Characterization of Hematopoietic PBX Interacting Protein (HPIP) as a novel regulator of mammary epithelial cell differentiation and anoikis resistance in breast cancer

Thesis submitted for the degree of

DOCTOR OF PHILOSPHY

IN BIOCHEMISTRY

By

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CERTIFICATE

This is to certify that the thesis entitled **"Characterization of Hematopoietic PBX Interacting Protein (HPIP) as a novel regulator of mammary epithelial cell differentiation and anoikis resistance in breast cancer"** submitted to the University of Hyderabad by **Mrs. Anju Dwivedi**, for the degree of Doctor of Philosophy, is based on the studies carried out by her under my supervision. I declare to the best of my knowledge that this has not been submitted earlier for the award or diploma from any other University or Institution.

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DECLARATION

I hereby declare that the work presented in this thesis is entirely original and was carried out by me in the Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad under the supervision of **Prof. Bramanandam Manavathi, PhD.** I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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Part of this thesis has been presented in the following conferences:

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Anju Dwivedi

List of Abbreviations

(A/B) polarity- Apical/basal (A/B) polarity
ADP- adenosine 5′-diphosphate
AKT: Protein kinase B
AML- Acute myeloid leukemia
ANOVA- Analysis Of variance
AO/EtBr- Acridine orange/ Ethidium Bromide
ATG- Autophagy related gene
BC: Breast cancer
BCALL- B-cell acute lymphoblastic leukemia
BCCs- breast cancer cells
Bit1- Bcl-2 inhibitor of transcription 1
bPRL: bovine prolactin
B-RAF- B-Raf proto-oncogene
CAFs- Cancer associated fibroblasts
ChIP- Chromatin immunoprecipitation assay
CLDs- Cytoplasmic lipid droplets
CM- cutaneous melanoma
CME- Clathrin-mediated endocytosis
Cox-2- Cyclooxygenase-2
CPD- Carboxypeptidase D
CPE- Carboxypeptidase E
CSF1- colony stimulating factor 1
Csn2- β-casein
CSR1- Cellular stress response 1
CTCF- CCCTC-binding factor
CTCs- Circulating tumor cells
DAB- 3,3′-Diaminobenzidine
DNA- Deoxyribonucleic acid
E- Embryonic Day
E2- 17β-estradiol
E_{2-} Estrogen,
EC- endometrial cancer
ECM- Extra cellular matrix
EEA1- Early endosome antigen 1
EMT: Epithelial to mesenchymal transition
EOC- epithelial ovarian cancer
ER- Estrogen receptor
ERα- estrogen receptor alpha
Erβ- estrogen receptor beta
Esr1- Estrogen receptor alpha gene
FAK: Focal adhesion kinase
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
GAS element- Gamma interferon activation site
GATA1- GATA binding protein 1
GEF- Guanine Exchange Factor
GFP- Green florescent protein
GH- Growth hormone GH
Ghr- Growth hormone receptor
GSK3β- Glycogen synthase kinase 3 beta
HCC: Hepatocellular carcinoma

HER2- Human epidermal growth factor receptor 2
HOX- Homeobox genes
HPIP/PBXIP1- Hematopoietic PBX interacting protein or pre-B-cell leukemia homeobox transcription factor 1 (PBX1)-interacting protein
IAEC- Institutional Animal Ethical Committee
Igf1-insulin-like growth factor 1
IHC- Immunohistochemistry
IRS1- Insulin Receptor Substrate1
JAK2: Janus kinase 2
LC3B: Microtubule-associated protein 1A/1B-light chain 3
LDL- Low-density lipoprotein
MAPK: Mitogen-activated protein kinase
MBC- Metastatic breast cancer
MDM2- Mouse double minute 2 homolog
MG- Mammary gland
miR-148a: microRNA-148a
MMP11- Matrix Metallopeptidase 11
MMPs- Matrix metallo proteases
MPS- Mean puncta size
mRNA- messenger Ribonucleic Acid
MTHFR- 5, 10-methylenetetrahydrofolate reductase
mTOR- Mammalian target of rapamyci
NFκB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
P_4- Progesterone
PBS- Phosphate-buffered saline
PBX: Pre-B-cell leukemia homeobox protein
PBX1 : Pre-B-cell leukemia homeobox protein1
PBX2: Pre-B-cell leukemia homeobox protein2
PBX3: Pre-B-cell leukemia homeobox protein3
PDTC- Pyrrolidine dithiocarbamate
Pgr- Progesterone receptor
PGRiA- Progesterone receptors isoforms A
PGRiB- Progesterone receptors isoforms B
PI3K : Phosphatidylinositol 3 kinase
PMSF- Phenylmethylsulfonyl fluoride
poly-HEMA- 2-Hydroxyethyl methacrylate
PRKCA- Protein kinase C alpha
PRL: Prolactin
PRLR: Prolactin receptor
PTEN- Phosphatase and TENsin homolog deleted on chromosome 10
PTHLH- Parathyroid hormone-related protein
PXR- Pregnane 6 receptor
Rab- Ras-associated binding
Rab5a: Ras-related protein Rab-5A
RANKL- Receptor activator of nuclear factor kappa-B ligand
RCC- renal cell carcinoma
RNF126: Ring finger protein 126
SIRPA- Signal regulatory protein alpha
Smad2- Mothers against decapentaplegic homolog 2
SOCS- Suppressors of cytokine signaling
STAT5: Signal transducer and activator of transcription 5
TBK1- TANK binding kinase 1

TEB- Terminal end bud
TGF-β- Transforming growth factor beta
TGIF2- TGFB Induced Factor Homeobox 2
TNBC- Triple negative breast cancer
TrkB- Tropomyosin receptor kinase B
Wap- acidic whey protein

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CHAPTER I

Role of HPIP in mammary gland development and prolactin autocrine signaling

1. Introduction

The mammary gland is a characteristic feature of mammals, differentiating them from other animals. The name Mammalia is coined after the mammary gland. It is an unusual organ that undergoes dynamic changes during puberty and the reproductive cycle. It functions to make and secrete milk for the nourishment of the newborn [1,2]. They have a unique anatomical structure that undergoes repetitive expansions throughout their menstrual cycles, and drastic variations occur in shape and function though pregnancy, lactation, and involution [1]. In contrast to specific mammalian organs, which grows in an embryonic stage, the mammary gland (MG) evolves specifically after birth. Altogether the crucial cell lineages in the mammary gland are well-established at the embryonic stage, leading to mammary rudiment [3,4]. The mammary rudiment is virtually idle at birth, unlike other essential tissues (e.g., cardiac, muscular, gastric). However, noticeable growing changes can be observed just after birth during the prepubertal phase, after puberty, and throughout the reproductive cycle. The mammary rudiment is also known as anlage, which develops gradually due to hormonal action and starts branching into the fat pad, leading to the formation of ducts, alveolar development, functional differentiation, regression, and redevelopment. Mammary glands are multifaceted secretory organelles that comprise many types of cells: epithelial cells, adipocytes, vascular endothelial cells, fibroblasts, and immunological cells. Mammary epithelium mainly comprises two significant cell types: basal and luminal. The basal epithelium forms the gland's outer layer. It mainly consists of myoepithelial cells and stem cells that create various cell types, luminal epithelium on the other hand, is responsible for the formation of ducts and secretory alveoli and consists of cells with their own unique hormonal receptor. There are three phases of MG development: embryonic, pubertal, and reproductive (Fig.1). The mammary gland cells undergo proliferation, differentiation, or apoptosis in response to stimuli throughout these stages, leading to symbolic remodeling of the glandular tissue structure.

1.1. Embryonic mammary gland development

Mammary glands are epidermal appendages derived from the ectoderm. The mammary gland has two cellular compartments in the embryo: epithelial and stromal. The epithelial and stromal tissue is originated from the ectoderm and mesoderm, respectively. The development of the murine glands begins with the formation of bilateral stripes, also known as milk lines, on the ventral side of the embryo at embryonic day (E) 10, which are composed of multilayered ectoderm extending from the anterior to the forelimb bud, hindlimb bud on the anterior surface of the embryo. By embryonic day E11.5, the mammary line has differentiated into five sets of placodes at fixed positions, which undergoes asynchronously development with 3 emerging first, followed by 4, then 1 and 5 simultaneously, finally, the one pair formation take place [5,6]. Placodes are characterized histologically by a thick layer of ectoderm made up of columnar- shaped cells that form when ectodermal cells migrate and aggregate as surface clusters along the mammary line [7]. Throughout the first trimester of human pregnancy, the mammary lines develop and give rise to one pair of placodes [8]. These placodes proliferate and give rise to primary mammary buds that grow downward into the mesenchyme by the regulatory factor released and enlarge in size and move from the dorsal to ventral side. These mesenchymal cells differentiate to form fibroblast, adipocytes, smooth muscles, and capillary endothelial cells. By the beginning of the second trimester of pregnancy, secondary epithelial buds appear from the edge of the central mammary bud. These secondary epithelial buds grow vertically around the mesenchyme and give rise to the lactiferous duct. The epithelial lining of the lactiferous duct is divided into two layers: the first layer, which is near to the lumen, has a secretory role, and the second layer, which is beneath the lumen, develops into myoepithelial cells. During the third trimester, mammary pit formation occurs, which in future becomes the nipple area of the breast. The proliferation of mesoderm further delineates the mammary pit. The mesoderm initiates proliferating by receiving stimulus from invaginating ectoderm in this region. The nipple is made up of smooth muscle fibers aligned circularly and longitudinally. In male mice, mesenchymal tissue surrounding the stalk begins to

condense until it forms a depreciated ductal system [9]. Altogether, these regulatory mechanism at the end of this development stage, form a basic ductal system that bestows the foundation for mammary growth through puberty.

1.2. Pubertal mammary gland development

Sexual dimorphism of the MG is developed during an embryonic stage in mice, whereas in the human case, it starts at the pubertal stage. The nipple formation and sexual dimorphism require parathyroid hormone-related protein (PTHLH) signaling. In contrast to mice, humans exhibit sexual dimorphism through a very peculiar mechanism. The male and female glands go through a similar development until puberty; their size varies under hormones [8]. In the case of humans, both male and female breast has normal growth, but as hormones play a vital role in their size difference, as made evident by the occurrence of gynecomastia, the benign outgrowth of breast tissue in males is a result of an imbalance between estrogen-androgen hormones. During birth, the mammary gland is a rudimentary ductal system capable of secreting milk in babies due to maternal hormone exposure, which subsides later. As the endocrine effect recedes, the gland goes through allometric growth in harmony with the overall body development till puberty. The MG go through expansive growth and branching, forming the bulbous duct-like structure in response to hormone and growth factors, filling the fat pad. These club-shaped bulbous ducts are terminal end bud (TEB) (Fig.1), which give rise to the primary ductal structure and regulate the surrounding stroma. Myoepithelial cells are formed when the cells located at the tip of the TEB cap undergo differentiation. These cells form the outer layer of the tubular ductal bilayer, which surrounds the inner luminal cells[10]. Further branching of primary ducts gives rise to secondary branches, which branch off the main ducts to form a tree structure that acquires around 60% of the surrounding fatty stroma. The formation of short under-developing tertiary branches occurs in response to cyclic ovary stimulation, but alveolar buds develop into fully functional milk-secreting units only when pregnancy hormones predominate [11]. The pubertal mammary gland in humans has a structure

resembling that of a mammary tree, but instead of having a TEB, it has lateral branches with terminal ducts, which gives rise to terminal ductal- lobular units, including many blind-ended ductules, called acini [8]. These acini structures are encapsulated in the fibroblastic, intralobular stroma that is far more evident in the human breast than the adipocytes-rich stroma of the rodent mammary tree.

1.3. Reproductive mammary gland development

This stage of the MG goes through three distinct development stages which are highly regulated by hormonal action 1) Pregnancy, 2) Lactation 3) involution. The mammary gland undergoes many changes in preparation for lactogenic differentiation and amelogenesis throughout this stage. The first stage in pregnancy is the proliferation and extensive branching of the primary duct into secondary and tertiary ductules, giving ductal stalk for the alveolar development and secondary transformation. The changes in the MG during puberty and the reproductive cycle (pregnancy to involution) are influenced by hormones and growth factors, such as growth hormone, prolactin, estrogen receptor, and progesterone (Fig.1). At the tip of TEBs, the epithelial cells propagate to produce alveolar buds that divide intensively and differentiate into well-defined alveoli, occupy the intraductal spaces, and later transform into milk-secreting lobules during lactation. During mid-pregnancy, increased vascularization is observed, which leads to the formation of a basket-like capillaries network around each alveolus [12]. Estrus cycles also contribute to these changes, including mild proliferation, differentiation, and partial secretion of milk proteins [11]. Lactation is a crucial phase of the mammalian reproduction system that involves a mother going through an intriguing physiological change that sustenance the survival and growth of her infant. Prolactin and oxytocin hormones are released from the pituitary gland, which is required to sustain milk secretion, with prolactin preserving the synthesis of milk products, while oxytocin promotes the letdown response that permits the newborn to extract milk from the mammary gland [13].

Lactating mammary gland over period switches to involution stage, a highly complicated

multi-step process where the lactating gland proceeds to appear morphologically like near pre-pregnant state. At this stage, the milk-secreting epithelial cells are removed, and the mammary tree is remodeled back into a ductal system. The central event in this stage involves a high degree of epithelial cell death, tissue remodeling and regeneration of the mammary adipose tissue [14].

1.4. Hormones and growth factors in mammary gland development

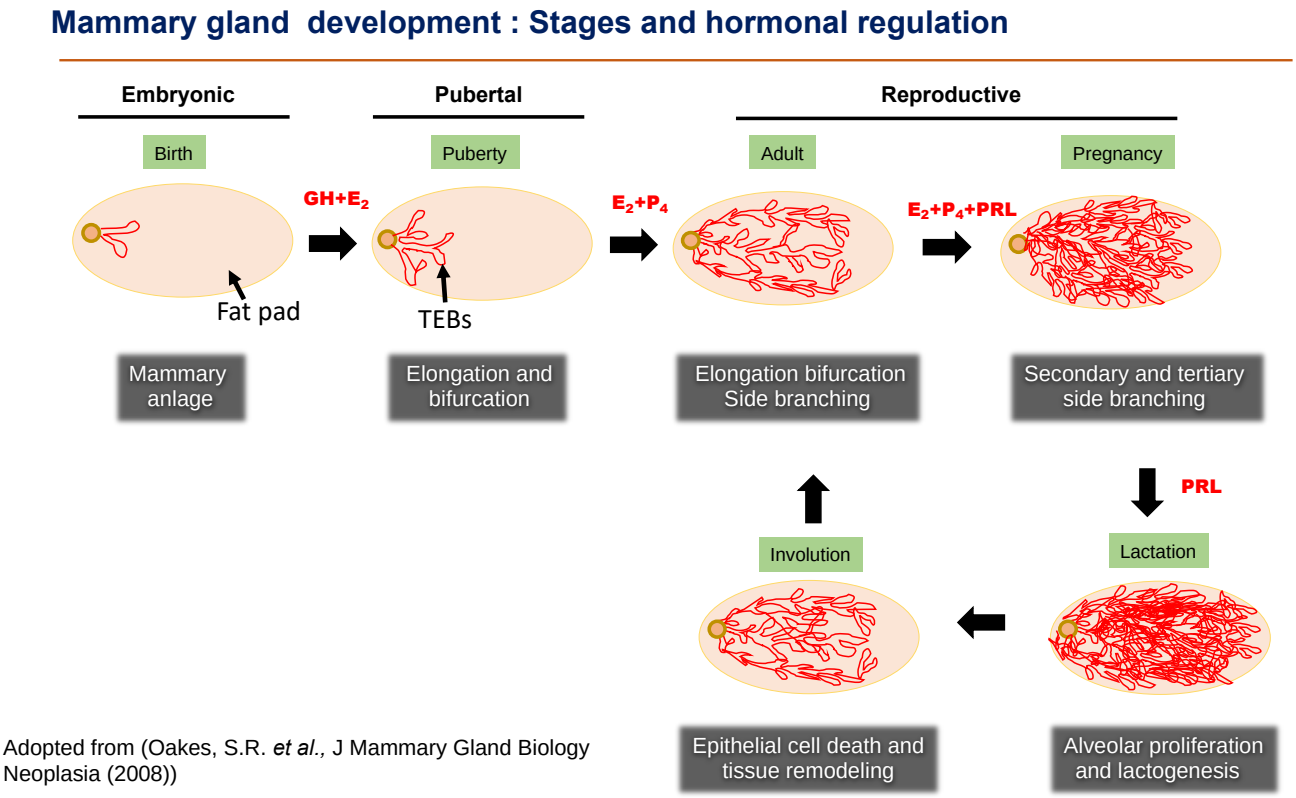
Earlier studies had demonstrated that pituitary extracts could regulate mammary gland function, as administration of pituitary extract on the mammary gland it enhanced mammogenesis and lactogenesis [15]. Later, it was discovered that growth hormone (GH) and prolactin (PRL) were responsible for these effects. Subsequently, further studies showed that *Gh*, insulin-like growth factor 1 (*Igf1*) or estrogen receptor alpha (*Esr1*) gene was important for pubertal mammary development, facilitating pathways responsible for ductal outgrowth and morphogenesis [16,17]. Interestingly, *Prl* or *Pgr* (progesterone receptor) gene-deficient mice show normal development as these genes are responsible for activating signaling pathways that regulate alveologenesis.

1.4.1. Growth hormone and insulin-like growth factor-1 in post-natal development

Growth hormones and insulin-like growth factor-1 play a very crucial role in post-natal development. The lack of *Gh* (growth hormone receptor) causes small mice and creates a model system for human Laron syndrome, a genetic disorder due to defect in GH that result in hereditary dwarfism [18]. The IGF1 mode of action on tissue growth is both endocrines as well as autocrine-paracrine. Its expression is site-specific, as in the case of mammary tissue it is expressed in the epithelial and stromal compartments. The *Gh* knock-out mice showed reduced expression of IGF1 in serum, delayed mammary gland development was observed with a subsequent underdeveloped mammary tree. GH and IGF1 are crucial for normal ductal growth and TEBs formation, an important event in post-natal mammary development [19].

1.4.2. Estrogen in pubertal mammary gland development

Estrogen, the ovarian hormone, is a crucial regular of MG development during puberty. The outgrowth of secondary and tertiary ductules happening during this period must create a functional mammary gland. The ovary releases estrogen, a soluble membrane ligand and stimulates gene expression by the intracellular receptor. Many studies confirmed the direct involvement of estrogen in MG development. In the first study using Elavax pellets, it has been shown that mammary gland development requires direct estrogen delivery to the gland. Elavax is a biologically compatible polymer with the tissue and can be implanted into it [20].



Adopted from (Oakes, S.R. *et al.,* J Mammary Gland Biology Neoplasia (2008))

Figure 1. Mammary gland development. Invasion of the fat pad by the mammary epithelium results in the formation of a tiny ductal network. After birth, the mouse's epithelium expands along with it, but the fat pad doesn't fill up until the ovarian hormones released during puberty cause it to do so (Estrogen E2, growth hormone GH). The ducts penetrate, branch, and finally fill the fat

pad with the onset of puberty. To stimulate tertiary branching and epithelium proliferation in the first stage of pregnancy, progesterone (P4) and prolactin (PRL) hormones are released. On the enlarged ductal tree, alveolar structures arise and later differentiate into lobular alveoli. This is when the lobular alveoli mature and the epithelium becomes secretory, ready to supply milk for suckling pups when they are born. Small pre-adipocytes have replaced the big fat cells, which have been dedifferentiated into pre-adipocytes at this point. Fat cells re-differentiate, and the mammary gland returns to its mature nulliparous condition when the secretory epithelium dies off by apoptotic necrosis.

Further, it has also been established that estrogen promotes ductal expansion in ovariectomized and intact mammary glands, and this ductal growth can be inhibited by tamoxifen, an estrogen receptor antagonist [21,22]. Estrogen receptors are expressed in the mammary gland's epithelial and stromal compartments. Estrogen and IGF1 show synergistic effects on ductal morphogenesis as they both together enhance the ductal outgrowth [17]. The intracellular estrogen receptors have two forms known as alpha and beta, which is encoded by *Esr1* and *Esr2*, respectively. The knock-out phenotype studies had shown that estrogen receptor alpha is crucial for proper ductal morphogenesis [23,24]. It was also observed that *Esr1* knock-out mice show a similar defect in mammary gland morphogenesis as in the case of *Igf1*. The lack of estrogen leads to the rudimentary ductal system and fails to form a functional mammary gland [23]. Apart from promoting cell growth during ductal morphogenesis, during pregnancy, it serves an important function in promoting the development and maintenance of alveolar cells [25]. Altogether these factors, including locally produced IGF1 in the stromal and epithelial compartment of the mammary, act through their receptor to promote ductal branching and TEB formation. Both estrogen and IGF1 works together to promote proliferation necessary for ductal morphogenesis.

1.4.3. Progesterone in reproductive mammary gland development

Progesterone is secreted from the ovary in all vertebrates. In mammals, progesterone hormone promotes epithelial growth, extensive side-branching and alveologenesis, which is essential for the mammary gland to become competent for lactation. In conjunction with prolactin, it also stimulates milk synthesis and differentiation of milk-related specialized structures like alveoli during lactation. The knock-out mice study of progesterone receptors revealed its importance in both processes. Due to the lack of progesterone, a simple epithelial tree in the mammary gland is observed. Even after experiencing the usual surge of growth at puberty in the mammary gland due to lack of progesterone, it shows no ductal proliferation or lobuloalveolar differentiation upon pregnancy [26,27]. Progesterone receptor (PGR) in the epithelial compartment is responsible for side-branching and alveologenesis [27]. Progesterone signaling happens by a paracrine pathway that is like estrogen signaling. Progesterone receptors have two receptor isoforms (PGRiA and PGRiB). They are expressed in the virgin and during pregnancy in the mammary gland, but PGRiA isoform expression is high compared to PGRiB [28]. To analyze the importance of each of these isoforms knock-out study was performed in reproductive tissues. Nevertheless, the loss of *Pgria* does not show any phenotypic effect in the mammary gland, but a drastic reduction inside branching and alveologenesis were observed upon loss of *Pgrib* during pregnancy [29,30]. These studies showed the critical function of PGRiB in MG development. However, the function of PGRiA is also important, which may be balanced by the existence of PGRiB. These two isoforms of progesterone regulate the expression of different sets of genes and the altered expression ratio of these two PGR have in breast cancer have different clinical outcomes [31,32].

