11 cells with HIP or PRL resulted in the differentiation of luminal progenitor ($EpCAM^{hi}/CD49f^{hi}$) cells into mature luminal ($EpCAM^{hi}/CD49f^{low}$) cells (Figure 3.8A). Indeed, we observed a significant ~30% of luminal progenitor cells differentiated into mature luminal cells in these samples (Figure 3.8B). 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 (Figure 3.8A&B). Moreover, histogram display of individual markers confirmed the decrease in expression of CD49f (Figure 3.8, C1) and the increase in expression of EpCAM (Figure 3.8, 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 (Figure 3.8, 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 (Figure 3.8D). Importantly, our data show that treatment of primary cells with either PRL or HIP induced the maturation of progenitor ($EpCAM^{hi}/CD49f^{hi}$) cells into mature luminal ($EpCAM^{hi}/CD49f^{low}$) 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 (Figure 3.8E). 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 48hr time point (Figure 3.8F). Histogram display of CD49f further showed that there was sustained expression of CD49f in J6 cells (Figure 3.8G) in comparison to HC11 cells whereas no significant change in EpCAM expression was found in the two different cell lines (Figure 3.8H). 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 EpCAM^{hi}/CD49f^{hi} to mature EpCAM^{hi}/CD49f^{low} cells (Figure 3.8I). This transition process was not further enhanced by the addition of PRL (Figure 3.8I). 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.Loss of Jak2 leads to centrosome amplification and DNA damage accumulation in mammary epithelial cells.

Polarized epithelial cells undergo temporary depolarization during proliferation. Orientation of cellular division, the axis of cell division, i.e. the orientation of the mitotic spindle is determined through positioning the mother/daughter centrosomes in their destined cortical membrane, which is determined through interactions between the astral microtubules and the cortical membrane. Centrosomes are subcellular organelles function as microtubule organization center (MTOC) for organization of the interphase microtubule elongation, organization of mitotic spindle and chromosome segregation during cell mitosis and serve as basal body for cilia development in somatic epithelial cells. I first set to check if the mitotic spindle assembly is affected when Jak2 expression is suppressed by siRNA or shRNA.

Importantly, Jak2-suppressed HC11 cells show abnormal centrosome numbers in comparison to control HC11 cells. Cells were stained with γ-Tubulin and pericentrin as markers of centrosomes, we found Jak2 KD HC11 cells exhibit multiple centrosomes (Figure 3.9). To eliminate any potential off-target effect of anti Jak2 shRNAs, we also used siRNA targeting Jak2 for Jak2 suppression. The efficacy of Jak2 siRNA was validated via both Jak2 probing (Figure S3.10A) and phsopho-STAT5 probing following 10 minutes of PRL stimulation (Figure S3.10B). When examining the mitotic spindle assembly after synchronization of the cells with double thymidine block in parental HC11 and Jak2-suppressed HC11 cells with shRNA or siRNA targeting Jak2, we found Jak2 KD cells to exhibit multipolar mitotic spindles (Figure 3.10). Quantification of centrosome aberrations in Jak2 KD cells showed more than 25% of the total cell population harbor multiple centrosomes in Jak2 KD cells (Figure 3.11). Of note, the presence of multipolar spindle during mitosis does not necessarily confer growth disadvantage to these Jak2 KD cells. MTT assays showed similar proliferation rate and cell viability of Jak2 KD cells compared to parental HC11 cells (data not shown).

During mitosis, excess copies of centrosomes might undergo clustering to form pseudo-bipolar mitotic spindles to complete mitosis(Fujiwara, Bandi et al. 2005), whereas it is not observed in these Jak2 KD cells in over 1,200 dividing cells containing multiple centrosomes (over 1,200 metaphase cells were counted in total for the quantification in figure 3.11). Those Jak2 KD cells harboring multiple centrosomes in G0 stage are either progenies inherited more than one copy of

centrosome during mitosis or resulted from defects in centrosome duplication. Given the low frequency of viable progenies from cells harboring multipolar mitotic spindles, the latter is more likely the case. We also examined the DNA contents of Jak2 KD cells by FACS. These analyses showed no difference between Jak2 KD cells and the parental HC11 cells with similar low percentage (<1% of the total cells, marginal and negligible) of tetraploid cells with double DNA contents were detected. This data excludes the possibility that the multiple centrosomes phenotype observed in Jak2 KD cells is the result of failure of cytokinesis (data not shown). Indeed, repeated rounds of cytokinesis failure in normal diploid cells cannot trigger either centrosome amplification or cellular transformation(Krzywicka-Racka and Sluder 2011).

Cells have developed a responsive machinery to ensure high fidelity heritage of DNA content to the progenies. Cell cycle progression is arrested upon detection of DNA damage, and each centrosome duplicates only once in one cell cycle for bipolar spindle assembly during mitosis. As a general consequence of de-regulated mitotic spindle organization during mitosis, delayed or unequal segregation of chromosomes introducing aneuploidy and further DNA damage to the cell, which is the predominant type of genomic instability found in human solid tumors(Storchova and Pellman 2004). To evaluate the detrimental effect of multiple centrosomes and multipolar mitotic spindle assembly observed in Jak2 KD cells, we evaluated nucleus DNA damages in those cells, using γH2AX as a marker of DNA double strand breaks, which is the most commonly occurring DNA damages during DNA hemostasis. We found more severe DNA damages in those Jak2 KD cells with both siRNA and

shRNA targeting Jak2(Figure 3.12). To exclude any potential off-target effect of siRNA or shRNAs, we also suppressed Jak2 activity using Jak2 kinase inhibitor II, which specifically inhibits the auto-phosphorylation of Jak2 during its activation. We found inhibitor treatment also leads to DNA damage accumulation similar with those in Jak2 KD cells (Figure 3.13). In addition to these previously discussed DNA damage response modulators like BRCA1, p53 and PARPs (refer to chapter 1), Jak2 is recently shown to localize to the centrosome as well(Jay, Hammer et al. 2015). However, the Jak2 antibodies used in this lab failed to detect the centrosome localization in both human and mouse cells (data not shown). We cannot conclude any causal/consequence relationship between the observed multiple centrosomes and DNA damage accumulation at this moment, however, involvement of Jak2 in regulation of centrosome duplication or DNA damage repair, and a step further, in maintenance of genome stability is implicated.

5.Breast cancer cells exhibit centrosome amplification and DNA damage accumulation.

Cancer cells show high frequency of centrosome aberrations, under most circumstances centrosome amplification and consequent genomic instability. We next examined the centrosome amplification and DNA damage accumulation in various breast cancer cell lines including T47D, MCF7, and MDA-MB-231 cells representing different breast cancer subtypes. We found centrosome amplification and DNA damages are most severe in high grade breast cancer cells. T47D, MCF7 (both belong

to the luminal subtype) and MDA-MB-231 (belongs to the TNBC subtype) cells were stained for centrosome formation. Our results show low frequency of excess centrosomes in the luminal T47D and MCF7 cells, however, in MDA-MB-231 cells, cells harboring multiple centrosomes are more frequent than in T47D and MCF7 cells (Figure 3.14). We next evaluated the DNA damage accumulation in these breast cancer cells by γH2AX staining. Our results showed universal DNA damage in all three breast cancer cell lines tested, with MDA-MB-231 exhibiting more severe DNA damages with stronger γH2AX staining in comparison to T47D and MCF7 cells (Figure 3.15).

One major DNA damage response gene is BRCA 1, mutations of BRCA1 predispose to breast and ovarian cancer. BRCA1 maintains genome integrity through its ability to repair DNA damage, G2/M checkpoint control and regulation of centrosome duplication. It localizes to centrosomes at all phases of the cell cycle(Broustas and Lieberman 2014). We examined its localization in these breast cancer cells. Our results showed the centrosome localization of BRCA1 is maintained in all three breast cancer cells tested (Figure 3.16). Our attempt to examine if BRCA1 localization is disrupted in events of Jak2 suppression was not successful due to lack of working antibody recognizing the mouse BRCA1 protein (Figure 3.17). In western blot probing for BRCA1 in both mice and human cell lysate, a weak band above 200kDa was detected, which is close to its calculated molecular weight of 207kDa. Although we observed an upregulation of BRCA1 in HC11 cells transfected with siRNA targeting Jak2, it remains skeptical due to the lack of positive control to validate the

efficacy and specificity of the antibody used in this study (data not shown).

In addition, Jak2 KD leads to a mesenchymal phenotype of HC11 cells. Quantitative RT-PCR was used to analyze the mRNA expression levels of various well known mesenchymal proteins including Slug, Snail, Vimentin, Zeb1 and Zeb2, in addition to the epithelial marker E-cadherin. We noticed a large suppression of E-cadherin and concomitant upregulation of mesenchymal markers in Jak2 KD cells in comparison to control HC11 cells. Furthermore, exogenous expression of human Jak2 cDNA in Jak2 KD cells partially suppressed the expression level of these mesenchymal markers and restored the expression of E-cadherin (Figure 3.18). In addition, cytokeratin 5 has been used as a marker of basal mammary epithelial cells. Normally, HC11 cells exhibit less than 10% CK5 positive cells. In contrast and importantly, the CK5 positive cells increased to over 50% in Jak2 KD HC11 cells (Figure 3.19), suggesting a dramatic phenotype change in the mammary epithelial phenotype following Jak2 suppression.

6. Exploring the role of PRL/Jak2 signaling in planar cell division

Maintenance of epithelial homeostasis in either monolayer or glandular morphology requires proper mitotic spindle orientation to establish the right axis of cell division. Oncogenic transformation such as Ras hyper-activation in epithelium leads to neoplasia characterized with multiple layering in epidermis and lumen filling in glandular structures in kidney and breast tissues, indicating loss of cell polarization

and disrupted planar cell division. In our 3D study, we observed PRL/Jak2 signaling is essential for proper acini development in mouse mammary epithelial cells. Cells demonstrated lumen filling phenotype mimicking oncogenic transformation in absence of PRL/Jak2 signaling. Of note, acini developed in the presence of PRL showed no sign of apoptosis, indicating the lumen expansion is probably achieved through controlled symmetric cell division. This suggested a potential role of PRL/Jak2 signaling in regulating the planar cell division during acini development. we hypothesize PRL/Jak2 mediates planar cell division during mammary acini development, for cells growing at the presence of PRL, the cell division falls in the plane parallel to base membrane, whereas orientation of cell division is randomized for mammary epithelial cells growing without PRL (Figure 3.20). We first tried immunofluorescent staining of centrosomes for mouse mammary epithelial cells cultured in 3D, we encountered technical difficulties including lack of working antibodies visualizing the centrosome and mitotic spindle and poor enrichment of metaphase mammary epithelial cells because the low serum culture condition is not favoring proliferation. We next adapted another system to visualize the dividing mammary epithelial cells. In brief, mammary epithelial cells were transfected with fluorescence labeled cell cycle indicators which discriminate the metaphase cells with GFP expression, those cells were then synchronized with thymidine block and plated in Matrigel as suspended single cell with or without PRL. Samples were visualized under confocal microscopy at various time points at 1 hour intervals after a quick fixation in 4% PFA. Again, very few proliferating cells were captured, and the cell

division plane cannot be evaluated in this system (Figure 3.21). However, it remains an interesting question to answer when new approaches to distinguish the dividing cells are available.

7. Examine the role of CDC42 in PRL/Jak2 signaling

Of the three members of the Rho family small GTPase, Rho, Rac, and CDC42, which play a crucial role in regulating the organization of the actin cytoskeleton in response to extracellular stimuli, CDC42 is critical for diverse cellular functions including the regulation of actin organization, cell polarity, intracellular membrane trafficking, transcription, cell cycle progression and cell transformation. CDC42 cycles between the active GTP-bound state and the GDP-bound inactive state in response to various signaling events. Previous studies revealed a phosphoinositides distribution pattern featuring high PtdIns(3,4,5)P3 (PIP3) at the basal membrane and low concentration of PtdIns(4,5)P2 (PIP2) at the apical membrane (Yu, O'Brien et al. 2003). It was postulated the tumor suppressor gene PTEN modulated apical segregation of phosphoinositides control epithelial morphogenesis through CDC42 in MDCK cells(Martin-Belmonte, Gassama et al. 2007). PTEN acts as dual-specificity phosphatase which preferentially dephosphorylates phosphoinositide substrates PIP3 to antagonize PI3K/Akt activity, as a target of oncoMiR MiR21(Melnik 2015),it is mutated in various cancers with high frequency (Hopkins, Hodakoski et al. 2014).PTEN cooperates with p53(Nakanishi, Kitagishi et al. 2014) and BRCA1(Minami, Nakanishi et al. 2014) in DNA damage repair and tumor

suppression. Mammary epithelium might utilize the similar mechanism during morphogenesis. Our study has confirmed a promotional role of PRL in polarization of mammary epithelial cells, we are interested to explore if PRL regulates CDC42 activity regulation during mammary epithelial cell polarity establishment as transient ectopic expression of PTEN in Jak2-suppressed HC11 cells partially restored the junction organization at the presence of PRL (Figure 3.22). We first ran GST pull down assays to determine whether PRL will induce CDC42 activation or inhibition in HC11 cells. HC11 cells were lysed after stimulation with PRL for different time intervals and CDC42-GTP was pulled down with GST-PAK-CRIB beads (recombinant protein purified from the proteinase null BL21 *E.Coli*, consisting of GST fused to the CDC42 binding domain of the human p21 activated kinase 1 protein and coupled to glutathione beads), HC11 cells treated with EGF was used as positive control for CDC42 activation. As a backup plan for this experiment in case the basal level of CDC42 activation in HC11 cells before PRL stimulation is too low to be detected, and PRL further inhibits the cdc42 activity, we compared the basal level of CDC42-GTP between HC11 cells and Jak2 KD cells to determine whether high/low CDC42 activation is needed for proper junction organization. To our surprise, CDC42-GTP level is high in all samples and no change was detected after these described manipulations (data not shown).

