THE NOVEL PRESENCE AND ROLE OF INOSITOL POLYPHOSPHATE 4-PHOSPHATASE IN THE CELL NUCLEUS AND EXTRACELLULAR MILIEU

Damini Vatsa

*Delhi Technological University, Delhi, India

E-mail ID: vatsa.damini@gmail.com

ABSTRACT

Phosphoinositides are phospholipids bearing phosphorylated inositol rings. They are small lipid secondary messengers which together, through their synchronized consortium, bring about complex intracellular cascades which are involved in the regulation of cell metabolism, endocytosis & exocytosis, membrane trafficking, cytoskeleton re-arrangements, nuclear events, cell proliferation & differentiation. The regulators of phosphoinositides i.e. Kinases and phosphatases can transform Phosphatidylinositol by phosphorylating or de-phosphorylating it at three positions 3', 4' & 5'-OH in an alternate manner and can produce seven different species of phosphoinositides. Every phosphoinositides & its specific regulator have a definitive organelle to which they localize and are involved in maintaining distinct roles in various intracellular cascades. Phosphoinositides are known to impart organelle identity. Hence, their location is in co-ordination with their role. PI3Kinase signalling pathway is one pathway involving various phosphoinositides and their kinases and phosphatases, all mediating a cascade regulating diverse cellular functions including metabolism, growth, proliferation, survival, transcription and protein synthesis. The key mediators of this pathway are PI3kinase, AKT, PDK1, INPP5, INPP4A/INPP4B, and PTEN. This pathway is the most mutated pathway associated with cancer pathogenesis. Many key mediators (i.e. some classes of PI3kinase, PTEN, AKT and PDK1) and phosphoinositides (i.e. PtdIns(3,4,5)P_3, PtdIns(3,4)P_2) involved in PI3K signalling pathway have already been found in the nucleus and their dynamic presence in nucleus has been

associated with the regulation of cell's proliferation and differentiation. PTEN which is emerging as a cellular regulator with multiple functions has already been found in cytoplasm, nucleus and secreted out of the cells classically and in nano-sized vesicles called exosomes. Its role as a tumour suppressor and its significance in maintaining cell proliferation and differentiation at all the locations has been validated. In this thesis, another key mediator of PI3K pathway, which also plays a crucial role in endosomes/phagosomes formation and neurological pathologies i.e. INPP4A has been reported for the first time to be present inside the nucleus as well as in the cell's extracellular milieu. Nuclear INPP4A was found to have a higher molecular weight than its cytoplasmic counterpart. Overexpression studies revealed an inhibitory role of nuclear INPP4A on cell's proliferation rate. The translocation of INPP4A in the nucleus increases on serum starvation and its presence in nucleus is highest in G0/G1 phase. The secreted INPP4A is glycosylated and is secreted via non-classical pathway of secretion. These findings are novel and will likely have implications in tumor biology.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Phosphatidylinostiol (PtdIns) are phospholipids that have a phosphatidic acid backbone which is linked to myo-inositol ring via phosphate bond. It represents only 5-10% of the total lipids in cell yet it acts as a powerful substrate for producing various acute response mediators controlling different aspects of cell's life signalling pathways (Riehle *et al*., 2013). PtdIns are typically found as a minor component on the cytosolic side of the eukaryotic plasma membranes. PtdIns were first identified only after the revolutionary study done by Jordi Folch in 1940s in which he was able to identify inositol linked phospholipids in bovine brain where they are most abundant (Folch, J. 1949). By 1980s, it was clear that these PtdIns are found not only in brain sections but in all tissues and cells in different fractions (Balla, T. 2013). Being lipids, PtdIns attracted little interest of researchers until 1950s, when a groundbreaking study was done by Mabel and Lowell Hokin which led to the discovery of phosphoinositides. Experiment of Hokin and Hokin was called as "phospholipid effect" in which they were able to see change in the total turnover of phospholipids within the cells when cells were exposed to exocrine hormones (Hokin *et al*., 1955a; 1955b). It was only after this that it was realised that PtdIns is the precursor to phosphoinositides which are key regulators of essential biological processes occurring in both plant and animal cells.

Phosphoinositides are produced by reversible phosphorylation at either position 3, 4 or 5 of hydroxyl group on myo-inositol head group of the PtdIns. This reversible phosphorylation is brought about by two key phosphoinositide regulators-kinases and phosphatases. This rapid interconversions of phosphates at position 3,4 and 5 produces seven isoforms of phosphoinositides namely phosphatidylinositol 3-phosphate (PtdIns-3-P), PtdIns-4-P, PtdIns-5-P, Phosphatidylinositol-3, 5-bisphosphate (PtdIns-3, 5-P_2), PtdIns-4, 5-P_2, PtdIns-3,4-P_2, and phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3). As phosphoinositides are very small and can undergo rapid interconversions when stimulated, they act as perfect regulatory mediators of intracellular signal transduction. Apart from signal transduction, phosphoinositides also play a major role in intracellular membrane trafficking, cytoskeleton remodelling, nuclear events, control of cell growth and survival, proliferation and apoptosis etc. PtdIns(4)P is the most important phosphoinositides in the Golgi complex region which determines its normal structure and function (Di Paolo *et al*., 2006). PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are also vital

for endocytosis as they are required for the recruitment of other endocytic proteins. PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are de-phosphorylated by inositol polyphosphate 5-phosphatase(INPP5) and inositol polyphosphate 4-phosphatase (INPP4) to produce PtdIns(3)P on early endosomes which further helps in the processing of endosome within cell. PtdIns(3)P are also essential for their interaction and recruitment of Rab proteins to endosomes. (Di Paolo *et al*., 2006). PtdIns(3,4,5)P_3 has Akt as its direct effector molecule. Akt is a part of PI3K signalling pathway which has its major role in cell proliferation.

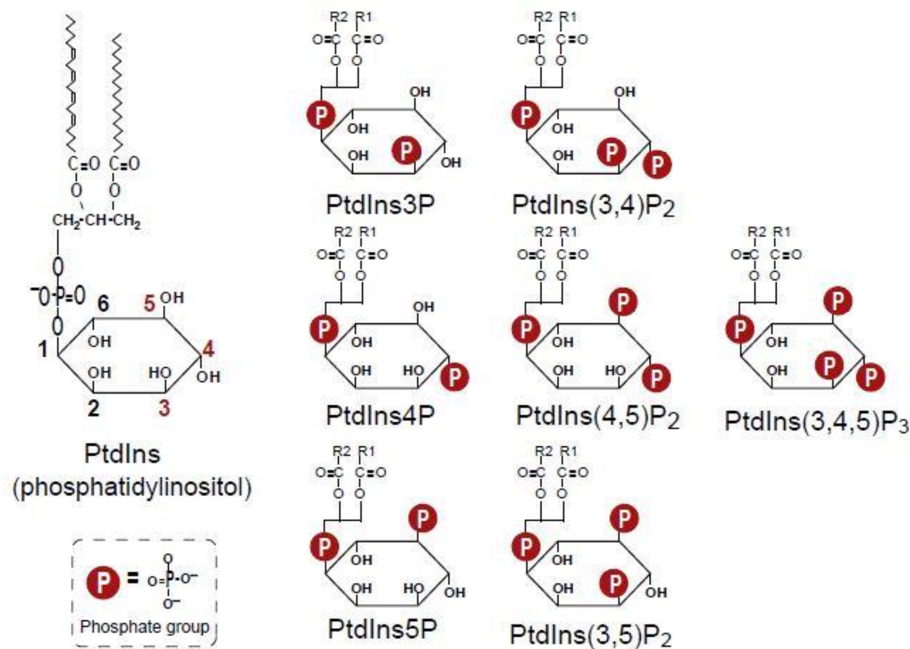


Fig. 1 Structure of PtdIns and its phosphoinositides (Sasaki *et al*., 2009)

Different phosphoinositides are found localized in different organelles of the cell. This localization is coordinated by the integrated action of specific kinases and phosphatases on phosphoinositides in the specific subcellular compartment of the cell. This establishes an organelle identity code by phosphoinositides on cell organelles (Lietha, D., 2011). Intracellular signalling triggers the specific regulators of the specific phosphoinositide to bring about a change in the phospholipid composition of the organelles so that the organelles can initiate their targeted functions (Balla, T. 2013). For example, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are found majorly docked on the plasma membrane and act like secondary messengers initiating intracellular cascades; PtdIns(3)P & PtdIns(3,5)P_2 are mostly found on endosomes and have a major role to play in protein trafficking. The presence of different phosphoinositides in

cytoplasm and cytoplasmic organelles has been well understood but new emerging studies are reporting the presence of different phosphoinositides in the new cellular locations i.e. nucleus as well as in cell's secretions. PtdIns(4,5)P_2 has already been found in nucleus along with its kinases and phosphatases (Cocco *et al*., 1987; Divecha *et al*., 1991). A replica of cytoplasmic pathway in which Diacylglycerol(DAG) and Ins(1,4,5)P_3 are produced on hydrolysis of PtdIns(4,5)P_2, has also been found in nucleus. This pathway may be involved in regulation of transcription and proliferation (Divecha *et al*., 1991; Martelli *et al*., 1992). Another pathway which also may be replicating inside the nucleus is PI3K/Akt pathway. Phosphoinositides of this pathway, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 have been found in nuclear matrix (Lindsay *et al.,* 2006; Elong Edimo *et al*., 2013; Davis *et al*., 2015). Nuclear PtdIns(3,4,5)P_3 is said to originate from PtdIns(4,5)P_2 in the nucleus by the action of nuclear Class I PI3Kinase (Davis *et al*., 2015). Its cytoplasmic inhibitors i.e. PTEN & SHIP-2 have also been found in nucleus (Elong Edimo *et al*., 2013; Lian *et a*l., 2005). Although their role is not as clearly known as in the cytoplasm. Nuclear SHIP-2 may hydrolyze PtdIns(3,4,5)P_3 as well as PtdIns(4,5)P_2. Silencing SHIP-2 leads to reduced Akt activation but increased PtdIns(3,4,5)P_3 accumulation in nucleus (Prasad, 2009, Elong Edimo *et al*., 2011). Nuclear PTEN plays a role in DNA repair mechanism by recruiting RAD51 at the sites of DNA Repair (Bassi *et a*l., 2013). Also, activation of PTEN is associated with depletion in the levels of nuclear Akt which induces Cell cycle arrest (Mistafa *et al*., 2010). PTEN also acts in a phosphorylation independent mechanism in the nucleus.

The other aspect of the study is the extracellular vesicles, namely microvesicles and exosomes. Microvesicles and exosomes are nano-sized vesicles secreted by cells. They carry proteins, DNA/RNA, miRNA in their matrix or embedded in their bilayer and are a means of intracellular communication between a donor cell and recipient cells. Exosomes play a role in inducing an immune response by acting like antigen-presenting vesicles ((Bobrie *et al*., 2011). They are also secreted by neurons and microglia cells and they mediate myelin formation, neurite outgrowth and neuronal survival (Wang *et al*., 2011). Exosomes, in airways, increase proinflammatory cytokines in asthmatic patients (Qazi *et al*., 2010). Cancer cells also secrete exosomes which promote angiogenesis and migration ((Rak, 2010). Exosomes (40-100nm in size) are vesicles that originate via multivesicular endosome fusion with plasma membrane in cells and the vesicles which bud from the plasma membrane, are referred to as microvesicles (upto around

1000 nm in diameter). Many proteins have been characterised to be secreted in exosomes/microvesicles. PTEN, present in PI3K pathway, has been implicated to be secreted by cells and exosomes uptake experiments showed its role in control of cell proliferation.
The understanding of phosphoinositides action mechanism in the nucleus and exosomes is crucial in particular as it will open a novel understanding of signalling cascades and action mechanism by which cell maintains its homeostasis.

Hence, in this thesis, I was able to decipher a small part of the big nuclear phosphoinositide signalling puzzle. Through this thesis, I aimed to identify and characterize another key regulator of PI3K signalling pathway, Inositol polyphosphate 4-phosphatase type I (INPP4A) in the nucleus and in cell's extracellular secretions. These are completely novel findings and ones that merits in-depth study, as its understanding can have tremendous potential for development in the field of cell cycle and related diseases. SHIP-2 & PTEN, inhibitors of the same PI3K/Akt pathway have been found in the nucleus and their role in nucleus has been linked to maintaining cellular proliferation. Hence, I also explored the role of nuclear INPP4A in regulating the proliferation of cells. Hint of INPP4A being secreted from the cells came from the preliminary finding in lab where INPP4A was seen in the serum and Broncho alveolar lavage fluid (BALF) of mice and human subjects hence, we sought to investigate its secretion and characterize the secreted INPP4A.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

2.1 PI3Kinase Signalling Pathway

PI3K signalling pathway has been known to play a crucial role in various metabolic pathways of cells such as growth, proliferation, apoptosis, differentiation, insulin regulation, cytoskeleton rearrangement (Hawkins *et al.*, 2006). Being involved in the core metabolic functions regulating cellular homeostasis, it is evident that dysregulation of this pathway has been reported in the pathogenesis of various diseases such as cancer, autoimmune disease, cardiovascular problems, and diabetes. Having oncogenes (e.g. PI3K, Akt) and tumour suppressors (e.g. PTEN, INPP4B), this pathway has been found to be mutated in almost 15-30% cancers (Hawkins *et al.*, 2006; Riehle *et al.*, 2013, Engelman *et al.*, 2006)..

PtdIns(4)P and PtdIns(4,5)P_2 are the most abundant phosphoinositide in cells which are constitutively expressed on the plasma membrane (Di Paolo *et al.*, 2006). PI3K signalling is initiated when plasma membrane receptors such as Receptor tyrosine kinase (RTK) and/or GPCR are stimulated by growth hormones such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin, and insulin like growth factor I (IGF-I) (Riehle *et al.*, 2013; Hawkins *et al.*, 2006). Stimulation of RTK and/or GPCR causes phosphorylation and activation of PI3kinase. In multicellular eukaryotes, there are three classes of PI3Kinase which have been classified according to their substrate and sequence. Class I PI3Kinase is involved in the PI3K signalling pathway and uses PtdIns(4,5)P_2 as its substrate. A Subunit of Class I, Class IA PI3Kinase is activated by the RTK whereas Class IB PI3Kinase is activated by the GPCR. Class IA PI3Kinase consists of two domains- p85 regulatory subunit containing Src Homolgy 2 (SH2) domain which interacts with RTK and another p110 catalytic subunit which is inhibited by p85 regulatory subunit. Interaction of SH2 domain in p85 with RTK, releases p110 catalytic domain from inhibition and p85-p110 unit recruits its substrate PtdIns(4,5)P_2 and phosphorylates it. Class IB PI3K consists of a p101 regulatory subunit and a p110γ catalytic subunit. It has no p85 subunit hence it is not able to interact with RTK. Class II PI3Kinase has only p110 regulatory subunit and uses Phosphatidylinositol as its major substrate producing PtdIns(3)P but also produces PtdIns(3,4)P_2 . It has been majorly found to play a role in the membrane trafficking and receptor internalization. Class III PI3Kinase (also known as Vps34),

has been found to play a role in controlling protein trafficking between Golgi to vacuole (Engelman *et a*l., 2006; Bridges *et al*., 2015). PI3Kinase I, on activation by stimulated RTK and/or GPCR phosphorylates 3'-OH on the inositol ring of PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3 on the plasma membrane. PtdIns(3,4,5)P_3 is produced only under stimulus and its level in cell is strictly controlled by the cell as it acts as a major secondary lipid messenger activating many effector molecules leading to the activation of various metabolic pathways. The basal PtdIns(3,4,5)P_3 levels in cell are extremely low but stimulation by growth hormones leads to 40-fold increase in the cellular levels of PtdIns(3,4,5)P_3 (Riehle *et al*., 2013). However, it is only a transient increase. To regulate the increased cellular levels of PtdIns(3,4,5)P_3, it is de-phosphorylated by two mechanisms. In one mechanism, PTEN (phosphatase and tensin homolog) , a lipid 3-phosphatase dephosphorylates 3'-OH on the inositol ring of PtdIns(3,4,5)P_3 to revert it back to PtdIns(4,5)P_2. PTEN is known widely as a tumour suppressor and is reported to be highly mutated in various types of common cancers (Di Paolo *et al*., 2006; Engelman *et al*., 2006). In another mechanism, SHIP1/2, a 5-phosphatase dephosphorylates 5'-OH on the inositol ring of PtdIns(3,4,5)P_3 to produce PtdIns(3,4)P_2. SHIP-1 is majorly expressed in the hematopoietic cells whereas SHIP-2 is produced in all other cell types (Riehle *et al*., 2013). PtdIns(3,4)P_2 shares many common effector molecules with PtdIns(3,4,5)P_3 hence it may lead to further activation of various downstream cascades(Hawkins *et al*., 2006). Complete shutdown of PtdIns(3,4,5)P_3 cellular levels is hence achieved by INPP4,a 4-phosphatase which dephosphorylates PtdIns(3,4)P_2 to PtdIns(3)P. As per some evidences, PTEN is more involved in controlling the basal level of PtdIns(3,4,5)P_3 whereas SHIPs and INPP4 control stimulus derived increase(Riehle *et al*., 2013).