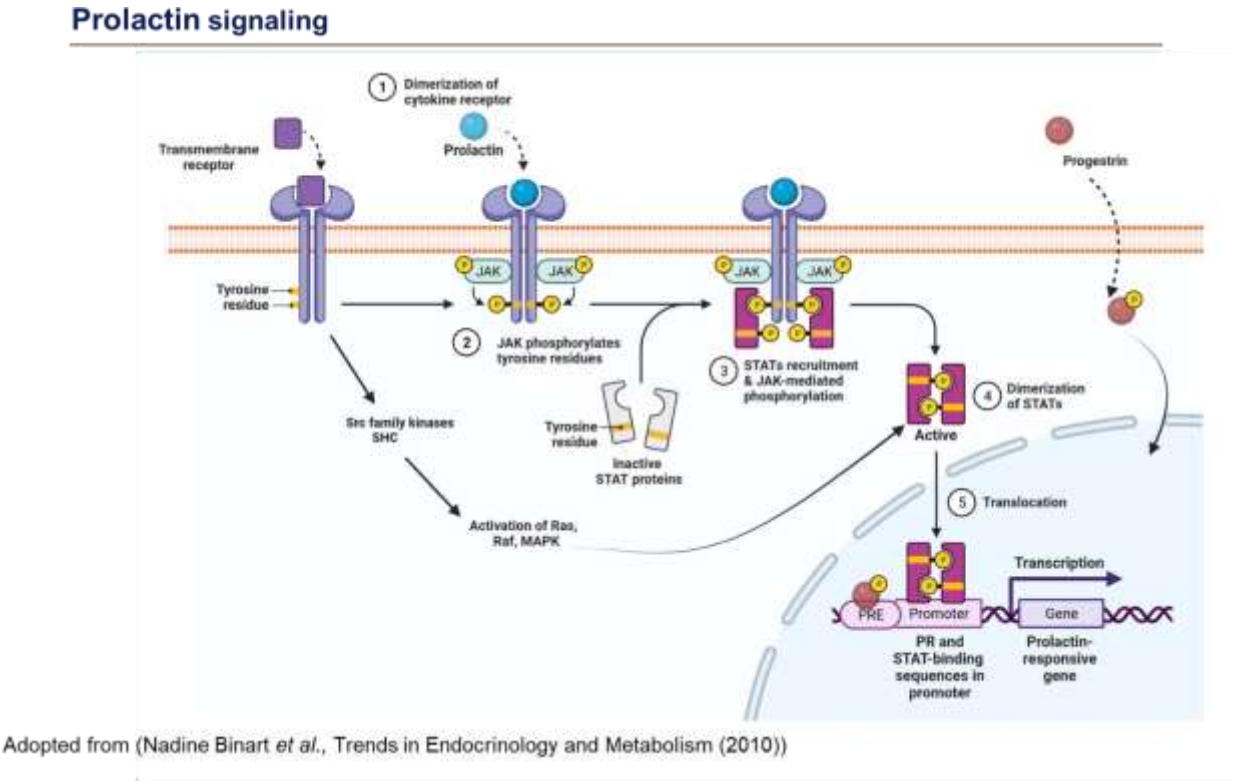
1.4.4. Prolactin signaling in lactation

PRL (Prolactin) is a small polypeptide hormone secreted from the anterior pituitary gland and other sites like mammary epithelium (in both rodents and humans), decidua and uterine myometrium (in humans) is a well-known site for the extra pituitary PRL secretion and processing

[33]. The mammary gland is one of the principal sites for the synthesis and secretion of PRL. It acts in both endocrine and autocrine ways and serves a significant role in the evolution of specialized cells (lactotrophs and acidophilic cells) and organs for milk production and secretion in mammals [34]. Prolactin is a known regulator of mammary gland development at three stages throughout the life history of females. It helps in organogenesis by promoting the formation of the primary ductal system from terminal end buds during the pubertal stage of mammary gland development [35]. It contributes significantly to the maturation of the primary ductal system into a fully nonpregnant gland. The mammary gland can be characterized by distinct features like terminal end buds, mammary trees (a highly branched structure) and lobular buds. During pregnancy, the mammary gland experiences enlargement of lobular buds into the lobuloalveolar system and extensive side branching [36,37]. These physiological variation in the MG is due to PRL, placental lactogens, and progesterone hormone.

Interestingly progesterone level subsides after the birth of a newborn, and PRL stimulates the secretion of milk protein gene expression and is followed by lactation. Studies have proven that PRL and the PRL receptor and transcription factors (STAT proteins) are critical for mammary gland regulation. PRLR belongs to the class I cytokine receptor superfamily. PRL binds to its receptors, causing homodimerization and leads to the activation of multiple signaling pathways, including Jak/Stat, MAPK, phosphoinositide (PI) 3 Kinase, and JAK2. The activation of the JAK2/STAT5 signaling pathway plays a vital role in forming alveologenesis. The knock-out study of Jak2 established this finding-/- and *Stat5-/-* mice where the same defect in MG was observed as in the case of *Prlr-/-* glands. Further studies illustrate that conditional lack of *Stat5* greatly impacts alveolar cells upon PRL-induced differentiation, proving that this signaling cascade is required for pregnancy [38-40]. This signaling cascade (PRLR/JAK2/STAT5) is also essential for the expression of milk protein like Beta-casein (*Csn2*) and acidic whey protein (*Wap*), which contain STAT5 responsive elements in their promoters (Fig. 2). There are several negative and positive regulators in this pathway, like integrins (the central ECM receptors necessary for full STAT5 activation [41].

Signal regulatory protein alpha (SIRPA), a transmembrane glycoprotein, acts as a mediator between integrin and PRLR. During lactation, it helps form a complex consisting of JAK2 and synchronizes the response of PRLR and integrin interaction with several ECM components [42]. RANKL is a downstream target of both PRLR, and PGR as homozygous deletion of either genos shows the same phenotype, disruption in these genes results in reduced side-branching and defective alveologenesis [43]. Besides, the expression of Rankl in virgin mice can prompt the characteristic features of pregnancy, resulting in side-branching and alveologenesis. Generally, PRL and progesterone are responsible for this kind of physiological change [44]. The suppressors of cytokine signaling (SOCS) family are a known negative regulator of PRLR signaling. Further *Socs1-/-* knock-out mice studies revealed better alveolar development and milk synthesis during pregnancy. Hence, out of many SIRPA, RANKL and SOCS are three good examples demonstrating the intricacy of the signaling pathways that conduct cells' response to PRL [45]. Altogether, these signaling pathways show a fascinating, highly intricate, but finely tuned transcriptional program that coordinates numerous signaling cascades and finally regulates the alveolar differentiation and milk production. Overall, the lactation phase plays a vital role in the evolution of mammals. Progesterone (P4) and PRL together contribute to these crucial reproductive phenomena by participating in an intricate and entangled relationship.



Adopted from (Nadine Binart *et al.*, Trends in Endocrinology and Metabolism (2010))

Figure 2. Crosstalk among growth hormone (GH), progesterone receptor (PGR) and the prolactin receptor (PRLR) in mammary epithelial cells signaling. Prolactin and progesterone are critical STAT protein regulators. The activation of PRLR by prolactin initiates multiple signaling cascades. Activation of JAK2 kinase, stimulates STAT5. Though PRLR, activation of the MAPK pathway can also result in activation of STATs. The activation of PGR by progesterone results in the activation of PGR genes, which include the downstream components of the PRLR signaling cascade (e.g., STAT5a). Additionally, progesterone augments prolactin mediated STAT5 activation via the JAK2 and MAPK pathways. Additionally, activation of Src family kinases provide a hormone-induced input to PRLR signaling (through STAT phosphorylation).

1.5. Role of miRNAs in mammary gland development

MicroRNAs (miRNAs) are non- coding endogenous RNAs of 18-25 bp long length. miRNAs were first discovered in eukaryotes, and these non-coding RNAs are currently recognized as crucial

regulators for most biological functions [46,47]. Furthermore, these miRNAs regulate several biological processes like hematopoiesis, organogenesis, body development, cell differentiation and proliferation, carcinogenesis, cell death, and various other processes by binding to 3'UTR of mRNAs and controlling their expression [48,49]. miRNAs also play an important role in MG development by modulating mammary cell proliferation, differentiation, and physiological function. Recent studies revealed the importance of miR-212/132 in mouse MG development [50]. Moreover, it has been revealed that miR-1266 and miR-616 also affect milk production and milk protein synthesis by regulating 5, 10-methylenetetrahydrofolate reductase (MTHFR) [51]. Some miRNAs help in remodeling of epithelium in involuting MG such as miR-424(322)/503 cluster and showed deterioration of secretory acini in the absence of miR-424(322)/503 [52]. Previously, several similar studies reported differential expression of miRNAs throughout the lactation and possible involvement of miRNAs in the physiological activity of the MG [53,54]. Using library establishment application, miR-23a, miR-24, and miR- 13 were identified to be present in breast fat cells and breast tissue and might play an essential role in lactation and overall MG development [55-57]. Also, MiR-155 is reported to involve in cell proliferation and apoptosis by controlling RhoA during mammary gland development [58,59].

Gene knock-out studies revealed that miR-101a is involved in the β-casein expression and differentiation of mammary epithelial cells. miR-101a and miR-199a* are also entangled in the expression and regulation of mammary gland differentiation by regulating Cox-2 (cyclooxygenase-2) [60-63]. In mammary gland development, different miRNAs are expressed throughout the development process. In one study, miR-129-5p and mir-126 were shown to target IGF-1(important in pubertal mammary gland development) and progesterone receptor (a major component and crucial for lactation), respectively, in mammary gland epithelial cells[64,65]. Furthermore, let-7g is shown to be involved in MG development by targeting Tgfbr1and, also influencing epithelial cells during lactation [64]. The recent studies confirmed that miR-139 and miR-15/miR-16 are differentially expressed throughout the cow mammary gland development and

might control the expression of growth hormone receptor (GH), but a further detailed investigation is required to delineate the mechanism [66,67]. Altogether, this evidence indicates that miRNAs play a critical role in mammary cell fate and gland development, lactation, involution, and the synthesis of milk ingredients [68-70].

1.6. Hematopoietic PBX interaction protein

Hematopoietic PBX interaction protein (HPIP), also known as pre-B-cell leukemia homeobox interacting protein (PBXIP1), was initially identified as a PBX1 interacting protein that acts as a repressor for the PBX1 transcription factor [71]. *HPIP* gene is located on chromosome 1 at position q21.3, and it encodes a 731 amino acid residue protein that does not have homology with any known protein. HPIP could also interact with other PBX family members such as PBX2 and PBX3. It also inhibits the transcriptional activity of E2A-PBX by preventing the binding of PBX-HOX complexes to DNA [71]. Interestingly, the expression of both HPIP and PBX1 is upregulated in early progenitor cells, e.g., CD34+ [71]. This strongly implies that HPIP plays a vital role in the early stage of hematopoiesis. Likewise, HPIP expression correlates with PBX1 in hematopoietic organs like bone marrow, spleen, tonsils, and lymph node tissues.

1.6.1. HPIP role in the developmental process

HPIP-Pbx is also expressed in other organ tissues and early embryos, insinuating that their function is not constrained to the hematopoietic system only, and it may also have roles in the developmental process. Recently HPIP is involved in erythroid differentiation and exhibits stem cell activity by controlling the PI3K/AKT/GSK3β signaling pathway [72] (Fig. 3). Besides, GATA1 transcription factors (erythroid-specific) bind to the HPIP promoter and enhance its expression in a CTCF-dependent manner [72]. HPIP also plays an essential role in germ cell proliferation by hindering the functional interaction between ERβ and Tex11 [73]. Tex11 is a gene involved in spermatogenesis, and its expression is elevated during spermatogonia and early spermatocytes

[74,75]. HPIP also negatively regulates the transcriptional activity of estrogen receptor ERα and interacts with microtubules in breast cancer [76]. The steroid hormone, 17β- estradiol (E2), is crucial for several cellular processes like cell proliferation, differentiation, apoptosis and is also needed for reproduction and mammary gland development. The ratio of these two nuclear hormone receptors, estrogen receptor α and β (ERs) is essential for HPIP function [77], although exact mechanism of action has not yet been determined. Additionally, HPIP has been linked to neural development, embryogenesis, and endometrial decidualization in women who had multiple implantation failures[78,79]. HPIP overexpression has been also associated with psychiatric disorders [78]. However, comprehensive research is urgently needed to address its role in diverse cellular processes. It is also necessary to examine its mechanistic functions.

1.6.2. HPIP role in oncogenesis

HPIP, a scaffold protein, stimulates estrogen signaling in an ERα-dependent manner and recruits p85 subunit of PI3K and Src kinase to form a complex with ERα, which further activates AKT and MAPK [76,78] (Fig. 3). Additionally, studies have also shown that estrogen-activated kinase TBK1 phosphorylates HPIP, leading to its degradation in an MDM2-dependent manner[79]. HPIP is mainly localized in the cytosol but can shuffle between cytosol and nucleus.

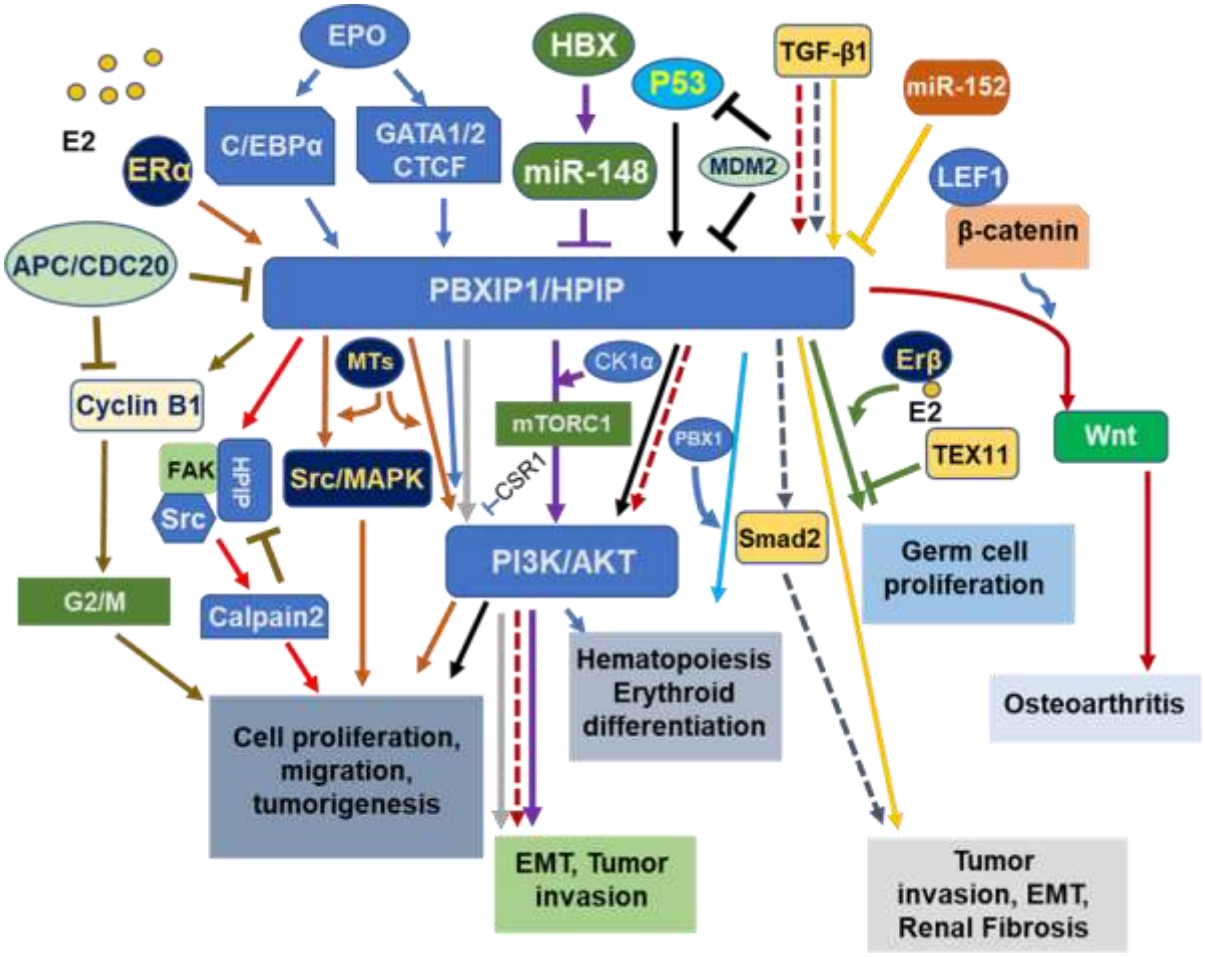


Figure 3: HPIP signaling in cellular and physiological functions. HPIP modulates PI3K/AKT/GSK3 to drive erythroid differentiation and hematopoietic stem cell activity. The erythroid lineage-specific transcription factor GATA1 binds to the HPIP promoter and drives CTCF-dependent gene transcription. HPIP interacts with FAK-Src to activate Calpain2, stimulates Src/MAPK pathway promotes G2/M phase transition and Wnt signaling which results in Epithelial-Mesenchymal Transition, migration, tumorigenesis, proliferation, and osteoarthritis. It also interacts with microtubules in breast cancer cells and inhibits ER transcriptional activity. HPIP is phosphorylated by the estrogen-activated kinase TBK1, which leads to MDM2-dependent HPIP degradation in breast cancer cells. It has also been known as a new transcriptional target of the P53 protein, miR-148 and miR-152.

The C-terminal domain (aa 443-731) of HPIP contains a nuclear export signal that enables it to enter the nucleus [80]. It also regulates cell adhesion and migration though modulation of focal adhesion dynamics by activating the FAK/ERK/Calpain2 pathway to promote breast cancer metastasis [81]. Numerous studies have shown that HPIP is highly expressed in many cancers like gastric cancer, colorectal cancer, hepatocellular carcinoma (HCC), glioma, leiomyosarcomas, oral carcinoma, pancreatic cancer, renal cell carcinoma (RCC), epithelial ovarian cancer (EOC), lung cancer, cervical cancer, endometrial cancer (EC) [82-89]. HPIP serves as a prognostic marker for ovarian cancer as its expression is correlated with high-grade ovarian tumors. In addition to this, HPIP can also be a potential therapeutic target for cisplatin-resistant ovarian tumors [90]. In the case of breast cancer cells, HPIP sensitizes breast cancer cells to paclitaxel in a microtubule- dependent manner [91]. Recent studies have revealed that cellular stress response 1 (CSR1), a tumor suppressor gene inhibits cell proliferation, migration, and invasion by inactivating HPIP and its downstream signaling target like PI3K/AKT [92]. It also plays a very significant role in cell cycle progression. HPIP acts as both substrate and inhibitor for APC/C-Cdc20 complex required for the temporal stability of cyclin B1 though the G2/M transition and so controls mitosis and cell division [93]. HPIP also promotes hepatoma cell proliferation though activation of G2/M checkpoint by increasing cyclin B1 [93,94]. The accumulating evidence support that HPIP promotes cell proliferation, migration, anchorage-independent growth, and invasion of cancer cells though the activation of AKT/MAPK pathways [76,94,95]. Recent studies revealed that HPIP regulates EMT by activating the PI3K/AKT/GSK3β/SNAIL and TGF-β/Smad2 pathways in ovarian and lung cancer, respectively [87]. It has been shown that HPIP promotes carcinogenesis though a variety of signaling cascades, including TGF-beta1, PI3K/AKT, Wnt/mTOR, and the Sonic hedgehog signaling pathway [76,94,96-98]. Although HPIP plays a vital role in breast cancer development and progression, its other biological significances cannot be overruled. Recent research shows HPIP as an important biomarker for several malignancies. Therefore, HPIP is a promising therapeutic target in cancer.

1.7. Rationale and objective:

Hematopoietic PBX interaction protein (HPIP), also known as pre-B-cell leukemia homeobox interacting protein (PBXIP1), was discovered as an interacting partner of PBX1 and operated as a repressor for the transcription factor PBX1 [71]. The function of HPIP is not restricted to the hematopoietic system, and it also has a role in various other cellular processes. It is involved in erythroid differentiation and displays stem cell activity by modulating the PI3K/AKT/GSK3B signaling pathway [72]. Recent studies revealed that HPIP regulates epithelial-to-mesenchymal transition by activating the PI3K/AKT/GSK3β/SNAIL and TGF- β/Smad2 pathways in ovarian cancer and lung cancer, respectively [87]. Although HPIP's role in breast cancer development and its functional relationship with ERα is known, its biological significance in mammary gland development is yet to be documented. Prolactin (PRL)-induced phosphorylation of STAT5a (signal transducer and activator of transcription) is considered a key event during mammary gland development and, lactational differentiation. PRL also activates PI3K–AKT signaling though STAT5a [99,100], thereby significantly augmenting the pro- survival PI3K/AKT pathway during mammary gland development [101]. While PRL, STAT5a, and PI3K/AKT signaling play a pivotal role in preparing the mammary gland to lactate, molecular integrators and the precise molecular mechanisms underlying this physiological process remain largely unknown.

Objective:

To determine the role of HPIP in mammary epithelial cell differentiation

1.8. Materials and Methods

1.8.1. Laboratory Animals

Female BALB/c mice of different age groups were purchased from Jeeva Life Sciences (Hyderabad, Telangana, India). They were treated and handled according to the "Recommendations for the Handling of Laboratory Animals for Biomedical Research" complied by the Institutional Animal Ethical Committee (IAEC, approval no. UH/IAEC/BM/2014-I/17) as per the CPCSEA, India guidelines for Laboratory Animal Experiments in our Institute. In this study, we used 6-week-old mice for studying expression of HPIP gene during mammary gland development. We kept them for 10 days to mate, then separated the male and female mice in separate cages and counted that day as the first day of pregnancy. The gestation period for BALB/c mice is 20 days; we counted the zero day of lactation on the day the pups were born; for the involution stage, we removed the pups after 12 days of lactation and counted that day as the zero day of involution.

1.8.2. Cell culture

HC11 cells (kind gift from Dr RK Vadlamudi, University of Texas, San Antonio, USA) were grown at 37°C with 5% CO_2 in RPMI medium supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. MCF10A cells were grown in 1:1 DMEM-F12 medium supplemented with 5% horse serum, 10 µg/mL insulin, 20 ng/mL EGF, 0.5 µg/mL hydrocortisone and 100 ng/mL cholera toxin.

1.8.3. Stable expression or knock down of genes by lentiviral mediated viral transduction

HPIP silencing in either MCF10A or HC11 cells was carried out by transfecting HPIP-specific shRNA (Dharmacon, Lafayette, CO USA) along with packaging plasmids (pREV, VSV-G and pΔR in the ratio of 1:0.4:0.5) using Lipofectamine™ 2000 (Invitrogen, Waltham, Massachusetts, USA) in HEK293T cells as described previously [81]. Forty-eight hours post- transfection, viral

soups were collected and added to either MCF10A or HC11 cells. Subsequently, positive clones were selected by eliminating the untransfected cells using 1μg/mL puromycin. After verifying the HPIP knock-down by Western blotting, we used them for various studies. Similarly, ectopic expression of miR-148a in HC11 cells was carried out. When necessary, HC11 cells were treated with Flag-HPIP carrying viral soups.

1.8.5. Acini formation in 3D culture

The Poly-D-Lysine coated 8-well culture slides (BD Biosciences, Franklin Lakes, New Jersey, USA) were used for 3D culture. Each well was first coated with 50 μL growth factor reduced Matrigel® (BD Biosciences, USA) followed by 5,000 cells in 200 μL growth medium were seeded and allowed to attach for 1h. 200 μL growth media containing 10% Matrigel® was added on top, making a final concentration of 5% Matrigel® in complete growth medium. The morphology of the colonies was evaluated after 3 to 14 days for the samples. Cell staining was carried out as described before [102]. Antibodies used for immunostaining were anti-β-integrin (Cell Signaling Technology, USA). All images were analyzed and processed using laser scanning confocal microscopy (Zeiss 510 UV/Vis Meta).

1.8.5. Plasmid constructs

1.8.5.1. Cloning of *HPIP* 3'untranslated region (3'UTR)

HPIP 3'UTR comprised of 951 base pair regions downstream to stop codon of HPIP gene was PCR-amplified with specific primers (Table 1) using HeLa cDNA as template, then subsequently cloned into pMIR-REPORT luciferase reporter vector (Promega, USA). The plasmid construct was verified by DNA sequencing. The cloning details of pMNDUS-HPIP, which expresses Flag-HPIP, was described earlier (11).

1.8.5.2. Cloning of *miR-148a* gene and HPIP promoter

Genomic region of miR-148a located at chromosome 7 (~300 bp) was PCR-amplified using HeLa DNA as a template and gene-specific primers (Table 1). The PCR product-containing pre-miRNA was cloned into pGIPz lentiviral vector (Open Biosystems, USA) and sequence verified. Similarly, the *Hpip* promoter region of approximately ~2 kb was PCR-amplified using mouse mammary gland genomic DNA as a template and gene-specific primers (Table 1). The PCR product was subsequently cloned into pGL3-Luc vector (Promega, USA) and sequence verified.

1.8.6. Western blotting

Cells were lysed in NP40 lysis buffer (50 mM, Tris-Cl pH 7.4, 150 mM NaCl, 1% Nonidet P- 40, 0.5% sodium deoxycholate, 1 mM PMSF and 1X protease inhibitor cocktail) and subjected to SDS-PAGE using BIO-RAD electrophoresis units followed by Western blotting with following protein-specific antibodies: anti-HPIP (Abcam, USA), anti-β-casein (Santa Cruz Biotechnology, USA), anti-GAPDH and pAKT (Cell Signaling Technology, USA), anti- pSTAT5a (Cell Signaling Technology, USA), anti-STAT5a (Invitrogen, USA) (Table 2).

1.8.7. Whole-mount staining

For whole-mount staining, mammary glands dissected from virgin, pregnant, lactating and involution mice were spread on glass slides and fixed for 24 h in Carnoy's fixative (ethanol: chloroform: glacial acid, 6:3:1). Tissues were washed for 15 minutes in 70% ethanol, rehydrated in distilled water for 1 hour and stained overnight in carmine alum (2 g/L carmine, 5 g/L potassium sulfate). Next day, tissues were washed in 70% ethanol, dehydrated using absolute alcohol, cleared with xylene, and mounted using DPX resin. Images were captured using Sony series-S HD camera.