We understand the overall CDC42 activation level could not well reflect its spatial and temporal modulation. We tracked the CDC42 activation upon PRL administration by immunofluorescent staining. GTP-CDC42 can be stabilized after TCA

fixation(Hayashi, Yonemura et al. 1999, Nishimura and Yonemura 2006), which better preserves the active form comparing to the conventional PFA or methanol fixation. We stained CDC42 with antibodies from various vendors and none of them seemed working in our system (data not shown). Given the pivotal role CDC42 plays in various biological processes, it is always interesting for a revisit of this question using different approaches like use of dominant negative CDC42 constructs, tracing of activation and potential relocation of CDC42-GTP using live imaging (Nalbant, Hodgson et al. 2004), and antibody specific to phospholipids PI (4,5)P2 to evaluate the effect of PRL on phospholipids distribution(Elong Edimo, Ghosh et al. 2016).

8.Figures in chapter 3

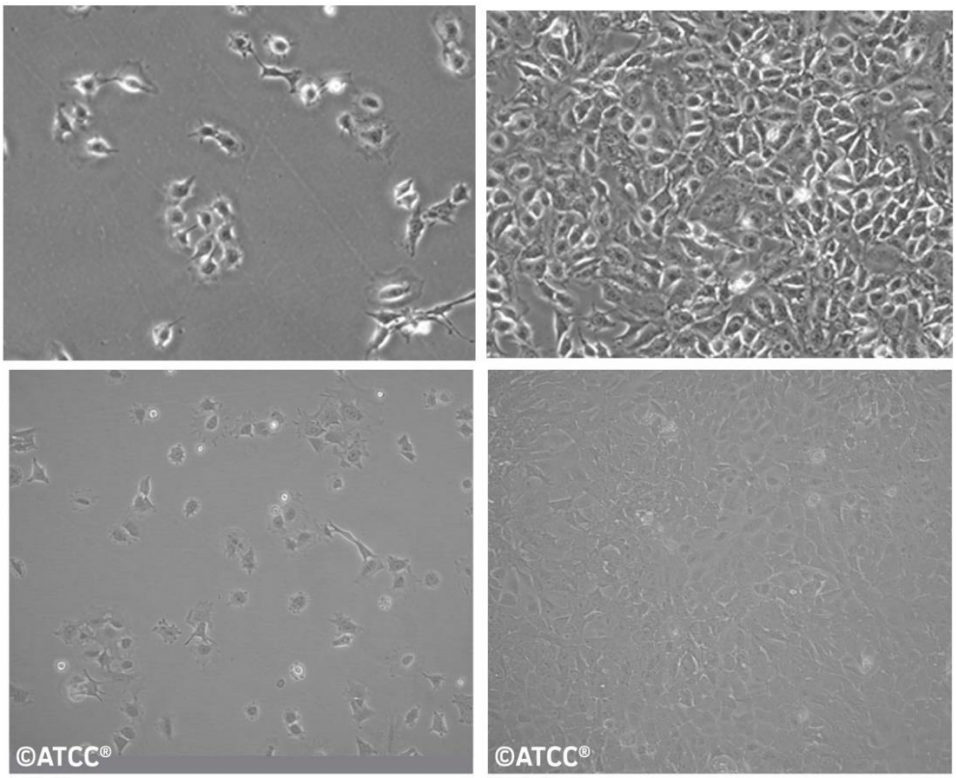

Figure3.1

Figure 3.1 Morphology of HC11 cells (top panels) cultured in 2D in comparison with ATCC reference images (bottom panels, ATCC number CRL-3062) growing at low (left) and high (right) densities in RPMI-1640 medium.

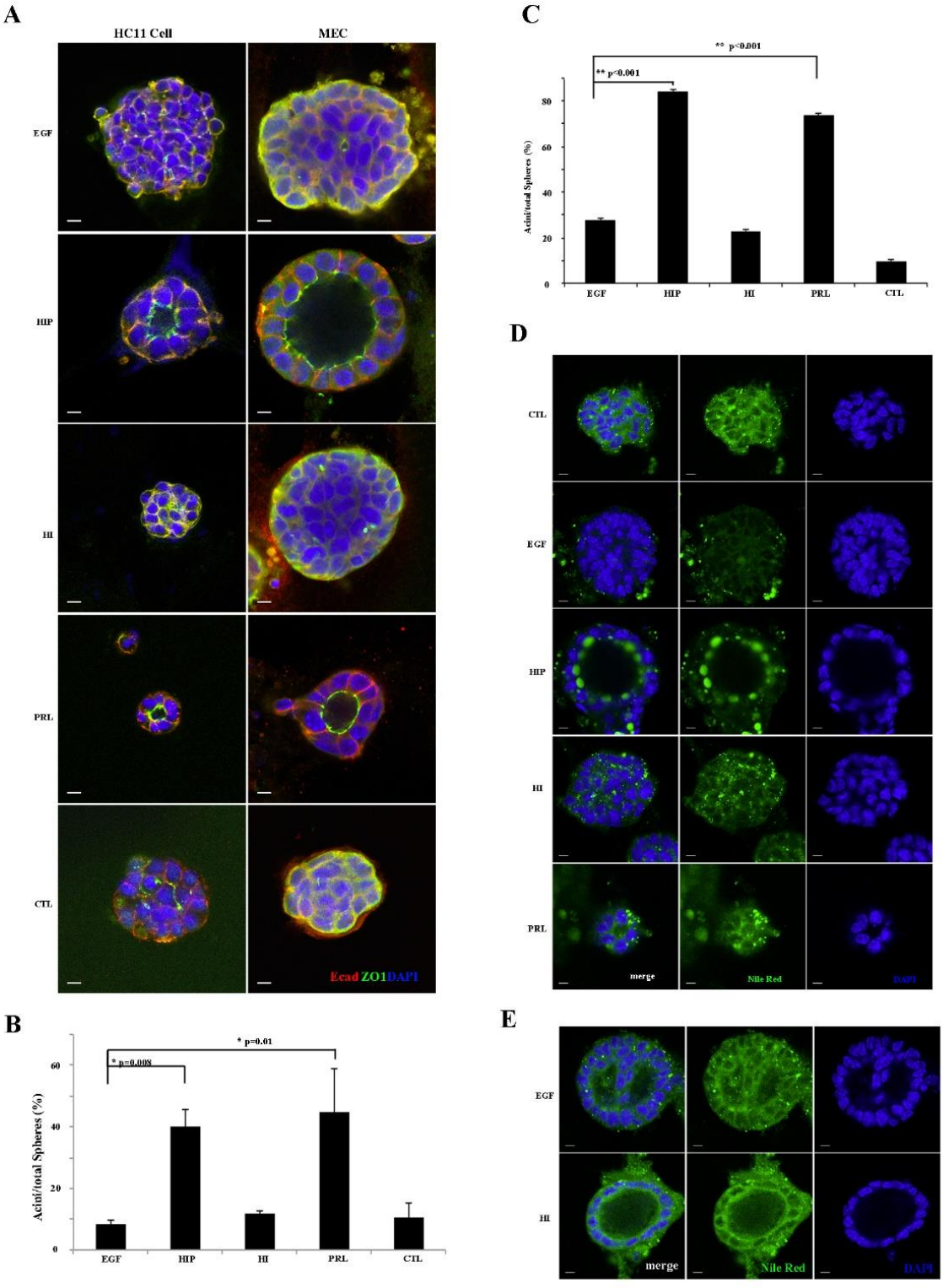
A
HC11 Cell
MEC
EGF
HIP
HI
PRL
CTL
Ecad ZO1 DAPI
B
* p=0.01
* p=0.008
Acini/total Spheres (%)
EGF
HIP
HI
PRL
CTL
C
** p<0.001
** p<0.001
Acini/total Spheres (%)
EGF
HIP
HI
PRL
CTL
D
CTL
EGF
HIP
HI
PRL
merge
Nile Red
DAPI
E
EGF
HI
merge
Nile Red
DAPI

Figure 3.2

Figure 3.2: PRL induces mammary acini morphogenesis (A) HC11 and primary mouse mammary epithelial cells grown in 3D culture (Materials and Methods) were stained with antibody to ZO1 (green) and E-cad (red). Nucleus was counter stained with DAPI (blue). (**B)** Percentage of acini/total mammospheres (>100 mammoshpere in duplicates) in HC11 cells and (**C**) in primary mammary epithelial cells. Error bars represent mean ± s.e.m. of 3 independent experiments. P values derived from unpaired two-tailed Student's *t*-test. (**D)** & **(E)** MECs in 3D culture were stained with 100ng/ml Nile Red for 20min and pseudo-colored in green. Nuclei were counter stained with DAPI. Scale bar, 10μm.

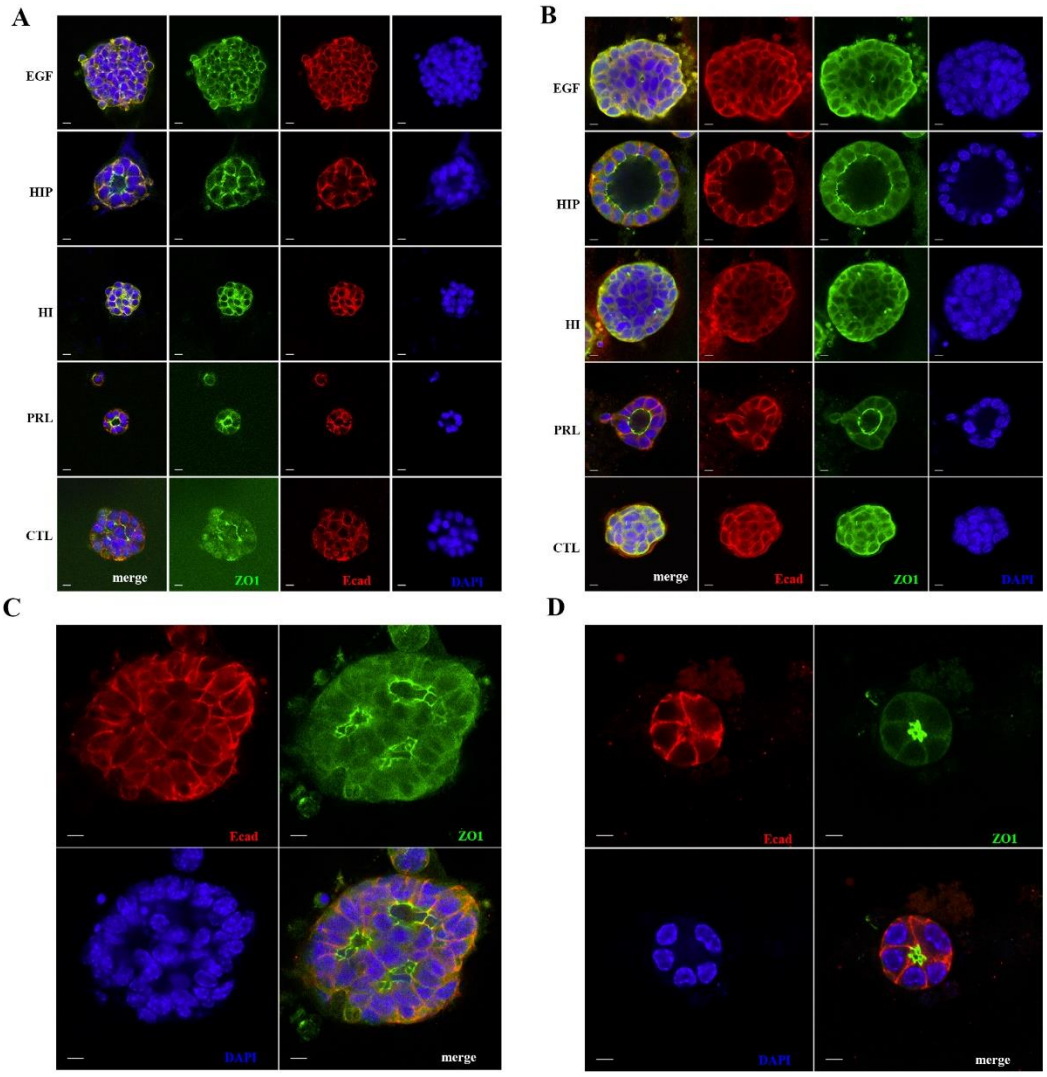

Figure 3.3

Figure 3.3: Mammary acini development in 3D is dependent on PRL

(A) HC11 cells and **(B)** primary MECs grown in 3D culture were stained for ZO-1 (green) and E-cad (red). **(C)** MECs grown in 3D culture in the presence of EGF or **(D)** in the presence of PRL were stained for ZO-1 (green) and E-cad (red). Merged images show nucleus counter-stained with DAPI (blue). Scale bar 10µm.