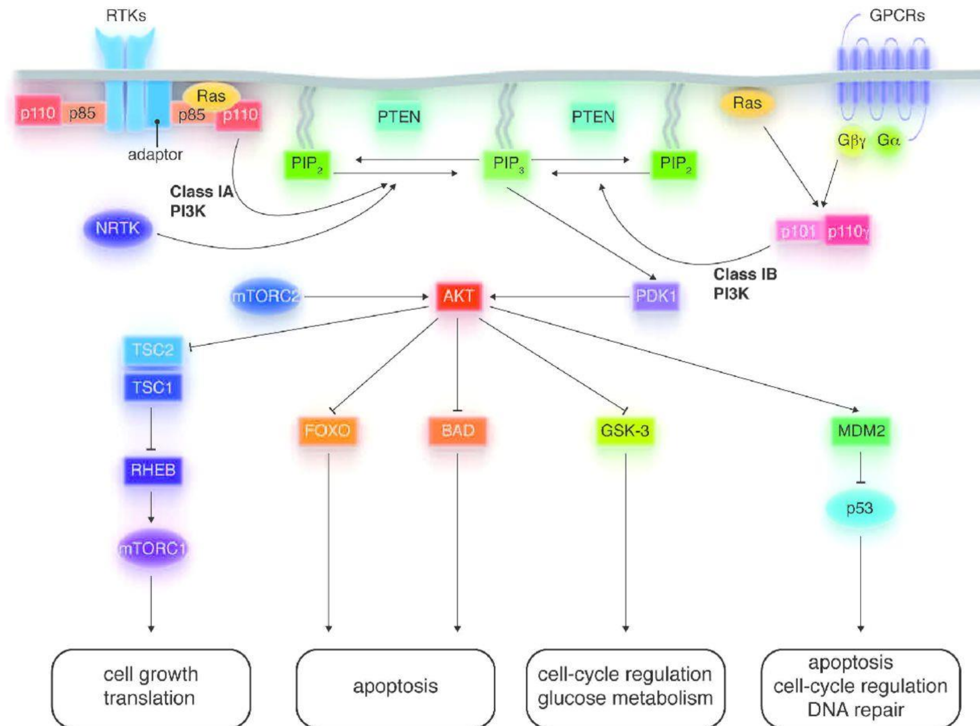


Fig. 2 PI3K Signalling pathway (Jabbour *et al*., 2014)

Among many effector molecules of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, the most extensively studied and a key regulator is Akt. Akt has been known to be the central to PI3K signalling pathway. Akt, a serine/threonine kinase has a crucial role to play in the cell growth and proliferation, cell metabolism, cell polarity and vesicle trafficking (Engelman *et al*., 2006). Activation of Akt involves multiple steps of phosphorylation. PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 binding to Akt brings about such conformational changes in Akt which recruits it to the plasma membrane and makes it available for further full activation by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of Rapamycin (mTOR). PDK1 and mTOR phosphorylate Akt at Threonine 308 and Serine 473, respectively (Bridges *et al*., 2015). Phosphorylated Akt then detaches from the plasma membrane and translocates to the different cellular regions to phosphorylate various targets facilitating activation of different pathways. Forkhead family of transcription factor (FOXO) is an important and prime target of phosphorylated Akt. FOXO mediates cell cycle progression inhibition and promotes pro-apoptotic proteins. FOXO inhibits Retinoblastoma-like protein 2 (RBL2) and p27kip1 thereby blocking cell cycle progression. Also, FOXO mediated pro-apoptotic proteins are BIM and Fas-ligand. Phosphorylated Akt blocks FOXO mediated inhibition/promotion by phosphorylating

FOXO and excluding it from nucleus. This releases the cell cycle inhibition and cell enters into G1/S phase of cell cycle (Riehle *et al*., 2013). Akt also blocks various other pro-apoptotic proteins such as BAD, NF-ßb (Riehle *et al*., 2013; Engelman *et al*., 2006). Akt also leads in the cell metabolism by playing a crucial role in the insulin regulation. In fat and muscle cells, Akt stimulation leads to the fusion of endosomes carrying glucose transporter GLUT4 with the plasma membrane so that concentration dependent glucose uptake can take place. Mutated Akt has been a major contributor towards Type-2 Diabetes. Also, Akt inhibits glycogen synthase inhibitor thereby leading to the expression of glycogen synthase (Engelman *et al*., 2006).

2.2 Significance of PtdIns(3,4)P_2

PtdIns(3,4)P_2 is produced from PtdIns(3,4,5)P_3 on de-phosphorylation by SHIP. Both PtdIns(3,4,5)P_2 and PtdIns(3,4)P_2 are plasma membrane bound phosphoinositides. Initially, PtdIns(3,4)P_2 specific roles were not known hence it was majorly neglected and considered just a by-product of de-phosphorylation step of PtdIns(3,4,5)P_3. But now many reports have been published studying the specific roles of PtdIns(3,4)P_2 in the cellular proliferation, motility, insulin sensitivity, neuronal dynamics, endocytosis (Li *et al*., 2015). PtdIns(3,4,5)P_3 cellular levels are very crucial as constant higher level of PtdIns(3,4,5)P_3 will continue to assist downstream pathways to the PI3K signalling pathway irrespective of any stimulus and may lead to cancer. The effect of higher level of PtdIns(3,4,5)P_3 are prolonged by PtdIns(3,4)P_2 as they share many effector molecules. PtdIns(3,4)P_2 brings about a more sustained signal and can last in cells upto 60 minutes (Ivetac *et al*., 2005; Li *et al*., 2015). Inhibition of PtdIns(3,4)P_2 is brought about by its specific phosphatase Inositol polyphosphate phosphatase(INPP4). De-phosphorylation of PtdIns(3,4)P_2 by INPP4 produces PtdIns(3)P. However, PtdIns(3)P is also very significantly produced by class II PI3Kinase and class III Kinase in cells (Engelman *et al*., 2006).

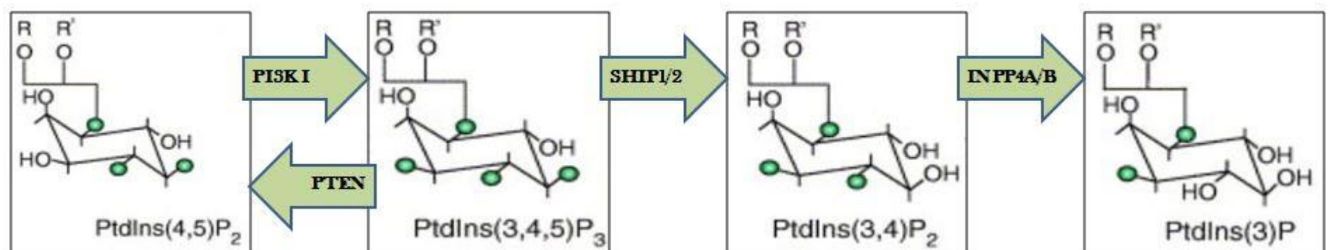


Fig. 3 PI3K signalling reaction

Akt and PDK1 are two common effector molecules among which many are shared by both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Full activation of Akt takes place only when it is phosphorylated by both (Ivetac *et al*., 2005). Akt has an equal affinity for both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Ivetac *et al*., 2005). There are reports which show that PtdIns(3,4)P_2 is essential for phosphorylation at Ser-473. The recruitment of Akt and phosphorylation by PDK1 at Thr-308 can be mediated by PtdIns(3,4,5)P_3 alone but full activation of Akt requires PtdIns(3,4)P_2 (Scheid *et al*., 2002; Ma *et al*., 2008). Rather, phosphorylation by PtdIns(3,4,5)P_3 alone brings about some inhibition of Akt activity (Scheid *et al*., 2002; Franke *et al*., 1997). Many reports suggests that Akt activation correlates with PtdIns(3,4)P_2 more than PtdIns(3,4,5)P_3 (Franke *et al*., 1997).

Other molecules shared by PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are Transporter associated with antigen processing (TAPP) proteins. TAPP proteins have been known to bind PtdIns(3,4)P_2 and their association inhibits Akt activity (Li *et al*., 2015). TAPP Knock in studies have been carried out where mutations were done in TAPP protein in such a way that it was not able to bind with PtdIns(3,4)P_2. These mice showed higher insulin sensitivity and enhanced proliferation due to enhanced Akt activation (Li *et al*., 2015). PDK1 (Alessi *et al*., 1997), PLCγ1 (Kavran *et al*., 1998) and Bam32/DAPP1 (Dowler *et al*., 1999; Al-Awan *et al*., 2010) are more molecules to which both PtdIns(3,4,)P_2 and PtdIns(3,4,5)P_3 bind.

PtdIns(3,4)P_2 is also found on early endosomes, phagosomes where PtdIns(3,4)P_2 is formed by SHIP1/2 and is required to produce PtdIns(3)P on early endosomes. PtdIns(3)P is crucial for Rab proteins recruitment and normal formation and maintenance of endocytic pathways (Posor *et al*., 2013; Ivetac *et al*., 2005,Nigorikawa *et al*., 2015, Posor *et al*., 2013).More knowledge on the importance of PtdIns(3,4)P_2 in maintaining cellular homeostasis can be gauged from the various studies investigating the effects of INPP4 deficiency which will be discussed later.

2.3 Inositol Polyphosphate 4-phosphatase: Structure

INPP4(E.C. 3.1.3.66) is a Mg^{2+} independent 4-phosphatase hydrolyzing PtdIns(3,4)P_2 (Gewinner *et al*., 2009; Ivetac *et al*., 2005, Ivetac *et al*., 2009) . However, INPP4 has been

reported to hydrolyze Ins(1,3,4)P3 and Ins(3,4)P_2 as well but the rate of hydrolysis is very slow (Norris *et al*., 1997; Norris *et al*., 1994). INPP4A (Inositol Polyphosphate 4-phosphatase type I) and INPP4B (Inositol Polyphosphate 4-phosphatase type II) are two 4-phosphatases. The genes encoding INPP4A and INPP4B are located at chromosome 2q11.2 and 4q31.21 respectively (Rynkiewicz *et al*., 2012). INPP4A and INPP4B share 37% amino-acid sequence similarity. The structure of INPP4A and INPP4B consists of N-terminal C2 domain, an uncharacterized central domain and C-terminal catalytic domain consisting of Inositol polyphosphate 4-phosphatase (IP4P) domain. The sequence similarity between INPP4A and INPP4B is the highest in the catalytic domain. The IP4P domain consists of a conserved CX_5R (Cys-Xaa5-Arg) catalytic motif (CKSAKDR) of the protein tyrosine phosphatase (PTPase) superfamily which is crucial to its phosphatase activity and mutation in cysteine renders it non-functional (Gewinner *et al*., 2009; Ivetac *et al*., 2009, Norris *et al*., 1997). The exact function of central domain is not known, however, it contains a PEST motif (proline, glutamate/aspartate, serine/threonine rich) which is prone to calpain mediated cleavage (Rynkiewicz *et al*., 2012; Norris *et al*., 1997). PEST sequence is present only in INPP4A and has not been found in INPP4B (Norris *et al*., 19974). C2 domain of INPP4 interacts with membrane in the calcium independent manner unlike PTEN (Rynkiewicz *et al*., 2012). INPP4A has two C2 domains. INPP4 binds to PtdIns(3,4)P_2 via C2 domains (Ivetac *et al*., 2005).

Fig. 4. Structure of INPP4

A variable C2 domain in both INPP4A and INPP4B is produced as a result of alternate splicing of pre-mRNA. The α-forms of INPP4A and INPP4B have hydrophilic C2 domain whereas β-forms are hydrophobic in nature. These splice isoforms develop when alternate splicing involves exon 23, 24 and 25 (Shern *et al*., 2001). The hydrophilic α-INPP4A and α -INPP4B are localized in the cytoplasm and are reported to play role in PI3K signalling pathway. But β - INPP4A and β–INPP4B are mainly docked to the plasma membrane and their function is largely unknown (Rynkiewicz *et al*., 2012). Apart from these, four splice isoforms of α-INPP4A have

been reported which differ in their alternative splicing between exon 15 and 16. The pre-dominant splice isoform 2 expressed in brain tissue is 106kDa (known as INPP4Aα1) .110kDa isoform 1 (known as INPP4Aα3) is majorly expressed in human platelets, MEG-01 megakaryocytes, and Jurkat T-cells (Shern *et al*., 2001; http://www.uniprot.org/uniprot/Q96PE3). Isoform 3 is 109kDa and the isoform 4 is β-INPP4A which is 107kDa (http://www.uniprot.org/uniprot/Q96PE3). Presence or absence of a particular splice isoforms is tissue dependent. The expression level of INPP4A and INPP4B in different tissues has been analyzed and it was seen that the highest expression was in the brain tissues. The order seen was skeletal muscle >heart >>brain =placenta=pancreas > liver> kidney > lung (Norris *et al*., 1997). However, the exact distinct functions of these spliceforms are still unknown.

2.4 Inositol Polyphosphate 4-phosphatase: localization and function

Specific distinct functions and localizations of INPP4A and INPP4B are not very well understood. However, both INPP4A and INPP4B are known to be involved in PI3K/Akt pathway. Only INPP4A also plays a crucial role in maintaining integrity of brain tissues and in endocytosis whereas only INPP4B has been established as a tumour suppressor.

2.4.1 INPP4A on early endocytic vesicles and phagosomes

INPP4A plays an essential role in endocytosis and formation of early and recycling endosomes. Rab proteins are necessary proteins which mediate both endocytosis and exocytosis. Rab 5 protein is involved in the endocytic internalization and fusion of early endosomes but for the recruitment of Rab5 protein on early endosomes, enrichment of PtdIns(3)P on early endosomes is of paramount importance(Ivetac *et al*., 2005). PtdIns(3)P enrichment on endocytic membrane is brought about majorly by class III PI3K and also by INPP4A. Class III PI3K phosphorylates Phosphatidylinositol and INPP4A hydrolyzes PtdIns(3,4)P_2 to produce PtdIns(3)P(Engelman *et al*., 2006; Ivetac *et al*., 2005). The significance of both Class III PI3K and INPP4A in this process can be understood from various reports showing abnormally formed early endosomes due to the absence of any of them. Studies show that loss of INPP4A leads to dilated early endosomes and inhibition of class III PI3K by wortmannin leads to enlarged endosomes (Ivetac

et al., 2005). Recruitment of INPP4A to early endosome is mediated via interaction with Rab5 protein through its C2 domain (Ivetac *et al*., 2005).

A recent published report showed an inhibitory role of INPP4A in phagosomes. Phagocytosis is a form of endocytosis performed by specific phagocytes such as macrophages, dendritic cells and B cells. In macrophages, it was seen that INPP4A deficient macrophages showed an increase in the phagocytic activity. An increase in PtdIns(3,4)P_2 and a considerable decrease in PtdIns(3)P on the phagosomes was seen in INPP4A deficient cells thereby implying that INPP4A is majorly responsible for the synthesis of PtdIns(3)P in phagosomes (Nigorikawa *et al*., 2015). Although co-localization of INPP4A and Rab5 proteins was seen on the phagosomes but it was suggested Rab5 protein is not responsible for the recruitment of INPP4A on phagosomes as INPP4A appeared on phagosomes before Rab5 proteins. It was rather suggested that PtdIns(3,4)P_2 was responsible for INPP4A recruitment as they both appeared simultaneously(Nigorikawa *et al*., 2015). Hence, the role of INPP4A on phagosomes is in contradiction with its role on early endosomes.

2.4.2 Role of INPP4A in maintaining neuronal integrity

The role of INPP4A in neurodegeneration and early postnatal mortality was identified long before it was discovered as a 4-phosphatase playing a role in PI3K signalling pathway. The study was done in two mice models. One was weeble mouse having a spontaneous single base pair mutation in INPP4A. The INPP4 mutant produced in weeble mice had only N-terminal of INPP4A and an absent IP4P domain. (Nystuen et al., 2001). Another model is the INPP4A knockout mice model. Both mice models die within a month of birth owing to extensive neurodegeneration (Nystuen *et al*., 2001; Sasaki *et al*., 2010; Billcliff *et al*., 2014). The neurodegeneration in the knockout mice is accompanied with involuntary movements (Sasaki *et al*., 2010; Billcliff *et al*., 2014). INPP4A is involved in the regulation of NMDA receptor localization and NMDAR-mediated postsynaptic excitatory current. In the absence of INPP4A, there is an accumulation of NMDA receptors at the post-synaptic surface of the neurons which leads to glutamate mediated excitotoxicity (Sasaki *et al*., 2010). Deletion in INPP4A chromosomal region has been linked to neurological disorders like schizophrenia (Karayiorgou *et al*., 2010) and temporal lobe epilepsy (Wang *et al*., 2012).

2.4.3 INPP4 mediated regulation of PI3K/Akt Signalling pathway

INPP4A and INPP4B both have an inhibitory role to play in the PI3K signalling pathway. They hydrolyze PtdIns(3,4)P_2 present on the plasma membrane leading to a complete shutdown of all the Akt mediated downstream pathways. INPP4A deficient mouse embryonic fibroblasts (MEF) were shown to have increased proliferation rate and decreased apoptotic rate. INPP4A deficient MEF when injected into mouse also led to the development of tumour. INPP4A controls Akt anchorage with plasma membrane hence its activation thereby may play a role in proliferation, survival and cancer (Ivetac *et al*., 2009). Cellular proliferation of megakaryocytes and NIH 3T3 cell line was reduced on over expression of the INPP4A (Vyas *et al*., 2000).But a direct role of INPP4A in any cancer has not been reported yet.

On the other hand, INPP4B has been declared as a tumour suppressor (Gewinner *et al*., 2009; Fedele *et al*., 2010; Agoulnik *et al*., 2011). The evidence that INPP4B may be playing a tumour suppressor started coming as early as in 2004 when *Johannsdottir* et al. showed loss of heterozygosity (LOH) in chromosomal region encoding for the INPP4B in the sporadic breast cancers (Johannsdottir *et al*., 2004). In 2005, *Naylor et al.* also showed a frequent deletion in advanced breast cancers at chromosome location 4q31.1-31.21 and chromosome location 4q31.3-q32.1 respectively (Naylor *et al*., 2005). The transcriptional profiling of malignant proerythroblast showed that INPP4B was silenced in them. Also, by overexpression of INPP4B in these malignant cells, phosphorylated Akt levels in the cells were reduced (Barnache *et al*., 2006). The human protein atlas now mentions the role of INPP4B in many common cancers such as breast cancer, testicular cancers, pancreatic, prostate cancer, urothelial and skin cancers (http://www.proteinatlas.org/ENSG00000109452-INPP4B/cancer).

In few reports, INPP4B has also been described as an oncogene. Knockdown of INPP4B inhibits proliferation in some melanomas (Chi *et al*., 2015), breast cancer (Gasser *et al*., 2014) and colon cancers (Guo *et al*., 2016). This oncogenic behaviour of INPP4B is not associated with PI3K/Akt but with serum- and glucocorticoid-regulated kinase 3 (SGK3) (Chi *et al*., 2015; Guo *et al*., 2016). SGK shares homology with Akt and are also activated by PI3K signalling pathway much like Akt (Brunet *et al*., 2001; Bruhn *et al*., 2010). There are three isoforms of SGK-SGK1, SGK2 and SGK3 (Moniz *et al*., 2013). Higher INPP4B levels have been associated with GSK derived melanomas (Chi *et al*., 2015).