1.8.8. Immunohistochemistry (IHC)

Mice carrying different stages of mammary glands were sacrificed. The glands were dissected and

fixed with 10% paraformaldehyde followed by embedding in paraffin blocks. After rehydration, 5 µM sections of representative tissue blocks were subjected to antigen retrieval in boiling buffer (10 mM sodium citrate and 10 mM citric acid) for 10 min. Then, sections were treated with a protein-blocking solution for 30 minutes and incubated with primary antibodies (HPIP and β-casein) at 1:100 dilution overnight at 4°C. After several rinses in PBS, the sections were incubated in a biotinylated secondary antibody for 30 minutes. The bound antibodies were detected using the Dako kit (Cat No. K405) (Agilent, USA). The slides were rinsed in PBS, exposed to diaminobenzidine, and counterstained with Mayer's hematoxylin. For immunofluorescence studies, we probed the mouse mammary tissue of pregnancy day 6 with anti-HPIP (primary antibody) followed by secondary antibody (anti-rabbit) linked to Alexa-546 and captured the images using a fluorescence microscope (Olympus, Singapore). DAPI was used as a nuclear marker.

1.8.9. Chromatin immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation was performed as described previously [103]. For ChIP in mammary tissue, dissected tissues were fixed with 7% formaldehyde for 10 min at room temperature, followed by glycine treatment to stop the cross-linking. After thorough washing in PBS, tissues were snap freeze in liquid nitrogen. Frozen tissues were lysed using lysis buffer and subjected to immunoprecipitation using antibodies directed against STAT5a (Invitrogen) or an isotype-matched control IgG. Input and bound chromatin was detected by quantitative reverse transcription-PCR (qRT-PCR) using primer sets surrounding high-affinity STAT5a- binding sites in target genes. Assay background was detected using primers directed against a non-promoter site in HPIP and was used for normalization.

1.8.10. Luciferase reporter assay

Luciferase assay was performed using the Bettelluceferin-Glo Luciferase Assay kit according to

the manufacturer's protocol (Promega USA). Approximately 200 ng of reporter plasmids (pGL3-*Hpip-Luc*), 500 ng of STAT5a-wt or STAT5a1*6mt (constitutively active Stat5) (kind gift from Dr Toshio Kitamura, University of Tokyo, Japan) and 50 ng of Renilla luciferase (internal control) were transfected. All transfections were performed using Lipofectamine-2000 (Invitrogen, USA) according to the manufacturer's instructions. The relative luciferase activity is represented in the bar diagram.

1.8.11. Real-time Quantitative Reverse Transcriptase PCR

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA, and RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's specifications. cDNA was diluted (1:10) for PCR reaction. The quantitative real-time PCR was performed using FastStart SYBR Green Master (Roche Applied Science, Mannheim, Germany) and LightCycler®96 Real-Time PCR System machine. The sequences of primer sets are listed in Table 1.

1.8.12. Prolactin antagonist treatments

HC11 cells were seeded in 60 mm plate for 24 h in conditioned media. Briefly, PBS wash cells were cultured in fresh conditioned media (without FBS) along with 5 μg/mL of Δ1–9-G129R-hPRL (PRLR antagonist, a kind gift from Dr. Vincent Goffin, INCERM, France) for 48 h, harvested and processed for Western blotting and qRT-PCR analysis.

1.8.13. Oil red O staining

Lipid droplet formation was performed as described earlier [104]. After removing the supernatant from the culture plates, cells were washed once with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 15 min at room temperature. The formalin was removed, and oil red O solution was filled into the culture plates. The plates were incubated at room temperature

for 30 min. They were then washed 3 times with distilled water. To elute the dye, 100% 2-propanol was added to the plates. The plates were incubated for 10 min at room temperature, 400 µL of the eluate was used for measurement at 510 nm UH5300 Hitachi spectrophotometer.

1.8.14. Statistical analysis

All the experiments were performed two to three times. The results are expressed as standard error mean, and the differences between groups were analyzed by one-way ANOVA using a sigma plot. Two-tailed unpaired Student's t-tests using Sigma plot were employed to evaluate the statistical significance.

1.8.15. Table1

List of primers used in the study

Sl. No.	Primer Name	Sequence	Restriction enzyme	Clone name
Site directed mutation				
1	HPIP 3'UTR SF1 FP	5'GGGTTG*GAGCTC* TACCCGGGAC 3'	*Sac* I	mtHPIP-1
2	HPIP 3'UTR SF1 RP	5'ATGCA*AAGCTT* GGTCAGCATGTGGG 3'	*Hind* III	
3	HPIP 3'UTR SF2 FP	5'ATGCA*GAGCTC* CCTTGACCCACATGC 3'	*Sac* I	mtHPIP - 2
4	HPIP 3'UTR SF2 RP	5'CTGCA*AAGCTT* TGCTACATCTCCCAG 3'	*Hind* III	
5	HPIP 3'UTR SF3 FP	5'ATGCA*GAGCTC* CTGGGAGATGTAGC 3'	*Sac* I	mtHPIP - 3
6	HPIP 3'UTR SF3 RP	5'CGCT*AAGCTT* TCCACATCACA 3'	*Hind* III	

Cloning				
7	Hsa miR-148a FP	5'TCGA*CTCGAG* GACCCGTTCCATTATCGGTCGC 3'	XhoI	pGipz-miR-148a
8	Hsa miR-148a RP	5'TCGA*ACGCGT* CTACAGTCAGGAGTCCACCAGGG 3'	BamHI	
9	HPIP 3'UTR WT FP	5'GGTG*GAGCTC* TACCCGGGAC 3'	*Sac* I	wtHPIP 3'UTR
10	HPIP 3'UTR WT RP	5'CGCT*AAGCTT* TCCACATCACA 3'	*Hind* III	
RT PCR				
11	*Hpip* FP	5' ATGGGTCTTCTGCTGGACAA 3'	NA	
12	*Hpip* RP	5' CAGGCTCTGAAGCTCTTCCTT 3'	NA	
13	*β - Actin* FP	5' AGCCATGTACGTAGCCATCC 3'	NA	
14	*β - Actin* RP	5' CTCTCAGCTGTGGTGGTGAA 3'	NA	
15	*β - casein* FP	5′-ACTCCAGCATCCAGTCACAGC-3′	NA	
16	*β - casein* RP	5′-AGGTGAGTCTGAGGAAAAGCC-3′	NA	
17	*Prl* FP	5 -CTCTCAGGCCATCTTGGAGAA-3	NA	
18	*Prl* RP	5 GGCTGACCCCTGGCTGTT-3	NA	
19	*Wap* FP	5' CCCTCGCTCAGAACCTAGAG 3'	NA	
20	*Wap* RP	5' TGTTGACAGGAGTTTTGCGG 3'	NA	
21	STAT5A BS *Wap* Pro FP	5' CATCTCTTCCTGCCCATGAC 3'	NA	
22	STAT5A BS *Wap* Pro RP	5' TCGGGCATACATTGAAAAGG 3'	NA	
23	STAT5a BS1 *miR148a* Pro FP	5' GATCTCCACAGCCCAAAAGC 3'	NA	

24	STAT5a BS1 *miR148a* Pro RP	5’ TCCGATTTAGATGCAGTTCACT 3’	NA	
25	STAT5a BS2 *miR148a* Pro FP2	5' TCC CGA TGA TAA ACCACT GGA 3'		
26	STAT5a BS2 *miR148a* Pro RP	5' CCG TGG TGC TGA CAG GTA A 3'		
27	STAT5a BS *Hpip* Pro FP	5'GAA AAG AAG GGG GTG GAG GG 3'		
28	STAT5a BS *Hpip* Pro RP	5'CAG CAT CTC ACT GCC TTA TTA GG 3'		
shRNA				
29	sHHPIP #1	ATGTTCTTAGCAGAGAGGC		PGipz-shHPIP
30	shHPIP # 2	AATTCTTTCCCATCTGTCT		

1.9. Results

1.9.1. HPIP is differentially expressed during mammary gland development

Estrogen receptor alpha (*Esr1* encodes ERα) is essential for mammary gland development as *Esr1* null mice develop rudimentary gland [105]. Previous studies demonstrated a regulatory role for HPIP on ERα functions in breast cancer cells [78], which led us to hypothesize a role for HPIP in mammary gland development. To test this hypothesis, we first analyzed HPIP expression during various stages of mammary gland development using IHC. The IHC data revealed a high-level expression of HPIP in pregnancy and lactation but was undetectable in the virgin and involution stages (Fig. 4A). Furthermore, immunofluorescence (IF) staining of HPIP in lactating gland revealed a clear cytoplasmic localization of HPIP in mammary epithelia (Fig. 4B). To further substantiate this finding, we examined the expression of HPIP at different stages of the mammary gland by qRT-PCR and Western blotting (Fig. 4C-D). Consistent with IHC data, HPIP (mRNA and protein) was undetectable in the virgin mammary gland. However, we could detect a marked increase in HPIP levels during the transition from pregnancy to lactation, but again undetectable in involution (Fig. 4C-D). Though *HPIP* expression starts at 6^{th} day of pregnancy and increases gradually during pregnancy, it peaks at 6^{th} day of lactation and declines as involution approaches (Fig. 4C). Consistent with this data, we also observed a differential expression of HPIP protein in parallel to its transcript (mRNA) during mammary gland development (Fig. 4D). Together our data show that lactation is associated with increased expression of HPIP.

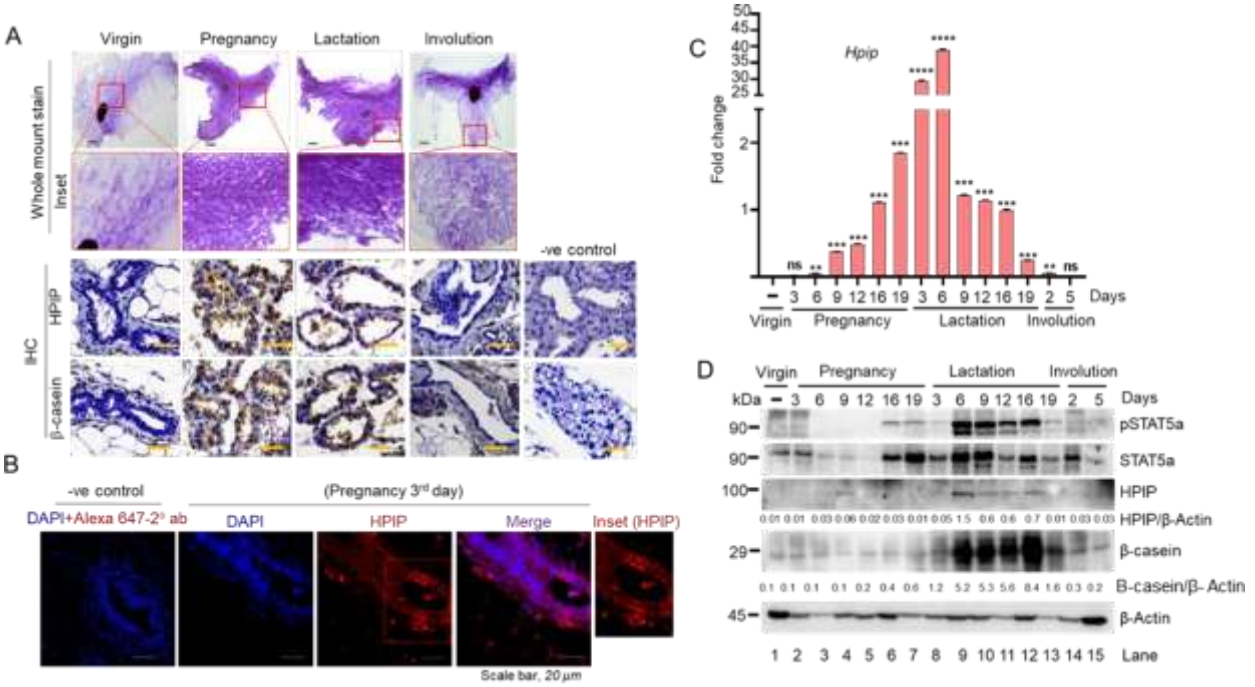


Figure 4: Differential expression of HPIP during mammary gland development. (A) Whole-mount staining of mouse mammary tissue at various developmental stages such as virgin (6 weeks), pregnancy (day 16), lactation (day 12) and involution (day 5) (upper panel, scale bar- 200 µm). Immunohistochemistry (IHC) images represent the expression of HPIP (middle panel) and β-casein (lower panel) in mammary tissue at various developmental stages as indicated. Scale bar- 20 µm (B) Fluorescence images represent the expression of HPIP in mouse mammary gland of pregnancy day 3. (HPIP, Alexa 647-red; nucleus stained with DAPI, blue), Scale bar- 20 µm. Alexa 647 (red)-tagged secondary antibody followed by DAPI stained tissue of pregnancy day 6th serves as a negative control. (C) Real-time qRT–PCR analysis of Hpip gene expression at various stages of mammary development as indicated. Gene expression levels were normalized to β-Actin. Data represents the mean ± SE.*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ measured by one-way ANOVA (n=3). (D) Western blot analysis demonstrates the differential expression of pSTAT5a, STAT5a, HPIP and β-casein at various stages of mammary gland development. β-Actin is a loading control.

1.9.2. *Hpip* is a PRL responsive gene

As PRL/STAT5a signaling regulates mammopoiesis/lactogenesis [16,106,107], the rise in HPIP expression during these stages led us to investigate whether this pathway may regulate HPIP. To that end, we used the immortalized, non-transformed HC11 mammary epithelial cell line that expresses the milk protein β-casein in response to bPRL (bovine prolactin) stimulation. HC11 cells were treated with bPRL for 24 h and *Hpip* expression was analyzed by qRT-PCR and Western blotting. As shown in (Fig. 5A-B) bPRL significantly induced the expression of *Hpip* and *β-casein* transcripts as well as proteins in HC11 cells. STAT5a is a transcriptional factor that regulates the expression of various genes during mammary gland development in response to bPRL. Therefore, we analyzed HPIP expression after STAT5a silencing in HC11 cells. STAT5a knock-down significantly reduced HPIP expression (Fig. 5C), indicating that PRL-STAT5a signaling regulates HPIP expression in HC11 cells.

Next, we verified whether STAT5a directly binds to the *Hpip* promoter, harboring one putative STAT5a binding site near the translational start site (Fig. 5D). To measure the STAT5a transcriptional activity on *Hpip* promoter, we next performed promoter probe Luciferase reporter assay in HC11 cells using *Hpip* promoter (2.3 kb) cloned into pGL3 vector (Fig. 5E). As compared to empty vector-treated cells, STAT5a-transfected cells displayed ~30-fold increased luciferase activity. Furthermore, transfection of a constitutively active STAT5a mutant (STAT5a1*6mt) showed an even higher induction of luciferase activity (~120 fold) (Fig. 5E). To further confirm the direct binding of STAT5a to the *Hpip* promoter, we performed a ChIP assay. Consistent with the luciferase data, ChIP assay demonstrated that STAT5a readily recruits to GAS element on *Hpip* promoter similar to *Wap* promoter, a known target of STAT5a and the recruitment of STAT5a is further enhanced by bPRL treatment (Fig. 5F). In support of this finding, ChIP on *ex vivo* mammary gland tissue further demonstrated that STAT5a binds to the *Hpip* promoter during lactation compared to other stages of mammary gland development (Fig. 5G). Together our data suggest that *Hpip* is a PRL-responsive gene, and its expression is dependent on STAT5a activity.

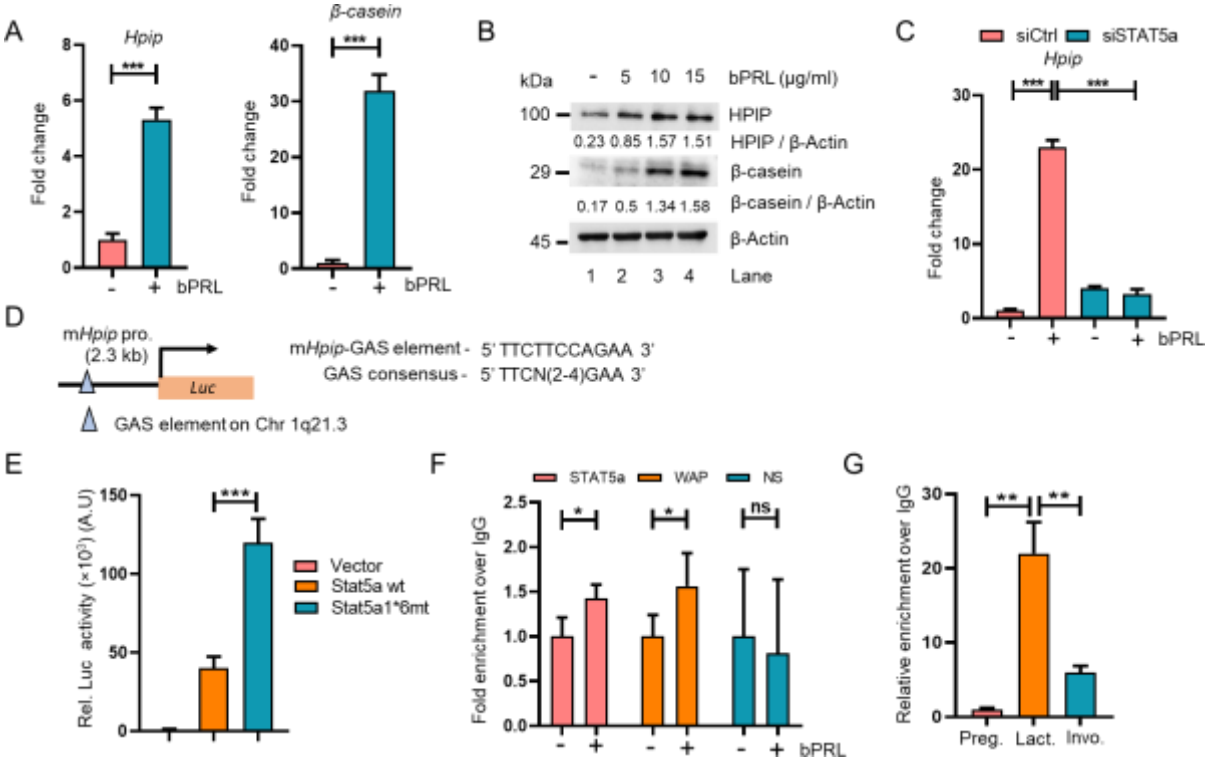


Figure 5: Hpip is a PRL-responsive gene. (A) Real-time qRT-PCR analysis showing the expression of the *Hpip* and *β-casein* in HC11 cells in response to bPRL (5 µg/mL) treatment for 48 h. (B) Western blotting analysis showing the expression of HPIP and β-casein in HC11 cells in response to PRL (5 -15 µg/mL, 48 h) treatment. β-Actin serves as a loading control. (C) The *Hpip* expression upon STAT5a knock-down in HC11 cells in response to bPRL was assessed by qRT-PCR. (D) Physical map of mouse *Hpip* promoter (2.3 kb) fused with Luciferase (Luc) gene. GAS element in the *Hpip* promoter was located at Ch 3:54 (left panel). Comparison of the *Hpip* promoter with consensus GAS sequence (right panel). (E) Luciferase assay demonstrating the effect of either wild type STAT5a or active mutant ofSTAT5a (or STAT5a1*6mt) on the activity of *Hpip* promoter in HC11 cells. (F) STAT5a enrichment onto Hpip promoter in HC11 cells in response to bPRL treatment (5 µg/mL, 48 h) was assessed by ChIP assay. *Wap* gene promoter was used as a positive control. NS, a non-specific region, was used as a negative control. (G) ChIP demonstrating STAT5a enrichment onto *Hpip* promoter at various stages of mammary gland development as

indicated (Preg.-pregnancy at 6th day, Lact.-lactation at 643th day, Inv.- involution at 2nd day). Data represent the mean ± SE. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 measured by student t-test. (n=3)

1.9.3. HPIP promotes mammary acini formation and lactogenic differentiation in HC11 cells

During the pregnancy/lactation stage, mammary epithelial cells undergo complex morphological and phenotypic programs that acquire apical/basal (A/B) polarity and acini formation for proper lactation. Prolactin is the primary signal for establishing cellular polarity and acini formation [108]. Using 3D culture of mammary organoids with either primary mammary epithelial cells or mammary cell lines, hormones, growth factors, extracellular matrix proteins, and intracellular signaling proteins in mammary epithelial morphogenesis such as acini formation lactogenic differentiation has been extensively studied [11,109]. To assess the role of HPIP in these processes, we first investigated the consequence of HPIP silencing on mammary acini formation *in vitro* using MCF10A cell line, an immortalized, non-transformed human mammary epithelial cell line that has been extensively used for such studies [102]. MCF10A- shCtrl cells showed organized acini previously reported [102,110] (Fig. 6A). In contrast, MCF10A-shHPIP cells showed loss of formation of organized mammary acini (Fig.6A). Moreover, HPIP-silenced cells had fewer cells per acinus and smaller acini than shCtrl-MCF10A cells (Fig. 6B, D). To further validate these findings and address whether HPIP is necessary for mammary acini formation and alveologenesis in response to bPRL,we performed similar experiments using HC11 cell line, which expresses high levels of PRLR (MCF10A has very low levels of PRLR), in 3D Matrigel culture system. We silenced HPIP expression by HPIP-specific shRNA in HC11 cells using a lentiviral-mediated knock-down approach (Fig. 6C). We then performed acini formation assays in a 3D culture system in the presence or absence of bPRL (5 µg). Compared to shCtrl-HC11 cells, shHPIP-HC11 cells showed improper acini where epithelial cells were not cleared entirely in the lumen (Fig. 6E) (Video S1-S4). To enumerate these observations, we quantified the organized acini in HPIP- silenced cells.

As shown in (Fig. 6F-G), HPIP-silenced cells treated with bPRL showed a significant decrease in organized acini development compared to bPRL-treated control siRNA- HC11 cells indicating that HPIP expression by PRL is required for *in vitro* acinar formation (Fig. 6H).

Next, we evaluated lipid droplet formation as a function of lactogenic differentiation in response to bPRL upon HPIP silencing using HC11 cells. As shown in (Fig. 6I-J), oil red staining analysis revealed that HPIP silencing significantly reduced bPRL-induced lipid droplets. In support of this data, HPIP silencing significantly reduced β-casein expression in response to bPRL (Fig. 6K). Together this data suggests that HPIP is required for mammary acini formation and lactogenic differentiation in HC11 cells.

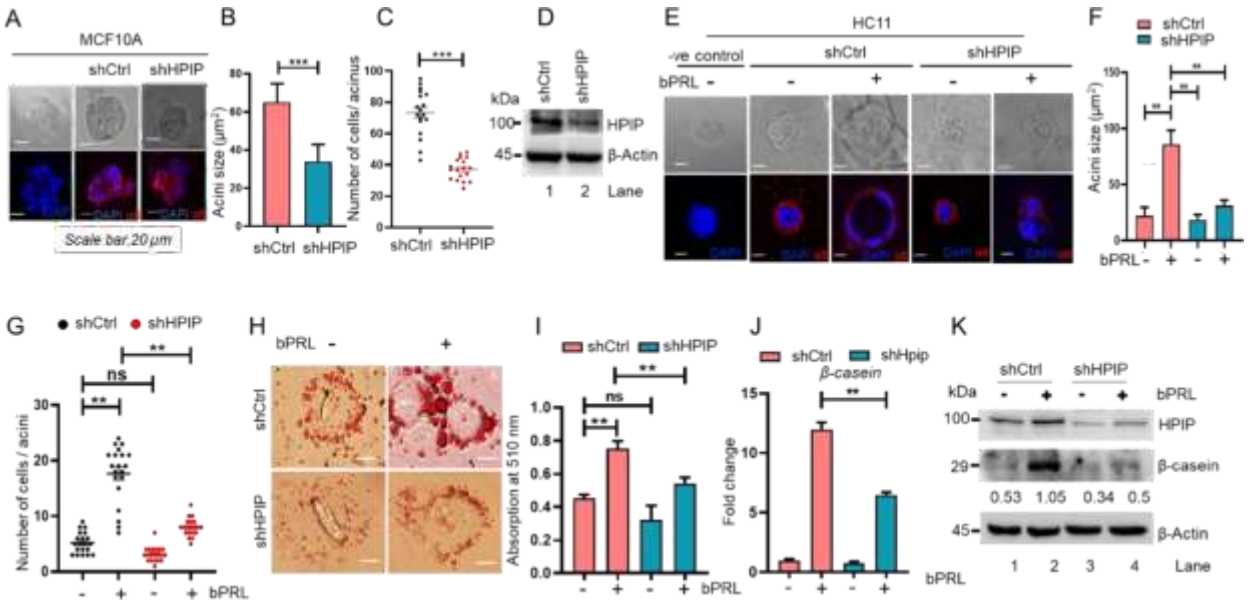


Figure 6: HPIP promotes mammary acini formation and lactogenic differentiation *in vitro.* (A) Acini formation assay with MCF10A cells. Representative phase-contrast and immunofluorescence images of MCF10A cells transfected with shCtrl or shHPIP. (B) The acinus size in shCtrl and shHPIP cells. (C) Quantification of the number of cells per acini in the presence and absence of HPIP. Acini formation assay with HC11 cells. (D) Western blot analysis demonstrates HPIP knock-down in HC11 cells. Effect of HPIP knock-down on (E) acini

morphology (F) acini size (G) number of cells per acini in response to bPRL. Acini were first probed with α6 antibody followed by staining with Alexa 647 (red)-tagged secondary antibody and DAPI (nuclear marker). Alexa 647 (red)-tagged secondary antibody followed by DAPI staining of acini serves as a negative control. Scale bar- 20 µm. (H-I) HPIP knock-down and bPRL on lipid droplet formation in HC11 cells measured by oil-red staining. Phase- contrast microscopic images represent the lipid droplets in HC11 cells, Scale bar- 10 µm (H). Quantification of lipid droplets by spectrophotometer analysis (I). (J-K) Expression of β-casein in HC11 cells upon HPIP knock-down in response to bPRL (5 µg/mL, 48 h) as analyzed by qRT-PCR (J) or Western blotting (K). β-Actin serves as a loading control. Data represent the mean ± SE. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ measured by two-way ANOVA and student t-test. (n=3).