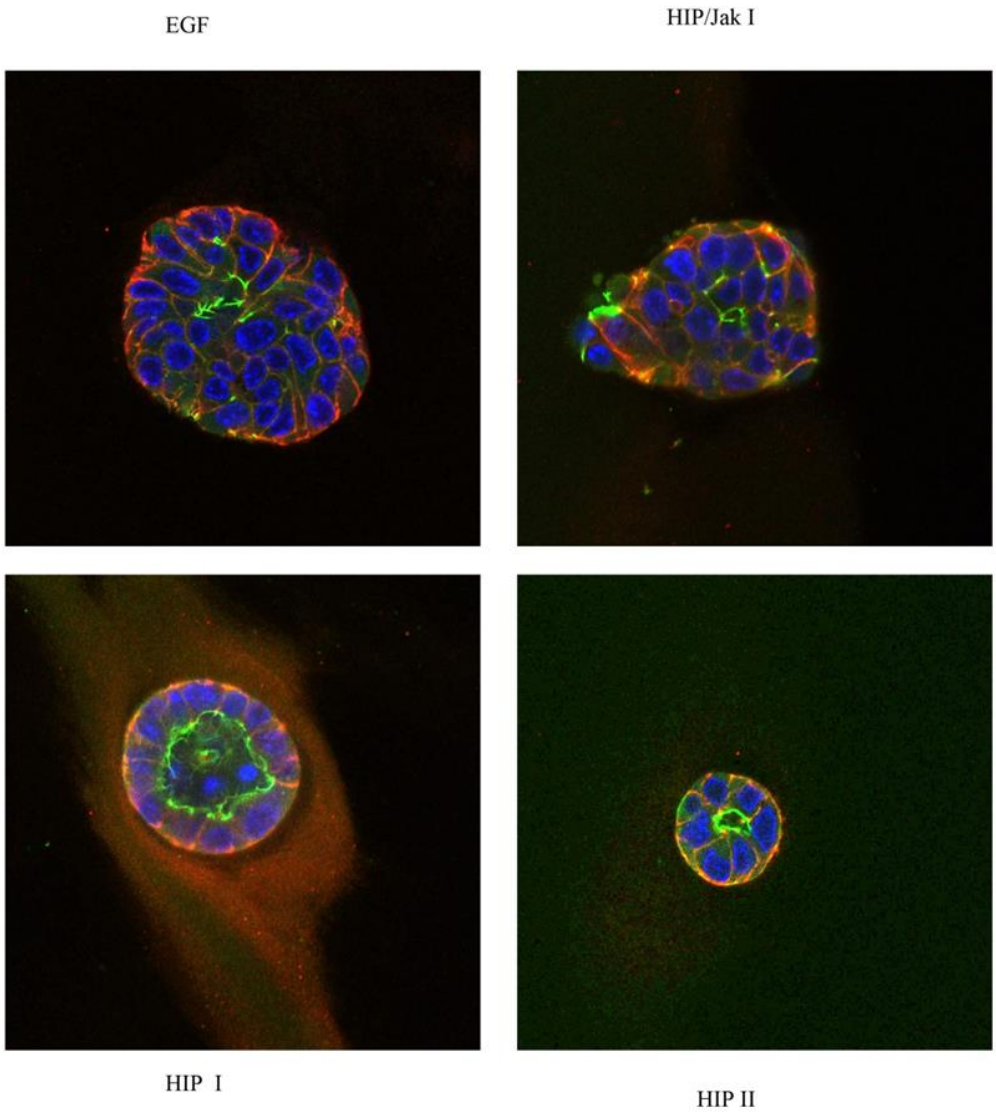

Figure 3.4

Figure 3.4 Jak2 Inhibitor treatment abolishes acini development at presence of PRL. Primary MECs grown in 3D culture in the presence of EGF (top left) or in the presence of HIP (the other 3 panels) were stained for ZO-1 (green) and E-cad (red), top right panel showed abolished acini development at presence of Jak2I, two representative acini were shown in bottom panels. Merged images show nucleus counter-stained with DAPI (blue).

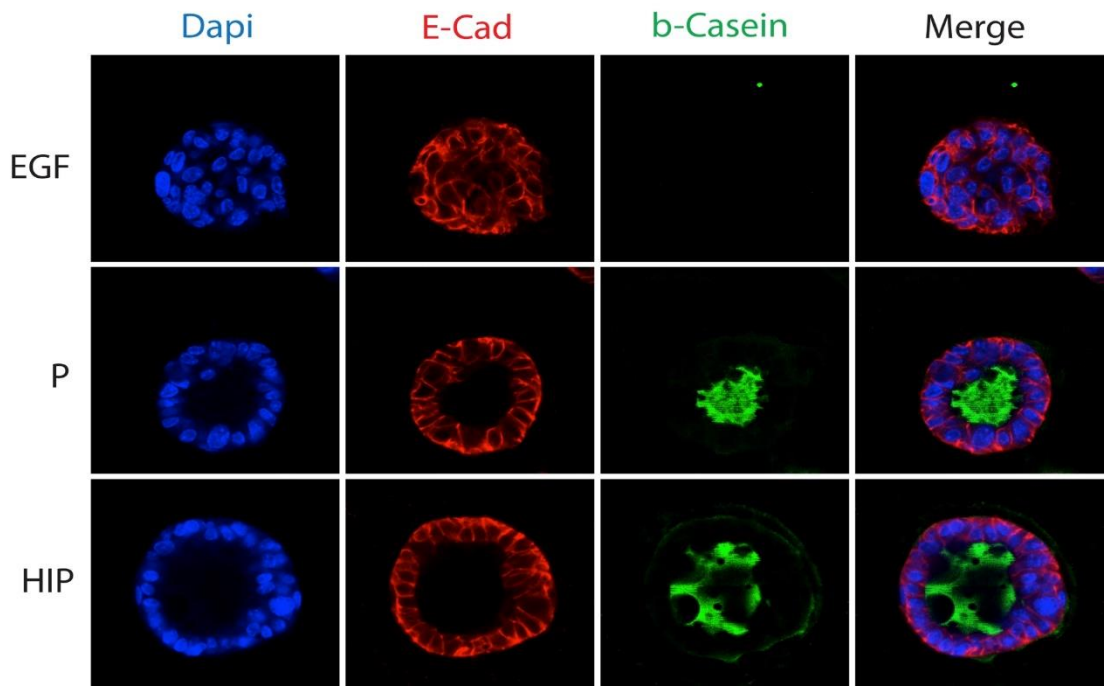

Figure 3.5

Figure 3.5: β-Casein staining in primary MECs isolated from mid-pregnancy mice. Primary MECs were isolated and plated in 3D Matrigel as described previously, cells were treated with EGF (top panel), PRL (middle panel) or HIP (bottom panel) respectively. β-Casein (green) expression were only detected at the presence of PRL in middle and bottom panels. Unpublished data, courtesy of Ms. Alaa Moamer.

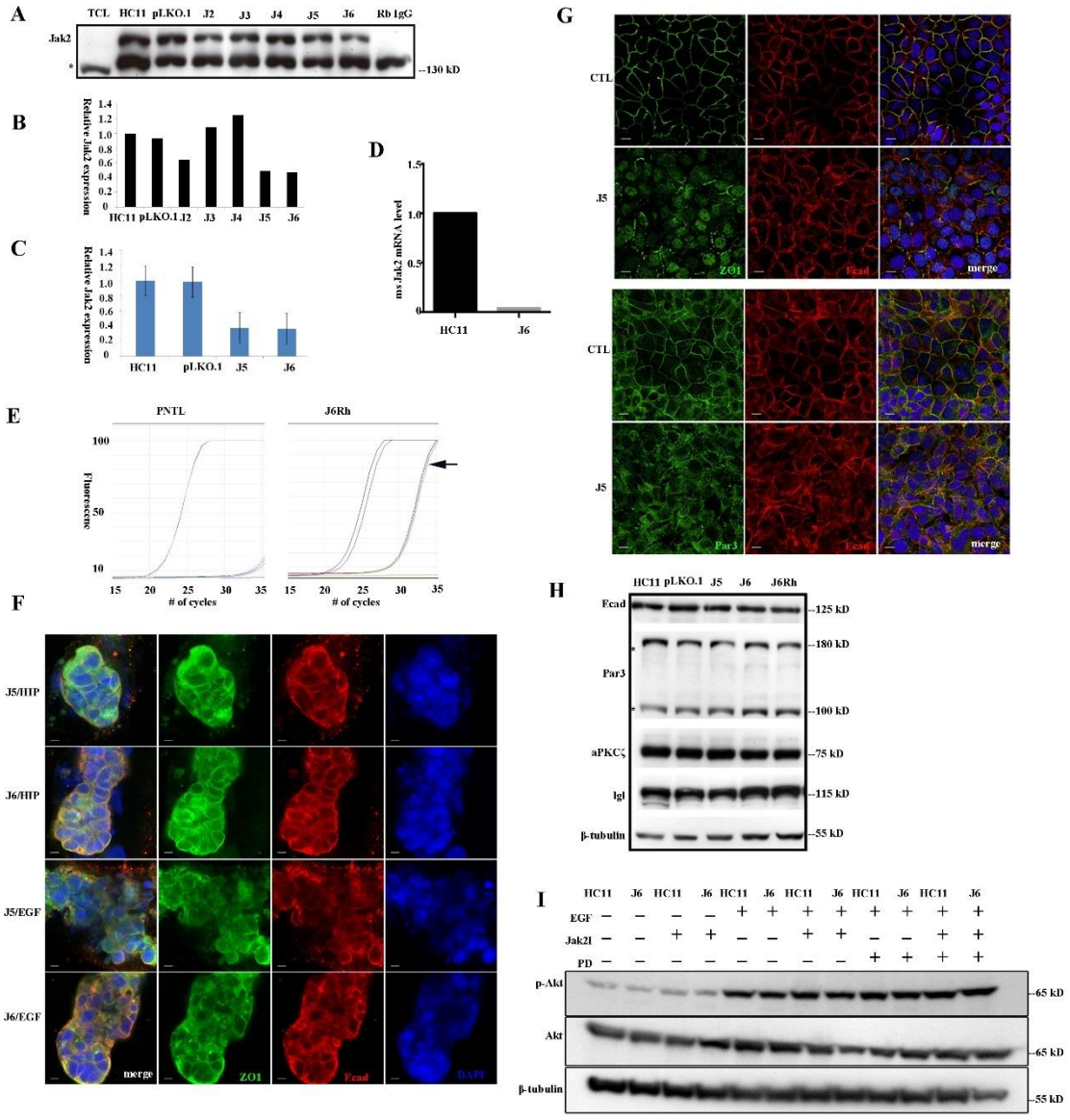
A
TCL HC11 pLKO.1 J2 J3 J4 J5 J6 Rb IgG
Jak2
*
~130 kD
B
Relative Jak2 expression
HC11 pLKO.1 J2 J3 J4 J5 J6
C
Relative Jak2 expression
HC11 pLKO.1 J5 J6
D
ms-Jak2 mRNA level
HC11 J6
E
PNTL
J6Rh
Fluorescence
# of cycles
F
J5/HIP
J6/HIP
J5/EGF
J6/EGF
merge
ZO1
Ecad
DAPI
G
CTL
J5
ZO1
Ecad
merge
Par3
H
HC11 pLKO.1 J5 J6 J6Rh
Ecad
Par3
aPKCζ
Igl
β-tubulin
125 kD
180 kD
100 kD
75 kD
115 kD
55 kD
I
HC11 J6 HC11 J6 HC11 J6 HC11 J6 HC11 J6 HC11 J6
EGF
Jak2I
PD
p-Akt
Akt
β-tubulin
65 kD
65 kD
55 kD

Figure 3.6

Figure 3.6 Construction of Jak2 KD HC11 cells

(A) Western blotting of Jak2 protein in HC11 cells, vector transfected cells (pLKO.1) and stable cell lines expressing shRNA targeting mouse Jak2 (J2, J3, J4, J5 and J6) after immunoprecipitation with antibody against Jak2. RbIgG indicates immunoprecipitation of HC11 cell lysates using control rabbit IgG. The star (*) indicates a non-specific band. **(B)** Representative densitometry of the Jak2/non-specific band ratio in the various clones normalized to HC11 cells. **(C)** Quantification of Jak2 knockdown in pLKO.1 vector transfected HC11 cells (pLKO.1), J5 and J6 cell lines normalized to HC11 cells. **(D)** Quantification of mouse Jak2 mRNA levels in HC11, J6 cells using qRT-PCR. **(E)** qPCR fluorescent curve showing the amplification of human Jak2 in J6Rh cell line, left peak represents the amplicon of GAPDH, and arrow indicates the human Jak2 amplicon. **(F)** Representative images of J5 and J6 cells grown in 3D culture in HIP or EGF were stained for ZO-1 (green) and E-cad (red). Nucleus was counter-stained with DAPI (blue). Scale bar 10μm. (**G**) HC11 cells transfected with control pLKO.1 vector (CTL) and J5 cells were stained for ZO-1 (green) and E-cad (red) (Top panel) or for Par3 (green) and E-cad (red) (Bottom panel). Merged images show nucleus counter-stained with DAPI (blue). Scale bar 10μm. (**H**) Western blot analysis of various junction proteins (as indicated) in HC11, CTL, J5, J6 and J6Rh cells. The stars (*) indicate the 180 kDa and 100 kDa forms of Par3. **(I)** Phospho-Akt (Ser473) probing in HC11 and J6 cells with the indicated treatments. Western blotting of Akt and β-tubulin were performed for loading control.