2.4.4 INPP4 in lung pathogenesis

Recently, INPP4B has been shown to be lost or non-expressive in Lung squamous cell carcinoma (SSC) and adenocarcinoma (ADCA). The report analysed the protein expression of PI3K signalling mediators i.e. INPP4B, pAkt, PTEN, PDPK1 in 180 human cases of SSC and ADCA. They found that 19% of the cases showed a loss of INPP4B whereas 47% showed no INPP4B expression (Stjernström *et al*., 2014). INPP4A has been associated with asthma pathogenesis. In the very first report, *Sharma et al* showed INPP4A as a novel marker to be involved in asthma pathogenesis and a SNP in the PEST sequence in INPP4A was linked with atopic asthma (Sharma *et al*., 2008). In yet another study, calpain mediated proteolysis of INPP4A was seen in allergic airway inflammation. It was shown that INPP4A modulation in allergic airway inflammation models greatly affected the airway inflammation and asthmatic phenotype. Knockdown of INPP4A vastly exacerbated the aggressive airway inflammation symptoms however; overexpression of INPP4A abated the symptoms and asthma phenotype. Also, knockdown of INPP4A in naive cells led to the spontaneous airway hyper responsiveness (Aich *et al*., 2012a). Oxidative stress in airway hyper responsiveness and asthma leads to the accumulation of PtdIns(3,4)P_2 in lung cells which further activates PI3K signalling pathway. PI3K signalling pathway leads to an increase in oxidative stress. INPP4A which is a specific phosphatase of PtdIns(3,4)P_2 is subjected to calpain mediated proteolysis in airway inflammation. Hence, a vicious loop sets in motion thereby aggravating the symptoms of airway hyper responsiveness, airway obstruction and inflammation (Aich *et al*., 2012b).

2.5 PI3K Signalling mediators in Cell Nucleus

Many phosphoinositides have been found to localize in the nucleus and play a role in activation of transcription factors, maintaining nuclear structure, regulating transport of RNA across nuclear membrane, DNA repair mechanisms. There has been found no similarity or relation between the cytoplasmic phosphoinositide signalling cascades or nuclear cascades. The major breakthrough in the field of nuclear phosphoinositide came when PtdIns(4)P and PtdIns(4,5)P_2 were found in nucleus (Cocco *et al*., 1987; Divecha *et al*., 1991). Soon, more research led to the discovery of nuclear PtdIns(3,4,5)P_3 and its regulators kinases (i.e. PI3K) and phosphatases (i.e. SHIP2, PTEN) in nucleus (Shah *et al*., 2013; Martelli *et al*., 2001;,Elong Edimo *et al*.,

2013;Lindsay *et al*., 2006; Bassi *et al*., 2013). The exact functions, localization or signal cascades followed by these phosphoinositides in the nucleus is not yet very well understood. It was found that not many PIs co-localize in the nuclear envelope but are rather found inside the nucleus clustering in a nuclear structure called as interchromatin structure (Boronenkov *et al*., 1998). The level of phosphoinositides inside the nucleus is very dynamic and is subjected to change vastly as compared to total cellular phosphoinositide concentration under certain stimulation, cell cycle progression, irradiation damage and oxidative stress (Shah *et al*., 2013) hence, the regulation of phosphoinositide functions inside the nucleus must be well-synchronized.

PtdIns(4,5)P_2 is the most expressed PI in cell. It acts a precursor to many downstream pathways mediating cellular proliferation, transcriptional regulation, metabolism, calcium regulation & differentiation. Hydrolyzes of Plasma membrane bound PtdIns(4,5)P_2 by Phospholipase C(PLC) produces Diacylglycerol (DAG) and Ins(1,4,5)P_3. Both the secondary messengers are water soluble and translocate into cytoplasm initiating many downstream processes. PtdIns(4,5)P_2 has also been found to localize in nuclear speckles (Boronenkov *et al*., 1998). Nuclear PtdIns(4,5)P_2 is majorly produced from PtdIns(4)P by PIP5K and some may be produced by PIP4K from PtdIns(5)P (Keune *et al*., 2011). Some isoforms of both PI5K (i.e. PIP5Kα) (Boronenkov *et al*., 1998) and PI4K have been found in nucleus and it was also found the retinoblastoma protein interacts with PI5K and activates it (Divecha *et al*., 2002). On stimulus such as cell-cycle progression or insulin growth factor etc., Diacylglycerol (DAG) and Ins(1,4,5)P_3 may be produced in nucleus by the hydrolyzes of Nuclear PtdIns(4,5)P_2 by nuclear PLC (Divecha *et al*., 1991; Martelli *et al*., 1992). DAG (Divecha *et al*., 1991) and various isoforms of PLC have been found in nucleus. (Martelli *et al*., 1992). Increase in DAG leads to increase in PKC in nucleus and PKC is involved in phorphorylating various nuclear proteins (Shah *et al*., 2013). Another secondary messenger released Ins(1,4,5)P_3 has its receptors on nuclear envelope which release calcium in nucleus(Rodrigues *et al*., 2008; 2007; 2009). Increase in calcium in nucleus leads to an increase in transcriptional factors regulating proliferation (Rodrigues *et al*., 2007; 2009).

Various key molecules of another pathway initiated by PtdIns(4,5)P_2 have been found in nucleus. PtdIns (3,4,5)P_3 have been found to localize in the nuclear matrix (Boronenkov *et al*., 1998; Lindsay *et al*., 2006). Only class I and class II PI3Kinases have been found in nucleus.

p110β catalytic isoform of class I PI3Kinase is predominantly found in the nucleus of mouse embryonic fibroblasts (MEF) (Marqués *et al*., 2008) and is involved in S-phase progression, DNA repair (Kumar *et al*., 2010) and replication (Marqués *et al*., 2009). Class II C2α is found in nuclear speckles and is involved in the regulation of mRNA transcription(Didichenko *et al*., 2001) whereas Class II C2β is found on the nuclear envelope and is majorly responsible for the production of PtdIns(3)P and PtdIns(3,4)P_2 inside the nucleus(Sindic *et al*., 2006). In another study, PTEN and SHIP2 were both found in the pig aorta vascular smooth muscle cell nuclei. This study revealed the production of PtdIns(3,4)P_2 from PtdIns(3,4,5)P_3 in nucleus. (Déléris *et al*., 2003) The mechanism of PI3K activation in nucleus is not very well understood but there are reports showing Nerve growth factor (NGF) stimulation activates a nuclear GTPase called as PIKE which activates nuclear PI3K. NGF stimulates PIKE by nuclear translocation of PLCϒ1. PDK1 has nuclear export sequence (NES) and it is exported out as soon as it enters in the nucleus. PI3K phosphorylates PDK1 at ser396 which is near to the NES sequence and this phosphorylation alters NES rendering it ineffective (Shah et al., 2013; Martelli *et al*., 2001). Although, it is not known whether phosphorylated nuclear PDK1 activates nuclear Akt.

Akt presence in the nucleus has been studied in many cancers including lung and breast tumours. There are three isoforms of Akt-Akt1, Akt2 and Akt3. Akt1 and Akt2 translocates to the nucleus on stimulation in the cytoplasmic pathway (Santi *et al*., 2010). Phosphorylated Akt1/2 detaches from PM and translocates to the different cellular locations including nucleus. But Akt3 pre-dominantly is present in the nucleus and nuclear envelope. Phosphorylation by T-cell leukemia-1 (TCL1) proteins has been demonstrated to drive the localization of akt in nucleus (Pekarsky *et al*., 2000). Nuclear Akt has many substrates in nucleus. Nuclear Akt co-relates with Cyclin D1 thereby regulating cell proliferation (Badve *et al*., 2010). It also blocks the apoptotic progression of cells. Akt in nucleus targets Acinus, which is a nuclear factor required for apoptotic chromatin condensation. Acinus is activated via cleavage by caspase-3. Akt phosphorylation on acinus provided resistance to acinus from cleavage thereby inhibiting apoptotic progression of cell (Sahara *et al*., 1999; Hu *et al*., 2005). Apart from its substrates, it has also been found that many enzymes (i.e. kinases and phosphatases) interact with other molecules in the nucleus in a non-enzymatic manner. Akt interacts with ErbB-3 binding protein (Ebp1) and B23 in a non-phosphorylating manner. Association of Ebp1 with Akt enhances its

anti-apoptotic activity (Ahn *et al*., 2006). B23 has its role in ribosome biogenesis, cell cycle progression, DNA repair and response to stress (Colombo *et al*., 2011).

SHIP2, a 5-phosphatase plays an inhibitory role in PI3K signalling pathway. SHIP-2 hydrolyzes PtdIns(3,4,5)P_3 to produce PtdIns(3,4)P_2 in the PI3K signalling pathway. This 5-phosphatase was found to localize in nuclei of cells in 2003 for the first time in vascular smooth muscle cells (Déléris *et al*., 2003). It has also been found in nucleus and nuclear speckles in the human actrocytoma N1 cells. Translocation of SHIP2 into nucleus is mediated by its partner proteins, lamin A/C and PR130 which are also found in nucleus (Elong Edimo *et al*., 2011; Norris *et al*., 1995). Variation was seen in the function of SHIP2 in the nucleus i.e. nuclear SHIP2 is phosphorylated on serine whereas cytoplasmic SHIP2 is phosphorylated at threonine. Also, in Nucleus, SHIP2 was hydrolyzing PtdIns(4,5)P_2 present in nuclear speckles instead of PtdIns(3,4,5)P_3 (Norris *et al*., 1995). On silencing SHIP2, levels of activated Akt decreased reducing cell migration and adhesion (Prasad, 2009). This was probably because of decrease in PtdIns(3,4)P_2 needed for full activation of Akt (Davis *et al*., 2015). On the other hand, reducing the expression of SHIP2 lead to the accumulation of PtdIns(3,4,5)P_3 and induced Akt phosphorylation (Elong Edima *et al*., 2011).

2.5.1 PTEN- A Lead

PTEN has nuclear as well as cytoplasmic localization as well as found in exosomes (Chung *et al*., 2005; Gabriel *et al*., 2013). Discovery of nuclear PTEN was done in 1999 in normal primary neurons and endothelial cells (Sano *et al*., 1999). With more research and reports, nuclear PTEN role was described in cellular proliferation and differentiation as an antagonist (Lian *et al*., 2005). The expression of nuclear PTEN is not constitutive in all cells but is expressed only under stimulus such as cell cycle arrest, stress, cellular differentiation etc. (Lachyankar et al., 2000;Ginn-Pease *et al*., 2003) and becomes absent in advanced stage tumours (Lian *et al*., 2005). It was found that PTEN has no signal peptide driving its translocation into the nucleus, however, SUMOylation, a post translational modification can mediate the entry of PTEN into the nucleus of cells (Bassi *et al*., 2013). SUMOylation of PTEN at K254 is necessary not only for its translocation but also for nuclear PTEN activity. In nucleus, PTEN in involved in the DNA repair mechanisms through its phosphatase- activity independent mechanisms (Bassi *et*

al., 2013). Recently, It was reported that PTEN associates with Anaphase-promoting complex (APC) thereby promoting the association of APC with CDH1 which enhances APC-CDHI tumour suppressor activity. This association of PTEN takes place in a phosphatase-independent manner (Mounir *et al*., 2009).

It was only recently in 2012, it was discovered that PTEN is also secreted in the exosomes. It was found that this secreted form of PTEN has an active phosphatase catalytic activity and reduced the level of pAkt when taken up by the recipient cells. It also reduced the rate of proliferation of recipient cells (Gabriel *et al*., 2013; Putz *et al*., 2012). The regulation of release of PTEN in the exosomes is mediated by Nedd4 Family Interacting Protein 1(Ndfip 1) (Putz *et al*., 2012). In another study, it was shown that PTEN is secreted only by cancer cells and not by the normal cells (Gabriel *et al*., 2013). This secreted PTEN may have a therapeutic effect on recipient cells as exosomes are secreted from cells under strict control and generally, are secreted to maintain or preserve the homeostasis of the tissue. No report is available as to account what type of cells generally secrete PTEN, or under what exact conditions PTEN is secreted or what cells are true recipient cells in the body.

Inspite of various reports since 1970's investigating the presence and function of various phosphoinositides and all the key mediators of PI3K signalling pathway in the nucleus & extracellular secretions, a clear understanding of their mechanism as to how these molecules translocate into the nucleus or are secreted and their exact functions has not been deduced till today.

CHAPTER 3

MATERIAL AND METHODS

MATERIAL AND METHODS

OBJECTIVE I: To confirm the presence of INPP4A In Nucleus & Investigating its role

3.1 IMMUNOBLOTTING FOR DETECTION OF INPP4A IN THE NUCLEUS OF CELLS

3.1.1 Preparation of Nuclear And Cytoplasmic Protein Extracts –

A. MATERIAL-

CHEMICALS	CELL LYSIS BUFFER(10ml)	NUCLEUS LYSIS BUFFER(10ml)
200mM Hepes, pH 7.9	500 µl	1000 µl
1M $MgCl_2$	15 µl	15 µl
250 mM KCl	360 µl	-
100% Glycerol	-	2500 µl
NP-40	25 µl	-
5M NaCl	-	840 µl
0.5M EDTA	2 µl	2 µl
5mM EGTA	200 µl	200 µl
100 Mm DTT	100 µl	100 µl
MQ water	8798 µl	5343 µl

*DTT & PI were added in 1:100 dilution just before use.

B. PROTOCOL-

i. Cells (such as NHBE, BEAS-2B, and A549) were harvested in 1X PBS.

ii. Cells were centrifuged for pelleting down the cells. They were spun at 5,000 rpm at 4°C for 10 minutes.

iii. Supernatant was discarded and 60 µl of cell membrane lysis buffer (CLB) was added to each sample and mixed well with pipette.

iv. Cells were slightly tapped on ice for 3-5 minutes while in CLB.

v. Cells were centrifuged at 7500 rpm for 15minutes at 4°C.

vi. Supernatant was aspirated and transferred to another eppendorf. This supernatant was labelled as the cytoplasmic extract of the cells.

vii. The pellet so obtained was washed in PBS at 7500 rpm for 5 min at 4°C.

viii. 60 µl of Nuclear lysis buffer (NLB) was added to the pellet and mixed well.

ix. Eppendorf containing pellet was firmly tapped for 30 minutes to break apart the nucleus and release its contents.

x. The solution was centrifuged at 13,000rpm for 1 hour at 4°C. The supernatant was labelled as the nuclear extract (NE) of the cells.

xi. The extracts were then stored at -80°C for long term till further used.

3.1.2 Procedure for estimation of protein concentration- BCA Protein Assay

A. MATERIAL:

- 1mg ml^{-1} BSA stock
- $CuSO_4$ solution
- Protein samples
- Milli-Q water
- Bicinchoninic acid (BCA) (sigma chemical Co.)
- 96-well plate, pipettes, tips
- Micro plate reader SYNERGY/H1

B. PROTOCOL:

i. Bovine serum albumin (BSA) standards were prepared. From 1mg ml^{-1} of BSA stock, serial (5-8) dilutions with a range of 0.1-1 µg $µl^{-1}$ were made in duplicates in 96 well plate.

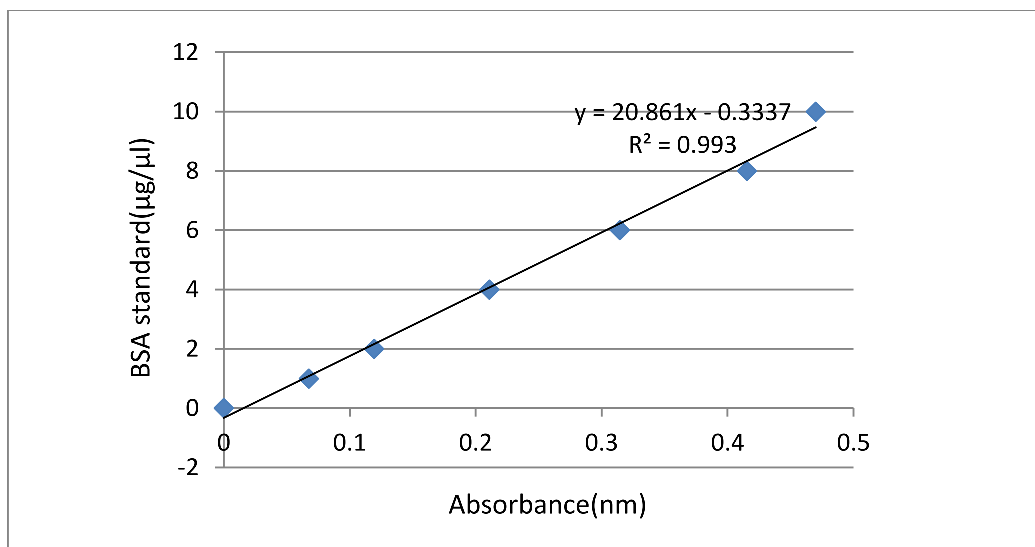
ii. 1 µl of each unknown protein sample was pipetted into 2 wells of micro plate and the volume was made up to 10 µl by Millique water. Millique water was used as a blank solution for standard curve and protein samples, respectively.

iii. BCA working reagent (WR). The total volume of WR needed was calculated (Sample to WR ratio was 1: 10 i.e. for each protein sample and BSA standard, 100µl of WR was required). WR was prepared by mixing 50 parts of BCA solution with 1 part of $CuSO_4$.

iv. 100µl of BCA WR was added to each well, covered with aluminium foil and incubated for 20-30 min at 37 °C until purple colour developed.

v. The absorbance was read at 562nm in a Micro plate reader SYNERGY/H1.

vi. The unknown protein concentration was calculated as- average of the duplicate values were calculated and subtracted from blank sample value. Using Microsoft office an Excel Data tool, Graph was plotted for BSA standards (a representative graph has been shown). From the graph, r^2 value was calculated ($r^2 \approx 1$) and with the help of slope equation, unknown protein concentrations were calculated. An example of BCA standard graph-



3.1.3 Preparation of protein samples for SDS-PAGE

A. MATERIALS-

- 5X loading dye- 10% SDS, glycerol,0.5 M Tris-Cl, pH 6.8,0.5 % (w/v) bromophenol blue dye, β -Mercaptoethanol, Millique water
- PCR Strip tubes
- Protein samples to be loaded.