1.9.4. miR-148a negatively regulates HPIP expression in HC11 cells

Emerging studies have revealed that miRNAs play a role in lactogenic differentiation and mammary gland development [111]. Previous studies revealed that miR-148a inhibits HPIP expression by binding to its 3'UTR region [94]. To address whether miR-148a restrains HPIP protein synthesis and its mammary-associated functions, we first cloned ~ 900 bp 3'UTR of *HPIP* in a reporter vector, pMIR-Luc vector. We also mutated miR-148a binding region in 3'UTR of *HPIP* to generate pMIR-mt-HPIP3'UTR (mutant) plasmid construct (Fig. 7A). pMIR-wt-*HPIP*3'UTR or pMIR-mt-HPIP3'UTR and miR-148a or empty pGIPz vector were co-transfected in HC11 cells, and the reporter assay was performed. As shown in (Fig. 7B), miR-148a expression induced a significant decrease in wt-*HPIP*3'UTR levels but not in the mtHPIP-3'UTR levels compared to control cells. Further supporting this data, miR-148a ectopic expression markedly decreased HPIP protein levels in HC11 cells (Fig. 7C). Next, we evaluated miR-148a expression levels during mammary gland development. Since previous studies reported miR-148a as an inhibitor of HPIP, a result that we confirmed in HC11 cells (Fig. 7C), we predicted low levels of miR-148a in pregnancy and lactation stages of MG development when HPIP levels are elevated (Fig. 4).

Counter intuitively, *miR-148a* levels also peaked during pregnancy (P) and lactation (L), similar to the *Hpip*. However, both gene- products exhibited distinct expression kinetics as *miR-148a* levels peaked at P16-19 when *Hpip* levels were still low, while the latter peaked at L6 when *miR-148a* levels had already started to decline (Fig. 7D). The high *miR-148a* expression levels during pregnancy and lactation suggested PRL may also regulate it. Indeed, bPRL treatment enhanced *miR-148a* levels in HC11 cells (Fig. 7E). Furthermore, the ChIP assay demonstrated STAT5a recruitment onto miR-148a promoter, harboring a STAT5a binding element (Fig. 7F-G). Together these results indicate that *miR-148a* is a PRL-responsive gene that negatively regulates HPIP expression.

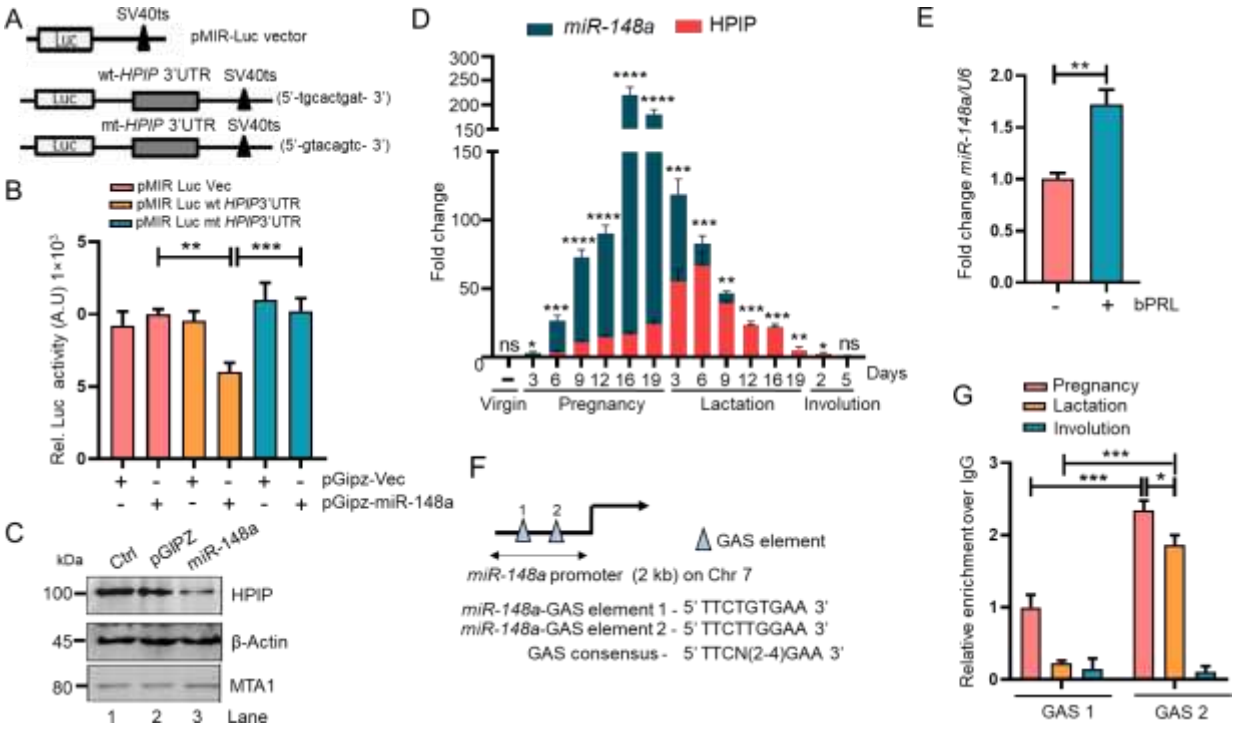


Figure 7: miR-148a negatively regulates HPIP expression in HC11 cells. (A) Physical map of HPIP 3'UTR (0.9 kb) fused with Luc gene (in pMIR-Luc vector). (B) Luciferase assay demonstrating the effect of miR-148a (cloned into pGIPz expression vector) on either wild type or mutant HPIP 3'UTR in HC11 cells. (C) Western blotting analysis of HPIP expression upon miR-148a ectopic expression in HC11 cells. MTA1 and β-Actin serve as off-target and loading

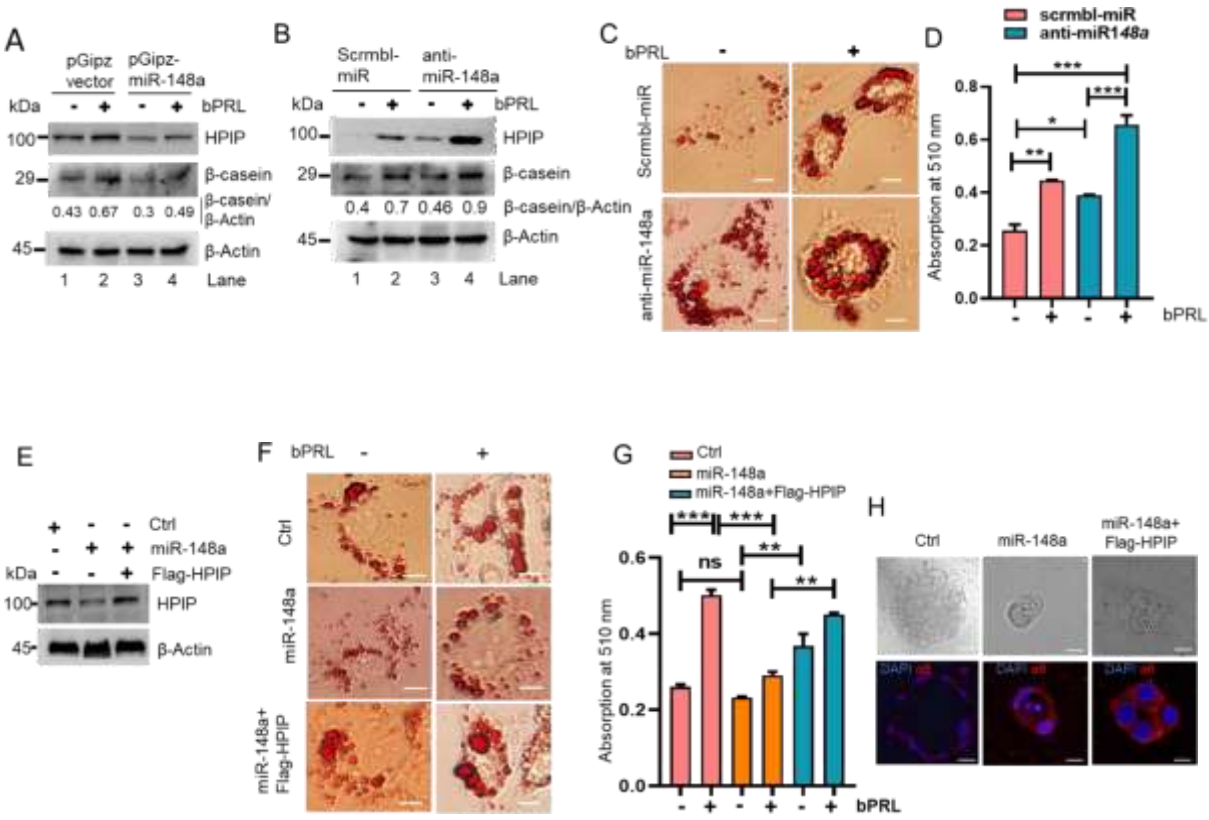
controls, respectively. (D) qRT-PCR analysis of Hpip and miR-148a expression in mouse mammary tissue at various stages of gland development. (E) qRT-PCR analysis showing the expression of miR-148a in HC11 cells in response to bPRL treatment (5 µg/mL, 48 h). (F) Physical map of mouse miR-148a promoter (2.0 kb) located on Ch 7 harbors two GAS elements, GAS1 and GAS2. Comparison of GAS1 and GAS2 with consensus GAS sequence. (G) ChIP assay demonstrating STAT5a enrichment on miR-148a promoter at various stages of mammary gland development (pregnancy at 6^{th} day, lactation at 6^{th} day, involution at 2^{nd} day). Data represent the mean ± SE. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ measured by multiple unpaired t-test and student t-test. (n=3).

1.9.5. miR-148a regulates milk protein synthesis by controlling HPIP expression

We next aimed to address whether the miR-148a-induced reduction in HPIP expression resulted in lactational differentiation defects. To check this, we first ectopically expressed miR-148a in HC11 cells and subsequently analyzed the PRL-responsiveness of β-casein synthesis, a marker for lactational differentiation of HC11 cells. As shown in (Fig. 8A), miR-148a-induced down regulation of HPIP expression prevented β-casein protein synthesis in response to bPRL stimulation. As a mirror image, ectopic expression of anti-miR-148a, an inhibitor of miR-148a, increased basal and PRL-induced HPIP levels, resulting in a parallel increase in β-casein synthesis (Fig. 8B).

A significant amount of cytoplasmic lipid droplets (CLDs) is accumulated during lactation in mammary epithelial cells [112]. Earlier studies have explored lipid droplet formation in HC11 cells to study the role of genes involved in this process [113]. Therefore, we next performed lipid droplet formation by oil red staining assay. In support of previous reports, we also found a significant increase in lipid droplets formation upon bPRL treatment in HC11 cells as compared to control cells (Fig. 8C-D). Furthermore, anti-miR-148a treatment significantly increased lipid droplet formation in HC11 cells (Fig. 8C-D). We next performed rescue experiments to check whether

ectopic HPIP expression can compensate for miR-148a-induced differentiation defects in HC11 cells. As shown in (Fig. 8E-G), miR-148a inhibited bPRL-induced lactogenic differentiation. However, HPIP ectopic expression partially rescued this effect. Since miR-148a restrains HPIP expression, we next analyzed whether miR-148a also affects HPIP-mediated acini formation. Results showed that as compared to control cells, miR-148a-transfected HC11 cells formed smaller and defective acini (Fig. 8H). To enumerate these observations, we quantified the organized acini in this experiment. miR-148a-transfected cells showed a significant decrease in the size and number of cells per acini compared to bPRL treated control- HC11 cells (Fig. 8 I -J; Video S5-S7). Conversely, ectopic expression of Flag-HPIP partially restored the organized acini. Together these data indicate that miR-148a inhibits HPIP-mediated acini formation and lactogenic differentiation in HC11 cells.



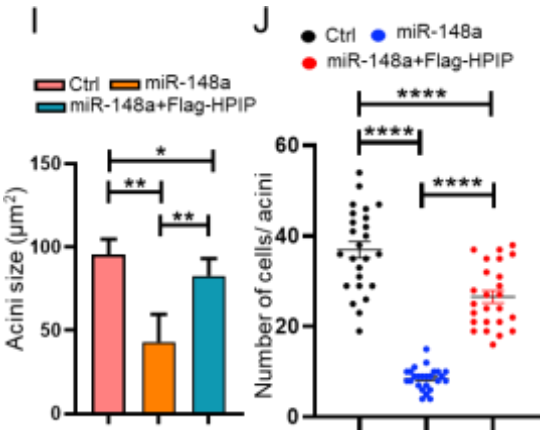


Figure 8: Inhibition of miR-148a upregulates HPIP and β-casein synthesis. Western blotting analysis showing the effect of miR-148a (cloned into pGIPZ expression vector) ectopic expression (A) or anti-miR-148a treatment (B) on β-casein synthesis in HC11 cells in response to bPRL treatment (5 µg/mL, 48 h). (C) Representative phase-contrast microscopic images of HC11 cells transfected with either scramble-Control or anti-miR-148a followed by treatment with bPRL (5 µg/mL) for 48 h. (D) Quantification of lipid droplets from the lipid droplet assay, Scale bar- 10 µm (C). (E) Western blot analysis of Hpip expression upon miR-148a ectopic expression in HC11 cells. (F) Representative phase- contrast microscopic images of oil-red stain in HC11 cells transfected with vector control, miR-148a or miR-148a and Flag-HPIP followed by treatment with bPRL (5 µg/mL, 48 h). Scale bar- 10 µm (G) Quantification of lipid droplets from the lipid droplet assay (F). (H-J) Effect of miR-148a alone or miR- 148a and Flag-HPIP (rescue) on acini morphology. Scale bar- 10 µm (H), acini size (I), number of cells per acini (J) in HC11 cells in response to bPRL. Data represent the mean ± SE. *, $p<0.05$; **, $p<0.01$;***, $p<0.001$; ****, $p<0.0001$ measured by student t-test and two-way ANOVA. (n=3)

1.9.6. HPIP mediates activation of STAT5a and PI3K/AKT signaling in response to prolactin treatment

Next, we investigated the molecular mechanism that underlies HPIP-mediated lactogenic differentiation. PRL receptor (PRLR)-induced phosphorylation of STAT5a is a key event in

functional mammary development and lactational differentiation [16,99,114]. Furthermore, PRL has also been shown to activate PI3K/AKT signaling though STAT5a [99-101]. HPIP also activates PI3K/AKT signaling in cancer cells and hematopoietic stem cells [72,76]. Because of these earlier reports, we hypothesized that HPIP might regulate lactogenic differentiation via PI3K/AKT signaling. With this notion, we treated HC11 cells with bPRL and analyzed STAT5a activation upon HPIP depletion. Although the decrease in STAT5a activation in HPIP-depleted cells was relatively modest compared to control cells, it was much more pronounced (2-3 folds) on AKT activation, irrespective of the phosphorylation site monitored (S473 and T308) (Fig. 9A). Furthermore, HPIP-induced activation of STAT5a and AKT and also β-casein expression were markedly downregulated upon treatment with the PI3K-specific inhibitor LY290402 (Fig. 9B). In support of this data, anti-miR-148a treatment potentiated the activation of these signaling molecules, which is accompanied by elevation of HPIP and β-casein levels in response to bPRL treatment in HC11 cells (Fig. 9C). Collectively our results indicate that HPIP promotes lactogenic differentiation via PI3K/AKT/STAT5a signaling pathway.

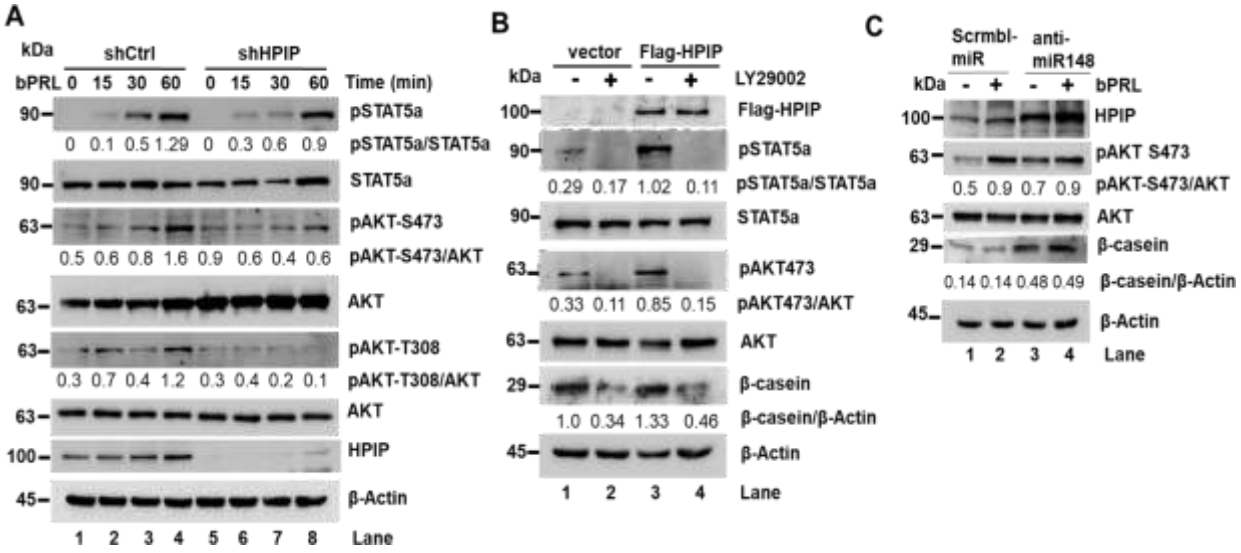


Figure 9: HPIP mediates the activation of STAT5a and PI3K/AKT signaling in response to prolactin in HC11 cells. (A) HC11 cells transfected with either shCtrl or shHPIP were treated

with bPRL (5 µg/mL) for various time points, and cell lysates analyzed by Western blotting and probed as indicated. (B) HC11 cells were transfected with a control vector or Flag-HPIP construct (human HPIP expressed on pMNDUS lentiviral vector) (11) followed by treatment with PI3K-specific inhibitor LY290492 (10 µM) for 24 h and protein lysates were analyzed by Western blotting and probed as indicated. (C) Western blotting analysis showing the effect of scramble-miR or anti-miR-148a on AKT activation and β-casein expression upon bPRL treatment (5 µg/mL, 24 h) in HC11 cells. β-Actin served as a loading control.

1.9.7. HPIP induces autocrine PRL signaling in HC11 cells

Although the pituitary is the primary source of PRL synthesis, various extra pituitary sources of PRL have been documented, leading to autocrine/paracrine PRLR signaling in various peripheral organs, including the mammary gland [99]. A more recent study pointed to the role of the AKT pathway in PRL synthesis and autocrine signaling in the mammary gland [99]. Based on these previous reports, we hypothesized that HPIP, as an upstream regulator of PI3K/AKT signaling, might promote PRL autocrine signaling in mammary epithelial cells. We analyzed PRL synthesis in HC11 cells by altering HPIP expression to address this issue. While ectopic expression of Flag-HPIP increased *Prl* transcript levels by 2-fold compared to control, depletion of HPIP significantly reduced it in HC11 cells (Fig. 10A-B). In support of this, the ectopic expression of miR-148a, which inhibits HPIP expression, significantly decreased *Prl* expression in HC11 cells (Fig. 10C), suggesting that HPIP promotes PRL synthesis miR-148a inhibits it. Δ1–9-G129R-hPRL is an engineered mutant of human PRL that acts as a pure competitive antagonist of the PRLR [115]. It was shown to block PRL-PRLR signaling in many preclinical settings irrespective of the PRL source (exogenous or locally produced). To further confirm that HPIP triggered autocrine PRL signaling in HC11 cells, we studied the effect of Δ1–9-G129R-hPRL on *Prl* and *β-casein* expression. Although ectopic expression of Flag-HPIP increased *Prl* and *β-casein* transcript levels significantly, Δ1–9-G129R-hPRL treatment abrogated it (Fig. 10D). Furthermore, Δ1–9-G129R-

hPRL ablated HPIP-induced STAT5a as well as AKT activation as indicated by reduced phosphorylation of these molecules and also β- casein synthesis (Fig. 10E). Together these data suggest that HPIP promotes PRL autocrine signaling while miR-148a opposes it in HC11 cells.

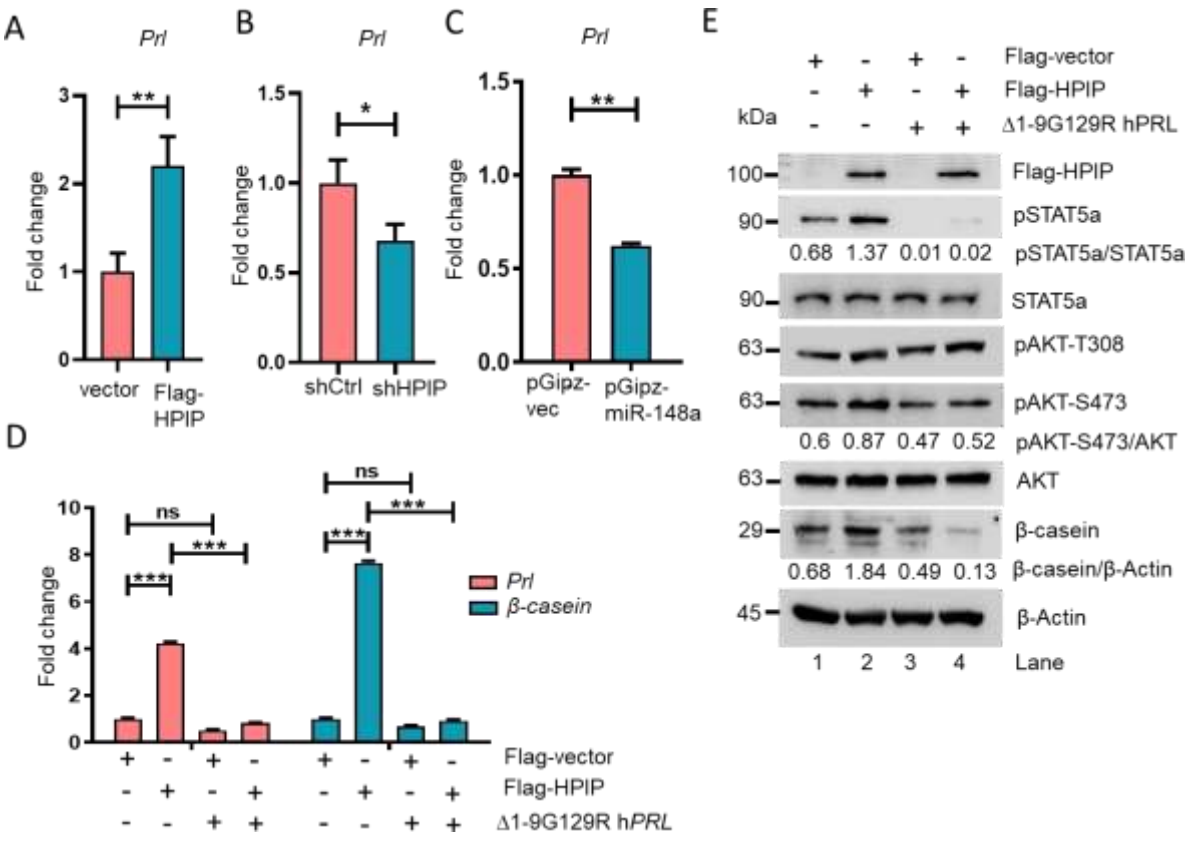


Figure 10: HPIP induces prolactin autocrine signaling in HC11 cells. (A-B) Effect of HPIP ectopic expression or knock-down on Prl expression in HC11 cells were analyzed by qRT-PCR. (C) HC11 cells were transfected with miR-148a and, 48 h post-transfection Prl expression were analyzed by qRT-PCR. (D) the qRT-PCR analysis demonstrates the effect of PRL peptide, i.e., Δ1–9-G129R-hPRL, on HPIP- induced Prl and β-casein synthesis in HC11 cells. (E) HC11 cells were transfected with Flag-HPIP (expressed on pMNDUS lentiviral vector) (11) followed by PRL peptide i.e., Δ1–9-G129R-hPRL treatment for 24 h, and protein extracts were subjected to immunoblotting as indicated. Data represent the mean ± SE. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ measured by student t-test.