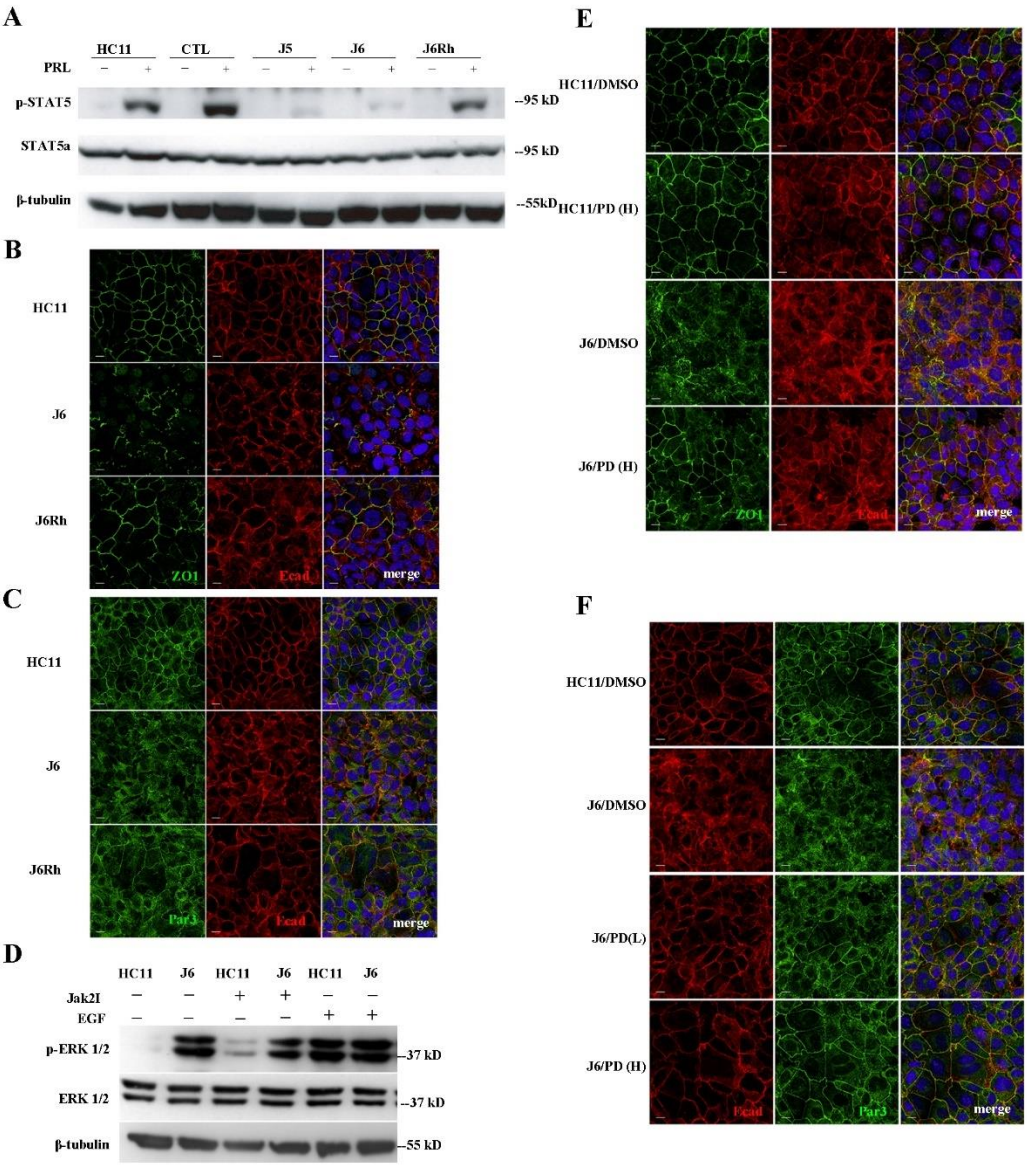
A
HC11
CTL
J5
J6
J6Rh
PRL
p-STAT5
STAT5a
β-tubulin
--95 kD
--95 kD
--55kD
B
HC11
J6
J6Rh
ZO1
Ecad
merge
C
HC11
J6
J6Rh
Par3
Ecad
merge
D
HC11
J6
HC11
J6
HC11
J6
Jak2I
EGF
p-ERK 1/2
ERK 1/2
β-tubulin
--37 kD
--37 kD
--55 kD
E
HC11/DMSO
HC11/PD (H)
J6/DMSO
J6/PD (H)
ZO1
Ecad
merge
F
HC11/DMSO
J6/DMSO
J6/PD(L)
J6/PD (H)
Ecad
Par3
merge

Figure 3.7

Figure 3.7: 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 10min. 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 10μm. **(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 50ng/ml for 15min and Jak2 kinase inhibitor (Jak2I) was used at 20μM for an overnight period. Membranes were probed for Erk1/2 and β-tubulin. **(E) & (F)** HC11 and J6 cells were subjected to 200nM (L) or 500nM (H) PD0325901 or DMSO mock treatment for 24hrs before staining for ZO1 (green) and E-cad (red) (E) or Par3 (green) and E-cad (red) (F). Scale bar 10μm. Merged images show nucleus counter-stained with DAPI (blue).

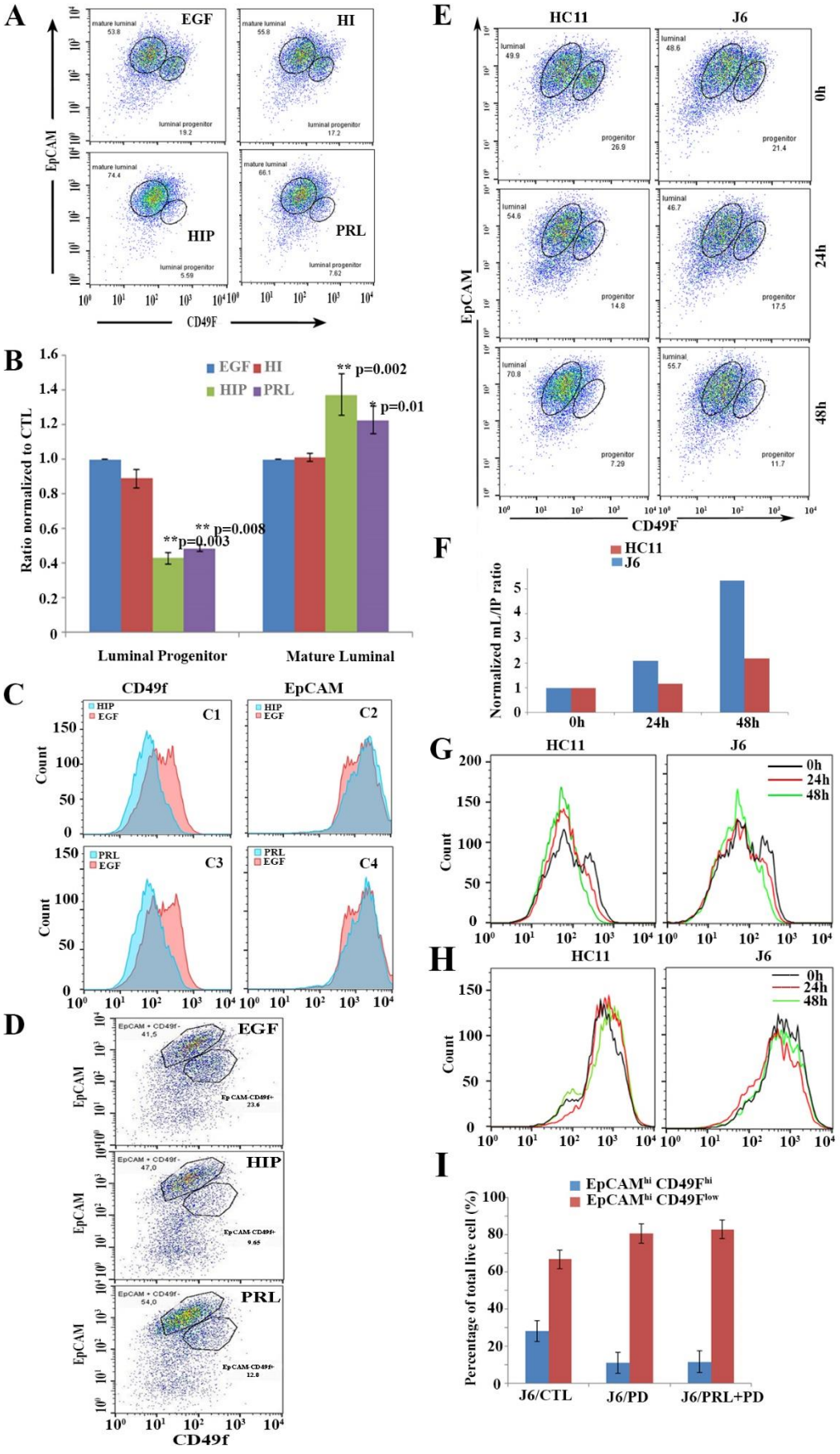
A
EGF
HI
HIP
PRL
EpCAM
CD49F
mature luminal
luminal progenitor
B
EGF HI
HIP PRL
Ratio normalized to CTL
** p=0.002
* p=0.01
** p=0.008
**p=0.003
Luminal Progenitor
Mature Luminal
C
CD49f
EpCAM
HIP
EGF
PRL
C1
C2
C3
C4
Count
D
EGF
HIP
PRL
EpCAM
CD49f
E
HC11
J6
luminal
progenitor
0h
24h
48h
F
HC11
J6
Normalized mL/lP ratio
G
HC11
J6
0h
24h
48h
Count
H
HC11
J6
0h
24h
48h
Count
I
EpCAMhi CD49Fhi
EpCAMhi CD49Flow
Percentage of total live cell (%)
J6/CTL
J6/PD
J6/PRL+PD

Figure 3.8

Figure 3.8: PRL/Jak2 promotes mammary luminal linage differentiation

(A) Dot plots of representative EpCAM and CD49f expression profile in HC11 cells following the indicated treatments (n=4). **(B)** Quantification of progenitor maturation in HC11 cells following the indicated treatments. Percentage of luminal progenitors and mature luminal cells under the indicated treatments were normalized to control EGF treated cells. Error bars represent mean ± s.e.m. of 3 independent experiments. P values derived from unpaired two-tailed Student's *t*-test. **(C)** Representative histogram display of CD49f (C1) and EpCAM (C2) expression profile in EGF, HIP and PRL (C3 & C4) treated HC11 cells (n=4). **(D)** Dot plots of representative EpCAM and CD49f expression profile in MECs with the indicated treatments for 48 hours. **(E)** Dot plots of representative EpCAM and CD49f expression profile in HC11 and J6 cells treated with HIP for 24hrs and 48hrs (n=4). **(F)** Ratio of mature luminal cells/luminal progenitors (mL/lP) in HC11 and J6 cells treated with HIP for 24hrs and 48hrs. All ratios normalized to time point 0hr (n=3). **(G)** Representative histogram display of CD49f expression profile in HC11(left) and J6 (right) cells treated with HIP for 24hrs and 48hrs (n=4). **(H)** Representative histogram display of EpCAM expression profile in HC11(left) and J6 (right) cells treated with HIP for 24hrs and 48hrs (n=4). **(I)** Percentages of luminal progenitors and mature luminal cells in J6 treated for 24hrs with DMSO (J6/CTL), 1μM PD0325901 (J6/PD), or 2ug/ml PRL and 1μM PD0325901 (J6/PRL+PD). Error bars represent mean ± s.e.m. from 3 independent experiments. P values derived from unpaired two-tailed Student's *t*-test.

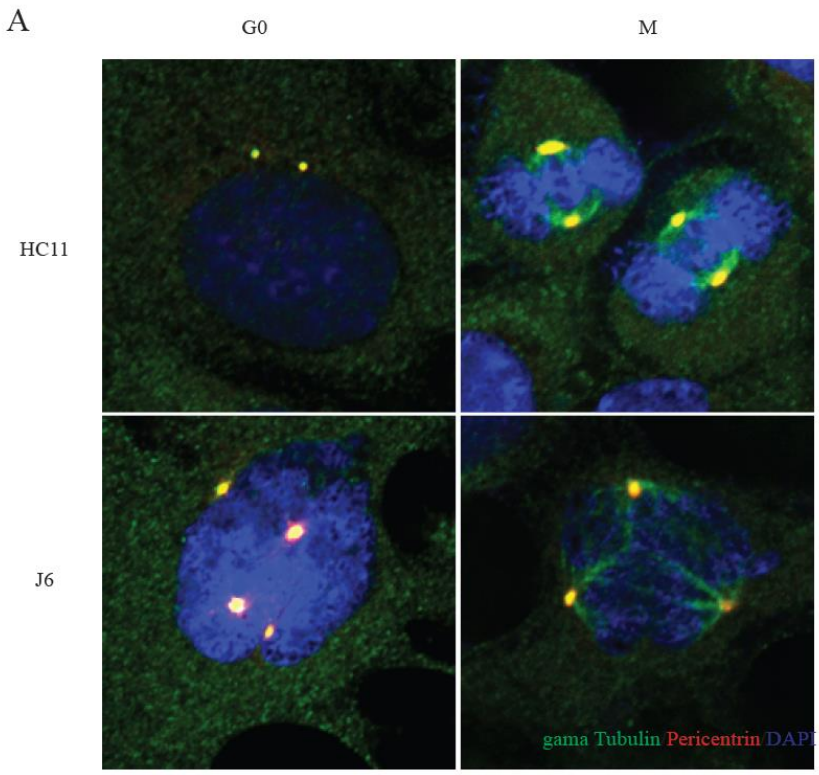

Figure 3.9

Figure 3.9 multiple centrosomes in Jak2 KD cells. representative images of centrosome staining with pericentrin (red) and γ-Tubulin (green) in parental HC11 cells and Jak2 KD cells (J6) during G0(left panel) and metaphase (right panel). Nucleus was stained with DAPI in blue.