B. PROTOCOL-

i. Calculated Volume of protein sample (as through BCA protein Assay) was taken in strip tubes. Protein loading dye was added (final concentration of the dye in the solution should be 1X).
ii. Strips tubes were given a quick spin.
iii. Sample was heated to 100 °C for 5 minutes so as to denature the proteins.
iv. Samples were put on ice for precipitating vapours.
v. Protein sample was given a quick spin and loaded on gel.

3.1.4 SDS-PAGE

A. MATERIALS-

- Biorad Electrophoresis Chambers, casting stands, casting frames, combs (usually 10-well), and glass plates (thickness 1.5mm).
- Casting gels-

	RESOLVING GEL(8%, 15ml for 2 gel)	**STACKING GEL(10ml for 2 gels)**
WATER	6.9ml	6.8ml
30% Acrylamide/Bis-acrylamide	4.0 ml	1.66ml
1.5Mm Tris(pH 8.8)	3.8ml	-
1mM Tris (pH 6.8)	-	1.26ml
10% SDS	150μl	100 μl
10%APS	150 μl	100 μl
TEMED	9 μl	10 μl

- Running buffer (1l) – 3.02g Tris, 18.3g glycine, 1g SDS make it 1litre with MQ Water.
- Pageruler™ Prestained Protein ladder(26616, 250 µl)

B. PROTOCOL-

Preparing the gel:

i. Two glass plates i.e. the supporter plate and the short plate were clamped and set in the casting frame which was set the casting stands. The leakage was checked by adding water in between the plates. Water was removed thereafter.

ii. The gel solutions (as described above) were prepared in a separate small beaker. The solutions were swirled gently but thoroughly.

iii. Appropriate amount of Resolving gel solution was added between the glass plates.

iv. To level the top of the separating gel and to prevent contact with air, isopropanol was added onto the top of resolving gel. Gel was left to set for 20-30 minutes.

v. After resolving gel sets, isopropanol was removed and Stacking gel solution was added over it until it overflows. The well-forming comb was inserted carefully without trapping air bubbles under the teeth. It was also left to set for another 20-30 minutes.

vi. Once the gels have set completely, the glass plates containing gel were taken out of the casting frame and were placed in the Electrophoresis chamber. Combs were taken out carefully without rupturing the wells. Running buffer (electrophoresis buffer) was poured into the inner chamber until the buffer surface reaches the required level in the chamber.

vii. Prepared protein samples along with the protein ladder were loaded into the wells carefully.

viii. Top was covered and anodes were connected. Electrophoresis was run at appropriate voltage (80-100V).

3.1.5 Western Blotting-

A. MATERIALS-

- Transfer buffer (1l) -5.8g Tris, 2.9g Glycine, 0.32g SDS, 200ml methanol and make volume 1litre with MQ water.
- PVDF membrane (mdi(membrane technologies), 0.2µm pore size)
- Transfer apparatus system (BioRad)
- 1X TBST (0.1% tween-20 in Tris buffered saline)
- Ponceau S (0.1% ponceu in 5% acetic acid)
- 5% Bovine Serum Albumin (BSA) (Sigma Chemical Co.)
- Primary antibody- customized Inositol Polyphosphate 4-phosphatase (INPP4A), Tata Binding Protein (TBP) (abcam) and Alpha-tubulin (Sigma).
- Secondary antibody- Goat Anti-rabbit IgG-HRP conjugate and rabbit Anti-mouse IgG-HRP conjugate (Merck Millipore)

B. PROTOCOL-

Transferring the protein from the gel to the membrane

i. PVDF membrane was activated with methanol for 3-5 min and rinsed with transfer buffer.
ii. The cathode core (the black panel) of the Blot Module was placed in the shallow glass tray half-filled with transfer buffer. The two soaked blotting pads were placed in the cathode core, followed by one piece of blotting paper.
iii. The SDS-PAGE gel was removed from in-between the glass plates; the bottom ridge and the wells at top were removed by trimming with the gel knife.
iv. The gel was kept on the pad-blotting paper assembly in the cathode core.
v. The PVDF membrane was kept on the gel and any air bubble between gel and membrane were smoothed away using a smooth roller onto the membrane gently. Now, more blotting papers and blotting pads were kept on the membrane to make a transfer stack as in the fig

Fig. 5. The transfer stack

vi. The stack was kept into the transfer assembly and connected to the voltage system. The transfer was run at 30V overnight.

Immunoblotting

vii. Once the transfer was complete, proteins were visualized by staining with Ponceu S stain to ensure even transfer with no air pockets. Ponceu S was removed from the membrane by washing in MQ Water. Extra membrane was cut by scissors.

viii. Blocking: The blocking of membrane was done for 1-2 hr at RT with 5% BSA on shaker. Blocking was done to reduce non-specific binding of Primary antibody onto the membrane.

ix. Primary Antibody Staining: The membranes was stained with the following dilutions of primary antibody in blocking buffer for following time periods-

Primary antibody	Dilutions	Time for incubation
Customized INPP4A	1:1500	2hrs
TBP	1:500	Overnight
α- Tubulin	1:2000	2hrs

x. The membrane was washed thrice with 1X TBST, 5 min each on shaker.

xi. The membranes was then incubated with the following dilutions of secondary antibody in blocking buffer for following time periods-

Primary antibody	Secondary antibody	Dilution for 2°	Time for incubation
INPP4A	Goat Anti-rabbit	1:6000	1hr
TBP	Rabbit Anti-mouse	1:4000	2hrs

α- Tubulin	Rabbit Anti-mouse	1:4000	2hrs

xii. The membrane was washed 3-5 times with 1X TBST, 5 min on shaker.

3.1.6 Detection of Immunoblotting

- By DAB-H_2O_2 method-

A. MATERIALS-

- DAB Solution (15ml): Take 13.5ml 100mM Tris-Cl. Add 9mg of DAB. Vortex. Add 1.5ml 1M Immidazole. Just before use, add 30µl H_2O_2.

B. PROTOCOL-

i. Dab-Peroxide solution was prepared as per mentioned.
ii. The membrane was put in the Dab-peroxide solution till antibody-specific band appear as a brown band on Membrane.
iii. As to stop the reaction, the membrane was washed in water.

- By Enhanced chemiluminescence (ECL)

A. MATERIALS-

- SuperSignal™ West Pico Chemiluminescent Substrate (Cat. No.-34080)
- BIORAD ChemiDoc MP Imaging system

B. PROTOCOL-

i. Peroxide and Enhancer were added on the blots in 1:1 dilution.

- The solution was spread evenly over membrane blot and the membrane was put in the BIORAD ChemiDoc MP Imaging system as soon as possible for imaging Chemiluminscence.

The Dab-developed blots were thereafter dried, scanned and analyzed with Densitometer software- AlphaEaseFC 4.0. Chemiluminescence images from ChemiDoc were also analyzed by the same software.

3.2 IMMUNOPRECIPITATION DONE TO ENSURE THE PRESENCE OF INPP4A IN NUCLEAR EXTRACTS

A. MATERIAL:

- Nuclear extract of cells
- Customized INPP4A antibody
- Rabbit Isotype IgG
- Protein G Sepharose®, Fast Flow (Sigma)
- Sigma mini spin columns
- 3:2 BSA: skimmed milk blocking buffer
- RIPA buffer/ NLS buffer
- 5X loading Dye
- Immunoblotting materials and method as described in protocol 1.

B. PROTOCOL:

i. 500ug nuclear cell lysate of MCF7 cells was taken for each tube i.e. antibody and isotypic control. The lysates were incubated with primary antibody INPP4A and Isotype IgG at 4°C for 6 hours on rotatory shaker.

ii. 50ul of sepharose beads was taken in a mini spin column and sepharose beads were washed with RIPA/NLS buffer by centrifuging it at 2000rpm for 1 min to remove alcohol. Beads were blocked with 3:2 BSA: skimmed milk blocking buffer for 2 hours with gentle rocking at 4°C. After blocking, beads were washed once with RIPA/NLS buffer.

iii. Antigen-antibody mixture from step (i) were transferred to the mini spin columns containing blocked beads and were incubated with beads for 1–3 hours at 4°C with gentle rocking.

iv. Then, the pellets in mini spin columns were washed 3-5 times with RIPA/NLS buffer by centrifuging it for 1 minute at 2000rpm at 4°C. They were kept on ice during washes.

v. The pellets were resuspended in 50 µl 5 X laemelli buffer.

vi. The samples were heated to 95–100°C for 2–5 minutes and immediately put on ice for atleast 2 minutes.

vii. They were then centrifuged at 10,000rpm for 5 min at 4°C for elution.

viii. Samples were then loaded on 8% polyacrylamide gel and Immnuo blotting was done for the detection of immunoprecipitated INPP4A as purified from the nuclear lysates. Isotype IgG was used as a control. Same protocol was followed as described in protocol 1. Detection was done by Dab-H_2O_2.

3.3 IMMUNOCYTOCHEMISTRY(ICC) OF CELLS TO VISUALIZE INPP4A IN NUCLEUS OF CELLS

A. <u>MATERIAL:</u>

- 6-well plates, cover slips, slides
- 1X PBS and PBST(0.1% Tween-20)
- 2% paraformaldehyde
- 0.5% Triton X-100
- 5% BSA(in 0.1% PBST) for Blocking
- Primary antibody: Customized INPP4A primary antibody
- Rabbit Isotype IgG
- Anti-rabbit Alexa fluor-488 (Invitrogen)
- Anti-fade reagent containing DAPI

B. <u>PROTOCOL:</u>

i. MCF7 cells were seeded on coverslips in 6-well plate overnight.

ii. The following day, the media was removed from the cells and cells were washed 3 times with 1X PBS on shaker.

iii. Cells were fixed in 2% paraformaldehyde for 15 minutes at RT and then washed 3 times in 1X PBS to remove residual paraformaldehyde.

iv. Cells were permeabilized with 0.5% Triton X-100 for 15 min and then washed 3 times in 1X PBS on shaker.

v. Cells were blocked with 5% BSA (in 0.1% PBST) for 30 minutes at RT on shaker and washed once with PBS.
vi. Primary antibody: INPP4A antibody and Anti-rabbit Isotype was added on the fixed cells at 1:2000 concentration. Cells were incubated for 2 hours on shaking at RT.
vii. Cells were then washed 3 times with 1X PBS on shaker.
viii. Cells were then labelled with secondary antibody Alexa fluor 488 at 1:250 concentration and incubated for 60 minutes on shaker. This step was done in dark.
ix. Cells were washed 3 times with 1X PBS on shaker.
x. Coverslips were then mounted on slides with anti-fade reagent containing DAPI.
xi. Imaging was done in Leica DMI6000B

3.4 PREDICTING SUMOYLATION SITES

Protein sequence of different isoforms of INPP4A (INPP4A Isoform a, INPP4A Isoform b, INPP4A Isoform c and INPP4A Isoform d) were taken from NCBI database.

Using software SUMOplot™ Analysis Program, presence of Sumo sites were predicted in different isoforms of INPP4A was detected.

3.5 PROLIFERATION RATE ANALYSIS BY OVEREXPRESSING INPP4A

- IN CYTOPLASM-

A. MATERIAL:

- Cell line : MCF-7 cell line, MCF7-ST cell line (This cell line has been produced in our laboratory by stably transfecting MCF-7 cells with a plasmid containing GFP tagged INPP4A and its antibiotic selection was done by G418)
- CellTrace™ CFSE Cell Proliferation Kit (cat. No.- C34554)
- BD FACSCalibur™
- Immunoblotting materials and method as described in protocol 1.

B. PROTOCOL:

Immunoblotting-

Total cell lysates (TCLs) of MCF7 and MCF7-ST were made in RIPA buffer. They were run on 8 % SDS-PAGE gel and thereafter immunoblotted for custom INPP4A antibody (1:1500). Same protocol was followed as in protocol 1. β-actin (1:4000) was used as a control. Detection was done by DAB-H_2O_2.

Proliferation analysis by CFSE labelling:

i. MCF7 and MCF7-ST cells were incubated with CFSE dye (0.15µl/ml in 1X PBS) for 15 minutes at 37°C.CFSE was then immediately replaced with media and cells incubated again at 37°C for 45 minutes so that cells acclimatize themselves after CFSE labelling. This was taken as Day 0. Unstained and CFSE labelled Day 0 cells were run on BD FACSCalibur™. Unstained was used to set up instrument settings.
ii. Next day, CFSE cells labelled day 1 were run on same settings and similarly, other samples were run on subsequent days on same settings.
iii. With the help of CFSE fluorescence shift (FL4), rate of proliferation of cells was analyzed by statistical tools.

➢ IN NUCLEUS-

A. MATERIAL:

- Cell line: MCF-7 cell line
- ICC reagents as described in protocol 3.
- Ki67 antibody (cat. No. - ab16667),
- Secondary Antibody- Goat anti-rabbit Alexa fluor 546

B. PROTOCOL:

i. Mutants of MCF7 cells were created by the transient transfection of MCF7 by plasmids containing GFP tagged INPP4A and GFP tagged INPP4A-NLS.transfection was done by Invitrogen Lipofectamine® LTX Reagent with PLUS™ Reagent.
ii. ICC was done by the same protocol as described in protocol 3 .5% BSA Blocking was done for 30 min. The primary antibody used was Ki67 (1:200 in 5%BSA)

incubated overnight at 4°C. The secondary antibody was Goat anti-rabbit Alexa fluor 546 (1:250 in 5% BSA) incubated for 1 hour at RT.

iii. Image was taken by Leica DMI6000B

iv. Image analysis was done by R-language designed algorithm.

3.6 TO INVESTIGATE THE CONDITIONS UNDER WHICH INPP4A TRANSLOCATES TO THE NUCLEUS & ITS CO-RELATION WITH CELL CYCLE

3.6.1 Conditions of translocation of INPP4A

A. MATERIAL:

- Cell line : MCF-7 cell line.
- CellTrace™ CFSE Cell Proliferation Kit (cat. No.- C34554)
- BD FACSCalibur™
- Immunoblotting materials and method as described in protocol 1.
- Dab

B. PROTOCOL:

Immunoblotting-

i. MCF-7 cells were grown under serum starve conditions.

ii. Cells were harvested at different time points -0hr, 6hr, 15, 19hr, 24hr and 48hr under serum starve condition.

iii. Nuclear lysates (NE) of MCF-7 was made.

iv. They were run on 8 % SDS-PAGE gel and thereafter immunoblotted for custom INPP4A antibody (1:1500). Same protocol was followed as in protocol 1. TBP (1:500) was used as a control. Detection was done by DAB-H_2O_2.

Proliferation analysis by CFSE labelling:

iv. MCF7 were incubated with CFSE dye (0.15µl/ml in 1X PBS) for 15 minutes at 37°C.CFSE was then immediately replaced with media and cells incubated again at 37°C for 45 minutes so that cells acclimatize themselves after CFSE labelling. Cells

were then allowed to grow in reduced media having no serum. This was taken as Day 0. Unstained and CFSE labelled Day 0 cells were run on BD FACSCalibur™. Unstained was used to set up instrument settings.

v. Next day, CFSE cells labelled day 1 were run on same settings and similarly, other samples were run on subsequent days on same settings.
vi. With the help of CFSE fluorescence shift (FL4), rate of proliferation of cells was analyzed by statistical tools.

3.6.2 Expression of INPP4A with cell cycle

A. MATERIAL-

- CellTrace™ CFSE Cell Proliferation Kit (cat. No.- C34554)
- BD FACSCalibur™
- Immunoblotting materials and method as described in protocol 1.
- Dab

B. PROTOCOL-

Immunoblotting-

i. MCF-7 cells were grown under serum starve conditions for 48 hours.
ii. After 48hours, cells were released from serum starved conditions and now grown in serum rich media for the next 48hours.
iii. Cells were harvested at 0hr, 6 hr, 12 hr, 16hr, 22hr, 30hr, 36 hr, 42hr and 48hr post release from serum starved conditions.
iv. Harvested cells were washed in 1X PBS and stored at -80°C till used.
v. The nuclear extracts of these cells were prepared.
vi. They were run on 8 % SDS-PAGE gel and thereafter immunoblotted for custom INPP4A antibody (1:1500). Same protocol was followed as in protocol 1. TBP (1:500) was used as a control. Detection was done by DAB-H_2O_2.

Propidium Iodide Labelling of cells-

i. Cells were harvested at different time points- 0hr, 6 hr, 12 hr, 16hr, 22hr, 30hr, 36 hr, 42hr and 48hr post release from serum starved conditions.
ii. Spin the cells at 1000rpm for 3 minutes.
iii. Supernatant was discarded and washed once with 1 X ice cold PBS.
iv. Chilled 70% ethanol was added in a dropwise manner and cells were gently mixed. Cells were left on ice for 30 minutes for fixation.
v. Keep the cells at -20°C for 16 hours.
vi. The cells are spun down and ethanol was removed.
vii. Wash the cells twice with Ice cold 1X PBS.
viii. 4µl RNAse and 200µl cold PBS were added to each eppendorf and mixed gently.
ix. Add 4 µl of PI (5mg/ml stock) to each eppendorf and gently tapped to mix well.
x. Incubate for 30 minutes at RT in Dark.
xi. Spun down and discard the supernatant and add fresh 1X PBS and acquire.
xii. PI is read at 535nm.

OBJECTIVE II: To confirm the presence of INPP4A in Extracellular milieu in vitro

3.7 PROTEIN ISOLATION AND PURIFICATION FROM CELL CULTURED MEDIA AND IMMUNOBLOTTING FOR INPP4A

3.7.1 By Trichloroacetic Acid/Acetone Wash Method.

A. MATERIAL-

- 20% TCA(Trichloroacetic acid) in MilliQue Water.
- Ice chilled acetone
- Urea/ thiourea with CHAPS solution

B. PROTCOL-

i. Cells were cultured in media with no FBS for 24 hours. This media was collected by aspirating from the culture flasks. The adhered cells were not disturbed while aspirating supernatant media.
ii. The supernatant media was centrifuged at 3000g for 20 min at 4°C as to remove any dead cells or debris.
iii. The supernatant media in then transferred into another falcon.
iv. An equal volume of 20%TCA was added to it and incubated on ice for 3 hours without disturbing.
v. Now supernatant media was centrifuged at 10,016g for 1 hour at 4°C.
vi. Supernatant was discarded and the pellet was resuspended in 1ml ice chilled acetone. Pellet was mixed well with pipette and dispersed completely.
vii. The resuspended pellet was transferred into an eppendorf and centrifuged again at 15,000rpm for 45 minutes at 4°C.
viii. The acetone was removed completely and the pellet was resuspended in 35-50 µl of Urea/thiourea with CHAPS solution depending on amount of pellet obtained.
ix. It was stored for long term at -80°C until used.