1.10. Discussion

Hematopoietic PBX-interacting protein (HPIP/PBXIP1) is an estrogen receptor-interacting protein [76,78]. It regulates estrogen signaling in breast cancer cells. Despite these earlier reports, the actual role of HPIP in the context of physiological mammary gland development and functions is yet to be understood. In this study, we investigated the role of HPIP in mammary epithelial cell differentiation. By providing multiple lines of evidence in mammary epithelial and lactation-competent cell lines, we claim that HPIP plays a critical role in acini formation and lactogenic differentiation function by coordinating PRL signaling. HPIP expression is induced by PRL-STAT5a signaling. Interestingly, we also report that miR-148a restrains HPIP- mediated mammary functions. This study suggests a feed-forward loop where miR-148a, a product of PRL signaling, impede HPIP-mediated PRL autocrine signaling, which likely contributes to fine-tune the mammary function.

Pregnancy and lactation are essential steps during mammary gland development, with the PRL-JAK2-STAT5a signaling pathway playing a central role in these physiological processes, which together constitute a developmental switch essential for survival of lactating animals [116,117]. PRL stimulates STAT5a activation via PRLR and JAK pathways. The activated STAT5a, in turn, transcribes genes that are essential for alveologenesis and lactation [118,119]. PI3K has been shown to be one of the crucial STAT5a target genes transcribed during pregnancy and lactation stages [101]. Protein signaling via phosphorylation provides signal amplification, fostering the biochemical reactions and thus biological response. PI3K is an upstream regulator of AKT activation that offers cell proliferation, differentiation, and survival functions to mammary cells [120]. Therefore, the PI3K/AKT signaling pathway modulators can signal the developmental switch during mammary gland development. We found that HPIP promotes lactogenic differentiation via PI3K/AKT activation in this context. We find that HPIP promotes lactogenic differentiation via PI3K/AKT activation and potentiates STAT5a activation in HC11 cells. STAT5a/PI3K/AKT activation is sufficient for the onset of secretory protein synthesis at the initiation of pregnancy and lactation,

including β-casein and prolactin [99,114]. Notably, our results also indicate that HPIP overexpression is sufficient to drive the activation of STAT5a and β-casein synthesis in a PI3K/AKT-dependent manner. This highlights the influential regulatory role played by HPIP in secretory protein synthesis.

This study also provides evidence for the regulatory role of miR-148a in mammary lactation function. Several miRNAs are expressed in the mammary gland and play regulatory roles during developmental switch [68,111,121]. For example, let-7g-5p could inhibit mammary epithelial cell differentiation and β-casein protein synthesis directly targeting PRKCA [122]. Similarly, miR-221 regulates mammary cell proliferation by targeting STAT5a and IRS1 in the bovine mammary gland [123]. In the pregnancy and lactation stage, the abundance of miR-26a, miR-26b, miR-30c, were also reported [123-126]. Interestingly, a few studies showed the expression of miR-148a during lactation, and it was proposed to represent a measure of milk quality [124,125]. However, its regulatory role and functional relevance with the mammary gland was unknown. Intriguingly, miR-148a was previously reported as an inhibitor of HPIP in liver cancer cells [94]. In this study, we explored whether miR-148a inhibits HPIP-mediated mammary functions. Indeed, we found that miR-148a inhibits HPIP-mediated lactogenic differentiation as well as acini formation of HC11 cells. Mechanistic studies further revealed that miR-148a restrains HPIP-mediated PI3K/AKT activation and PRL synthesis. Considering these observations, we expected an inverse correlation of *miR-148a* expression with *Hpip* during pregnancy and lactation.

In contrast, we found that miR-148a is also expressed during pregnancy and lactation and is also a PRL response gene similar to HPIP. Although both *Hpip* and *miR-148a* are PRL- response genes, it is important to note that the kinetics of their expression during pregnancy (P) and lactation (L) is different. *miR-148a* levels peaked at P16-19 when *Hpip* levels were still low, while the latter peaked at L6 when *miR-148a* levels had already started to decline. Interestingly, the expression of both genes diminishes at the end of the lactation phase, perhaps due to back-to-normal pituitary PRL secretion resulting in down-regulation of STAT5a activation. In addition to HPIP, miR-148a

has few known targets such as PTEN [127], TGIF2 (TGFB-induced factor homeobox 2) [128] and drug-metabolizing-related PXR (pregnane 6 receptor) genes [129]. In light of these earlier reports, whether miR-148a targets those gene products in the mammary gland may not be ruled out. miR-148a is also reported to be present in milk exosomes [124,125]. From this study, we showed that miR-148a inhibits lactogenic differentiation. Based on these observations, we speculate that miR-148a is expressed as a counter-regulator to suppress the excess β-casein to be synthesized. In parallel, the mammary gland might have evolved to export miR-148a in the form of exosomes to fine-tune β-casein synthesis and hence, lactogenic differentiation. Therefore, we predict that miR-148a may have broader regulatory roles during mammary gland development and warrants future *in vivo* knock-out studies to understand those aspects better.

Another intriguing observation of this report is the role of HPIP in autocrine PRL signaling. Prolactin is typically regarded as a classical endocrine hormone secreted by the pituitary gland. It regulates multiple reproductive and metabolic functions [116,130]. It is now increasingly evident that PRL is also synthesized in several other tissues and cell types, as well as in the mammary gland and also in some breast and prostate cancer cell lines [130-133]. Further studies revealed the dependency of autocrine PRL signaling on PTEN-AKT signaling [99]. However, the intricate molecular mechanisms for the synthesis and secretion of autocrine prolactin are elusive. Using the gain of function and loss of function approach, we demonstrate that HPIP regulates *Prl* expression in HC11 cells via PI3K/AKT pathway. We also provide the first evidence that miR-148a opposes autocrine PRL signaling in mammary cells. Furthermore, based on studies involving the PRLR antagonist Δ1-G129R-hPRL, our results point to a model in which the PRL-HPIP-miR-148a loop controls PRL synthesis, AKT activation and β-casein synthesis. Together these findings imply an unpredicted mechanism underlying the autocrine PRL signaling in mammary cells (Fig. 8).

In conclusion, we report that *HPIP* and *miR-148a* are PRL-responsive genes in the mammary gland. Our findings implicate a 'feed-forward control loop' involving HPIP, miR- 148a and PI3K/AKT in regulating the autocrine PRL pathway, which contributes to lactogenic

differentiation of mammary cells. Further *in vivo* studies are warranted to precisely decipher the role of HPIP and miR-148a in alveologenesis and lactation during mammary gland development.

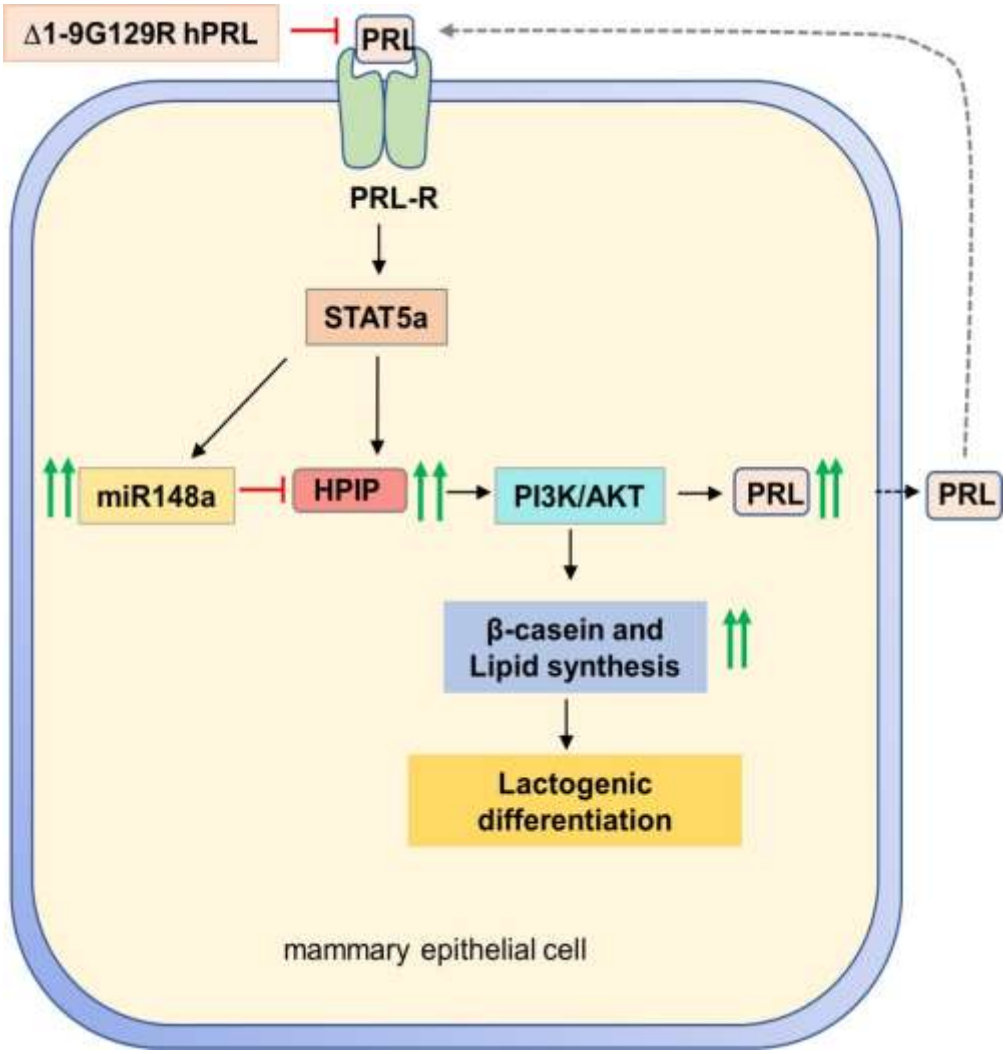


Figure 11: Model depicting HPIP-miR-148a-AKT axis in the regulation of PRL autocrine signaling and lactogenic differentiation. PRL binding to PRLR triggers the activation of the receptor, which leads to the activation of the JAK2-STAT5a signaling complex. The activated STAT5a translocate into the nucleus to turn on the transcription of both *Hpip* (*Pbxip1)* and *miR-148a* genes. HPIP, thus synthesized in turn, stimulates PRL synthesis via PI3K/AKT pathway. The secreted PRL will participate in autocrine signaling to promote lactogenic differentiation by operating positive feedback loop.

CHAPTER II

HPIP promotes cell survivability in suspension by autophagy in breast cancer cells

2. Introduction

Breast cancer is a leading cause of malignancy among women, and it is metastasis that causes most of the mortality in this population. Female breast cancer accounts for most of all cancer diagnoses in women, accounting for 6.9 % of all cancer deaths globally [134]. Similarly, 1 in 29 females has breast cancer in India, and 179,790 newly reported cases contribute 10% of overall cancer in 2020 [135]. However, lack of knowledge and delayed diagnosis led to a greater mortality rate in India than in western countries even if the number of new cases recorded is less (one- third than in Western countries) [136,137]. Interestingly, the lifestyle, food habits, late childbearing, increased alcohol, and contraceptive use appears to be the potential risk factors for advanced breast cancer incidences in urban areas compared to rural parts of the country. In India, women age group around 30-50y reported having more incidences of breast cancer than in the western countries of women with 50-70y age group. The cancers in the young are more likely to be aggressive and require early detection and much better therapies [138]. The therapeutic approaches that have emerged over the past 10–15 years have considered this variability, with an increased emphasis on more physiologically tailored medicines and reducing the intensity of treatment to mitigate its adverse effects. However, the tumor heterogeneity appears to drive the locoregional tumor burden or metastatic patterns and could impact the cancer treatment [139]. The majority of early breast cancers are treatable, meaning that they are contained within the breast. Improvements in multimodal therapy have increased the likelihood of cure in approximately 70–80 percent of patients. Advanced (metastatic) illness, on the other hand, is not considered treatable with the present therapeutic choices accessible to clinicians.

The primary aim of treatment is to prolong survival and control symptoms while maintaining or improving quality of life (i.e., enhanced quality-adjusted life expectancy) with the least amount of treatment-associated toxicity. Treatment for breast cancer is divided into two primary categories: localized treatment and systemic therapy. Breast cancer histological and molecular characteristics have a significant role in determining which treatments are most effective

[140]. Numerous molecular abnormalities contribute to breast carcinogenesis, and several classifications have been devised to categorize tumors according to their molecular modifications [141]. When Perou and Sorlie1 first published their intrinsic variety of breast cancer in 2000, they recognized four subtypes: luminal A, luminal B (expressing the estrogen receptor (ER), basal-like and human epidermal growth factor receptor 2 (HER2)-enriched (without ER expression) [142]. As a result of this classification, clinical therapy of breast cancer has changed away from tumor burden-based techniques and biology-based approaches. The surrogate classification of five subtypes-based histology and molecular features is currently used in clinical practice more often in the United States. ER-positive and progesterone receptor (PR)- positive breast cancers are distinguished from triple-negative breast cancers, which are defined as tumors that do not express the ER, PR, or (-ER, -PR, -HER2) (TNBC) [143]. TNBC is therefore pose major obstacle in the current drug regimen available only personalized medicine as a choice of treatment.

2.1. Metastasis

Metastasis is the real culprit behind the high mortality rate of cancer patients. The term "metastasis" refers to the growth of secondary tumors outside of the primary cancer site. Several steps are involved in the metastasis, including EMT, localized invasion, resistance to anoikis, intravasation, transport though circulation, extravasation and colonization on secondary site [144]. Even though cancer metastasis is a leading cause of treatment failure and mortality, the molecular mechanism remains elusive. On a daily basis, patients with cancer produce vast numbers of cancer cells, while melanoma research in animal models suggests that just 0.1 percent of tumor cells spread [145]. Malignancy is defined by the ability of cancer cells to invade and seed distant tissues, resulting in metastases, as a central feature. More than 90% of cancer patients die as a result of the disease spreading to other organs [146],[151]. Metastatic breast cancer accounts for 6-10% of all newly diagnosed cases, and it is expected that 20-30% of all breast cancers will progress to this stage. Despite recent breakthroughs in the treatment of metastatic breast cancer (MBC), it is still

considered as an incurable illness with a survival rate of 18 to 30 months [147]. For the time being, detection of breast cancer metastasis is based on biopsy, radiographic examination, investigation of circulating tumor cells (CTCs) and serum tumor markers [148],[149]. Cancer growth and metastasis can be halted or even reversed if we understand the dynamics of this process.

2.2. Role of extracellular matrix (ECM) in cancer progression

The non-cellular three-dimensional macromolecular network is the extracellular matrix (ECM). These components form a network by binding to one other and the cell surface receptors. This network aids cell survival in all tissues and organs. The interstitial matrix and basement membrane are made up of these components (BM). The basement membrane is an extracellular matrix (ECM) that divides the epithelium or endothelium from the stroma. The basement membrane is made of type IV collagen, fibronectin, laminins, and linker proteins like nidogen and entacin are less permeable and more condensed than the interstitial matrix. The biomechanical properties of ECM can change under pathological situations, which significantly impacts cell migration. Finally, interactions between cells and the extracellular matrix (ECM) allow cells and tissues to adapt to their surroundings [150,151]. During normal and pathological situations, several matrix-degrading enzymes are constantly rebuilding ECM. ECM remodeling, which also include the basement membrane, is one of the master switches for cancer invasion, neo-angiogenesis, and metastasis [152]. ECM also plays a crucial function in maintaining stem cell characteristics and the control of stem cell development. Stem cells are found in a microenvironment known as a niche, and they play an essential role in tissue regeneration and maintenance [153]. As a mediator of cell-ECM interaction, Integrin receptors offer transducing signals and physical linkages with the cytoskeleton from the ECM to cell protein modification activities such as proliferation, migration, and survival [154]. A niche in cancer is a unique local microenvironment that includes CAFs, immune cells, non-CSC cancer cells, blood, and lymphatic vessels, ECM, growth factors, and cytokines. Interactions between tumor cells and stroma enhance cancer development and metastasis by releasing chemo-

attractants from distant organs [155]. Cancer cells break down the epithelial basement membrane and contact the tumor stroma in the early stages of cancer spread. Tumor cells penetrate the stroma and enter the blood arteries as clusters/sheets (collective model) or single cells (individual model) [156].

Matrix metalloproteases (MMPs) are required for ECM degradation and are therefore involved in metastasis [157]. MMP2 and MMP9, for example, break down type IV collagen to aid invasion in retinoblastoma. In malignancies, MMP9 induces angiogenesis [158]. MMP9 and MMP2 overexpression has been seen in patients with head and neck squamous cell carcinomas with lymph node involvement [159,160]. Bone metastases are a common occurrence in several malignancies. Some elements that release growth factors in the ECM promote bone deterioration by removing growth factors. Metastatic outgrowth is aided by bone degradation[152]. The predominant component of ECM in the liver is collagen IV. Hepatic metastasis has been linked to collagen-IV binding to integrins (particularly integrin-2). Collagen-IV protects cancer cells in the liver from anoikis [161].

2.3. Anoikis, a detachment induced cell death

Anoikis is a Greek term that signifies "homelessness" or "loss of home." It is a type of apoptosis triggered by the loss of cell adherence to the ECM. Anoikis occurs when a normal epithelial cell is detached from its ECM. The mitochondrial (intrinsic) and cell death receptor (extrinsic) pathways are the two apoptotic processes [162,163]. Anoikis is necessary for tissue homeostasis because it eliminates misplaced endothelium / epithelial cells, preventing them from seeding inappropriately. Resistance to anoikis is a defining feature of the EMT phenomenon and a requirement for metastasis [154]. Resistance to anoikis also increases circulating tumor cells (CTCs), making recurrence and metastasis easier [164]. According to a recent study, the highly glycosylated mucin protein (MUC1), which is overexpressed in all types of epithelial cancer cells, can delay the onset of anoikis in response to cell adhesion loss [165]. Furthermore, insulin receptor or insulin-like growth factor receptor activation plays a vital role in cancer cell resistance to anoikis. The specific part of p53 in anoikis has yet to be determined; nevertheless, p53 has been shown to play

a protective role in anoikis via activation of ECM and expression of the integrin gene. E-cadherin is another component linked to anoikis. E-cadherin knock- down increases cancer cell resistance to anoikis, resulting in the EMT phenomenon. EMT is induced by both the TGF- and Wnt pathways, which leads to anoikis resistance [166]. Bit1 (Bcl-2 inhibitor of transcription 1), on the other hand, increases apoptosis. Bit1 is released to the cytosol after cell attachment is lost, where it interacts with the transcriptional regulator amino- terminal enhancer of split (AES) to trigger a caspase-independent form of apoptosis. Anoikis resistance and anchorage-independent growth are enhanced by Bit1 downregulation in malignancies like lung adenocarcinoma, resulting in tumorigenicity and metastasis [167]. Platelets promote anoikis resistance and metastasis by interacting with extravasating tumor cells or single tumor cells. Platelets are activated by cancer cells secreting adenosine 5′-diphosphate (ADP), which causes the production of pro-angiogenic and pro-tumorigenic substances [168]. We now have a better knowledge of how anoikis affects cancer growth and metastasis at the molecular level, thanks to recent findings. When cancer cells manage to detach from the primary tumor and escape anoikis, they have an uncontrolled development at secondary places in the body. Several variables have been discovered as modulators of anoikis resistance, including cell adhesion molecules, growth proteins, oxidative stress, stemness, autophagy, non-coding RNAs, and signaling pathways [169]. In this study, we discuss autophagy as one of the factors and its involvement in mediating anoikis resistance.

2.4. Role of autophagy in anoikis resistance

Autophagy, a natural process of self-digestion of long lived nonfunctional, malformed protein and organelles by lysosome in all cells. Depending on the context and stimuli, autophagy can serve as cell survival or a cell death mechanism exploited for cancer therapy [170]. Loss of attachment to the extracellular matrix (ECM) triggers the process of anoikis, which occurs in normal cells to maintain homeostasis. Cancer cells are notably resistant to anoikis, allowing them to spread outside their initial environment and develop new tumors. Autophagy appears to contribute

substantially to anoikis resistance of tumor cells. Debnath et al. executed several studies to elucidate the role of autophagy in cell survival after ECM loss [171-173]. They confirmed that ECM loss rapidly promoted autophagy in various cell lines [174]. Moreover, ATG knock- down (autophagy inhibition) reduced cell survival in cells that can (apoptosis-sensitive) and cannot (apoptosis-defective) undergo apoptosis, indicating autophagy as a survival mechanism in anoikis cells. These findings gave a possible explanation for cancer cell's capacity to evade anoikis-induced cell death, as well as a prospective therapeutic target [171]. Nevertheless, it is becoming increasingly likely that these pathways are involved in tumor cell dormancy. Because metastatic lesions at places other than the primary tumor cause most cancer fatalities, since recurrence might occur years after primary cancer has been treated, the causative cells are likely to remain latent at distant places indefinitely. Suppose autophagy could be inhibited to limit cellular dormancy or perhaps start cell death in latent cells in this condition. Therefore, it may be a promising therapeutic and preventative target for metastatic diseases [172].

2.5. Endocytosis

Cellular processes such as sorting, recycling, activating, silencing of membrane receptors are regulated by endocytosis. It has a wide range of functions, including signaling, proliferation, cell dynamics regulation, autophagy and defense [175]. Endocytosis can occur by clathrin or non-clathrin-mediated pathways [176]. Low-density lipoprotein (LDL), nutrients and pathogens, transferrin and growth factors, antibodies, receptors, etc., are all transported by clathrin- mediated endocytosis (CME). Phagocytosis and pinocytosis, two other key endocytosis processes, are essential in internalizing fluids and compounds. Recent research suggests that lipid rafts and proteins associated with rafts are transported by non-classical endocytic routes[177-179]. Many, but not all, cell types have caveolae, the most frequent non-clathrin-endocytosis [179].

An important part of the early endosome's production and maintenance is played by RAB5 and early endosome antigen 1 (EEA1). RAB35, RAB4, RAB10, RAB11, and RAB22a are required

for the normal recycling of clathrin-dependent payloads back to the plasma membrane [180]. As molecular switches for vesicular transport, small GTPases of Ras-associated binding (Rab) govern endocytic trafficking [181] ,[182]. There has been an increase in the number of studies focusing on the involvement of Rab GTPases during cancer, and several members of this family have been found to be overexpressed in breast and ovarian malignancies[182-185]. Deregulation of Rab GTPases, which play a critical role in integrin trafficking and recycling, is linked to cancer formation and progression[183-185]. Endocytosis of integrins regulates cell motility in numerous cell types, like fibroblasts and cancerous cell lines, such as melanoma [186],[187]. Rear-end detachment of focal adhesion and ECM breakdown is enhanced by integrin endocytosis, which in turn increases migration of cells[188]. It has been shown that several Rab GTPases control the endocytosis and intracellular trafficking of integrins [189],[187]. Integrin recycling is intimately linked to Rab GTPase activities in tumorigenesis. For example, Rab5 helps tumorigenesis by guiding the integrin-recycling vesicles and encouraging cell motility [190]. Furthermore, integrin endocytosis by Rab21 has been revealed to increase cell adhesion and motility [191].