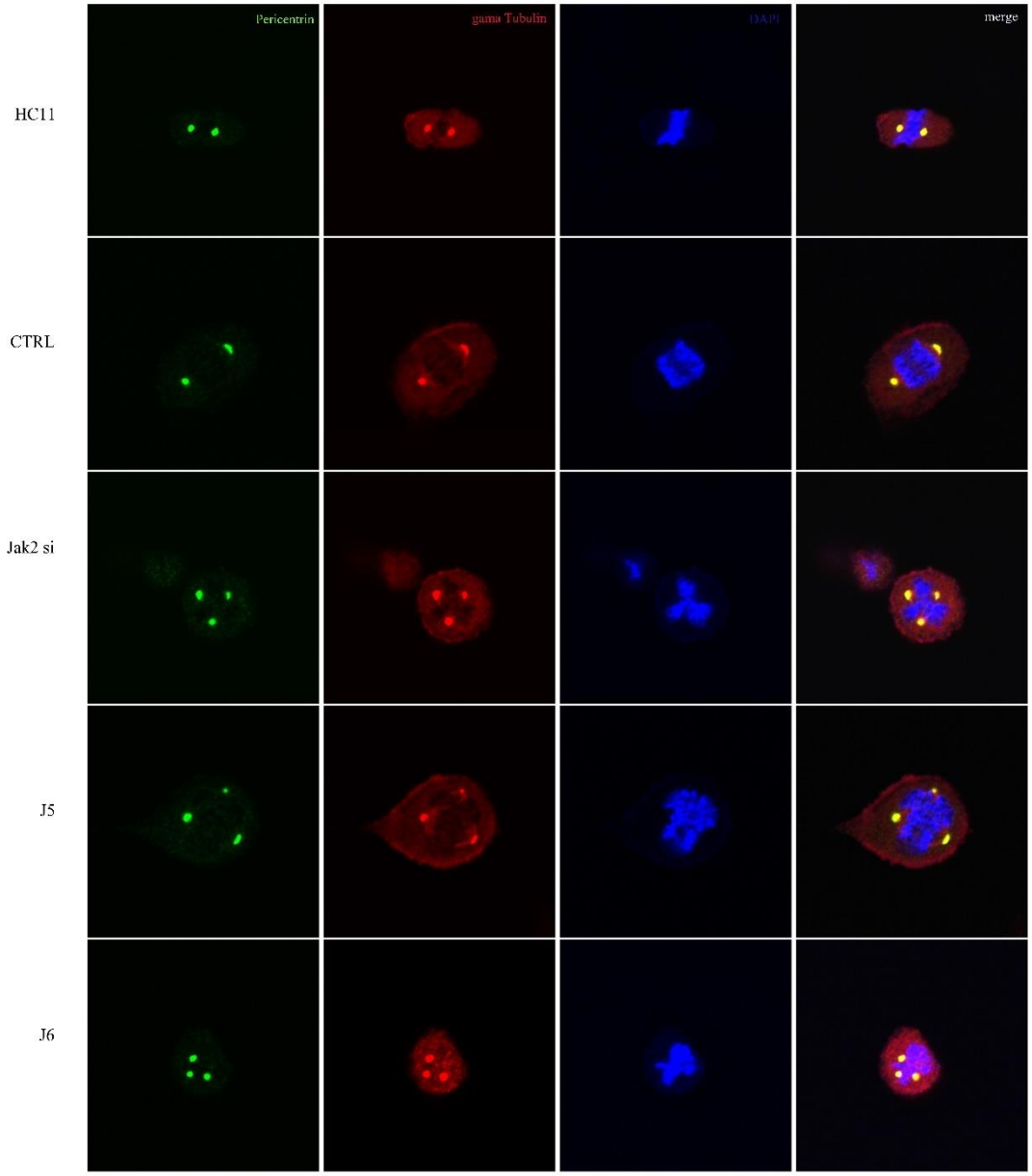

Figure 3.10

Figure 3.10: Loss of Jak2 leads to multiple polar spindles in dividing cells Jak2 KD cells by siRNA or shRNA targeting Jak2 transfection exhibit multiple centrosomes comparing to normal centrosomes in parental HC11 cells or vector transfected control cells. Pericentrin (green) and γ-Tubulin(red) were used for centrosome staining.

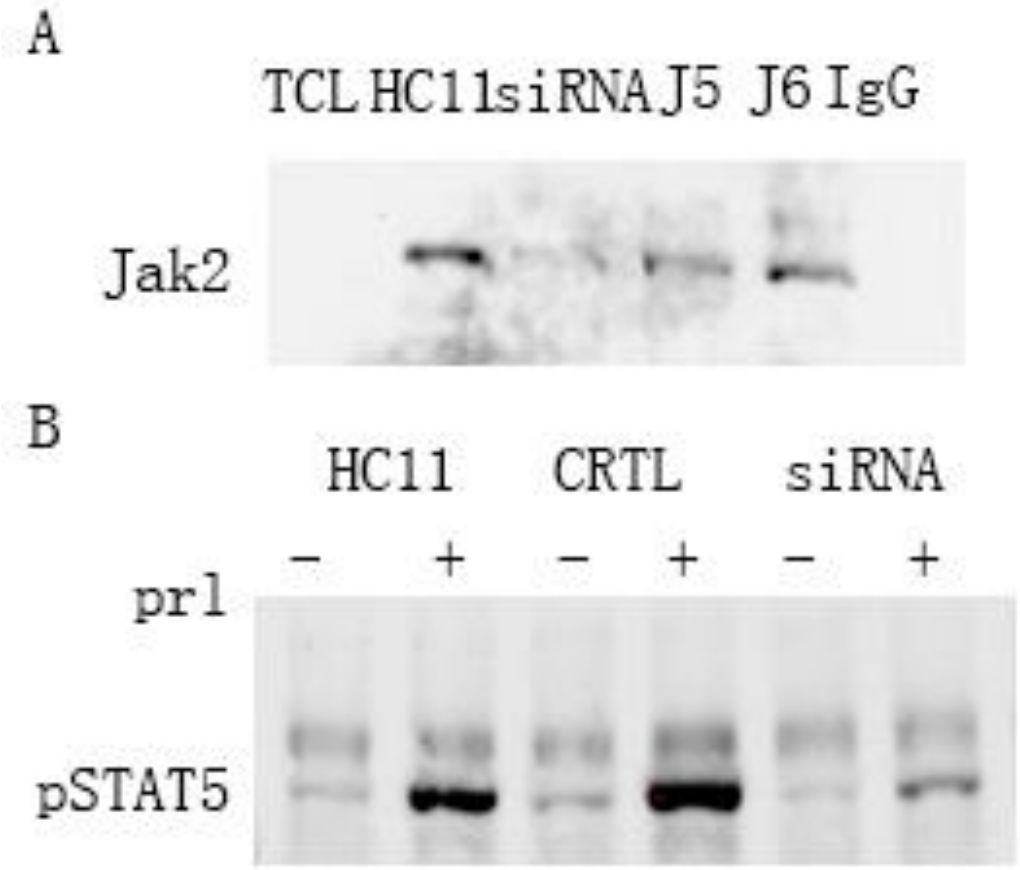

Figure S3.10

Figure S3.10 Validation of Jak2 siRNA efficiency. (A) Western blotting of Jak2 protein in HC11 cells, Jak2 siRNA transfected HC11 cells (siRNA), after immunoprecipitation with antibody against Jak2. TCL, total cell lysate, IgG indicates immunoprecipitation of HC11 cell lysates using control rabbit IgG. (B) Western blot showing Stat5 phosphorylation in HC11, scrambled siRNA (CTRL) and Jak2 siRNA transfected (siRNA) HC11 cells following PRL stimulation for 10min.

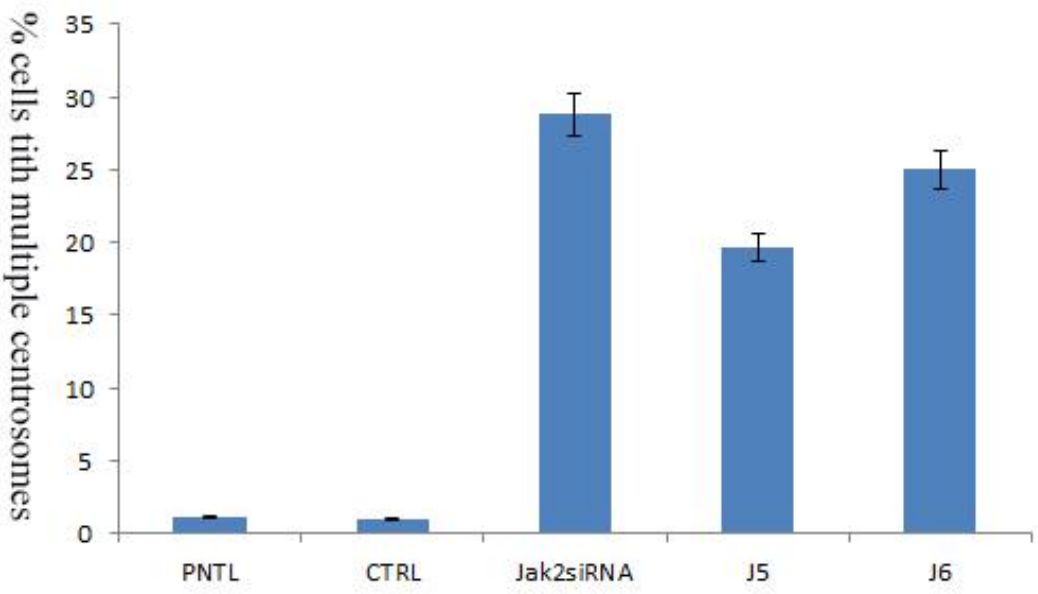

Figure 3.11

Figure 3.11: Quantification of multiple centrosomes in Jak2 KD cells. over 20% of total Jak2 KD cells by siRNA or shRNA targeting Jak2 transfection contain multiple centrosomes comparing to parental HC11 cells or pLKO vector transfected control cells.

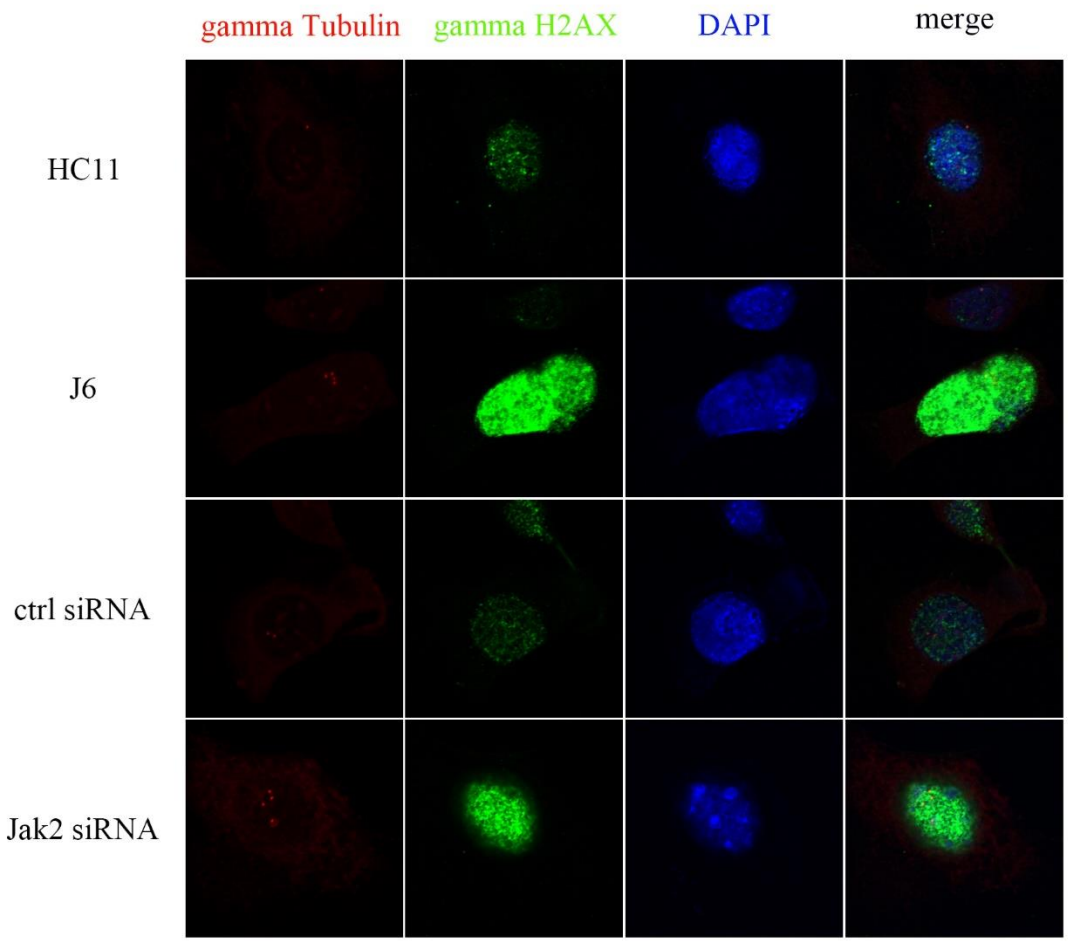

Figure 3.12

Figure 3.12: Loss of Jak2 leads to accumulation of DNA damages in the nucleus. Jak2 was knocked down by siRNA or shRNA (J6) targeting Jak2 and stained with γ-Tubulin (red) and γH2AX (green) antibodies. Increased γH2AX staining detected in Jak2 KD cells comparing to parental HC11 cells or HC11 cells transfected with control siRNA.