3.7.2 By using Merck Amicon Ultra-15 Centrifugal Filter units

A. MATERIAL:

- Amicon Ultra-15 Centrifugal Filter Units
- 0.1mM DTT
- Proteainase Inhibitor(PI)

B. PROTOCOL:

i. Cells were cultured in Opti-MEM® Reduced Serum Medium for 24 hours. This media was collected by aspirating from the culture flasks. The adhered cells were not disturbed while aspirating supernatant media.
ii. The supernatant media was centrifuged at 3000g for 20 min at 4°C as to remove any dead cells or debris.

iii. The supernatant media was transferred in the Amicon Ultra-15 Centrifugal Filter Units.
iv. This was centrifuged at 4000 rpm for 15-20 minutes at 4°C till only 50-80µl volume of media was left in filter.
v. The filtrate was discarded.
vi. The left over media in filter was transferred into an eppendorf and PI, DTT were added at 1:100 concentration according to the volume of filtered media.
vii. This can be stored for long term at -80°C until used.

3.7.3 Immunoblotting

The purified protein from the supernatant culture media was run in the SDS-PAGE and Immunoblotted for determining the presence of INPP4A. Same protocol was followed as in protocol 1. No loading control was used.

3.8 PREDICTING N-/0- GLYCOSYLATION IN INPP4A ISOFORMS

Protein sequence of different isoforms of INPP4A (INPP4A Isoform a, INPP4A Isoform b, INPP4A Isoform c and INPP4A Isoform d) were taken from NCBI database.

Using softwwere NetNGlyc 1.0 Server and NetOGlyc 4.0 Server, presence of N-linked and O-linked glycosylation was predicted in different isoforms of INPP4A was detected.

3.9 DETERMINING IF SECRETED INPP4A WAS GLYCOSYLATED

A. MATERIAL:

- Cells & Supernatant media of cells cultured in OptiMEM media
- Amicon Ultra-15 Centrifugal Filter Units
- PNGase kit (Cat. No.- P0704L)
- MilliQ water
- 37°C incubator
- Dry heater (upto 100°C)

For SDS-PAGE & western blotting-

- SDS-PAGE apparatus & buffers (as mentioned in protocol 1)
- Immunoblots reagents and antibodies (as mentioned in protocol1)

B. PROTOCOL:

i. Cell line used- ST-MCF7 & MCF7.

ii. Cells were seeded in complete media for them to grow well and acclimatize.

iii. After 24 hours, complete media was exchanged with OptiMEM media and cells were allowed to grow and secrete in this media for next 24 hours.

iv. After 24 hours, the media was aspirated and collected.

v. The cells were also trypsinized and collected.

vi. The supernatant media aspirated was filtered using Amicon Ultra-15 Centrifugal Filter Units. (as described in point) to capture secreted proteins which were more than 100kDa.

vii. Total cell lysates was made using RIPA buffer.

PNGase deglycosylation protocol-

viii. From the PNGase kit, 10X glycoprotein denaturing buffer was added to the filtered supernatant media obtained in step v. such that the final concentration should be 1X. For eg- 4μl to be added to a final volume of 40μl. The volume was made by H_2O.

ix. These samples were denatured by heating at 100°C for 10 minutes.

x. Then, 10X G7 reaction buffer and 10% NP-40 were added to these samples such that there final concentration should be 1X. The volume was made by H_2O.

xi. 1.5μl -2μl PNGase enzyme was added. (NOTE- PNGase F enzyme final concentration should be less than $1/10^{th}$ of total reaction to keep the final glycerol concentration equal to or less than 5 %.)

xii. Incubate the samples at 37°C for 1 hour.

xiii. To stop the reaction, keep on ice for some time and add 1X loading dye.

SDS-PAGE & western blotting-

xiv. Same protocol as followed in protocol 1. No Loading control was used.

xv. Detection was done by ECL

3.10 PREDICTING SIGNAL PEPTIDE IN INPP4A ISOFORMS

Protein sequence of different isoforms of INPP4A (INPP4A Isoform a, INPP4A Isoform b, INPP4A Isoform c and INPP4A Isoform d) were taken from NCBI database.

Using software SignalP 4.1 Server, presence of signal peptide in different isoforms of INPP4A was detected.

3.11 ISOLATING EXOSOMES FROM CELL CULTURE SUPERNATANT MEDIA AND IDENTIFYING THE ASSOCIATION OF INPP4A WITH SECRETED EXOSOMES

A. MATERIAL:

- Ultracentrifugation machine
- Okaridge tubes
- Ultracentrifugation tubes
- 1X PBS

For Western blotting apparatus-

- Blocking buffer- 5% Skimmed milk (in PBST(0.05% tween-20))
- Primary Antibodies- Customized inpp4a antibody, anti-TGFP(1:1000), Tsg-101.
- Secondary Antibodies- rabbit Anti-mouse IgG-HRP conjugate (Merck Millipore)(1:6000)
- Rest all the reagents and materials for SDS-PAGE as described in protocol 1.

B. PROTOCOL:

Isolating exosomes-

i. For Isolation of exosomes from cell culture media supernatant, cells were grown in media containing 10% FBS which was devoid of its exosomes. Exosomes were removed from FBS by ultracentrifuging FBS for 19hours at 1, 00,000g at 4°C.This step was performed under sterile conditions.
ii. Supernatant media of cell culture flasks were collected aseptically without disturbing the adhered cells.
iii. Supernatant was centrifuged at 300g for 10 minutes at 4°C as to remove any dead cells or debris that may interfere in later steps.
iv. The supernatant of this step was carefully transferred into another new oakridge tube and the pellet was preserved and labelled as 300g pellet.
v. Supernatant was again centrifuged at 3000g for 20 minutes at 4°C.
vi. The supernatant of this step was carefully transferred into another new oakridge tube and this pellet was also preserved and labelled as 3000g pellet.
vii. The supernatant was then centrifuged at 10,000g for 40 minutes as to remove micro particles/micro vesicles.
viii. The supernatant of this step was carefully transferred into another new oakridge tube and the pellet was again preserved and labelled as 10,000g pellet
ix. The supernatant was then ultracentrifuged at 1, 00,000g for 2hrs at 4°C as to obtain exosomes.
x. Supernatant of this step was discarded and pellet was resuspended properly in 1ml of cold 1X PBS.
xi. It was again ultracentrifuged at 1, 00,000g for 1 hr at 4°C.
xii. Supernatant was discarded and pellet was resuspended in the residual volume of 1X PBS.

Immunoblotting-

i. All steps in SDS-PAGE remain the same as described in protocol 1.
ii. Samples loaded-: exosomes isolated and the different pellets collected at different differential centrifugation speeds.

ANTIBODIES	BLOCKING BUFFER	PRIMARY DILUTION AND TIME	SECONDARY DILUTION AND TIME
Anti-GFP	5% skimmed milk in 1X PBST(0.05%tween20) for 45 minutes at RT	1:1000 dilution used for 2 hours at RT	Anti-mouse at 1:6000 dilution for 45 minutes at RT.
Tsg			

iii. Detection was done by ECL.

CHAPTER 4

RESULTS & DISCUSSION

RESULTS & DISCUSSION

The cellular localization of PIs and their regulators i.e. kinases and phosphatases is very specific as PIs are known to impart a cellular identity to the organelle in which they pre-dominate (Di Paolo *et al*., 2006). The localization of PtdIns(3,4)P_2 within the cell has been limited to cytoplasm and early endosomes but recently, PtdIns(3,4)P_2 was reported to be present in nucleus as well(Sindic *et al*., 2006; Déléris *et al*., 2003). Also, many key mediators of PI3K signalling pathway i.e. Class I & II PI3K, PTEN, PDK1, SHIP2 & Akt have been reported to be present in the nucleus of cell (Davis *et al*., 2015). This led us to investigate the presence of INPP4A in the nucleus of the cells. Also, previous findings in our lab found the presence of INPP4A in BALF fluid of mice which gave us a lead to explore the possibility of the presence of INPP4A in cell's extracellular milieu.

Investigations for the presence of nuclear INPP4A were done in 5 cell lines- Normal human bronchial epithelial cells (NHBE) which are the Bronchial epithelial cells established from the epithelial lining of airways; BEAS-2B cells are epithelial cells taken from the normal human bronchial epithelium of a non-cancerous individual. These cells were made immortal by infecting with an adenovirus 12-SV40 virus hybrid (Ad12SV40); A549 cells are adenocarcinomic human alveolar basal epithelial cells taken from the explanted tumour of a 58-year-old caucasian male; MCF 10A is a breast epithelial non-tumourogenic cell line whereas MCF-7 is a breast cancer cell line established from a 69-year-old Caucasian woman. Lung cell lines were chosen for the study as previous reports from our lab have shown association of INPP4A with asthma (Aich et al., 2012; Sharma *et al*., 2008). Breast cell lines were chosen for easy transfection studies. Also, INPP4B was first regarded as a tumour suppressor in breast carcinoma (Fedele *et al*., 2010; Gewinner *et al*., 2009). The antibody used for the detection of INPP4A in Immunoblots and ICC is customized INPP4A from Imgenex made to detect the epitope "CPPEGTYGKVET" on C-terminus.

OBJECTIVE I: To confirm the presence of INPP4A In Nucleus & Investigating its role

We employed various methods to confirm our preliminary observation that INPP4A localizes to the nucleus of the cells.

4.1 IMMUNOBLOTTING FOR DETECTION OF INPP4A IN NUCLEAR EXTRACTS

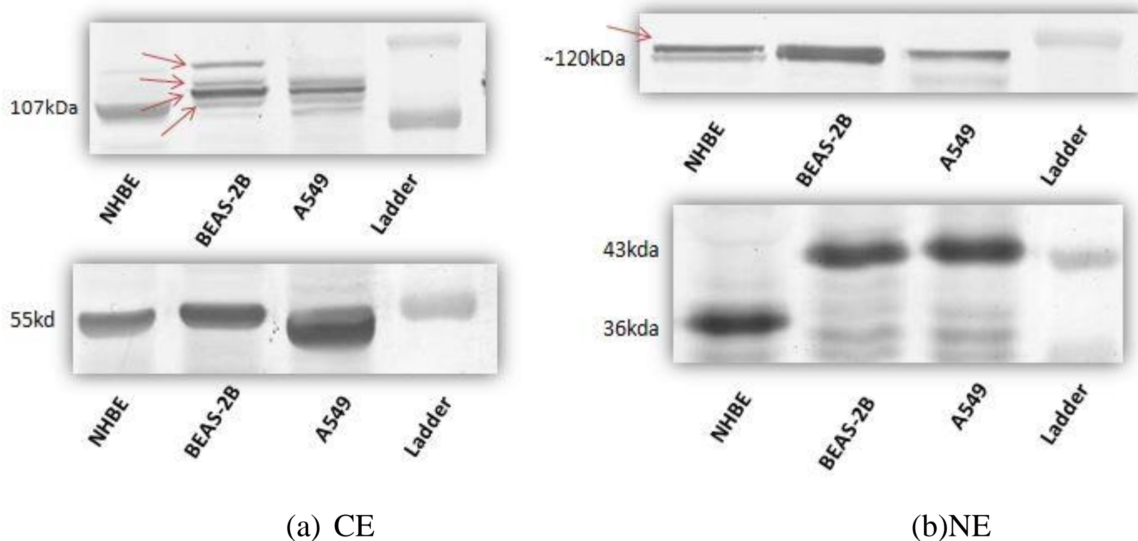
Immunoblotting was done to detect the presence of INPP4A in the nuclear extracts and cytoplasmic extracts of the above mentioned cell lines although the presence of INPP4A in cytoplasm is widely known. Customized INPP4A was used for Immunoblotting. α-tubulin and Tata binding protein (TBP) were used as loading controls for cytoplasmic and nuclear extracts respectively.

In our results, blot of cytoplasmic extracts showed known isoforms of INPP4A (100-110kDa). Isoform I of INPP4A (INPP4Aα3) is 110kDa which represents the major form of INPP4A that is expressed in human platelets, MEG-01 megakaryocytes, and Jurkat T-cells (Shearn *et al.,* 2001). Isoform II (INPP4Aα2) of INPP4A is a 106 kDa form that represents the major form of the enzyme expressed in human, rat and mouse brain (Norris *et al.,* 1995). Isoform III (INPP4Aα1) is a 109kDa form and Isoform IV (INPP4β) is a 107kDa form. (Norris *et al.,* 1997). The expression of these isoforms is tissue dependent hence, not all isoforms can be seen in all the types of cells. In our results also, we can see that Beas-2b, A549 and NHBE are all expressing different isoforms of INPP4A with different expression levels (Fig. 6(a)).

Our novel finding detects that INPP4A is also being expressed in the nucleus of the cells hereby detected in the nuclear extracts of the cells by Immunoblotting. In nuclear extracts, only one isoform of INPP4A has been detected which has a higher molecular weight (~120kDa) as can be seen in Fig.6 (b). This remains consistent in all the cell types analyzed. PIs are widely known to provide a unique lipid signature to each organelle in the cell and so are their regulators. So far, INPP4A has been known to be present only in cytoplasm and its function is to de-phosphorylate cytoplasmic PtdIns(3,4)P_2. But this is for the first time that we are showing that INPP4A is also

present in the nucleus. A new location of INPP4A in cell can have a new function or a new mechanism of function as well.

Addition of nearly 10-15kDa Mol. Wt. is unlikely to be due to alternate splicing as seen in already known spliceoforms of INPP4A. It has been hypothesized that this addition could be due to SUMOylation. SUMOylation is a post-translational modification which involves the covalent addition of “SUMO” proteins or small proteins (~100 amino acids) onto other proteins adding upto a Mol Wt. of 12kDa. Mostly SUMOylated proteins are involved in nucleoa-cytosolic transport, transcriptional regulation, protein stability, apoptosis and progression through cell cycle. Widely known tumour suppressor PTEN which is also involved in the same pathway as INPP4A, has also been found in the nucleus. The nuclear PTEN is also 20kDa higher than the cytoplasmic PTEN and it was found that this increase is due to SUMOylation at k254 amino acid on nuclear PTEN. The nuclear localization and tumour suppressor function of nuclear PTEN has been linked to its SUMOylation (Bassi *et al*., 2013)



(a) CE (b)NE

Fig. 6 (a) Different isoforms (shown by arrows) of INPP4A can be seen in cytoplasmic extracts of cell lines. (b) Nuclear extracts of cells showing 120kDa nuclear INPP4A.

4.2IMMUNOPRECIPITATION OF INPP4A IN NUCLEAR EXTRACTS

This step was done as a confirmatory experiment for the presence of INPP4A in the nuclear lysates. In the approach taken, free, non-bound INPP4A enzyme present in the nuclear lysates

were immunoprecipitated with customized anti-INPP4A antibody from IMGENEX and purified INPP4A was run on SDS-PAGE and western blotting was performed to detect INPP4A. Anti-rabbit IgG was used as a control for the same. The results show that the protein purified by IP is indeed INPP4A as no corresponding band was seen in the control (Fig. 7). Hence, it was confirmed that the INPP4A is present in nucleus of the cells.

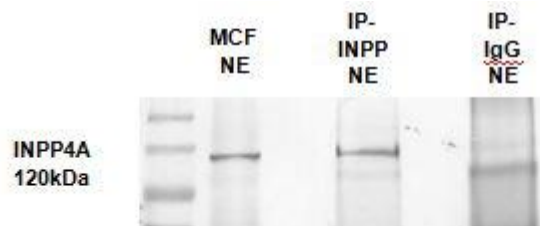


Fig. 7. Immunoblot showing nuclear extract of cells, IP isolated INPP4A and Isotype IgG in order. Immunoblotting was performed for INPP4A. No INPP4A band is seen in Isotype IgG.

4.3 IMMUNOCYTOCHEMISTRY (ICC) OF CELLS TO VISUALIZE INPP4A IN NUCLEUS

Immunocytochemistry was done to further confirm the nuclear localization of INPP4A and not due to the contamination from the cytoplasmic extracts. ICC was done by two antibodies – customized INPP4A antibody from IMGENEX and Sigma INPP4A (Cat. No. - HPA001628). Two antibodies were used to make sure that the detection by one antibody is not non- specific. The merged image shows the co-localization of DAPI and Alexa flour-488 labelled INPP4A antibody.

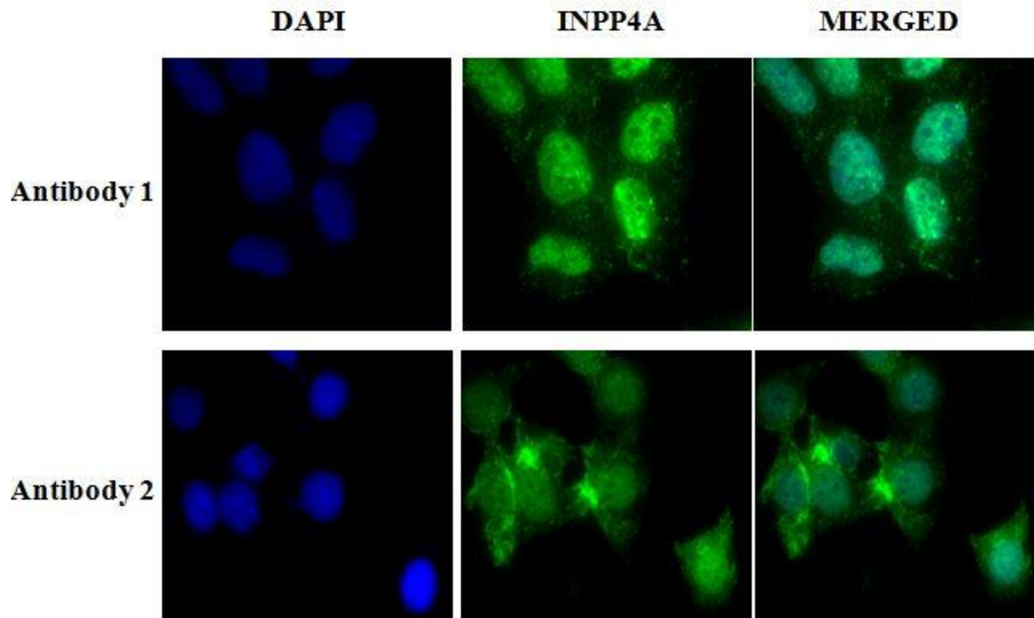


Fig. 8 ICC image showing co-localization of INPP4A with DAPI

4.4 PREDICTING SUMOYLATION SITES ON INPP4A

After confirming the presence of INPP4A in nucleus, predicted the sites for SUMOylation in known INPP4A isoforms through online software- SUMOplot™ Analysis Program. SUMOylation mostly occurs on a consensus sequence ψ-K-X-E/D where ψ is a large hydrophobic amino acid (such as Isoleucine, Leucine or Valine), K is the modified lysine on which SUMO proteins are added, X is any amino acid and E is the glutamic acid. There are three SUMOs available- SUMO-1, SUMO-2 and SUMO-3. SUMO-1 is mostly seen conjugated to some protein whereas SUMO-2 & SUMO-3 are available feely in the cell. SUMO attachment to a protein adds almost 11kDa polypeptide to the protein.