2.6. Rab5 protein

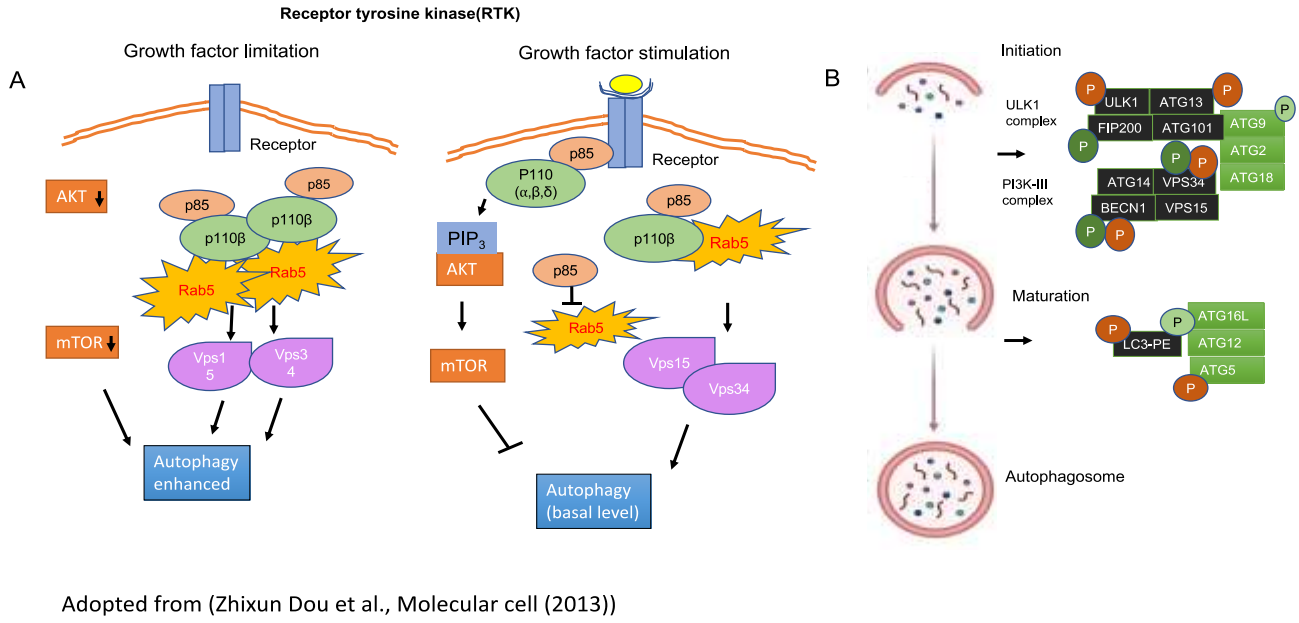
Rabs are small GTP-bound proteins that govern intracellular signaling pathways and belong to the Ras superfamily [182]. In structure, Rab proteins resemble Ras and some other GTP-bound proteins. Rab5 is one of the most important and well-studied members. Rab5 exist in two conformations: GTP-bound Rab5, which is activated, and guanosine diphosphate-bound (GDP) Rab5, which is inactivated [192]. When Rab5 is activated, it participates in vesicle transport, membrane trafficking, and signaling cascades through interactions with its effector molecules[193].

In most research, Rab5 is Rab5A which is located on chromosome 3 at position p24.3 and encodes for 23.658 kD protein [194]. S34N, Q79L, A30P, G78I, and C-terminal and N- terminal truncation variants of Rab5 are currently being studied. Inhibition of early endosome fusion is prevented by ectopic expression of the dominant-negative Rab5-S34N [195]. As a result of loss of

GTPase activity in Rab5-Q79L, Rab5 become constitutively active. Endosomal fusion is induced when Rab5-Q79L is overexpressed while endosome expansion is suppressed [196]. To maintain adequate endocytosis, Rab5 and Rab7 must be always present in adequate amount in the cell. Rabex-5 activates Rab5 which in turn binds numerous effector proteins, including early endosome antigen 1 (EEA1), to stimulate endosome fusion after it is recruited to the endosomal membrane [197]. It has been suggested that Rabs play other roles besides just governing cell growth and death. During the migration of both normal and cancerous cells, Rab5 is an essential modulator and promotes cell motility. As an opposing force, Rab5 is involved in downstream integrin signaling cascades, including proteins like Ras and Rho family GTPases, and promotes focal adhesion disintegration. Research into Rab5's regulatory and effector molecules is critical to understanding how cancer cells use endosome control to develop more aggressive features and become metastatic [198]. Rab5 has been shown to participate in autophagosome production in a model of Huntington's disease where it regulates autophagy to remove toxic mutant protein Huntingtin[199]. Also, Rab5 is reported to be crucial for autophagy induction during growth factor limitation.

Previously, it was demonstrated that cells upon growth factors stimulation have at least two p110β/p85 pools. One of these pools is linked to growth factor receptor signaling complexes at plasma membrane and inhibits autophagy though kinase activity of Akt/mTOR. A second intracellular pool of p110β/p85 binds to the small GTPase Rab5, which in turn stimulate basal autophagy in a kinase-independent manner. The interaction between p110β and Rab5 shields Rab5-GTP from the GAP activity of p85a. As a result, the total amount of activated Rab5 increases, which in turn promotes interaction between Rab5 and its effectors, such as Vps34 and promotes basal autophagy. Upon an inadequate supply of growth factors, the p110β/p85 signaling molecules become dissociated from the growth factor receptor, which in turn causes 58 an increase in the interaction between the signaling molecule and Rab5, results in enhanced autophagy [200]. Rab5 can interact with Beclin1 when Vps34 is present. There is an autophagosome-forming complex that includes Vps34 and Beclin1. These findings imply that Rab5 is a component of the

Beclin1 and Vps34 macromolecular complex that controls autophagosome formation [201].



Adopted from (Zhixun Dou et al., Molecular cell (2013))

Figure 12: Role of Rab5 proteins in autophagy. (A) A schematic representation of the interactions between autophagy initiation complex and the early endocytic protein Rab5 is depicted in the figure. In the earliest stages of autophagosome formation, a group of Rab5 small GTPases play an essential role by providing a diverse range of membrane sources for the pre-autophagosome structure (PAS). Because of its interactions with the PI3K-III-BECN1 complex, Rab5 plays a role in the autophagic stage of the cell's life, while AKT and mTOR signaling inhibit autophagy formation. (B) The autophagosome assembly process is depicted in this flowchart, from initiation to autophagosome formation and the factors involved in the process.

2.7. RNF126

RNF126 is one of the RNF family of E3 ubiquitin ligase. It contains Ring finger and Zn finger at the C- and N-termini respectively. It has been reported to regulate cell cycle, cell proliferation, and also DNA repair [202],[203],[204]. Interestingly, RNF126 has been identified as an oncogenic factor in many cancers, like breast, gastric, prostate, and ovarian cancer [205],[206],[207]. Additionally, it has been

discovered that RNF126 is a component of the endosomal complex [208] and is essential for cell survivability upon ECM loss [209]. However, its role in autophagy is scarce.

2.8. Targeting anoikis resistance as a cancer therapeutic strategy

Macro-autophagy (after this referred to as autophagy), an evolutionarily conserved lysosomal mechanism in which cells digest their cytoplasmic contents, can contribute to anoikis in the normal cells [210]. For instance, during mammary gland development anoikis can be induced by autophagy. Anoikis resistance's mechanisms are still a mystery, and therefore quest continues to identify potential targets conferring anoikis resistance [211]. Anoikis resistance develops during cancer progression and drive the metastasis, hence it serves as a potential therapeutic target for metastatic cancers. If anoikis-resistance drivers are identified and characterized, they can be targeted for anoikis induction and thus apoptosis in cancer cells. The presence of hypoxia and low pH in the tumor environment contributes to anoikis evasion and resistance to therapy [212-216]. The metabolic landscape of the tumor microenvironment is reprogrammed by an acidic environment, boosting tumor proliferation and resistance to anoikis and metastasis [217]. Patients with metastatic cancer may benefit from combining bicarbonate administration to lower cellular acidosis with other currently available treatments to avoid the acidic microenvironment [218]. Several medications like metformin and piplartine that stimulate anoikis though regulating oncogenic or metabolic signaling pathways have been documented, but these drugs do not specifically target anoikis-resistant cells [169]. Salinomycin is one such drug reported to induce anoikis sensitivity in BC cell line MDA-MB-231 cells though inhibition of STAT3 activation [213].

2.9. HPIP

Hematopoietic PBX-interacting protein (HPIP), also known as pre-B-cell leukemia homeobox interacting protein (PBXIP1), was initially identified as a PBX1 interacting protein that acts as a repressor for the PBX1 transcription factor, suggesting a role for HPIP in primitive stages of

hematopoiesis. It is involved in erythroid differentiation and displays stem cell activity by modulating the PI3K/AKT/GSK3β signaling pathway. It also regulates cell adhesion and migration though modulation of focal adhesion dynamics by activating the FAK/ERK/Calpain2 pathway to promote breast cancer metastasis [81]. Numerous studies have shown that HPIP is highly expressed in gastric cancer, colorectal cancer, hepatocellular carcinoma (HCC), glioma, thyroid cancer, leiomyosarcomas, oral carcinoma, and ovarian cancer [76,82,83,90,95,97,219-222]. The accumulating evidence support that HPIP promotes cell proliferation, migration, anchorage-independent growth, and invasion of cancer cells though the activation of AKT/MAPK pathways [76,78,82,95]. HPIP is mainly localized in the cytosol but can shuffle between cytosol and nucleus. Recent studies revealed that HPIP regulates epithelial-to-mesenchymal transition by activating the PI3K/AKT/GSK3β/SNAIL and TGF-β/Smad2 pathways in ovarian cancer and lung cancer, respectively [87]. Given its complex role in cancer progression by modulating various signaling pathways like PI3K/AKT and FAK/ERK/Calpain2, its role in anoikis resistance is yet to be investigated.

2.10. Rationale and objectives:

Normal cells undergo apoptosis (anoikis) when their attachment to the extracellular matrix is compromised. Because of this, invasive and metastatic cancer cells frequently develop anoikis resistance, allowing them to grow in suspension and metastasize to secondary organs in the body [223]. Cancer cells gain resistance to anoikis primarily though the activation of pro-survival signaling pathways and autophagy. Given the fact that HPIP has been shown to activate various signaling proteins, we hypothesized that it might be involved in anoikis resistance as well. Therefore, we framed the following objective to characterize the role of HPIP in anoikis resistance in breast cancer cells (BCCs).

Objective:

To investigate the role of HPIP in anoikis resistance in breast cancer cells

2.11. Materials and methods

2.11.1. Cell culture

MCF7,4T1 and MDA-MB231 cell lines (obtained from NCCS, Pune) were grown at 37°C with 5% CO_2 in a humidified incubator in DMEM medium supplemented with 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin.

2.11.2. Suspension culture and spheroid formation assay

60 mm tissue culture plates were coated with 1.3 ml of poly-HEMA (35 mg ml−1 in 95% ethanol) and kept overnight in a laminar flow hood at room temperature to dry. Cells were trypsinized into a single cell suspension, and 8×10^5 cells were seeded on poly-HEMA-coated plates. After 48 h, the cells were harvested by centrifugation and processed for cell viability, RT-PCR analysis, and protein analysis. For the spheroid assay, we followed a previously standardized protocol [224]. Single-cell suspensions were seeded in precoated poly-HEMA six- well culture plates (10,000 cells per well in 2 ml) with DMEM-F12 (Gibco Life Technologies) in serum free medium supplemented with FGF (Peprotech Europe, London) 20 ng/mL, EGF (Sigma Aldrich) 10 ng/mL and N2 supplement (Gibco Life Technologies) 1X/ml. After 4–7 days of growth, light microscopy is used to capture the spheroid formation. The data is represented in the bar graph.

2.11.3. Real-time Quantitative Reverse Transcriptase PCR

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA, and RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's specifications. cDNA was diluted (1:10) for PCR reaction. The quantitative real-time PCR was performed using FastStart SYBR Green Master (Roche Applied Science, Mannheim, Germany) and LightCycler®96 Real-Time PCR System machine. The sequences of primer sets are listed in Table 1.

2.11.4. Western blotting

Cells were lysed in NP40 lysis buffer (50 mM, Tris-Cl pH 7.4, 150 mM NaCl, 1% Nonidet P- 40, 0.5% sodium deoxycholate, 1 mM PMSF and 1x protease inhibitor cocktail) and subjected to SDS-PAGE using BIO-RAD electrophoresis units followed by Western blotting with following protein-specific antibodies: anti-HPIP (Bethyl Laboratories, USA), anti-LC3BII (Cell Signaling Technology, USA), anti-RNF126 (Abcam, USA), Rab5 (Cell Signaling Technology, USA), anti-NFkB, pIKK and IKK (Santa Cruz BioTech, USA) (Table 2).

2.11.5. Generation of shHPIP or shRNF126 expressing stable clones

Gene (HPIP or RNF126) silencing in either MDA-MB-231 or 4T1 cells was carried out by transfecting gene specific (HPIP and RNF126) shRNAs (Dharmacon, Lafayette, CO USA) along with packaging plasmids (pREV, VSV-G and pΔR in the ratio of 1:0.4:0.5) using Lipofectamine™ 2000 (Invitrogen, Waltham, Massachusetts, USA) in HEK293T cells as described previously [81]. Forty-eight hours post-transfection, viral soups were collected and added to either MDA-MB-231 cells or 4T1 cells. Subsequently, positive clones were selected by eliminating the untransfected cells using 1 µg/mL puromycin. After verifying the HPIP or RNF126 knock-down by Western blotting, we used them for various studies. When necessary, cells were treated with viral soups carrying pMNDUS-HPIP which express Flag-HPIP.

2.11.6. Cell survival Assay

Cells were seeded in 96 well plate (Ultra-low cluster plate, Corning) at the density of 3000 cells/well in 100 µL of cultured medium for a specified period (24-96 h) at 37 °C and 5% CO_2, after adding 10 µL of WST-1 mixture to each well, cells were incubated for 2-4 h at 37 °C in a CO_2 incubator. Before reading the plate, it was gently mixed for 1 min to homogeneous distribution of the colored substance, and absorbance was measured at 450 nm wavelength data was quantified and represented in a graph.

2.11.7. Apoptotic assay (AO/EtBr Staining)

The conventional acridine orange/ethidium bromide (AO/EtBr) staining was followed to differentiate the live from apoptotic cells. Briefly, ctrl or HPIP knock-down cells were stained with acridine orange (50 µg/mL) and ethidium bromide (50 µg/mL) for 20 min and analyzed under a fluorescence microscope LASER beam excitation at 488 nm and 550 nm. The data was quantified and represented in a graph.

2.11.8. Fluorescence imaging

Cells were grown in poly-Hema coated plate for 48 h. Afterwards, cells were seeded atop a lysine-coated coverslip for 1 h. They were fixed for 20 min with 4% paraformaldehyde (PFA) at room temperature. After fixation, cells were washed thrice with PBS and permeabilized with 0.1% Triton X-100 for 20 min, followed by additional PBS washes. Cells were then incubated with primary antibodies overnight at 4 ^{o}C, followed by PBS washes thrice each 15 min. It was then incubated with Alexa FluorTM 548 and Alexa FluorTM 488 (Life Technologies) diluted 1:200 in PBS for 1 h and were washed with PBS. For nuclear staining, the cells were incubated with DAPI for 5 min at room temperature. Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Jena, Germany) were used for measuring Immunofluorescence.

2.11.9. Colony formation assay (Clonogenic assay)

Cells were transfected with shCtrl or shHPIP were cultured in suspension condition for 48 h. Colony formation assays were performed in 6 well plates by seeding 500 cells in 1 mL per well. Fresh 1 mL of DMEM medium supplemented with 10% FBS was also added to the plates and were kept for two weeks at 37 °C in 5% humified CO2 incubator. Every third day, fresh cell growth medium was added. Crystal violet solution was used to stain the colonies (Sigma, 0.5 mg/mL), and images were taken by Kodak Image Station 2000R (Eastman Kodak Company, New Haven, CT, USA), data was quantified and represented in a graph.

2.11.10. Luciferase reporter assay

Luciferase assay was executed using the Bettel luceferin-Glo Luciferase Assay kit according to the manufacturer's protocol (Promega USA). Approximately 200 ng of reporter plasmids (pGL3-*HPIP-Luc*), and 50 ng of Renilla luciferase (internal control) were transfected. All transfections were performed using Lipofectamine-2000 (Invitrogen, USA) according to the manufacturer's instructions. The relative luciferase activity is represented in the bar diagram.

2.11.11. Pyrrolidine Dithiocarbamate (PDTC) treatment

Cells were trypsinized into a single cell suspension before plating 8×10^5 cells on poly-HEMA-coated dishes. PDTC (Pyrrolidine dithiocarbamate) were dissolved in DMSO and 30 µM was added to the medium while seeding the cells. After 48 hours, the cells were centrifuged and collected for cell viability, RT-PCR and western blot analysis.

2.11.12. Cycloheximide pulse chase experiment

Equal number of HPIP (stably knockdown) and RNF126 (ectopically expressed) cells ($1x10^6$) were seeded into 60 mm poly-HEMA coated dishes, allowed in suspension for 48 h. Next day cells were treated with cycloheximide (25 µM) for the indicated time points, western blotting was then carried out as indicated after collecting in RIPA buffer. Band intensities were quantified using ImageJ software and then half-life ($t_{1/2}$) was calculated using graph-pad prism software.

2.11.13. Autophagy determination

Equal amount of protein was loaded in the wells of SDS-PAGE gel along with molecular weight marker. After immunoblotting the densitometry of LC3BI and LC3BII were analyzed by using ImageJ software. The ratio between LC3BII and LC3BI in the samples represent the level of autophagy flux. By using fluorescence microscopy, autophagy flux was also determined by counting the LC3BII puncta. An increase in the number of LC3 puncta is a measure of how many

autophagosomes would have been degraded during the treatment period when a lysosome inhibitor was present compared to when the inhibitor was not present [225].

2.11.14. *In vivo* tumor growth and metastasis

Female nude mice of 4–5-week-old age groups were inoculated with 1×10^6 4T1 cells (shCtrl or shHPIP) subcutaneously for tumor growth or though tail vein for metastasis study, respectively, in 100 µl of sterile medium. In tumor growth study, matrigel was used along with sterile medium in 1:1 ratio. Every three days after cell injection, tumor volumes were measured by a digital caliper. The tumor tissues were collected 30–35 days after injection. To determine the tumor weight, GFP intensity was measured in the stable cells. (The pGIPz vector backbone in stable cells causes GFP fluorescence). Tumor volume= (length x width2)/2, where length is the largest tumor diameter and width is the perpendicular tumor diameter. For metastasis study after 30 days of infusion, animals were dissected, and lungs were collected to image for GFP intensity to determine the metastatic ability of the cells. Tissues were dissected for histopathology studies.

2.11.15. Statistical analysis

All the experiments were performed three times. The results are expressed as standard error mean using a sigma plot. Two-tailed unpaired Student's t-tests using Sigma plot were employed to evaluate the statistical significance.

Table 2

List of antibodies used in the study

Name of Anti-body	Company Name	Catalog
HPIP	Sigma Aldrich	HPA006949
Flag tag	Cell Signaling Technology	8146S
β- Actin	Cell Signaling Technology	4967S
PBXIPI/HPIP	Bethyl Laboratories	A301-628A
Rab5	Abcam	ab18211
RNF126	Abcam	Ab234812
LC3BII	Cell Signaling Technology	2775

2.12. Results

2.12.1. Loss of HPIP expression affects cell survival and spheroid formation efficiency, features of anoikis resistance

Breast cancer cells were grown in poly-HEMA (2-hydroxyethyl methacrylate) coated plates to induce ECM loss and then checked for the ability of BC cells to survive in suspension cultures. Growing the cells in poly-HEMA coated plate is a well-established method to enrich the anoikis resistant cells [226,227]. These enriched anoikis resistant cells have the capability to form primary to tertiary spheroids or tumor spheres, also express pluripotency factors. After 2 days of growing cells in suspension, breast cancer cells (BCCs) were analyzed for HPIP expression and their ability to survive in suspension condition. We observed an elevated expression of HPIP in MDA-MB-231 than other breast cancer cell lines (Fig. 13A). In addition to this, 4T1 and MDA- MB-231 cells, both are triple negative breast cancer cell lines (TNBC), showed high efficiency to form spheroids and showed higher survival rate which is accompanied by less degree of apoptosis than MCF-7, BT474 and SK-BR3 cells (Fig. 13B-D). Further, to justify the function of HPIP in anoikis resistance, we silenced HPIP expression and performed cell survivability as well as spheroid formation assay in MDA-MB-231 cells. We found a significant decrease in the survivability of cells and spheroid formation ability upon loss of HPIP expression in suspension (Fig. 13E-H). Together, these finding imply that HPIP exhibit better suspension survival and spheroid formation potential of breast cancer cells in suspension culture.

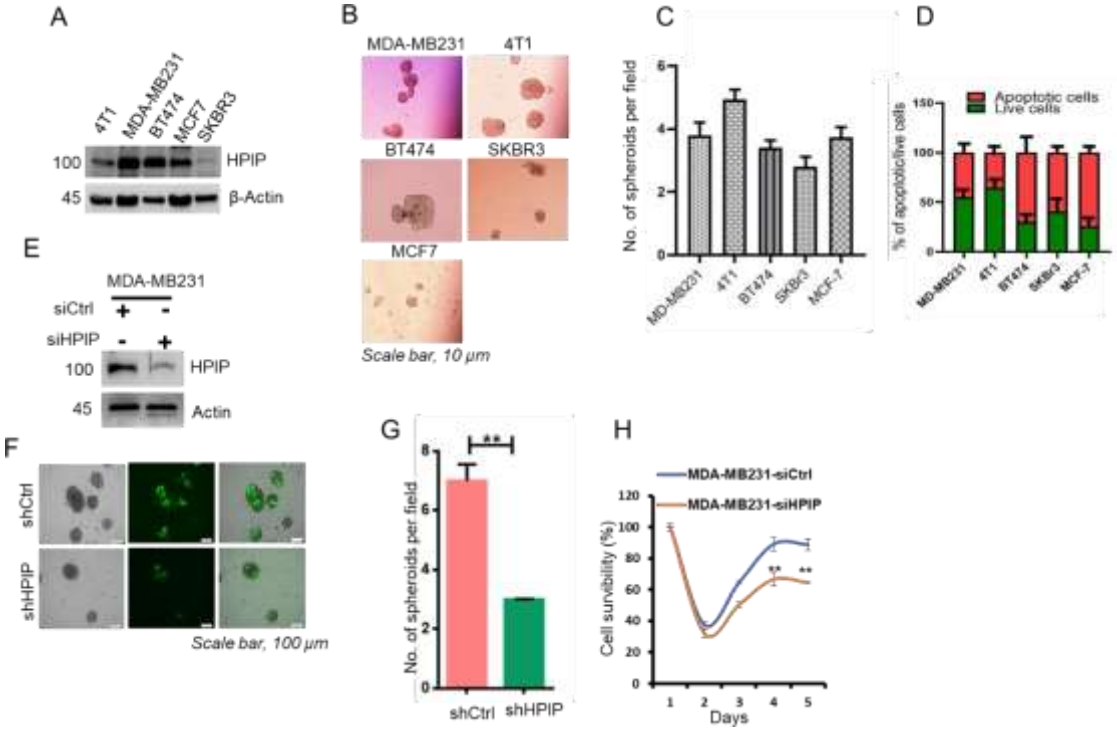


Figure 13: HPIP silencing confers anoikis resistance in breast cancer cells. (A) Cell lysates from 4T1, MDA-MB231, BT474, MCF7 and SKBR3 cells grown on poly-HEMA coated plates (mimics suspension) for two days were examined by western blot analysis for HPIP expression. β-Actin was used as a loading control. (B) Phase-contrast microscopic images of 4T1, MDA-MB231, BT474, SKBR3 and MCF7 cells in suspension (48 h); Magnification 10x. (C) Quantification of spheroid formation efficiency (SFE) of the indicated breast cancer cell lines. (D) The percentage of survival or apoptotic breast cancer cells (4T1, MDA-MB231, BT474, SKBR3 and MCF7) grown in suspension condition. (E) Western blot analysis of HPIP knock-down in MDA-MB231 cells. (F-G) Effect of HPIP knock-down on spheroid formation efficiency and cell survivability (H) of MDA-MB231 cells. Values are mean ± SEM of three independent experiments in each case *$p < 0.05$, **$p < 0.01$.