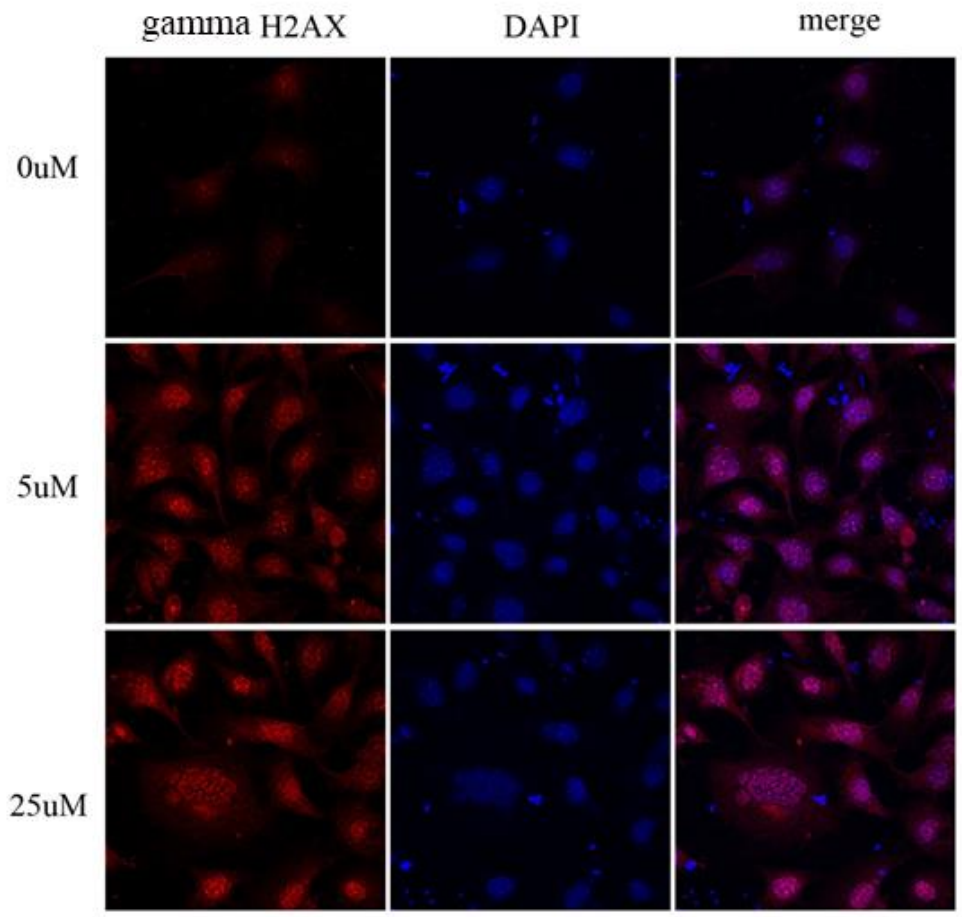

Figure 3.13

Figure 3.13: Jak2 kinase inhibitor treatment leads to DNA damage accumulation in HC11 cells. HC11 cells were plated on coverslips in 2D and stained with antibody against γH2AX (red), increased DNA damage detected in cells treated with Jak2 inhibitor at various concentrations.

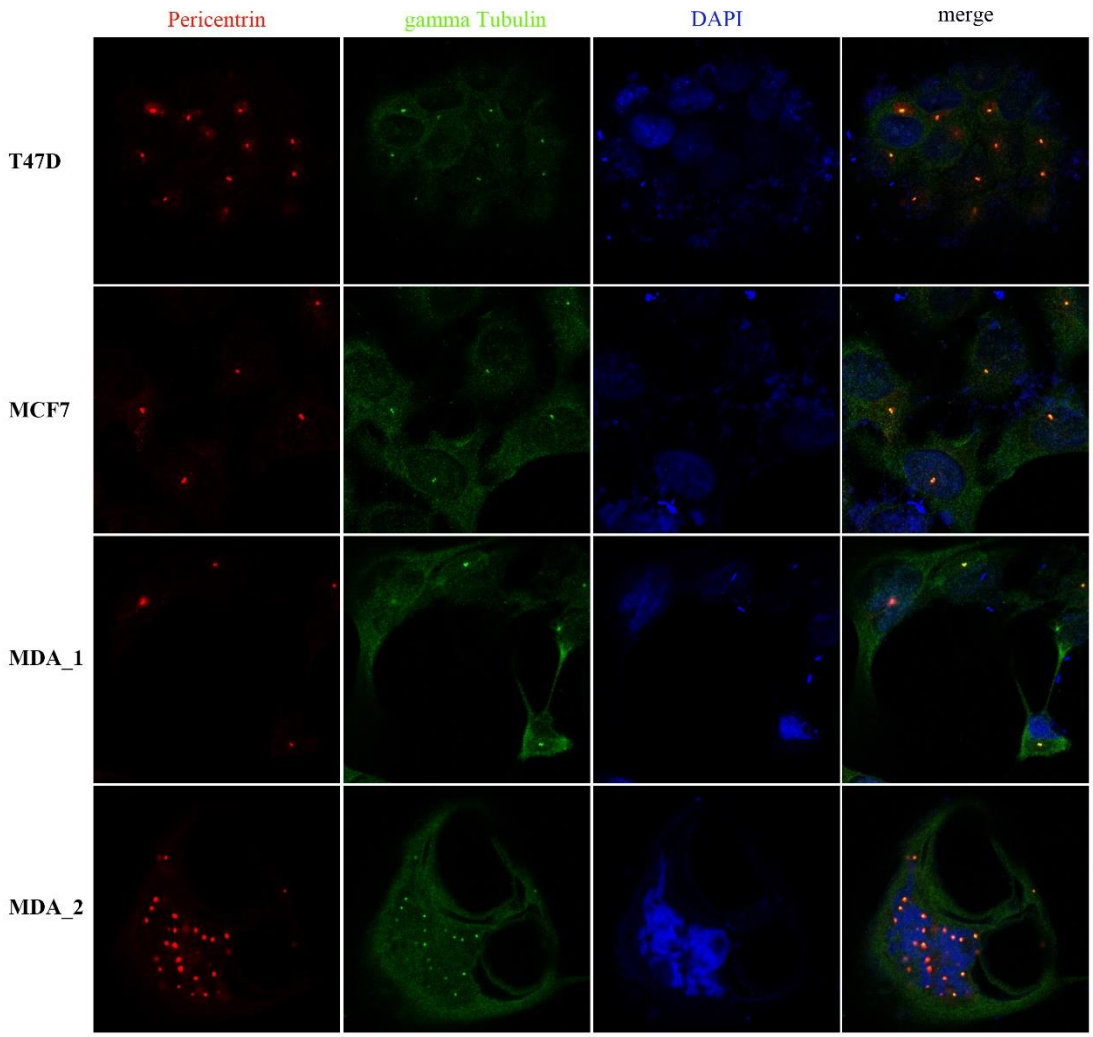

Figure 3.14

Figure 3.14: Breast cancer cells show various frequency of centrosome amplification. Cells were stained with pericentrin (red) and γ-Tubulin(green) to distinguish centrosome. Nucleus was stained with DAPI in blue. MDA-MB-231 cells harboring normal(MDA_1) or excess number of centrosomes(MDA_2) were presented.

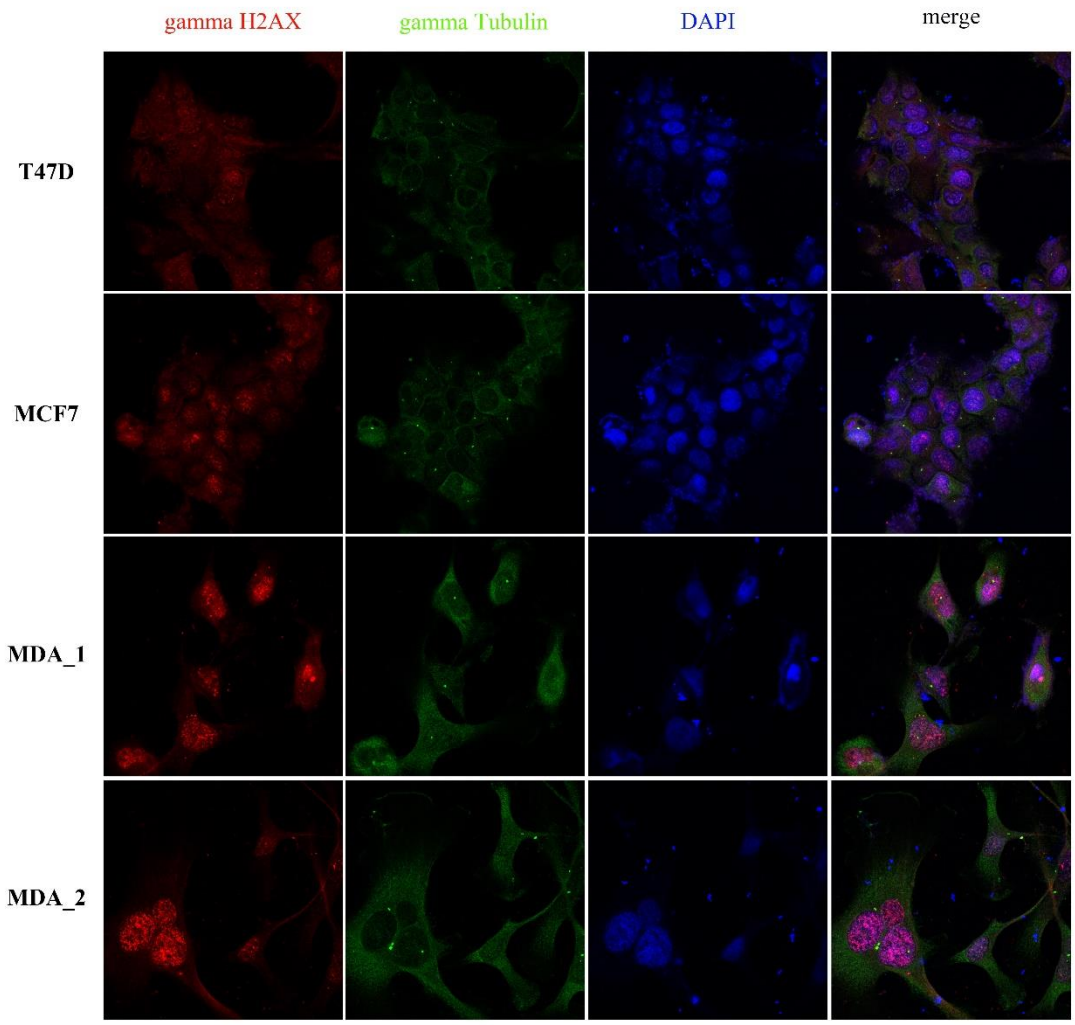

Figure 3.15

Figure 3.15 Various DNA damages in breast cancer cells. Cells were stained with γH2AX(red) and γ-Tubulin (green) antibodies.

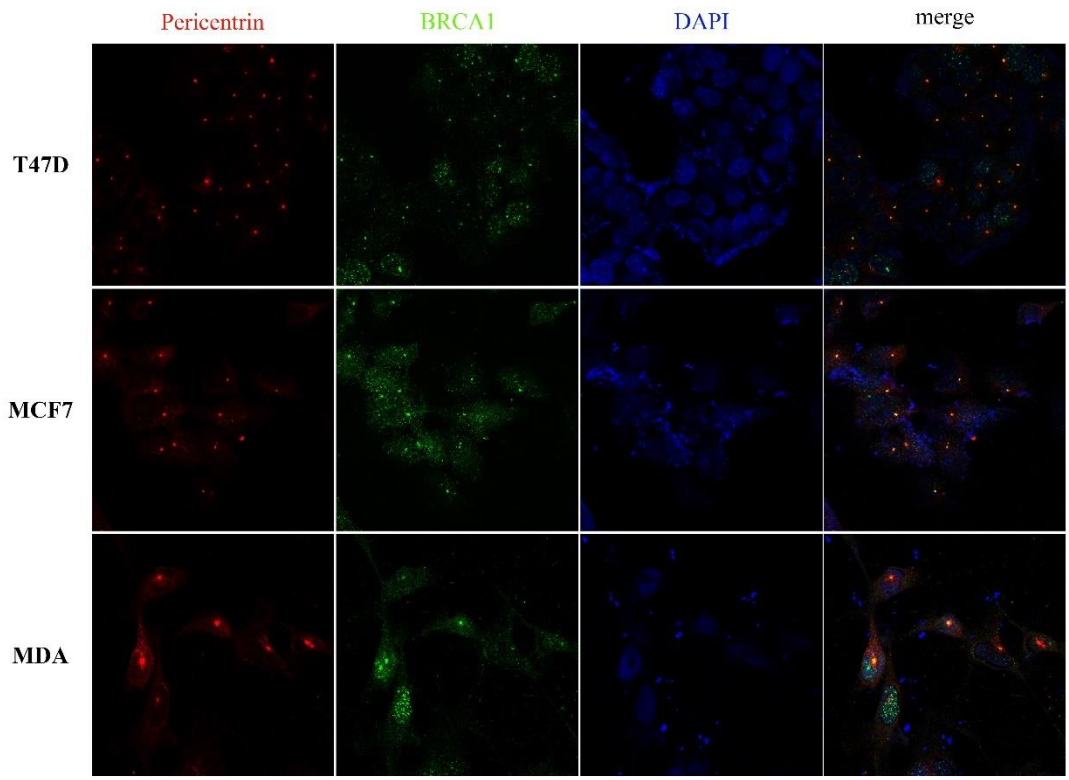

Figure 3.16

Figure 3.16 BRCA1 localizes to centrosome in breast cancer cells. breast cancer cells were cultured on coverslips in 2D, fixed with methanol and stained for pericentrin (red) and BRCA1(green), nucleus was stained with DAPI in blue.