SUMOplot™ Analysis Program is able to predict and score the sumoylation sites on any protein. The prediction is based on direct matching of consensus sequence in protein or some substitution in the consensus sequence in the protein.

FASTA sequence of four known INPP4A isoforms were taken from NCBI and put into the software. The result revealed the presence of various sumoylation sites on all the isoforms of INPP4A. The best possible site of sumoylation can be predicted with the help of score given. The highest and the closest to 1 score has been given to the best site.

		INPP4A-Isoform 1	
No.	Pos.	Group	Score
1	K437	FKASS **LKAD** KKLEF	0.91
2	K483	AHCQG **FKSG** GLRKK	0.68
3	K1096	PEGTY **GKVE** T	0.67
4	K493	LRKKL **HKFE** ETKKH	0.52
5	K284	GWQME **EKSD** QR PP	0.5
6	K441	SLKAD **KKLE** FVPTN	0.48
7	K499	KFEET **KKHF** EECCT	0.13

		INPP4A-Isoform 2	
No.	Pos.	Group	Score
1	K442	FKASS **LKAD** KKLEF	0.91
2	K488	AHCQG **FKSG** GLRKK	0.68
3	K1061	PEGTY **GKVE** T	0.67
4	K1046	FNSLQ **LKAF** PKHYR	0.56
5	K498	LRKKL **HKFE** ETKKH	0.52
6	K289	GWQME **EKSD** QR PP	0.5
7	K446	SLKAD **KKLE** FVPTN	0.48
8	K504	KFEET **KKHF** EECCT	0.13

		INPP4A-Isoform 3	
No.	Pos.	Group	Score
1	K442	FKASS **LKAD** KKLEF	0.91
2	K488	AHCQG **FKSG** GLRKK	0.68
3	K1096	PEGTY **GKVE** T	0.67
4	K1080	FNSLQ **LKAF** PKHY	0.56
5	K498	LRKKL **HKFE** ETKKH	0.52
6	K289	GWQME **EKSD** QR PP	0.5
7	K446	SLKAD **KKLE** FVPTN	0.48

		INPP4A-Isoform 4	
No.	Pos.	Group	Score
1	K442	FKASS **LKAD** KKLEF	0.91
2	K488	AHCQG **FKSG** GLRKK	0.68
3	K498	LRKKL **HKFE** ETKKH	0.52
4	K289	GWQME **EKSD** QR PP	0.5
5	K446	SLKAD **KKLE** FVPTN	0.48
6	K504	KFEET **KKHF** EECCT	0.13

Fig. 9. Predicted SUMO sites on different isoforms of INPP4A.

After confirming the presence of INPP4A in nucleus, we moved towards investigating the role of nuclear INPP4A

INPP4B has been widely accepted as tumour suppressor (Gewinner *et al*., 2009; Fedele *et al*., 2010). There are reports which show that the knockdown of INPP4A leads to an increase in the cellular proliferation and overexpression studies done in NIH 3T3 fibroblasts cells showed reduced cellular proliferation (Ivetac *et al*., 2009; Vyas *et al*., 2000). Although no direct role of INPP4A in cancer development has been reported but there are indications that INPP4A may affect cellular proliferation (Ivetac *et al*., 2009). Hence, we examined the effect of nuclear INPP4A on the cellular proliferation and cell cycle.

4.5 EFFECT OF INPP4A OVEREXPRESSION ON PROLIFERATION RATE OF CELLS

CYTOPLASMIC INPP4A-

The effect of cytoplasmic INPP4A overexpression was analyzed on the proliferation rate of cells.

MCF-7 cell line was taken and a new cell line MCF7-ST was created by stably transfecting wildtype MCF-7 cells with a plasmid expressing GFP tagged INPP4A and antibiotic selection done by G418. Immunoblotting was done to compare the levels of INPP4A expression in normal MCF-7 and MCF7-ST cell line. The densitometric results showed that MCF7-ST cell line was expressing cytoplasmic INPP4A at a higher level (Fig. 10 (a) & (b)).

Further, cells were labelled with CFSE dye and grown for 3 days. CFSE is used for proliferation studies via flow cytometer. Labelling of cells with CFSE helped to trace multiple generations of the cells by dye dilution. As the cells divided, CFSE fluorescence per cell as seen in flow cytometer, decreased. This decrease in the fluorescence was quantified and compared. The result showed that the proliferation rate of Normal MCF-7 cells expressing less INPP4A was higher than the proliferation rate of ST-MCF cells expressing higher INPP4A. This confirms that higher expression of INPP4A can inhibit cell proliferation. INPP4A being an antagonist to PI3K signalling pathway inhibits the cell’s growth.

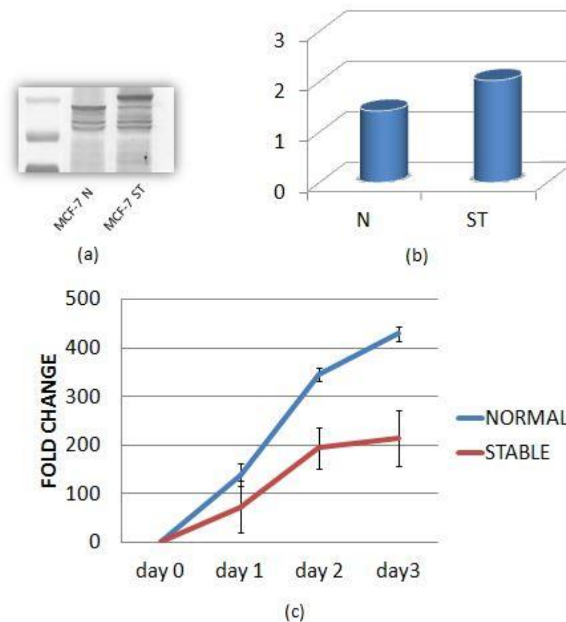


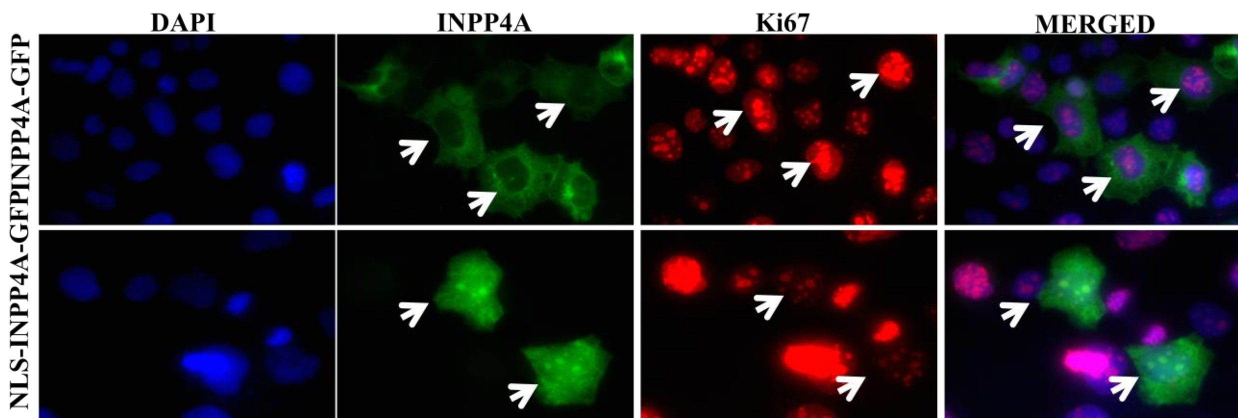
Fig.10 (a) Immunoblot showing INPP4A detection in MCF7 & ST-MCF7. (b) Densitometric analysis reveals overexpression of INPP4A in ST-MCF7. (c) Graph showing CFSE fluorescence fold change in ST-MCF & MCF7. MCF7 showing a higher fold change i.e. more cellular divisions.

NUCLEAR INPP4A-

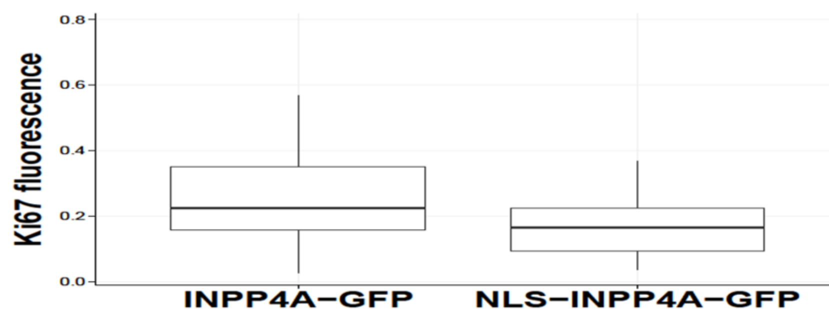
For this experiment, MCF-7 cells were transiently transfected with two plasmids containing GFP tagged INPP4A and GFP tagged INPP4A-NLS. INPP4A-GFP plasmid will express the exogenous INPP4A predominantly in the cytoplasm of the transfected cells whereas NLS-INPP4A-GFP plasmid will express the exogenous INPP4A predominantly in nucleus due to the addition of NLS (nuclear localization signal). This was done to understand the difference in the effect of overexpressed INPP4A in cell's cytoplasm and nucleus on cell's proliferation rate. Proliferation rate was analyzed by Ki67 proliferation rate index. Ki67 is a marker for growing cells in a cell population. Ki67 is a nuclear protein expressed only during the interphase with no or little expression during G0 phase of cell cycle. Also during interphase, lesser expression of ki67 is seen during G1 and S phase and it is at its peak during mitosis and then further reduces after telophase (Jonat *et al.*, 2011). ICC of transfected cells was done for nuclear ki67 staining. Intensity of fluorescence of Ki67-alexa fluor 647 was calculated with the help of an algorithm designed using R-language. The algorithm detected cells by their blue (DAPI) and green

(transfected-GFP) fluorescence and then calculated the intensity of red fluorescence (Ki67) and compared it between cells.

In our results, we analyzed that the ki67 fluorescence was higher in cells transfected with INPP4A-GFP than the cells transfected with NLS-INPP4A-GFP which implies that the cells expressing cytoplasmic INPP4A were proliferating more than the cells expressing nuclear INPP4A. Hence, we can say that the proliferation rate of cells transfected with NLS-INPP4A-GFP was less than the proliferation rate of cells transfected with INPP4A-GFP. The results showed that the overexpression of INPP4A in nucleus led to a reduced proliferation rate. This further supports the role of INPP4A in regulating the cellular proliferation. Overexpression of INPP4A in nucleus has a greater impact on suppression of proliferation of cells then overexpression in cytoplasmic INPP4A.



(a)



(b)

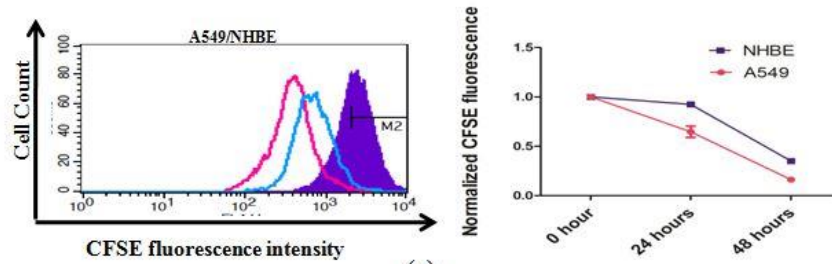
Fig. 11 (a) ICC images of cells transfected with GFP-INPP4A-NLS and GFP-INPP4A. DAPI shows the nucleus. The GFP-green signal shows transfected cells. The red signal shows Ki67 expression. Only GFP

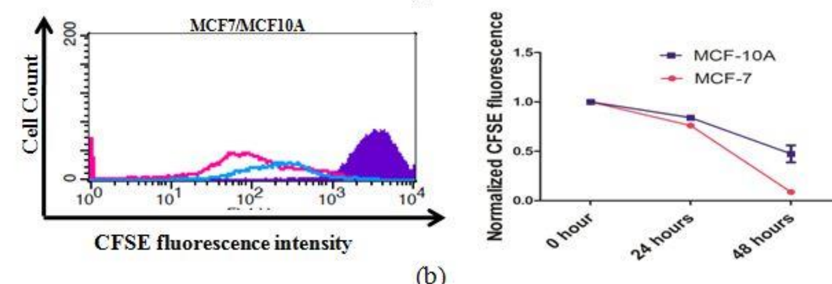
expressing transfected cells were considered. Arrow shows ki67 expression in transfected cells. (b) Graph showing ki67 fluorescence quantified using R-language algorithm. Cells transfected with NLS-INPP4A-GFP plasmids show less fluorescence.

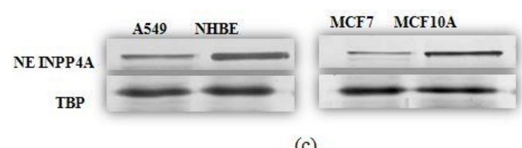
4.6 DIFFERENCE IN LEVELS OF NUCLEAR INPP4A EXPRESSION BETWEEN CANCEROUS AND NORMAL CELL TYPES

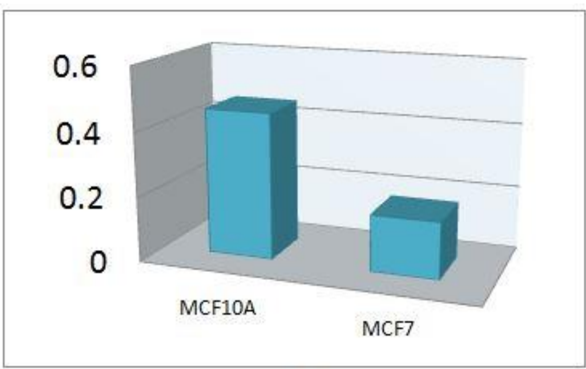
We further investigated and compared the endogenous expression of nuclear INPP4A in the cancerous and normal cell lines. Cancer cell lines are characterized by unregulated proliferation and anchorage independence. We used lung cancer epithelial cell line (A549) and breast cancer epithelial cell line (MCF-7) and compared them with lung normal epithelial cells (NHBE) and breast normal epithelial cells (MCF-10A). Immunoblotting and densitometric analysis were done to ascertain the endogenous expression of nuclear INPP4A. CFSE staining was done to analyze and compare the proliferation rate of normal and cancerous cells.

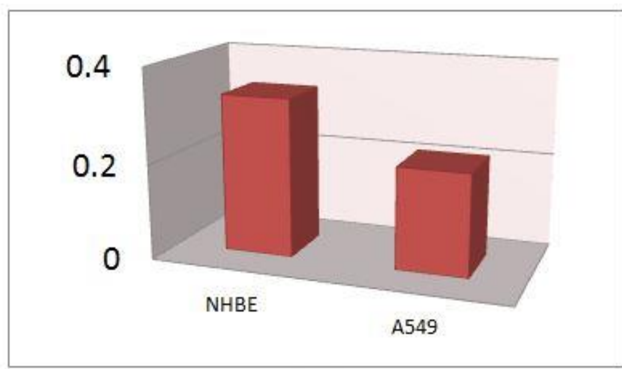
The result was in agreement to our previous result only. The Densitometric results revealed that the levels of endogenous nuclear INPP4A in lung epithelial cancer cell line A549 was significantly less as compared to that of endogenous nuclear INPP4A in non-cancerous epithelial cells (NHBE). Also, the same was seen between Breast epithelial cancer cell line (MCF7) and breast epithelial normal cells (MCF10A). CFSE staining analysis confirmed that the cancerous cell lines were having a higher proliferation rate as can be seen by the higher shift of CFSE fluorescence in case of cancerous cells. Hence, it can be said that higher proliferation rates are associated with the lower expression levels of nuclear INPP4A and nuclear INPP4A has a major role in regulating the cellular proliferation. Although the mechanism involved in this process is not yet known. In future, further experiments may also lead to INPP4A being recognised as a potential tumour suppressor, both in nucleus as well as in cytoplasm.
To normalize the levels of nuclear INPP4A in cells, Tata Binding protein (TBP) was used as a loading control.











(d) (e)

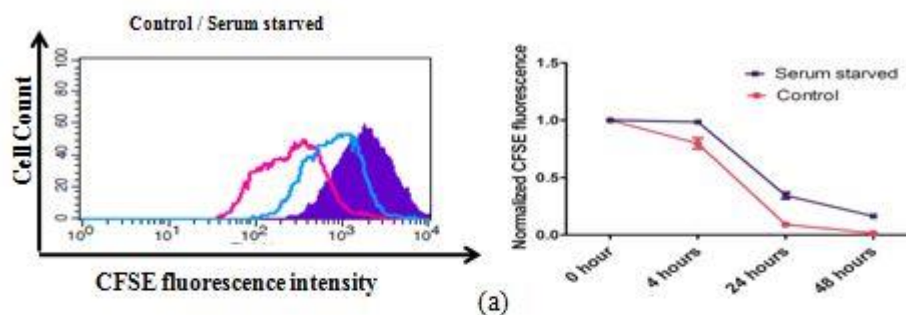
Fig. 12 (a) Higher CFSE fluorescence seen in NHBE (pink) then A549 (blue). Graph depicts CFSE fluorescence fold change higher in NHBE. (b) Higher CFSE fluorescence seen in MCF10A (pink) then MCF7 (blue). Graph depicts CFSE fluorescence fold change higher in MCF10A (c) Immunoblot showing INPP4A detection in Lung cell lines(NHBE,A549) and in breast cell lines (MCF10A, MCF7). Significant difference in the expression of nuclear INPP4A can be seen between cancerous (A549, MCF7) and normal (NHBE, MCF10A) cell line. (d) & (e) Densitometric analysis show higher expression of INPP4A in normal cell lines. Lung normal cell line NHBE expressing higher INPP4A then the Lung cancer cell line A549. Similar results can be seen in breast cell lines MCF10A, MCF7.