2.12.2. HPIP expression correlates with increased autophagy flux and anoikis resistance

Autophagy is a physiological process occurring in healthy and malignant cells and can function as either a tumor-suppressing or tumor-promoting factor [228]. It can control cancer stem cell viability,

cellular differentiation, and anoikis-resistance [229]. Based on the earlier reports that autophagy is one of the principal mechanisms for anoikis resistance, we investigated the function of HPIP in autophagy regulation. We suppressed HPIP both in MDA-MB231 as well as 4T1 cells. Cells were grown in attachment cultures or suspension cultures for 48 h and LC3 levels (the ration between LC3BII to LC3BI) were evaluated as an autophagy read-out. The data revealed a marked reduction in autophagy flux upon silencing of HPIP either in MDA- MB231 or 4T1 cells (Fig.14A-B). Next, we examined the effect of bafilomycin, which inhibits later stages of autophagy, upon silencing of HPIP on LC3B levels. Both in MDA-MB231 as well as in 4T1 cells, bafilomycin treatment triggered autophagy flux (L3BII/LC3BI) in control shRNA cells. Although HPIP silencing resulted in reduced autophagy flux in both cell lines, bafilomycin did not alter it (Fig. 14C-D). Together, these findings demonstrated that HPIP expression is needed for the induction of autophagy in suspension grown BC cells.

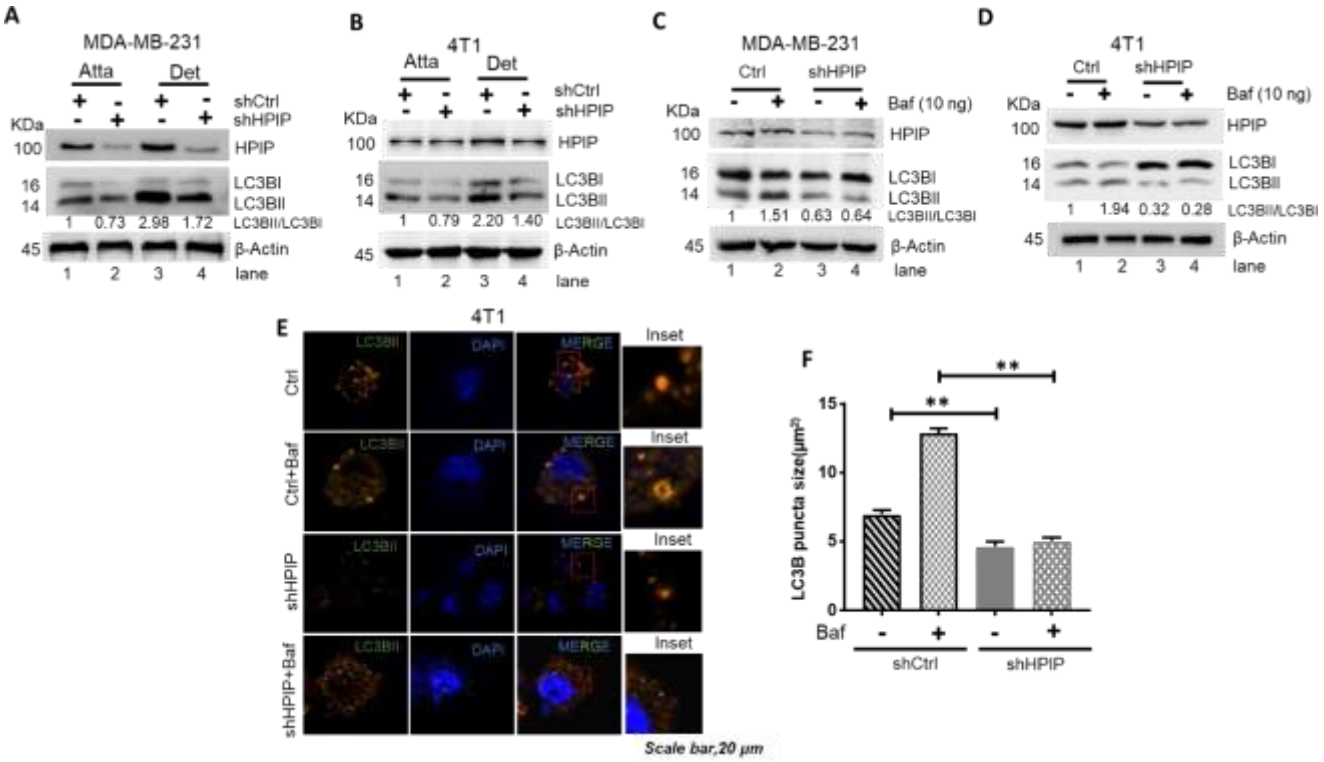


Figure 14: Effect of HPIP silencing on autophagy flux in BC cells. (A-B) shCtrl or shHPIP treated MDA-MB231 and 4T1 cells grown in adherent (Attached) or suspension (Detached) was analyzed for autophagy flux (LC3BII/LC3BI) by immuno blotting. β-actin was used as a loading control. (C-D) Upon HPIP silencing, MDA-MB231 and 4T1 cells grown in adherent (Attached) or suspension (Detached) were treated with bafilomycin (baf, 10 ng/mL) and was analyzed for autophagy flux (LC3BII/LC3BI) by western blotting. (E) Confocal microscopic images representing LC3B puncta in shCtrl or shHPIP-MDA-MB231 cells grown in suspension (Detached) and were treated with bafilomycin (baf, 10 ng/mL). AF-546-LC3B, scale bar 20 μm; magnification 63x. (F) Mean puncta size (MPS) for LC3B from data (E) was analyzed using ImageJ software ($n = 15$) and represented graphically. Values are mean ± SEM of three independent experiments in each case or representative of typical experiment, $*p < 0.05, **p < 0.01$.

2.12.3. HPIP promotes autophagy in breast cancer cells in response to suspension induced survival via Rab5 pathway

After establishing the role of HPIP in anoikis resistance, we next investigated the underlying mechanism. Previous studies showed the role of endocytosis and Rab5 in anoikis resistance [198]. An independent investigation from this lab by another investigator revealed the interaction between HPIP and Rab5 facilitating cell migration (unpublished data). These observations led us to investigate the role of HPIP-Rab5 axis in anoikis resistance. To test this, we followed both gene overexpression as well as silencing approach. Upon overexpression of HPIP we noticed an elevated levels of Rab5 protein in both adherent and suspension, but the effect is more pronounced in suspension cultures (detachment). Concomitantly, a marked increase in autophagy flux measured by LC3BII/I ratio was observed in HPIP overexpressed cells as compared to vector transfected cells (Fig.15A). We next assessed autophagy flux in HPIP silenced cells following ectopic expression of GFP tagged Rab5. The data revealed reduced autophagy flux in GFP-Rab5

transfected-HPIP silenced cells similar to HPIP silenced cells alone as compared to control cells. These data together indicate HPIP might trigger autophagy in response to suspension induced survival in breast cancer cells via Rab5.

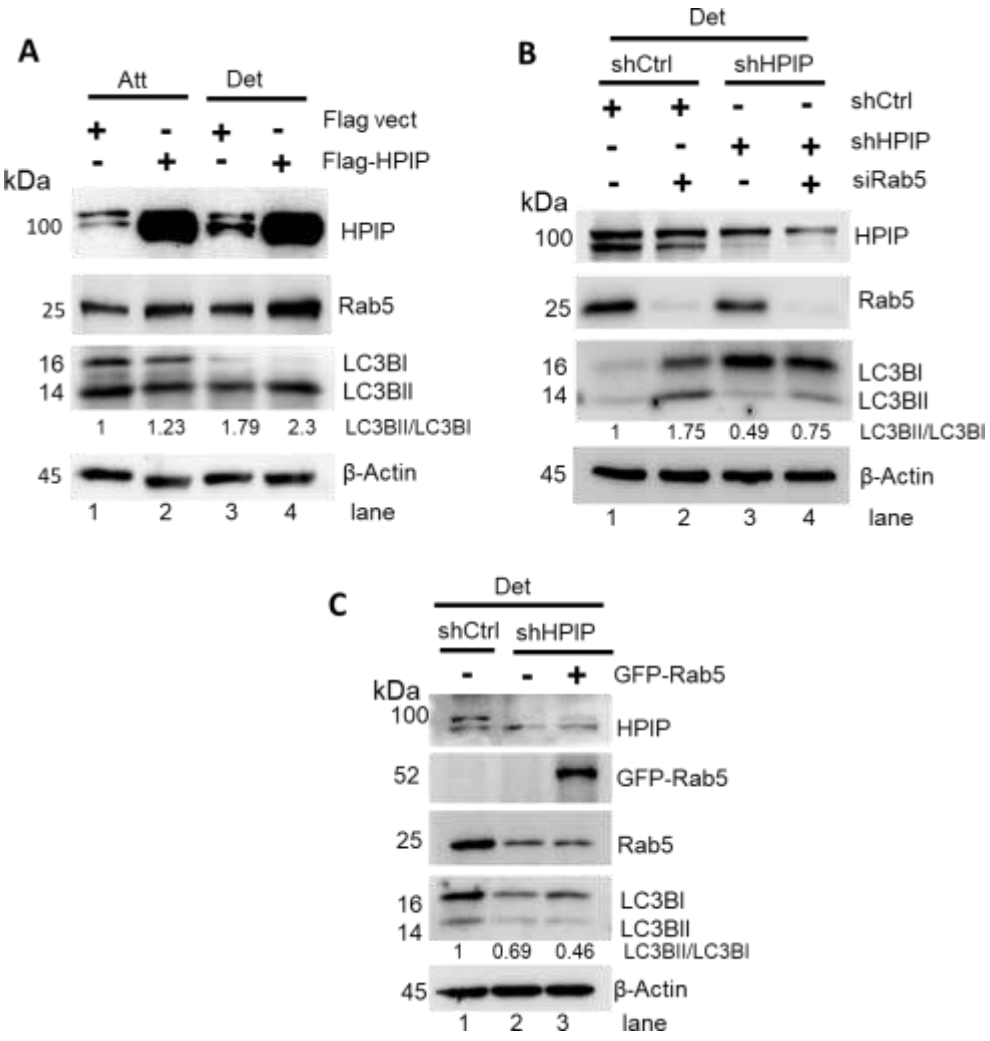


Figure 15: HPIP promotes autophagy in BC cells in response to suspension induced survival via Rab5 pathway. (A) Autophagy flux measured by LC3BII/I ratio in MDA-MB-231 cells upon ectopic expression of Flag-HPIP in adherent (attachment) or suspension (detachment) was analyzed by western blotting (WB). (B) Autophagy flux measured by LC3BII/I ratio in MDA-MB-231 cells in HPIP silenced MDA-MB-231 cells upon ectopic expression of GFP-Rab5. β-Actin served as a loading control.

2.12.4. HPIP antagonizes RNF126 mediated Rab5 degradation (stability) in response to suspension induced survival in breast cancer cells.

ECM detachment leads to reprogramming of various survival pathways and post-translational modification of several proteins. The ubiquitin-proteasome system (UPS) is one of the most important cellular mechanisms for protein degradation. The E3 ubiquitin ligase RNF126 is important for endosomal sorting and encourages cell survival after ECM detachment [208,230]. We have established that HPIP promotes anoikis resistance though Rab5-mediated autophagy. To further understand the underlying mechanism, we ascertained if RNF126 participate in this process based on its demonstrated role in endosome-mediated functions. To test this possibility, first we analyzed Rab5 protein levels upon overexpression of RNF126 in 4T1 cells. We found dipping in Rab5 level both in adherent as well as in suspension cultures of 4T1 cells albeit it is more pronounced in suspension cultures but is restored upon MG132 (proteasome inhibitor) treatment suggesting possible involvement of proteasomal pathway in controlling of Rab5 levels (Fig. 16A). However, HPIP levels were unaltered upon ectopic expression of GFP- RNF126. Consistent with this data, dose dependent expression of GFP-RNF126 (1- 4 µg) resulted a diminished expression of Rab5 but not HPIP (1-4 µg) (Fig 16B). We observed that even the lower dose (1 µg) of GFP-RNF126 could deplete Rab5 in 4T1 cells. Since HPIP ectopic expression triggered Rab5 expression (Fig 15A), we next examined if HPIP could antagonizes RNF126-mediated Rab5 degradation. We analyzed Rab5 levels following co- transfection of Flag-HPIP (1-4 µg) and GFP-RNF126 in 4T1 cells. The data revealed a gradual elevation of Rab5 levels in presence of Flag-HPIP and GFP-RNF126 (Fig 16C). This data indicates that HPIP antagonizes RNF126 mediated Rab5 degradation.

To further strengthen these findings, we measured the half-life of Rab5 upon HPIP silencing in 4T1 cells treated with cycloheximide, a protein translation inhibitor. The data revealed that Rab5 protein was stable with a half-life of ~4.6 h in control cells. However, it's levels declined upon HPIP silencing with a half-life of ~3.9 h (Fig 16D-E). Conversely, ectopic expression of

GFP-RNF126 resulted a marked reduction in the half-life of Rab5 from 6.9 h to 4.2 h in GFP-RNF126 over expressing cells (Fig 16F-G). Further confocal imaging analysis showed distribution of RNF126 in throughout the cytoplasm but a punctate pattern. Further we found a marked colocalization of Rab5 with RNF126 in 4T1 cells indicating possible interaction in endosome compartment (Fig. 16 H). Together these results suggest that RNF126 controls Rab5 levels possibly via proteasomal pathway, while HPIP antagonizes it.

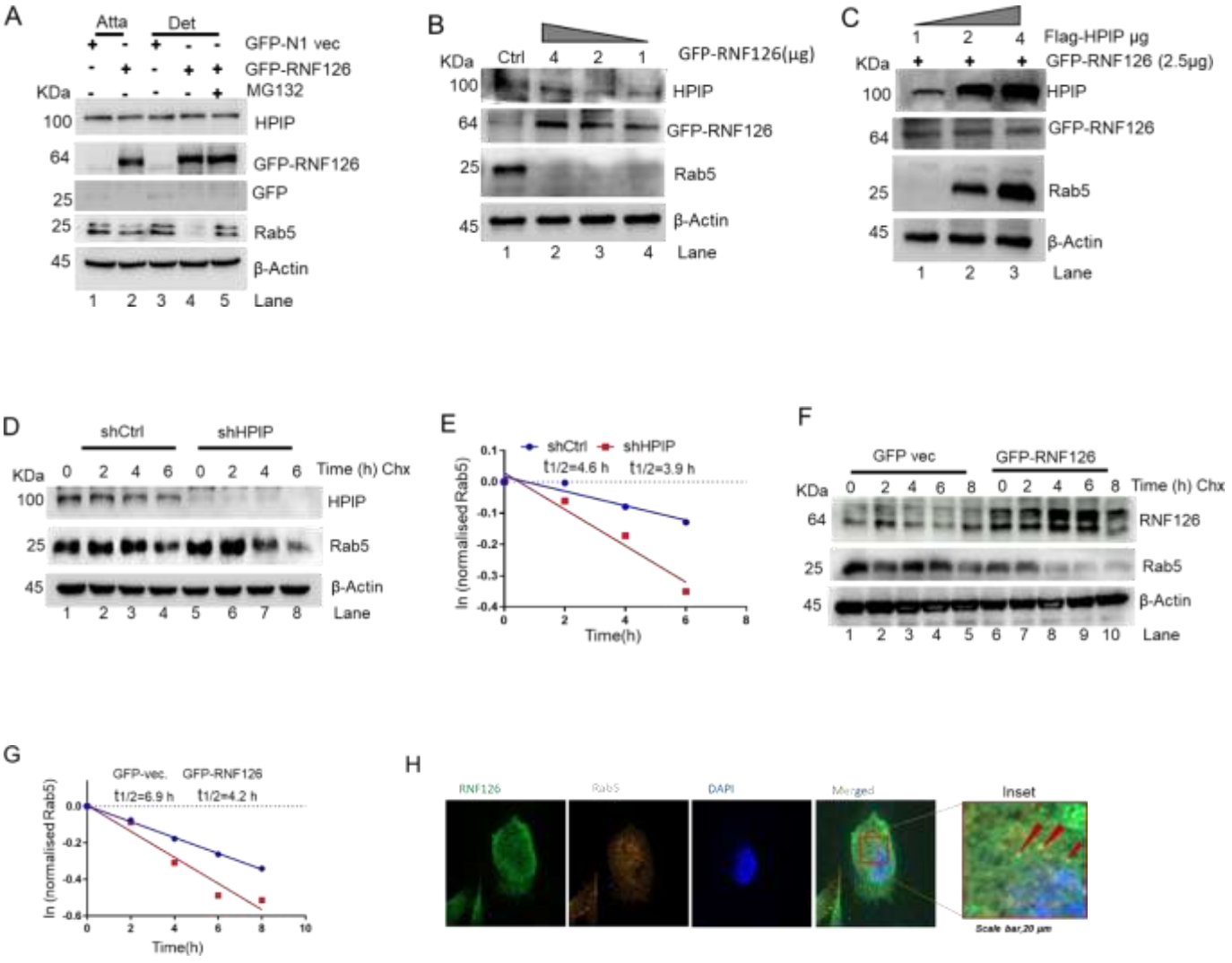


Figure 16: RNF126 affects Rab5 stability in suspension cultures of BC cells. (A) GFP or GFP-RNF126 transfected cells were grown in adherent (attachment) or suspension (detachment) cultures of 4T1 cells were examined by immuno blotting as indicated. 4T1 cells were transfected

with GFP-RNF126 (B) or Flag-HPIP (C) plasmid constructs at various concentrations (1-4 µg) and lysates were analyzed by WB as indicated. (D) shCtrl or shHPIP- 4T1 cells were treated with Cycloheximide (Chx) for various time points and lysates were analyzed by western blotting as indicated. (E) Quantification of Rab5 from fig D. (F) GFP-vec or GFP-RNF126- 4T1 cells were treated with Cycloheximide (Chx) for various time points and lysates were analyzed by western blotting as indicated. (G) Quantification of Rab5 from fig.F. (H) Colocalization of Rab5 and RNF126 in 4T1 cells analyzed by confocal microscopy. Scale bar, 10 µm. Magnification 63x.

2.12.5. NFkB upregulates HPIP upon ECM loss

We next investigated the expression of HPIP in suspension cultures. MCF10A, MDA-MB231 or MCF7 cells were grown in poly-HEMA coated plates from 24 to 48 h and cell lysates were analyzed by western blotting or qRT-PCR. The data revealed that HPIP expression is significantly increased both at transcript as well as protein level in all the cell lines tested (Fig. 17A-C). Previous studies have shown the activation of NFkB in suspension cultures [226]. Hence, we performed *in silico* analysis and found one NFkB binding site in the vicinity of HPIP gene regulatory region (promoter). Based on this information, we hypothesized that NFkB might regulate HPIP expression in suspension cultures of breast cancer cells. We validated our hypothesis by treating MDA-MB231 cells grown in adherent or suspension condition with PDTC, a specific inhibitor of NFkB. AS shown in Figure 17D, HPIP expression is downregulated upon NFkB inhibition. Consistent with this data, HPIP protein levels were also markedly reduced in response to treatment with PDTC (Fig 17E). To strengthen these findings, next we performed luciferase assay using HPIP-promoter-Luc construct. The data shows a significantly increased HPIP-promoter activity in cells grown in suspension cultures than in adherent cultures (Fig. 17F). Next cell viability assay was employed to assess the role of NFkB in HPIP-mediated cell survival in suspension cultures. The data revealed a decreased cell viability upon treatment with PDTC in control cells and this effect was further pronounced upon HPIP knock down in MDA-MB231 cells (fig.17G-H).

Together these data support that NFkB drives HPIP expression that in turn support anoikis resistance in BC cells.

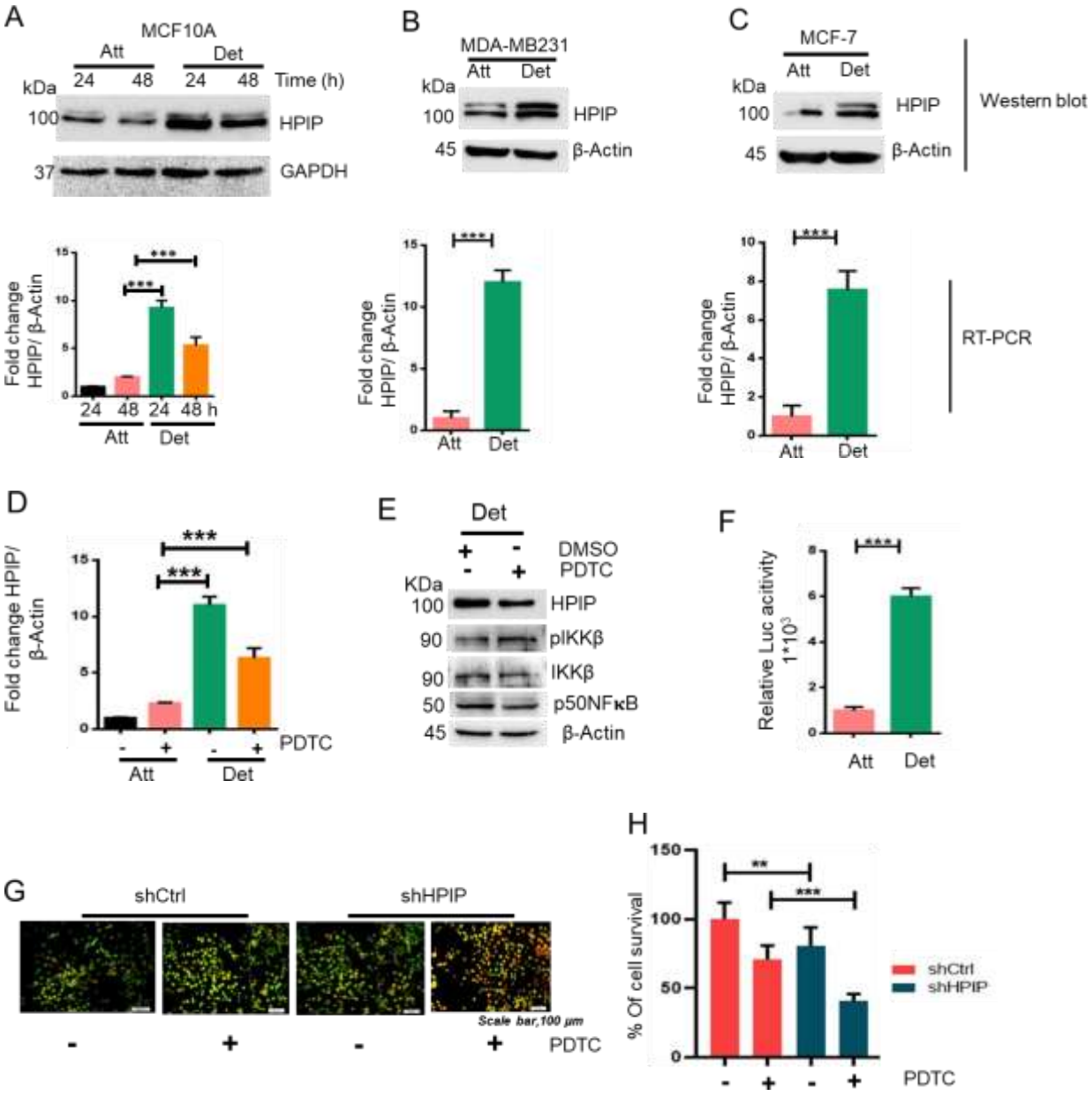


Figure 17: NFkB upregulates HPIP expression in suspension cultures in breast cancer cells. (A-C) MCF10A, MDA-MB231 or MCF7 cells were grown in poly-HEMA coated plates from 24 to 48 h and cell lysates were analyzed by western blotting or qRT-PCR. Att- attachment, Det- detachment. (D) qRT- PCR analysis showing HPIP expression in MDA-MB231 cells grown in adherent or detached condition followed by PDTC treatment. (E) Western blotting analysis

demonstrating HPIP expression in MDA- MB231 cells grown in suspension condition followed by PDTC treatment. β-Actin served as a loading control. (F) Luciferase assay demonstrating the activity of HPIP promoter in Att or Det cultures of MDA- MB231 cells. (G-H) Cell survival assay by acridine orange and ethidium bromide staining (AO/EtBr) in MDA-MB231 cells grown in detached condition following HPIP silencing and PDTC treatment. Quantification of cell survivability using Luna software. Values are mean ± SEM of three independent experiments in each case or representative of a typical experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.12.6. HPIP silencing affects clonogenic capacity, tumor formation and metastasis ability of breast cancer cells in response to suspension survival (ECM loss)

Thus far, we have established that HPIP is an essential regulator in anoikis resistance. We next performed *in vivo* experiments to determine if the observed decreased ability of HPIP-silenced cells to survive in suspension to form tumors and metastasize. We used breast cancer cell line 4T1 to undertake the experimental metastasis for this aim. Stable clones of 4T1 cells were generated by stably expressing control shRNA (4T1-shCtrl) or HPIP-targeting shRNA (4T1 shHPIP). pGipz vector, where shCtrl or shHPIP RNAs were cloned, expresses GFP to trace the cells expressing this vector in mice. First, we conducted a clonogenic assay to confirm the role of HPIP in in vitro colony formation. It was observed that HPIP silencing reduced the ability of cells to form colonies (Fig.18A-B). Similar results were also observed in MDA-MB231 cells (Fig.18A-B). Next, we evaluated the role of HPIP in invasion during the extravasation and colonization stages of metastasis. Cells were grown in suspension and after 48 h of incubation, spheroids were infused through tail vein into the mice for lung metastasis. High levels of GFP expression in the lungs of mice infused with 4T1-shCtrl cells demonstrated higher metastasis of cancer cells than HPIP knock-down cells after 21 days of injection (Fig.18C-D). Suppressing the expression of HPIP significantly reduced invasion of 4T1 cells in shHPIP treated mice (Fig. 18C-D). In addition, we examined HPIP's tumor-forming capacity by injecting subcutaneously 4T1 cells that are grown in

suspension condition for 48 h into nude mice and found that HPIP silencing impaired tumor forming ability in the same way as it hampered metastatic ability (Fig. 18E-F). Together this study supports the finding that HPIP promotes anoikis resistance in BC cells and thus metastasis.