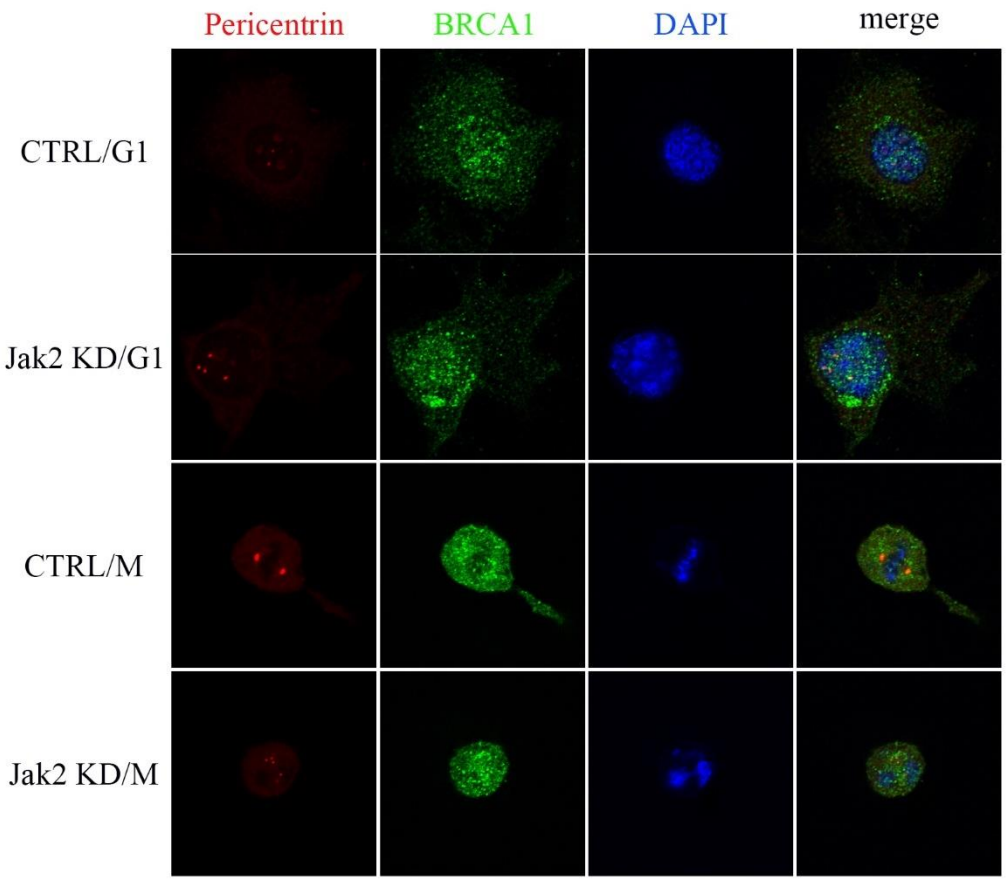

Figure 3.17

Figure 3.17: BRCA1 localization in HC11 cells. BRCA1 antibody cannot recognize the mouse BRCA1.Notice the multiple centrosome staining (red) in Jak2 KD cells.

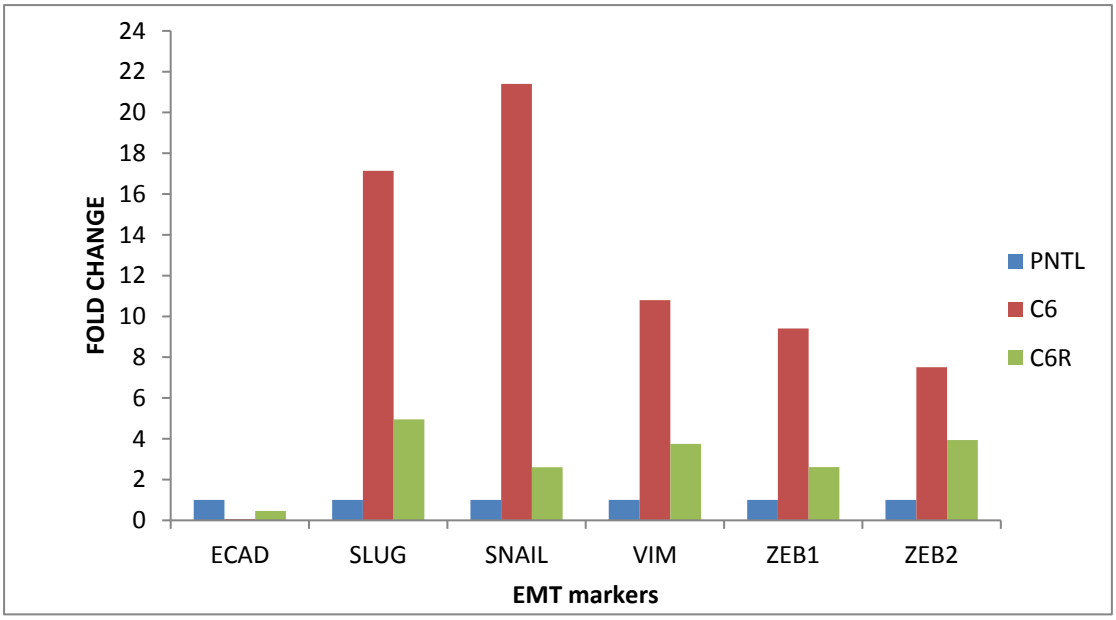

Figure 3.18

Figure 3.18: Loss of Jak2 induces EMT in HC11 cells. mRNA levels of various EMT markers were analyzed in parental HC11 cells (PNTL), Jak2 KD (C6) and Jak2 KD cells rescued with human Jak2 transfection (C6R). Downregulation of epithelial marker E cadherin (ECAD) and upregulation of Slug, Snail, Vimentin (VIM), Zeb1 and Zeb2 were detected in Jak2 KD cells. Phenotype is partially restored in Jak2 KD rescue cells.

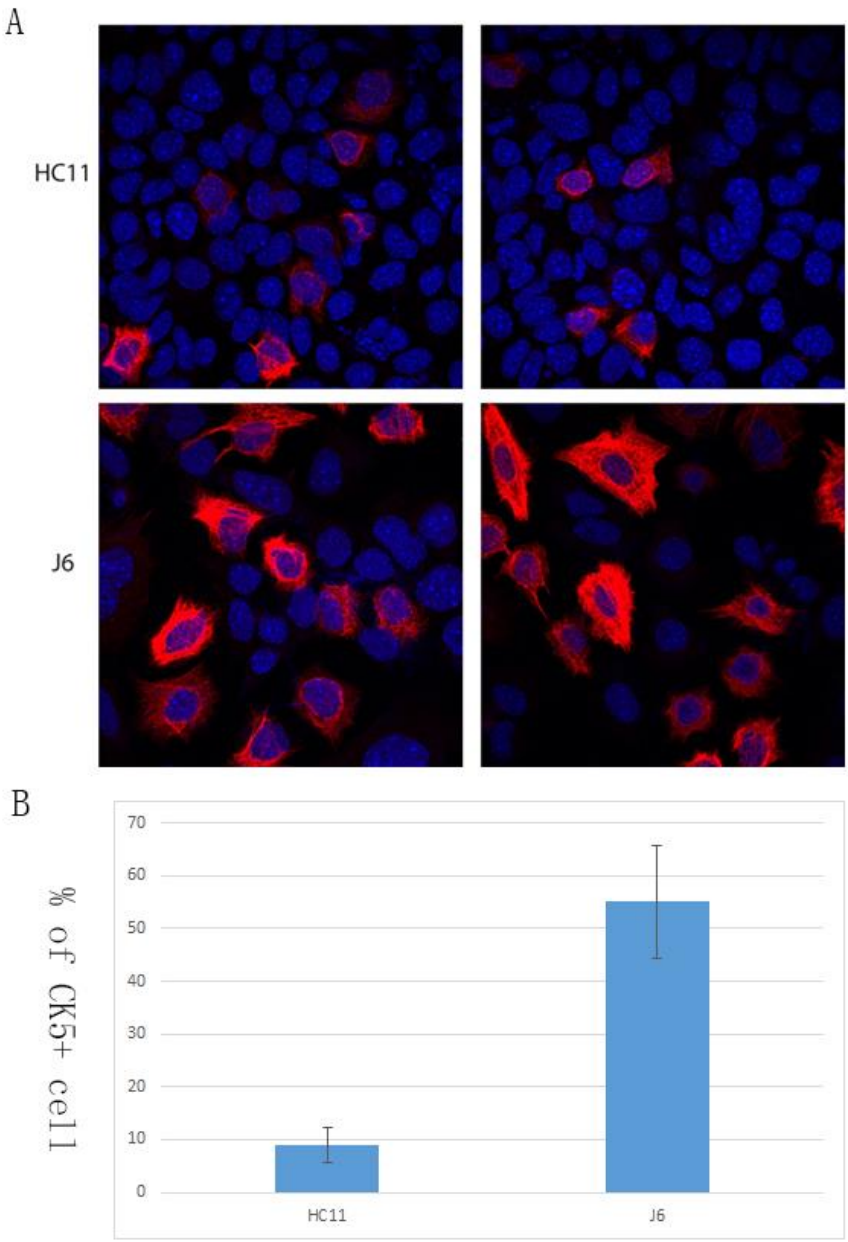

Figure 3.19

Figure 3.19: Increased CK5 positive cells in Jak2 KD cells. (A)Cells were stained with CK5 antibody(red) and DAPI for nucleus. Duplicate representative images for parental HC11(top panels) and Jak2 KD cells (J6, bottom panel) were presented. (B) Quantification of CK5 positive cells in parental HC11 cells and Jak2 KD (J6) cells (pValue <0.01).

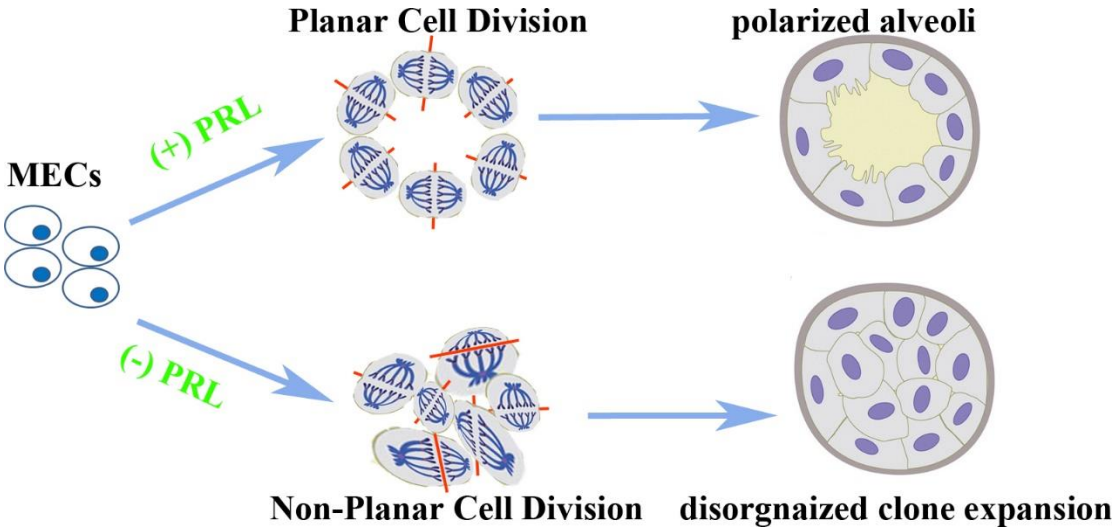

Figure 3.20

Figure 3.20 A schematic model of PRL mediated planar cell division during mammary acini formation. At the presence of PRL, all proliferating cells divide with uniform axis perpendicular to the basement membrane, and cells organize into polarized alveoli, however, proliferating cells with random axis of cell division leads to compacted cell clumps without PRL.

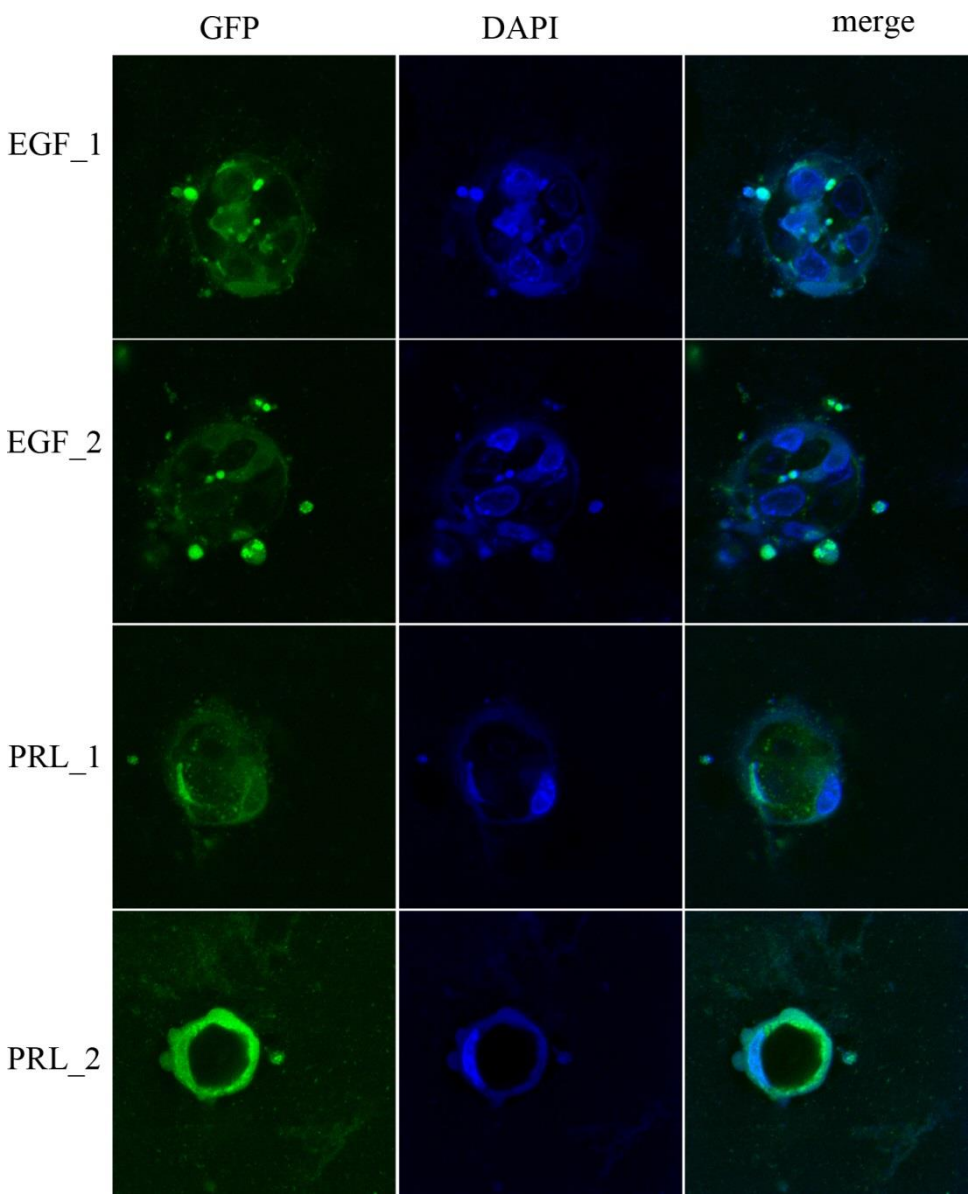

Figure 3.21

Figure 3.21: chasing the dividing cells in 3D. Primary mouse mammary epithelial cells were transfected with fluorescent labels as described and plated in Matrigel. Dividing cells were visualized with GFP fluorescence in confocal microscopy.