4.7 NUCLEAR INNP4A EXPRESSION WITH CELL CYCLE

SERUM STARVATION INCREASES NUCLEAR INPP4A-

Nuclear phosphoinositides shuttle between cytoplasm and the nucleus and their appearance in nucleus is associated with certain stimulus such as growth factor stimulus, cell cycle progression, stress such as oxidative stress, irradiation etc. Here, we investigated the affect of serum starvation on the nuclear INPP4A. Cells were serum starved for 48hours. These cells were harvested at different time points as they were serum starved in culture. Immunoblotting was performed to check the level of expression of INPP4A in their nuclear extracts. Also, Far Red CFSE staining and its flow cytometer analysis was done to analyze the proliferation rate of serum starved cells and cells being grown in complete media.

Nuclear INPP4A expression in nucleus was found to co-relate with serum starvation. When cells were serum starved for 48hours, the nuclear INPP4A expression increased as cells continue to survive in the serum starved conditions, with having a peak expression level at 48hours post serum starve. Flow cytometric analysis of CFSE staining showed that rate of proliferation of serum starved cells was less than the cells grown in complete media. It can be seen in Fig. 13(a) that at 48hour, CFSE staining of serum starved cells was very low depicting as the cells have almost ceased to grow whereas densitometric analysis shows the highest level of nuclear INPP4A expression at 48hour (Fig. 13(b)). This is in sync with the previous finding that level of INPP4A is associated with the cellular proliferation. As the cells struggle to survive in serum starve conditions, their rate of proliferation reduces and the cell cycle progression comes to a halt at 48hours into serum starve conditions which is also accompanied with the highest expression level of Nuclear INPP4A. Serum starvation can act as a stimulus under which INPP4A translocates into the nucleus and have an inhibitory role on the proliferation



(a)

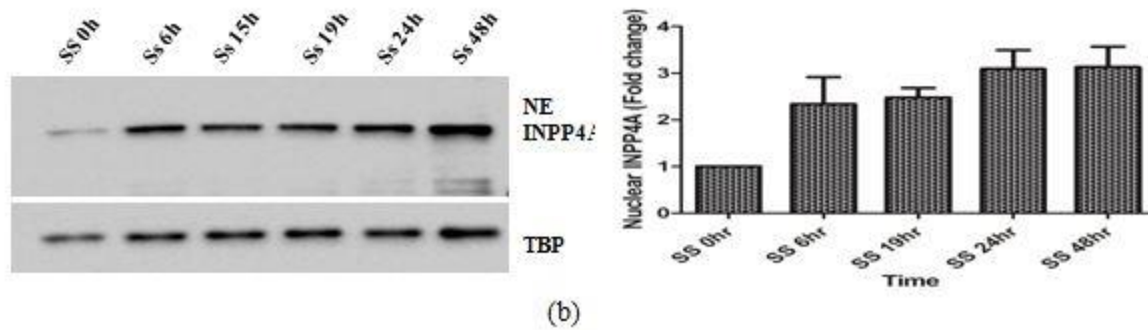


Fig. 13 (a) CFSE staining of serum starved cells versus cells grown in complete media. (b) Nuclear INPP4A expression of serum starved cells harvested at different time points.

PATTERN OF NUCLEAR INPP4A EXPRESSION ACROSS CELL CYCLE -

Serum starvation is a common method to synchronize the cells at G0/G1 phase. Serum starvation brings about a Growth restricted induced quiescence in cells (Rosner *et al*., 2013). In my study, Cells were serum starved for 48 hours to synchronize them at G0/G1 phase. The cells were then released from serum starved conditions and were grown in serum rich media. This released the cells from stress induced cell cycle arrest and cells entered into cell cycle through G2/M phase (Jabbour *et al*., 2014). These cells were then grown for next 48hours in serum rich media and were harvested at different time points. Immunoblotting was performed to examine the level of INPP4A in these cells harvested at different hours post serum starvation. Also, these cells were analyzed for the cell cycle phase via Propidium iodide (PI) staining protocol.

The results showed that a pattern of nuclear INPP4A expression was seen as the cell progresses through the cell cycle post serum starvation. It was seen that nuclear INPP4A expression is maximum at 0 hour when cells are released from serum stress, but then the expression decreased until at 30hours, where the nuclear INPP4A expression was lowest. Post 30hours into serum rich conditions, expression level of nuclear INPP4A again continued to increase. Propidium iodide staining used to mark the cell cycle phases post serum release and it co-relate with the levels of expression of the nuclear INPP4A with cell cycle phases. It was found that nuclear INPP4A expression was highest in G0/G1 phase and lowest in S-phase.

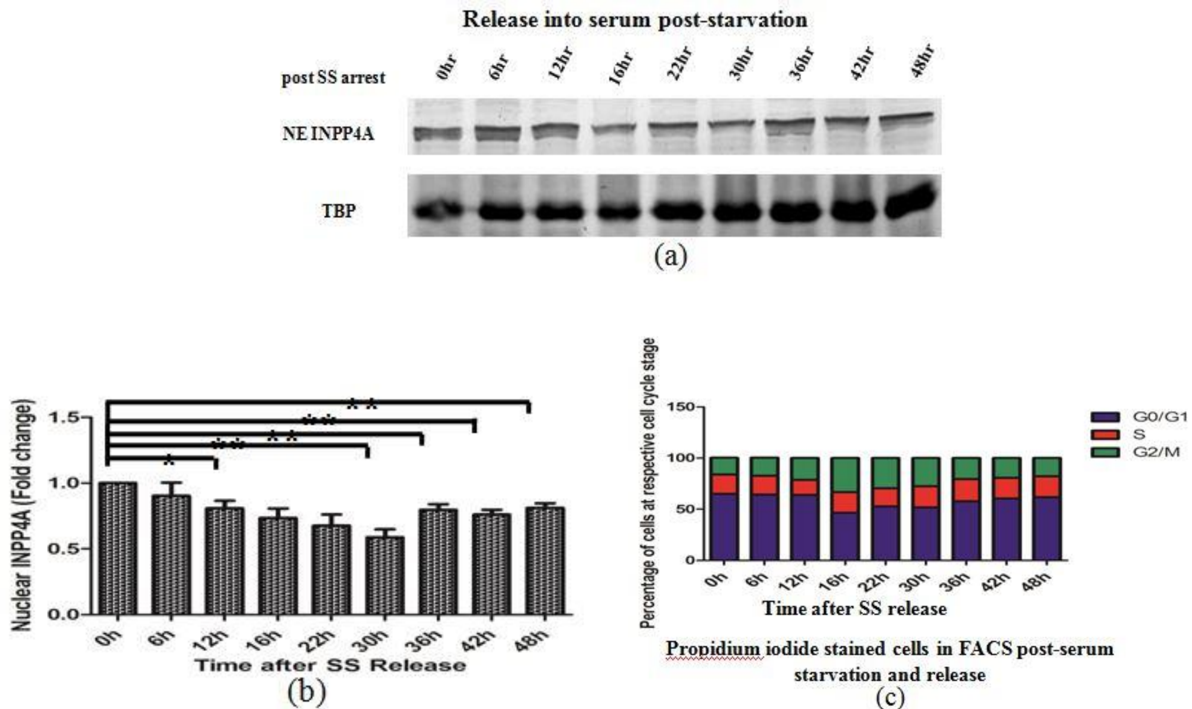


Fig. 14 (a) Immunoblot showing INPP4A detection in cells harvested at different hours post serum starve release. (b) Densitometric analysis of Immunoblot. Nuclear INPP4A levels were highest at 0 hour and lowest at 30 hour. (c) Graph showing percentage of cells in different cell cycle phases at different hours post serum starve release.

This result is similar to PTEN expression in nucleus. On post serum starve release, PTEN was also found to have a maximum expression in G0 /G1 phase and lowest in S phase (Lian *et al*., 2005). This shows the dynamic behaviour of INPP4A in nucleus. It is not constitutively expressed in nucleus but enters only under cell cycle stimulus. Hence, we can strongly say that nuclear INPP4A has an inhibitory role in controlling the proliferation of cells. Nuclear INPP4A shuttles between the cytoplasm and the nucleus or the novel variant we have found is expressed in nucleus only, is not yet known. However, the presence of INPP4A in nucleus was increased on serum starvation, a form of stress. Also, the expression of nuclear INPP4A reduces during cell cycle which shows an inhibitory effect of INPP4A on cell cycle.

OBJECTIVE II:To confirm the presence of INPP4A in Extracellular Milieu in vitro

We employed various methods to confirm our preliminary observation that INPP4A is secreted by the cell.

4.8 DETECTION OF INPP4A IN CELL'S EXTRACELLULAR MILIEU BY TCA PRECIPITATION METHOD AND MOLECULAR WEIGHT CUT-OFF FILTER BASED CONCENTRATION METHOD.

Preliminary results from the lab showed the presence of INPP4A in the body fluids like serum and broncho-alveolar lavage fluids (BALF) of both mice and human subjects. This indicated that INPP4A might be present outside of the cell as well. To confirm this hypothesis, an *in vitro* approach was used. Cell's extracellular secretions were obtained from the media in which cells were cultured and grown. The conditioned media used to culture cells contains 10% Fetal Bovine Serum (FBS) which is in itself a source of large number of proteins such as albumin, urea, TSH etc. While collecting supernatant culture media from cell culture flasks, these FBS proteins come in large quantity and cell's secreted proteins are masked by them. To avoid this masking and to be able to obtain pure cell-secreted proteins with no contamination from the conditioned media, cells were grown in two different media- Opti-MEM® which is a reduced serum media and media with no FBS at all i.e. plain media.

Protein precipitation from supernatant culture media was also done by two methods- Trichloroacetic acid (TCA/Acetone wash method) and by using Amicon Ultra-15 Centrifugal Filter Units (100k). Two methods were used due to different end products obtained in both the methods. The TCA/Acetone wash method denatures the protein rendering them in-effective for the functional analysis where the structure of protein needs to be conserved. The proteins precipitated from supernatant culture media by TCA/Acetone wash method were used only for immunoblotting. This supernatant culture media was conditioned media with no FBS (plain media).

Another method used to precipitate proteins from supernatant culture media is with the use of Amicon Ultra-15 Centrifugal Filter Units. The cells were grown in OptiMEM media to obtain secreted proteins from supernatant culture media using the centrifugal filters. The protocol is able to capture all the proteins present in media having Mol. Wt. more than 100kDa (Mol Wt. of INPP4A is107kDa) in their native state. The proteins concentrated via centrifugal filters can be used for both immunoblotting and functional analysis.

The protein precipitation by TCA/Acetone wash protocol is much better at capturing INPP4A protein as compared to the method using centrifugal filters. Centrifugal filters cause some loss of the captured proteins. This is evident from the Fig. 16 which shows Ecl image of the immunoblot of INPP4A as captured by TCA method and centrifugal filters. The band in TCA precipitated protein is denser and thicker then centrifugal filter precipitated protein.

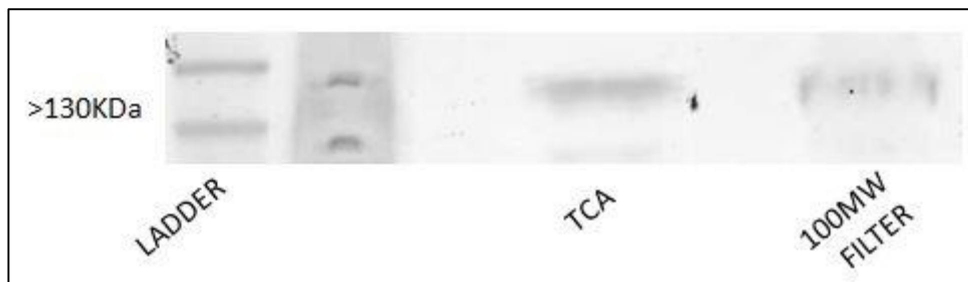


Fig. 15 Immunoblotting of INPP4A in TCA precipitated protein and Amicon Ultra-15 Centrifugal Filter precipitated protein

Conditioned media of different cell lines were collected and protein precipitation was done by both method- TCA/Acetone wash method and centrifugal filters. The cell line chosen were MCF-7, MCF-ST cell line, Beas-2b. Protein precipitated and filtered from these cell lines were immunoblotted for the presence of secreted INPP4A. INPP4A was being secreted by all the cell lines but in different measure. Although the role of secreted INPP4A is not known yet, neither any protein database states that INPP4A is a secretary protein so it is difficult to say if INPP4A is being constitutively secreted by the cells or secreted only under some stimulus. As can be seen in the Fig. 17, the expression of secreted INPP4A in MCF ST is higher than MCF Normal, due to stable constitutive expression of INPP4A in the former cell line. Beas-2b is secreting more INPP4A which is consistent with the reports of The Human Atlas also which shows the cytoplasmic endogenous expression of INPP4A in lung tissues is high

(http://www.proteinatlas.org/ENSG00000040933-INPP4A/tissue). A549 being cancerous cell line shows least expression or almost no expression of secreted INPP4A. Similar result can be seen in Fig. 17(b), showing Immunoblot containing protein precipitates with centrifugal filters.

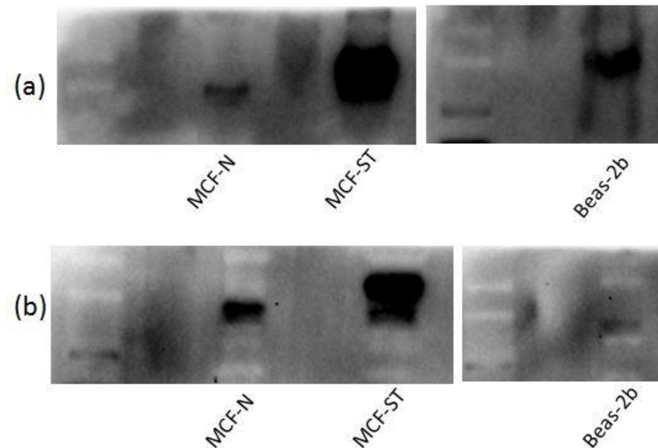


Fig 16 (a) Immunoblot of INPP4A staining of TCA precipitated proteins (b) Immunoblot of INPP4A staining of proteins concentrated by Mol. Wt. cut off filter

Secreted INPP4A was also seen to have a higher mol. Wt. (~130kDa). No isoform of INPP4A has been reported so far to have this high molecular wt. It can be hypothesized that this secretary INPP4A may be post translationaly modified and hence, runs slower on SDS-PAGE. Secretary proteins are generally glycosylated for providing protective function to extracellular proteins, thus we checked for glycosylation on INPP4A.

4.9 PREDICTING GLYCOSYLATION SITES IN INPP4A

Glycosylation is a common post translational modification in which glycans are added to the proteins, lipids, or other organic molecules. Addition of glycans strongly influences the function, cellular localization, protein quality and turnover of the protein. Different heterogeneous types of Glycosylation have been seen -N-linked Glycosylation where glycans are added to a nitrogen of asparagine or arginine side-chains present in the consenses sequence Asn-Xaa-ser/Thr (Xaa is any amino acid except proline); O-linked Glycosylation where glycans are added to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side-chains; C-linked glycans, where the sugar is attached to a carbon on the tryptophan side-chain. The glycosylation serves in protein folding; metabolism and transport;

maintenance of protein structure and cellular morphogenesis and other processes (Marino *et al.*, 2010).

We predicted the N-/O- linked glycosylation of different known isoforms of INPP4A using NetNGlyc 1.0 server and NetOGlyc 4.0 server.

N-linked glycosylation predictions-

The NetOglyc server produces predictions of neural network of type GalNAc O-glycosylation sites in mammalian proteins.

Asn-Xaa-Ser/Thr sequences in the INPP4A isoforms below are in blue highlight.

Asparagines which are predicted to be N-glycosylated are in red highlight.

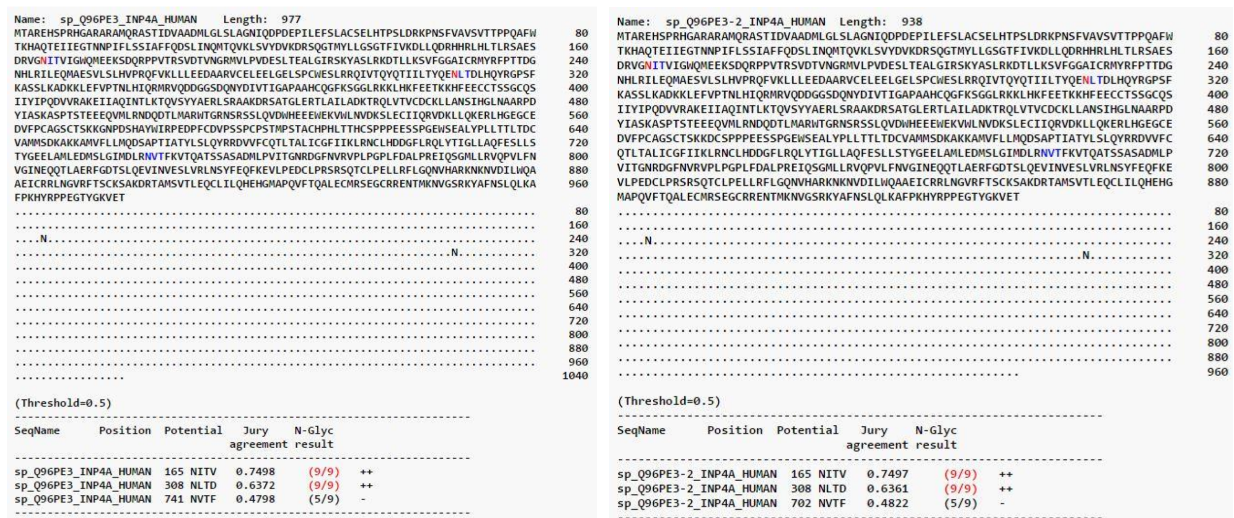


Fig. 17a Predicted result for N-glycosylation in Isoform I & II of INPP4A

Fig. 17b Predicted result for N-glycosylation in Isoform III & IV of INPP4A

The predicted results show that all the isoforms have atleast three predicted glycosylation sites. All isoforms have two common N- linked glycosylation sites at the position 165 and 308 which have the highest predicted rate of potential glycosylation. Other sites are present at 702 which is common to isoform II & IV.