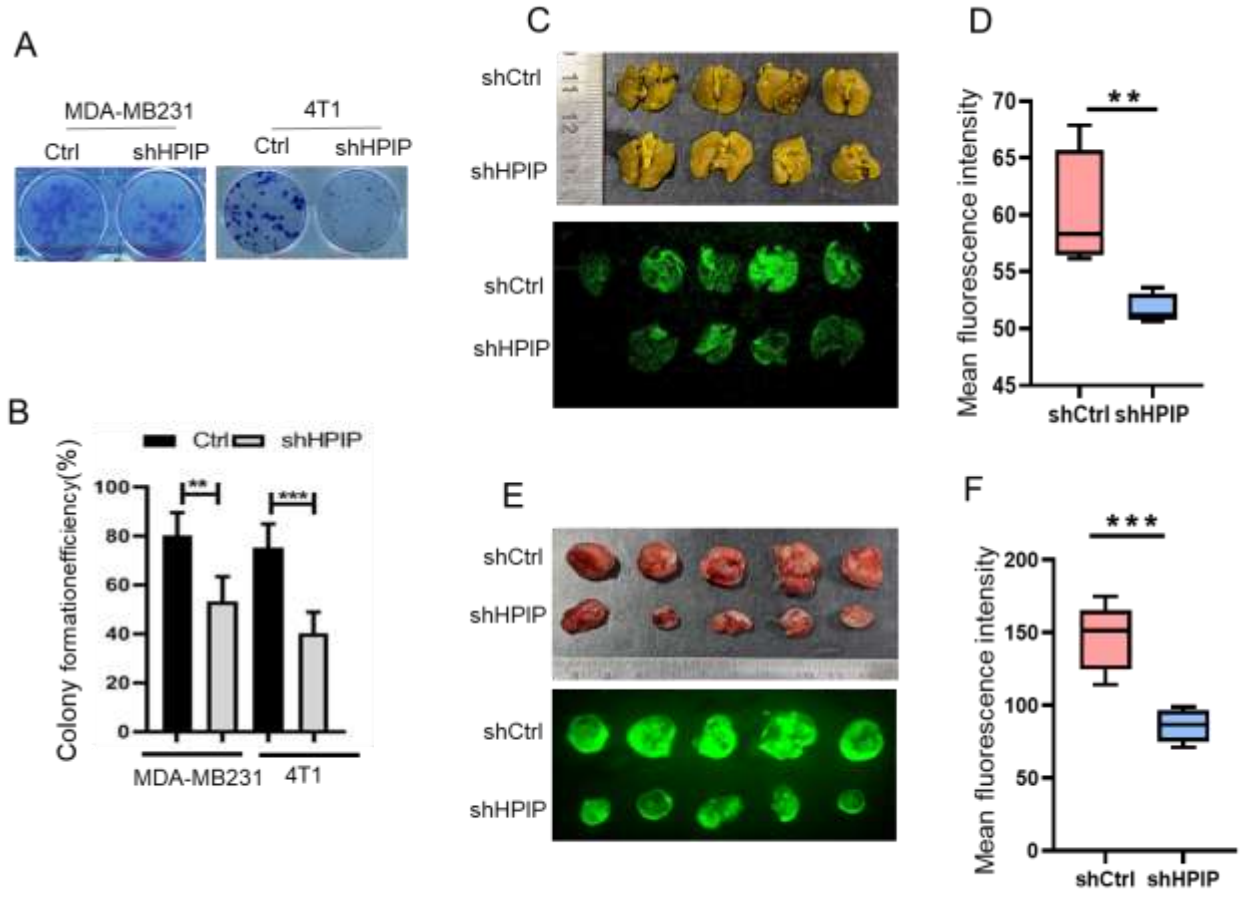


Figure 18: HPIP expression is required for colony forming ability, tumor formation and metastasis in BC cells that are primed in ECM deficiency condition. (A) Clonogenic assay demonstrating the effect of HPIP knock-down on colony forming ability of MDA-MB231 or 4T1 cells. (B) Quantification of data A. (C-F) Images of excised (C) lungs and (E) tumors after tail vein injection and subcutaneous implantation of shCtrl /shHPIP-4T1 cells into nude mice, respectively. (D, F) Mean fluorescence intensity of shCtrl /shHPIP-4T1 treated lungs or tumor in nude mice, respectively.

2.13. Discussion

Anoikis is a programmed cell death associated with loss of extracellular matrix to the cells and is hypothesized to act as a barrier to cellular transformation and metastatic activity. It is a vicious strategy explored by the cancer cells to circumvent the cellular adhesion regulated by the integrins, enabling cells to migrate, and spread more readily to establish metastasis at secondary sites. Therefore, understanding the anoikis process and the regulator of this phenomenon is imperative in clinical oncology as so far anoikis resistance inhibitors are not available.

In this study, we inspected the role of HPIP gene in anoikis resistance in breast cancer cells. We utilized triple negative breast cancer cells MDA-MB231 and 4T1 as model systems and adopted suspension cultures by growing cells in poly-HEMA coated cell culture plates (Debanth et al., 2008). Suspension condition was previously thought to be harmful to cells due to apoptotic induction and growth inhibition, however suspension state could promote cancer cell reattachment by potentiating Laminin A/C though cytoskeleton disruption [234]. An *in vitro* study revealed that cancer cells cultured in suspension induced microtubule-based micro tentacles to enhance reattachment [235]. The suspension state encourages cell survival and cancer progression in aggressive BC cells such as MDA-MB-231 and 4T1 cells. Indeed, we demonstrate BC cells cultured in suspension cultures had better spheroid formation and survival abilities. Normal epithelial cells tend to die after matrix detachment due to disruption of integrin- mediated cell survival signals, whereas tumor cells can survive due to genetic abnormalities (e.g., neurotrophic receptor tyrosine kinase B (TrkB), B-Raf proto-oncogene (B- RAF)) [236,237]. Suppression of ECM degradation (MMP11, ADAM family) and migration (CSF1) associated genes may promote proliferation and migration of BC cells in suspension. Single-molecule RNA sequencing confirmed significant enrichment for ECM genes in CTCs from pancreatic and breast cancers [238,239]. In line with the some of these earlier observations, our finding that HPIP gene is induced upon ECM loss is essential for suspension induced survival as loss of its expression dampened the spheroid formation ability, colony forming units, increased apoptosis, tumor forming ability and

metastasis capacity in nude mice. We have demonstrated these findings using both cell line based as well as *in vivo* mouse xenograft models. Earlier reports point to the role of HPIP gene in cell proliferation, migration, and metastasis. In addition to these properties, the current study bestows an additional functional property i.e., anoikis resistance to HPIP gene.

Next, we investigated the mechanism that underlies this process. Increased autophagy has been associated with suspension induced cell death in normal cells as well as in cancer cells [231,232]. In cancer cells, autophagy appears to play a 'double edged sword' role as it confers anoikis resistance rather than cell death [233, 234, 235]). There is a large amount of literature supporting this point [226]. Autophagy has shown both pro and anti-metastatic properties [233]. In the early stages of cancer, autophagy acts as an anti-metastatic agent by limiting necrosis and inflammation responses to cancer cells. Autophagy also reduces the invasion and migration of cancer cells from their origin sites in the initial stages of metastasis. However, in the later stages of metastasis, autophagy promotes cancer cell survival and colonization in secondary sites [227]. Autophagy appears to be a key mechanism for ECM-detached cancer cells to avoid anoikis [235]. In hepatocellular carcinoma (HCC), anoikis resistance and lung metastasis of HCC cells was primarily attributable for autophagy [236]. Autophagy has been shown to cause ECM detachment and inhibit the β1 integrin expression in cancer cells [226]. Our findings revealed that HPIP silencing increased apoptosis in suspension cultures of BC cells, which is accompanied by increased autophagy flux as evaluated by LC3B levels. Further we found that the autophagy flux induced by HPIP is dependent on Rab5 as Rab5 silencing severely affected it in breast cancer cells. Studies involving over-expression, silencing and rescue experiments supported this finding. Rab5 levels were induced in suspension cultures which are dependent on HPIP as loss of HPIP expression dampened it and this is further accompanied by decreased LC3B levels suggesting the HPIP-Rab5 pathway in controlling the suspension survival or anoikis resistance via autophagy.

Earlier studies demonstrated the importance of endocytosis and Rab5 in anoikis resistance [32]. Parallel study in our lab by another investigator demonstrated that HPIP interacts with Rab5

and aids in cell migration (in communication). Therefore, we decided to investigate the inter relationship between HPIP and Rab5 in anoikis resistance. We demonstrated that loss of HPIP expression results in decreased levels of Rab5 in breast cancer cells, which is accompanied by reduced autophagy flux implying the role of HPIP-Rab5 pathway in suspension induced survival of BC cells. Next, we investigated the mechanism underlie in HPIP-mediated Rab5 expression. RNF126 is an E3 ubiquitin ligase. It is known to participate in endosomal sorting and thereby it promotes cell survivability upon ECM detachment [33,34]. We ascertained if RNF126 could control Rab5 levels because of HPIP loss. In support of our hypothesis, overexpression of RNF126 led to diminished Rab5 levels, but restored upon either ectopic expression of HPIP or treatment with MG132, a proteasome inhibitor. Moreover, pulse-chase experiments using cycloheximide, a protein translation inhibitor, revealed increased half-life of Rab5 upon silencing of RNF126. Further, colocalization assay demonstrated a significant colocalization of Rab5 and RNF126 in BC cells. Together these observations imply that HPIP stabilizes Rab5 in suspension cultures by antagonizing RNF126 mediated degradation of Rab5. Whether RNF126 destabilizes Rab5 via ubiquitination requires further investigation.

Another intriguing observation in this study was induced expression of HPIP in suspension cultures of BC cells. We further explored the upstream signaling and transcription factors that drive HPIP transcription in suspension cultures. First, we have analyzed the expression of HPIP in both adherent and nonadherent cells by western blotting, and qRT-PCR and the data indicated that HPIP levels were significantly increased both at transcript as well as protein levels in MCF10A, MDA-MB231, and MCF-7 cell lines grown in suspension cultures. Previous reports suggested the activation of NFκB in suspension conditions [35]. Based on this information, we performed *in silico* analysis on *HPIP* promoter sequence and found one NFκB binding site in it. In support of our hypothesis that NFkB could drive *HPIP* transcription in suspension cultures. Promoter probe assay by luciferase reporter showed a significant elevation of *HPIP* promoter activity in suspension cultures as compared to attachment cultures. Moreover, pharmacological inhibitor studies

demonstrated that inhibiting NFκB activity by PDTC could abrogate HPIP expression both at the mRNA and protein levels, suggesting the direct involvement of NFkB in suspension induced expression of HPIP in BC cells. In support of this, functional analysis also showed HPIP-mediated cell survival of cells cultured in suspension was dependent on the NFkB activation. Together these data imply that NFkB could regulate HPIP expression in suspension cultures and ensure breast cancer cell survival in suspension (-ECM).

In conclusion, overall, these results demonstrate that HPIP is a novel anoikis resistance associated gene. We proved that NFkB could drive HPIP transcription in suspension cultures. HPIP thus induced could promote autophagy flux via Rab5-mediated pathway by antagonizing RNF126, which otherwise suppresses Rab5 function by destabilizing it. Together it establishes a new signaling pathway involving HPIP, Rab5 and NFkB regulating anoikis resistance and thus metastasis in breast cancer. Finally, this study envisages that anoikis resistance cells can be eliminated by targeting HPIP-Rab5-NFkB pathway and could be leveraged to build a new method for controlling breast cancer metastasis (Fig 19).

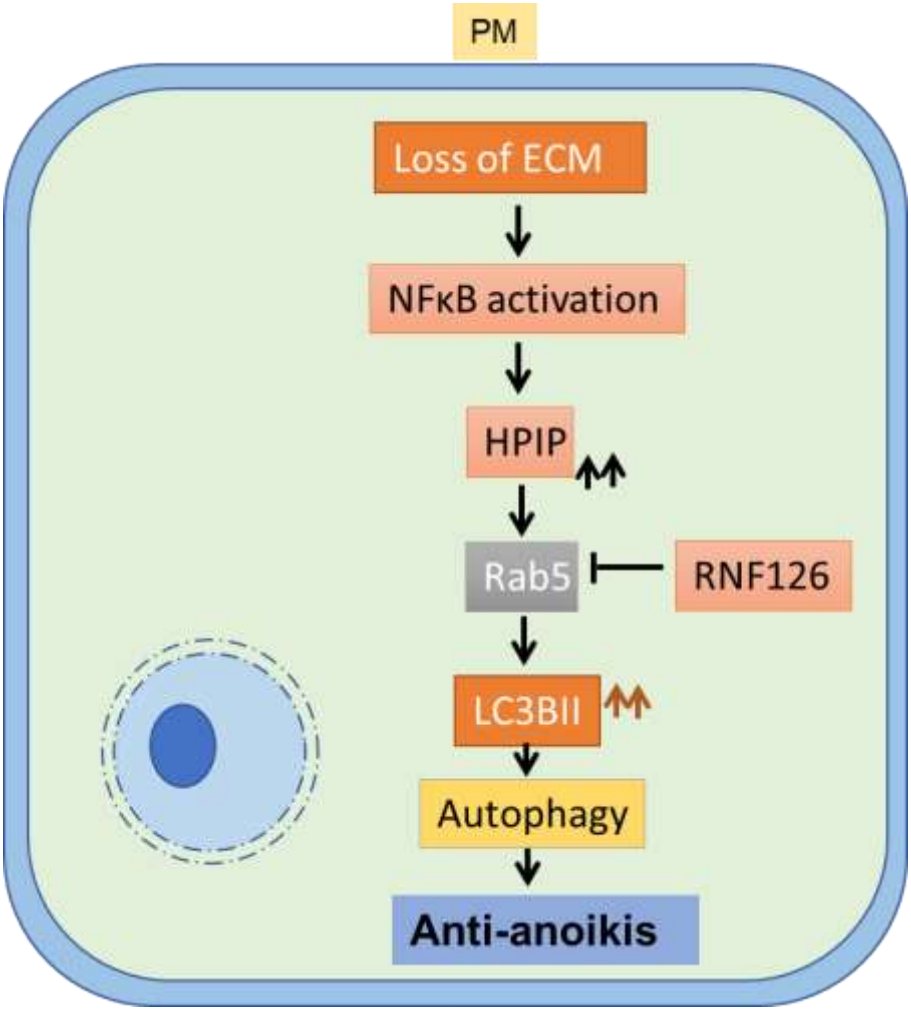


Figure 19: Model depicting HPIP-Rab5-RNF126 axis in the regulation of anoikis resistance in breast cancer cells. ECM loss triggers the activation of the NFkB that drives HPIP expression. HPIP in turn stabilizes Rab5 protein by antagonizing RNF126 (ubiquitin ligase) thus, promoting autophagy flux and anoikis resistance in breast cancer cells. PM, plasma membrane. Upward arrows denote increased levels of proteins, HPIP or LC3BII.

2.14. Conclusions

Overall, this study we investigated the role of HPIP gene in mammary lactogenic differentiation and its role in anoikis resistance in breast cancer cells. From the first objective, we report that HPIP is required for prolactin (PRL)- induced lactogenic differentiation *in vitro*. Molecular analysis of HPIP expression in mice revealed its induced expression at pregnancy and lactation stages of mammary gland. Moreover, PRL is a lactogenic hormone that controls pregnancy as well as lactation and induces Hpip/Pbxip1 expression in a signal transducer and activator of transcription 5a-dependent manner. Using mammary epithelial and lactogenic-competent cell lines, we further show that HPIP plays a regulatory role in PRL-mediated mammary epithelial cell differentiation, which is measured by acini formation, β-casein synthesis, and lipid droplet formation. Further mechanistic studies using pharmacological inhibitors revealed that HPIP modulates PRL induced β-casein synthesis via phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) activation. This study also identified HPIP as a critical regulator of autocrine PRL signaling as treatment with the PRL receptor antagonist Δ1-9-G129R-hPRL restrained HPIP- mediated PRL synthesis, AKT activation, and b-casein synthesis in cultured HC11 cells. Interestingly, we also uncovered that microRNA-148a (miR-148a) antagonizes HPIP mediated mammary epithelial cell differentiation. Together, our study identified HPIP as a critical regulator of PRL signaling and revealed a novel molecular circuitry involving PRL, HPIP, PI3K/AKT, and miR-148a that controls mammary epithelial cell differentiation *in vitro*.

From the second objective, we conclude that HPIP is an essential protooncogene that could bestow anoikis resistance to breast cancer cells by promoting protective autophagy. We showed that HPIP influences anoikis resistance by triggering Rab5/RNF126/LC3B signaling. Further our data revealed that NFkB drives *HPIP* transcription in suspension cultures of BC cells. HPIP thus induced stabilizes Rab5 by antagonizing RNF126 and mediates anoikis resistance in breast cancer cells.

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PUBLICATIONS

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Hematopoietic PBX-interacting protein is a novel regulator of mammary epithelial cell differentiation

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Hematopoietic PBX-interacting protein (HPIP, also known as PBXIP1) is an estrogen receptor (ER) interacting protein that regulates estrogen-mediated breast cancer cell proliferation and tumorigenesis. However, its functional significance in the context of mammary gland development is unexplored. Here, we report that HPIP is required for prolactin (PRL)-induced lactogenic differentiation *in vitro*. Molecular analysis of HPIP expression in mice revealed its induced expression at pregnancy and lactation stages of mammary gland. Moreover, PRL is a lactogenic hormone that controls pregnancy as well as lactation and induces *Hpip*/*Pbxip1* expression in a signal transducer and activator of transcription 5a-dependent manner. Using mammary epithelial and lactogenic-competent cell lines, we further show that HPIP plays a regulatory role in PRL-mediated mammary epithelial cell differentiation, which is measured by acini formation, β-casein synthesis, and lipid droplet formation. Further mechanistic studies using pharmacological inhibitors revealed that HPIP modulates PRL-induced β-casein synthesis via phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) activation. This study also identified HPIP as a critical regulator of autocrine PRL signaling as treatment with the PRL receptor antagonist Δ1-9-G129R-hPRL restrained HPIP-mediated PRL synthesis, AKT activation, and β-casein synthesis in cultured HC11 cells. Interestingly, we also uncovered that microRNA-148a (miR-148a) antagonizes HPIP-mediated mammary epithelial cell differentiation. Together, our study identified HPIP as a critical regulator of PRL signaling and revealed a novel molecular circuitry involving PRL, HPIP, PI3K/AKT, and miR-148a that controls mammary epithelial cell differentiation *in vitro*.

Introduction

Mammary glands are highly evolved, specialized exocrine glands made up of lobes, and ducts [1]. The alveoli (hollow cavities) in mammary glands are lined with milk-secreting cuboidal cells and are surrounded by myoepithelial cells. The alveolus undergoes development and differentiation under the control of

Abbreviations
AKT, protein kinase B; bPRL, bovine prolactin; HPIP, hematopoietic PBX-interacting protein; JAK2, Janus kinase 2; miR-148a, microRNA-148a; PI3K, phosphoinositide 3-kinase; PRL, Prolactin; PRLR, prolactin receptor; PTEN, phosphatase and tensin homolog deleted on chromosome 10; STAT5, signal transducer and activator of transcription 5.

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Review Article

Epithelial to Mesenchymal Transition (EMT): From a Developmental Instructor to Metastasis Propeller

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Abstract

Epithelial to mesenchymal transition (EMT) is an essential physiological process in all-multicellular organisms. It plays pivotal role in the normal development of an organism but is equally important for tumor metastasis. For example, during mammary gland development, EMT plays critical role in mammary epithelial cellular migration and the establishment of the new tissue. Epithelial cells in terminal end buds (TEBs) show higher plasticity and induce EMT. The EMT in TEBs is regulated by various factors like epidermal growth factor (EGF), insulin like growth factor-2 (IGF-2), Wnt-4 and hepatocyte growth Factor (HGF. Interestingly, cells at branching induce the expression of typical mesenchymal markers such as Vimentin and Snail. Besides its fundamental role in developmental process, a large body of data suggests that EMT is an important and integral process in breast tumor metastasis. Emerging studies revealed that EMT and partly tumor heterogeneity cause therapeutic resistance in breast cancer. Comprehending the complexity of the EMT may offer significant understandings that lead to the improvement of therapeutic targets for invasive cancer, and perhaps it can also be used as biomarkers pinpointing tumor subclasses with better likelihoods of relapse, metastasis and drug resistance leading to death. Herein we will review and provide critical comments on the role of EMT as a developmental instructor, metastasis propeller and master of therapeutic resistance using primarily breast as a model system.

ABBREVIATIONS

EMT: Epithelial to Mesenchymal Transition; MET: Mesenchymal to Epithelial Transition; TEBs: Epithelial cells in Terminal End Buds; CTC: Circulatory Tumor Cells; CSCs: Cancer Stem Cells; HGF: Hepatocyte Growth Factor/Scatter Factor (HGF/SF); IGF-2: Insulin Like Growth Factor- 2; ESRP: Epithelial Splicing Regulatory Protein; δEF1: Delta-Crystallin Enhancer Binding Factor; SIP1: Smad Interacting Protein 1

INTRODUCTION

For all multi-cellular organisms, epithelial to mesenchyme transition (EMT) is a critical process and plays a central role in normal development as it involves in embryogenesis, wound healing, embryo implantation and placenta formation [1]. Furthermore, several developmental process including gastrulation, neural crest formation and organogenesis requires EMT. During embryonic development, neuro-ectodermal epithelial cells undergo EMT and give rise to highly migratory neural crest cells. As a consequence, they dissociate and become motile and migrate to different parts of embryo, where they undergo further differentiation into different cell types. Various signaling pathways such as TGFβ-SMAD, Wnt, growth factor signaling provides essential instructions to the cells to occur EMT [2]. For example, embryos deficient in Wnt3 cannot undergo EMT. Furthermore, deficiency of TGF-beta family proteins like Nodal and Vg1, which mediates Wnt action can leads to defects in mesoderm formation due to absence of EMT [3-5].

EMT is an essential physiological process in all multicellular organisms, which plays important role in the normal development of an organism but is equally important for tumor metastasis to occur. Metastasis comprises of a series of steps that includes epithelial to mesenchymal transition (EMT), loss of cell adhesion, cell migration, and new blood vessels formation, intravasation into blood or lymphatic vessels and extravasation (Figure 1). These cells shows less epithelial features than mesenchymal and have self-renewal ability which is the indication of cellular heterogeneity of tumor which contains the sub-population of cancer cells which has cancer stem cells (CSCs) property and they can migrate from primary tumor site to distant secondary progression. In many cancers, the neoplastic cells exhibit EMT activation only when the tumor population is abundant with CSCs [6,7] on the other hand forced activation of EMT by means of chemical treatment increases the tumor initiating capacity and shows CSCs property [8,9].

Characterization of hematopoietic PBX interacting protein as a novel regulator of mammary epithelial cell differentiation and anoikis resistance in breast cancer

by Anju Dwivedi

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