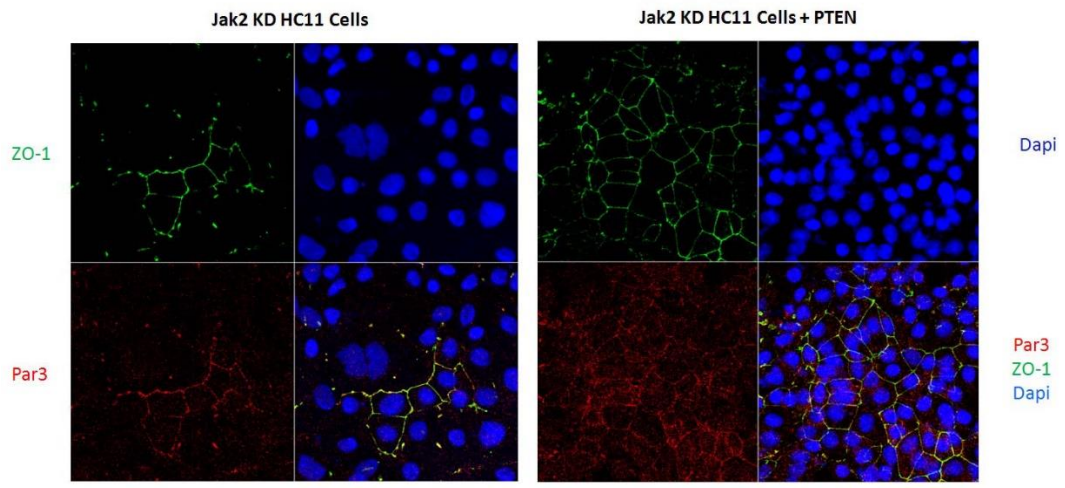

Figure 3.22

Figure 3.22 overexpression of PTEN rescued Par3 and ZO1 localization in Jak2 KD cells at the presence of PRL. Cells were stained with ZO1 (green) and Par3 (red), overexpression of PTEN in Jak2 KD cells partially restored the localization of both ZO1 and Par3 at presence of PRL (right panel). Nucleus was stained with DAPI in blue. Unpublished data, courtesy of Ms. Zhenqian Feng

Chapter 4: Discussion and Conclusions

In this study, various newly identified roles of the canonical lactogenic hormone PRL were described. PRL functions as a polarity cue during apical/basal polarization and terminal differentiation of mammary epithelial cells. PRL promotes the polarization of mammary epithelial cells via cellular junction organization and proper localization of polarity complex proteins. In addition, PRL promotes the maturation of luminal progenitor cells. PRL/Jak2 signaling counteract Erk1/2 activations to exert these effects. Loss of Jak2 confers mammary epithelial cells basal/mesenchymal phenotypes including up-regulation of cytokeratin 5 and EMT markers such as Slug, Snail, Vimentin and Zeb1/2. Blockage of PRL/Jak2 signaling leads to centrosome amplification and DNA damage accumulation, suggesting a crucial role of Jak2 signaling in maintenance of genomic stability. Although no supportive data is obtained in this study, the role of PRL in mediating planar cell division during mammary tissue homeostasis and its potential cross-talk with the CDC42 activation during polarization of mammary epithelial cells are strongly implicated. These non-confirmed speculations provide interesting directions for future study.

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 as an important regulator of mammary acini morphogenesis (Lo, Mori et al. 2012, Lee and Streuli 2014). 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 TGFβ (Ozdamar, Bose et al. 2005) , Erbb2 (Aranda, Haire et al. 2006) and Ephrin B1 (Lee, Nishanian et al. 2008) as well as NFκB (Becker-Weimann, Xiong et al. 2013) to interfere with mammary acini formation/organization. Thus, our results 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, Krajewska et al. 1996, Debnath, Mills et al. 2002, Mailleux, Overholtzer 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 a coordinated mechanism regulating lumen formation. Other than this proposed apoptosis involved hollowing, clump of mammary epithelial cells may develop into acinar structure through coordinated cell proliferation and polarization, i.e., through the process of controlled planar cell division in the presence of PRL as discussed in the results section. Indeed, PRL treated acini show well established polarity, whereas the lumen of these colonies is smaller in comparison to acini grown under HIP treatment conditions. Although lumen could develop in the absence of cell division under certain circumstances (Yu, Fang et al. 2007), minimum clonal expansion in our 3D culture model system is required from single cell to these small acini. This difference in lumen size implicates two potential roles of insulin and/or hydrocortisone during acini development: (1) insulin and hydrocortisone work alone or in combination to promote clonal expansion of the mammaspheres, providing more epithelial cells as building blocks for acini development, which is consistent with the observation that acini grown under HIP treatment have more cells than PRL treated acini. (2) insulin and hydrocortisone work alone or in combination is involved in apical membrane generation/expansion. As the most potent anabolic hormone(Saltiel and Kahn 2001), insulin promotes synthesis and storage of the major structural glycerophospholipids: phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns) and phosphatidic acid (PA)(van Meer, Voelker et al. 2008). Indeed, studies performed with MDCK cells do implicate lipid metabolism in apical membrane

generation, the orchestrated apical trafficking of sorted vesicles expanded the apical membrane (Bryant, Datta et al. 2010). Therefore, we propose that lipid metabolism downstream of insulin is potentially leading to the expanded lumen seen in HIP treated acini.

Furthermore, accumulation of lipid droplets was only observed in mammary epithelial cells treated with HIP points to a potential insulin/ hydrocortisone regulation on adipophilin, a lipid binding protein essential for maturation of cytoplasmic lipid droplets(Russell, Palmer et al. 2007).Further investigation is necessary to elucidate the interplay between PRL and insulin/hydrocortisone during acini development for successful lactation.

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 ($EpCAM^{low}/CD49f^{hi}$ or $EpCAM^{low}/CD49f^{low}$) as has been documented using primary mammary epithelial cells isolated from virgin mice. Together, these results indicate the enrichment of the luminal linage during pregnancy (Shehata, Teschendorff et al. 2012, Visvader and Stingl 2014). 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 Stat5a/b knockout mice show loss of mammary luminal cell population (Yamaji, Na et al. 2009). Together our results demonstrate that PRL hormone through Jak2 kinase integrates both cellular A/B polarization and mammary stem cell hierarchy. Further work should be directed to exploring the effect of PRL on different mammary stem cell sub-populations as well as on breast cancer stem cells.

Involvement of PRL in stem cell regulation is not yet fully addressed. A full understanding of the spatial and temporal PRLR expression pattern in the mammary gland is prerequisite to evaluate the ligand effect on different subpopulations of mammary epithelial cells. Although PRLR is required for RANKL mediated progesterone-driven expansion of adult mammary stem cells, implicating a pro-proliferation effect of PRL during this process, if any(Joshi, Jackson et al. 2010, Schramek, Leibbrandt et al. 2010). Direct effects of PRL on different mammary stem cell subpopulations are hard to evaluate due to lack of PRLR antibodies validating receptor expression in these cells(Sackmann-Sala, Guidotti et al. 2015). Alternative approaches to circumvent this limitation include mRNA profiling like RT-PCR, in-situ hybridization and cDNA microarray. In prostate cancer, local exogenous expression of PRL leads to amplification of basal/stem cell population as well as luminal cell populations which might derive from the amplified basal cells(Sackmann-Sala, Chiche et al. 2014). Again, the prevailing reports challenge our newly reported pro-differentiation effect of PRL. Given these discrepancies, it is plausible to postulate PRL and PRL receptor signaling function differently within

different cellular context and micro-environmental conditions, even within the same tissue.

The Erk1/2 signaling cascade regulates a variety of cellular processes by phosphorylating multiple target proteins (Yoon and Seger 2006, Lefloch, Pouyssegur et al. 2009). 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, Kayali 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.

Multiple mechanisms are utilized to maintain genomic integrity, ensuring the daughter cells will have the same DNA content as their parent during mitosis. These include the protein machinery for high-fidelity DNA replication and error-free repair of sporadic DNA damages, the controlled mitotic spindle assembly during mitosis for precise chromosome segregation, and multiple checkpoints to oversee the cell cycle

progression(Shen 2011). Genomic instability is a major driving force during tumorigenesis, and it could arise from any dysregulated mechanisms aforementioned. Loss of Jak2 in mammary epithelial cells led to centrosome amplification and accumulated DNA damages in the nucleus, these progenies resemble pre-cancerous cells featuring increased EMT marker and CK5 expression. Our findings suggest a protective role of PRL/Jak2 signaling from carcinogenesis via maintenance of genome stability. Although these descriptive results are far from elucidating the underlying signaling cross-talks in regulation of genomic stability, various potential links are subject to further investigation. Consistently, involvement of PRL/Jak2 signaling in maintenance of genomic integrity is implicated in a recent study, in which PRL is shown to attenuate age-associated alterations of metaphase-II chromosome morphology in aging mature bovine oocytes(Lebedeva, Singina et al. 2015). PRL is reported to confer cancer cell resistance to chemotherapy due to its oncogenic role. Alternatively, this can also be attributed to its role in maintenance of genomic integrity, as cancer cells might well benefit from the protective role of PRL counterstriking the detrimental effect on cellular DNA content to which most chemotherapy agents are targeting. A drawback of the study in centrosome amplification and DNA damage accumulation is that the effect of PRL is not evaluated, and one may well argue that Jak2 is essential for regulation of centrosome duplication and maintenance of genomic stability, whereas the upstream activator of Jak2 might not necessarily be PRL. Given the high sequence homology and structural identity of Jak1 and Jak2, other cytokine and growth factor signaling pathways

include Jak1 or other Jaks may also activate Jak2 in a non-selective manner(Wilks 2008). However, the ATP binding sites in Jak2 is very different from Jak1(Williams, Bamert et al. 2009), cross-activation of multiple Jaks was rarely reported. Plus, under normal conditions, basic physiological level of Jak2 activation is expected due to the promiscuous ligand binding of PRL receptors to other ligands like growth hormone, whereas in the Jak2 KD cells, the physiological level of Jak2 activation is depleted. Our lab recently restored Jak2 signaling in MDA-MB-231 cells by viral transfection of PRLR construct(Lopez-Ozuna, Hachim et al. 2016). In observance of the severe centrosome amplification in MDA-MB-231 cells, restoration of Jak2 signaling alone or in combination with PRL might be able to alleviate this phenotype, this provides a good platform to test whether PRL/Jak2 signaling is essential for regulation of centrosome duplication and maintenance of genomic stability.

Many questions arise from this study. Does the Jak2-suppressed HC11 cells carrying the centrosome amplification become more malignant or serve as a marker of cell abnormality and lead to cell death and further elimination of this damaged subpopulation? Another question is how much the p53 mutant genetic background of HC11 cells(Merlo, Venesio et al. 1993) contributes to the observed multiple centrosome phenotype. Although centrosome amplification is not observed in non-transfected HC11 cell, additional approaches is needed for clarification. Knock down of Jak2 in cells with different p53 genetic background and examine the centrosome duplication may fulfill this purpose well. T47D or MDA-MB-468

contains single mutated copy of p53 gene, MCF7 cells harbor wild type p53 gene(Casey, Lo-Hsueh et al. 1991). All these cell lines can be used to validate our finding in HC11 cells. Our most recent progress showed T47D cells exhibited centrosome defects after CRISPR^{TM} cellular knock out of PRLR (unpublished data), suggesting the critical role of PRL/Jak2 signaling in regulation of centrosome numbers in mammary epithelial cells. Further efforts will be directed to elucidate the signaling cross-talk in regulation of centrosome cycle during mitosis.

Finally, the results described here have important implications in expanding our understanding of the role of PRL in breast tumorigenesis. As lumen filling/repopulation 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. Investigation toward this direction is currently underway in this lab, PRL treatment of T47D cells cultured in 3D resulted in cleared lumen, whereas non-treated T47D cells developed into solid mammasphere under the same culture condition (unpublished data). Importantly, in a parallel study we found PRL, PRLR and Jak2 to be markers of favorable prognosis and their expression correlate with good patient outcome in relapse free survival and distant metastasis free survival(Hachim, Shams et al. 2016). These results combined with our findings that PRL promotes the maturation of luminal progenitor cells, thereby reduces cancer risk of mammary cells via exhaustion of the disease-prone stem cell pool, and induces mammary morphogenesis strongly implicate PRL as a tumor suppressor and highlight this pathway as an important therapeutic target against breast

cancer, In summary, our findings added to the expanding spectrum of PRL functions, its functions as a differentiation factor in mammary epithelial morphogenesis and guardian of genomic integrity provide molecular insight into the acknowledged protective role of breastfeeding against breast tumorigenesis.

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