O-linked glycosylation predictions-

```
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##source-version NetOGlyc 4.0.0.13
##date 15-11-16
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   423   423   0.0714345    .   .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   426   426   0.537314     .   .   #POSITIVE
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   433   433   0.641714     .   .   #POSITIVE
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   440   440   0.44281 .    .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   442   442   0.285195     .   .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   447   447   0.440084     .   .
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   484   484   0.871221     .   .   #POSITIVE
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   487   487   0.895027     .   .   #POSITIVE
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13   CARBOHYD   490   490   0.712975     .   .   #POSITIVE
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        612     612     0.5     .       .       #POSITIVE
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        627     627     0.0773265       .       .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        635     635     0.0281963       .       .
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        832     832     0.0266827       .       .
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        894     894     0.195808        .       .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        897     897     0.0420171       .       .
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        905     905     0.0276885       .       .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        907     907     0.0539152       .       .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        926     926     0.0882497       .       .
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SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        971     971     0.171866        .       .
SP_Q96PE3_INP4A_HUMAN   netOGlyc-4.0.0.13       CARBOHYD        977     977     0.0555527       .       .
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Fig. 18 Predicted result for O-glycosylation in Isoform I INPP4A

Only one isoform of INPP4A has been shown for O-linked glycosylation. The prediction says that there are more sites of O-linked glycosylation than N-linked. In isoform I itself, there are atleast 7 predicted O-linked glycosylation sites. This can be a possible explanation for the higher molecular weight of secreted INPP4A as seen in immunoblots.

4.10 DEMONSTRATING THE PRESENCE OF GLYCOSYLATION IN SECRETED INPP4A

To check if the Glycosylation predictions done by NetNGlyc 1.0 server and NetOGlyc 4.0 server holds true for the 130kDa isoform of secreted INPP4A, de-glycosylation assays were done to enzymatically cleave the glycans from the intact glycoproteins. Enzymatic deglycosylation will decrease the Mol. Wt. from the glycosylated protein (130kDa) bringing it down to the Mol Wt. of known INPP4A isoforms (106-110kDa). We used PNGase F (Peptide -

N-Glycosidase F), an amidase which preferably cuts between the innermost GlcNAc & asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins. PNGase F does not cleave N-glycans which contain fucose α (1, 3) linked to the core GlcNAc residues.

The supernatant culture media was collected and the protein was concentrated using Amicon Ultra-15 Centrifugal Filter Unit. The precipitated protein was denatured as it is improves the PNGase F enzyme accessibility onto the relaxed three-dimensional protein structure. PNGase F treatment was then done and detection of INPP4A was carried out by immunoblotting. Deglycosylation led to restoration of the molecular weight of secreted INPP4A to 107 KDa. Additionally, there was an improved detection of INPP4A, perhaps due to higher accessibility of the epitope after the removal of glycan moieties.

Fig. 19 Decrease in Mol Wt. after PNGase treatment in centrifugal filter used filtered supernatant culture media.

4.11 ABSENCE OF SIGNAL PEPTIDE IN INPP4A

After detection of INPP4A in the extracellular secretions of cell, we wanted to check if INPP4A has a signal peptide driving its secretion. The presence of signal peptide was predicted through bioinformatics tools. The N-terminus of the many newly translated proteins that are destined towards the secretory pathway have a short peptide of 5-30 amino acids which is called as signal peptide. These proteins are those that are destined either for certain organelles (golgi, endosomes or ER), to be inserted into most plasma membrane or to be secreted from the cell. The common structure of signal peptides consists of a positively charged N-region, then a hydrophobic H-region and a neutral but polar C-region. The (-3,-1)-rule states that the residues

at positions -3 and -1 (relative to the cleavage site) must be small and neutral for cleavage to occur correctly.

The presence of signal peptide was predicted with the help of SignalP 4.1 Server online software. Protein sequence of four isoforms of INPP4A as taken from Uniprot, were fed into SignalP 4.1 Server. The SignalP 4.1 server predicts the region of signal peptide bearing cleavage sites in amino acid sequences of both eukaryotes and prokaryotes. The software predicts the presence of cleavage sites and signal peptide through a combination of many different artificial neural networks. The software calculates a D-value on the basis of presence of cleavage sites and signal/non-signal peptide sequences. If the calculated D-value is above the threshold (D=0.45), then the software gives information about the site of signal peptide. The results for INPP4A isoforms were given in this way-

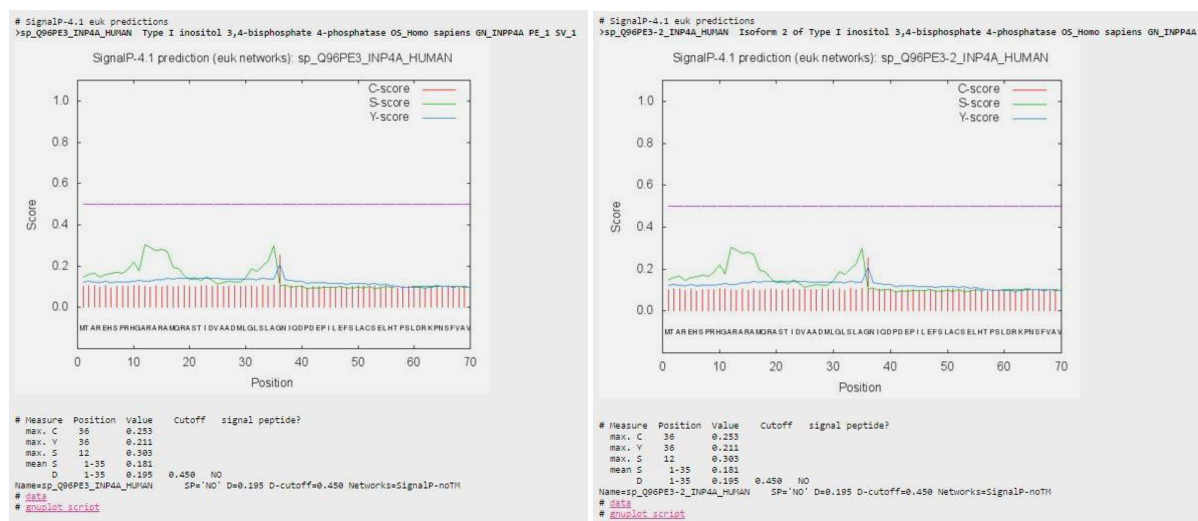


Fig. 20 signal peptide prediction of Isoform I & II of INPP4A

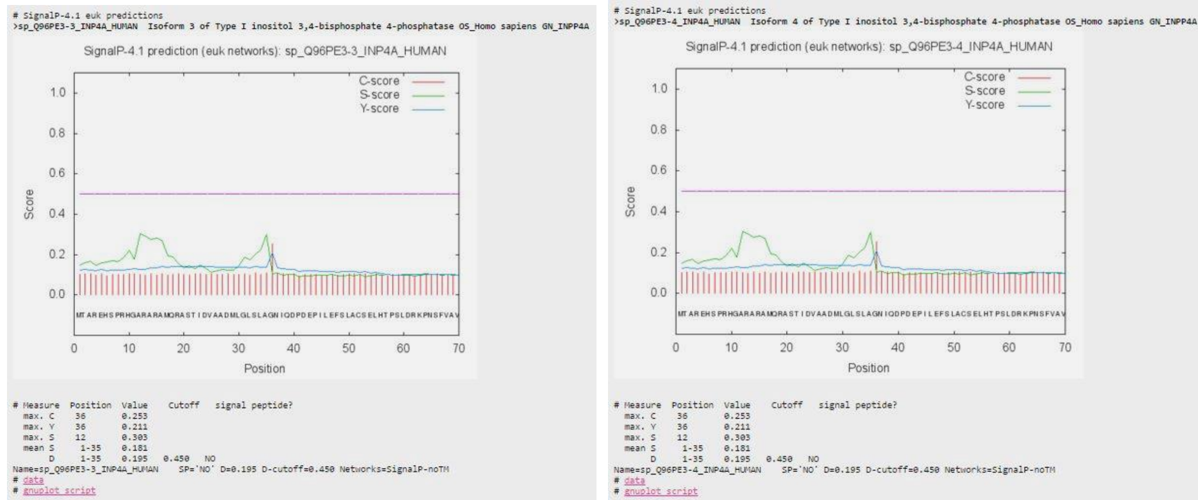


Fig. 20 signal peptide prediction of Isoform III & IV of INPP4A

The calculated D-value for all the isoforms was 0.195 and the threshold is set at 0.450 so the prediction of the signal-4.1 server was that there is no secretary signal peptide present in any of the isoforms of INPP4A.

The possible explanation for this result is that INPP4A is being secreted by a non-classical pathway of the cellular secretion. The presence of signal peptide on any secretary protein in the cell is required when the secretion of the protein is taking place through the classical pathway of secretion wherein proteins targeted for the extracellular space are translated by the ribosomes docked to the rough endoplasmic reticulum (RER). From ER, these proteins move to golgi and then enter into secretary vesicles which take these proteins to the plasma membrane to be release out of the cells. Glycosylation starts to take place from ER and golgi. Signal peptide is added in the ER only which helps its movement through various organelles and finally to the target. But the absence of signal peptide in INPP4A opens a completely new and fascinating area of research.

4.12 INPP4A SECRETION VIA NON-CLASSICAL PATHWAY

In the previous experiment, it was predicted that known Isoforms of INPP4A do not bear any signal peptide. To confirm the findings of the prediction analysis for the secreted INPP4A, an

experiment was performed to block the classical pathway of secretion and then check if INPP4A is still being secreted or not.

Brefeldin A (BFA), a known potent inhibitor of classical pathway was used to block classical Protein secretion in the cells- MCF7 and MCF7-ST. The cells were incubated with brefeldin A for 24 hours until there supernatant media was collected and protein precipitation was done by TCA.BFA is dissolved in Ethanol hence, ethanol was used as a vehicle control. The results showed that INPP4A was still being secreted in the extracellular secretions.

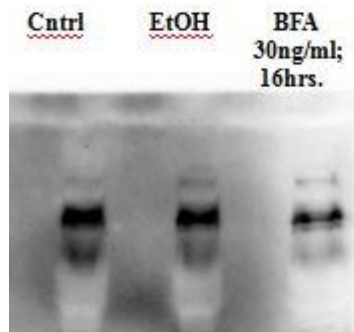


Fig. 21 Immunoblot showing INPP4A still being secreted after treatment of cells with BFA.

Through this experiment, we can understand that INPP4A is being secreted by some other pathway of secretion. The non-classical pathway is the secretary pathway for proteins which lack the conventional signal peptides and are excluded from classical secretary organelles such as the ER and the Golgi. This pathway was discovered 10 years ago and since then many proteins lacking in signal peptides have been found to be secreted by the cell through non-classical pathways. Some of the molecules are Il-1β, FGF1, FGF2, En2 etc. (Nickel,W. 2003) . An important mode of non- classical secretion is via the exosomes.

Not only this, there are reports showing that INPP4A does not co-localizes with golgi complex. Also, there was no co-localization seen with mannose-6-phosphate receptor which targets proteins from golgi to lysosomes. Rab-8 which is a secretary vesicle marker also showed no colocalization with INPP4A (Ivetac *et al*., 2005). These reports corroborate with our hypothesis that INPP4A is not being secreted via golgi mediated classical pathway but has a completely different mechanism of secretion. Also, it is not known whether this secretion is constitutive or takes place only under some specific conditions.

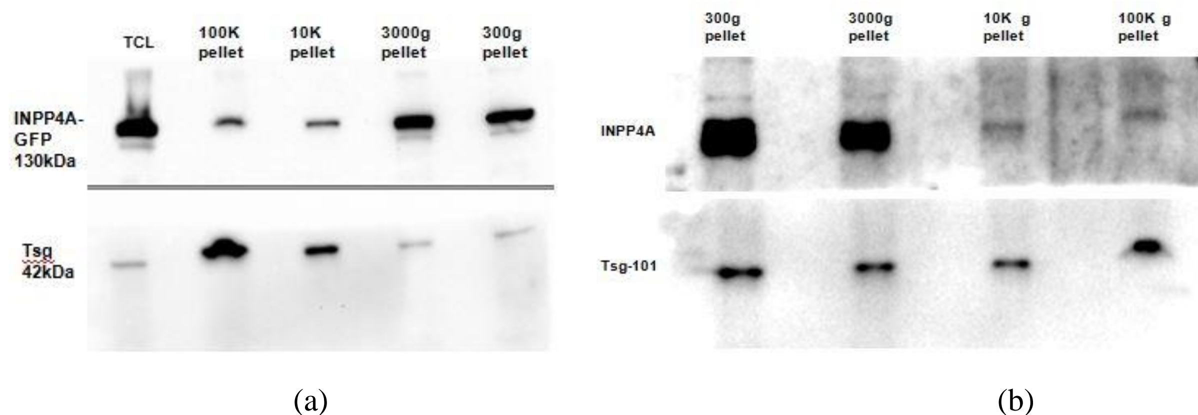
4.13 ISOLATING EXOSOMES FROM CELL CULTURE SUPERNATANT MEDIA AND PROBING FOR INPP4A

Exosomes/Microvesicles (EVs) mediated secretion is an upcoming and exciting area in the field of non-classical secretion. Exosomes are 30-100nm extracellular vesicles which are derived from multivesicular bodies (MVB). They carry RNA, protein or nucleic acid contents in their aqueous core or linked to their lipid membrane to be delivered to their target cells. We decided to check the presence of INPP4A in EVs. For this EVs of different sizes were purified from supernatant culture media using differential ultracentrifugation method. For the isolation of EVs, cells were grown in a media containing filtered FBS. Before using FBS in conditioned media, vesicles present in FBS were removed by ultracentrifuging the FBS at 1, 00,000 g for 20hours at 4°C. This filtered FBS was then added to the conditioned media to be used for culturing the cells. This was done to prevent bovine exosomes to mask the exosomes secreted by cells in culture media. Cells were grown in this media for 24hours before collecting.

EVs secreted by cells display various types and size ranges. EVs which are in the size range of few nanometers to few micrometers (the largest) and arise directly from PM of the cells are often called as microparticles/microvesicles or ectosomes. Exosomes are EVs which are smaller than 100nm (Kowal *et al*., 2016).

Classically used differential centrifugation protocol was used to isolate EVs from the supernatant culture media. Differential ultracentrifugation protocol involves centrifuging the supernatant at different centrifugation speeds to remove EVs of different sizes and to collect exosomes as the end product. All the pellets of each centrifugation speed in the protocol were collected to see the INPP4A expression level in the pelleted materials through immunoblotting. The result showed that INPP4A is present in all the pellets with varying expression levels. Tsg-101 has been used as a control marker for EVs in immunoblotting. The cell lines used for this is MCF-ST cell line expressing GFP tagged INPP4A and wildtype MCF7 cells probed for endogenously expressed INPP4A.

Pelleted material collected at 300g contains mostly dead cells or debris (apoptotic/necrotic cell fragments). Pelleted material collected at 10,000g contains EVs which are mostly greater than 200nm or in the size range of 200-150nm. 1, 00,000g pellet material finally captures the exosomal vesicle population which is in the size range of <150nm (Kowal *et al*., 2016). Our results show that INPP4A is expressed in both microvesicless (10,000g pellet) and exosomes (1,00,000g pellet). Both endogenous INPP4A and GFP tagged INPP4A are detected in these extracellular vesicles. .Since INPP4A is known to localize to the plasma membrane and the endosomes, both of which are sites of origins of the extracellular vesicles: plasma membrane for microvesicles and endosomes for exosomes – the presence of INPP4A in these vesicles in an interesting observation. Extracellular INPP4A could be an important mediator for carrying about regulation of the PI3K signalling beyond the cellular boundaries, at a broader level.



(a) (b)

Fig. 22 Immunoblot of INPP4A staining showing different pellet contents isolated during exosome isolation protocol.

It is yet not known whether INPP4A is secreted packed inside the lumen of these EVs or is present on its lipid bilayer. The origin of these Exosomes is also not known however, INPP4A do not co-localizes to the Golgi body and BFA blocking was not able to block the release of INPP4A in extracellular secretions. These findings discard the possibility of INPP4A secretions via any classical pathway. Four pathways which secrete proteins through a pathway other then classical pathway have been reported. First pathway involves the fusion of lysosomal contents with the plasma membrane (Stinchcombe *et al*., 2004). IL-1β is secreted via this method (Rubartelli *et al*., 1990). Another pathway is which involves ABC transporters (Cleves and Kelly, 1996). FGF-1 and FGF-2 are secreted via ABC transporters (Prudovsky *et al*., 2002; (Schäfer *et al*., 2004). Another pathway is via fusion of multivesicular bodies with plasma

membrane e (Stoorvogel *et al*., 2002). Galectin-3 is secreted via this pathway (Thery *et al*., 2001). Fourth Pathway is via membrane blebbing (Freyssinet, 2003).

Exosomes are a means of intracellular signalling. Exosomes are capable of altering the behaviour of the recipient cells. They are capable for modulating many recipient cells at once and they are considered as a great means in drug delivery. Exosomes are secreted by stem cells, haematopoietic, epithelial, neuronal and cancerous cells. Exosomes play a role in initiating an immune response by offering MHC molecules to APC or T-cells/ B-cells (Muntasell *et al*., 2007). Cancerous cells secret exosomes which play anti-tumourogenic or immunosuppressive role (Park *et al*., 2010). Stem cells secret exosomes to act like paracrine modulators (Sahoo *et al*., 2011). Even pathogens use exosomes to establish a suitable environment in the body for them. Secreted PTEN was discovered few years ago but not much reports are available for the same. It has been established that this secreted PTEN has an inhibitory role on recipient's cellular proliferation via regulating pAkt levels.

One can see the resemblance between PTEN and INPP4A, both being the regulators of the same pathway, both being found in nucleus and extracellular secretions. INPP4A, by localizing to these non-canonical sites, could be regulating the PT3K signalling and the tissue homeostasis in a diverse manner.

CHAPTER 5

CONCLUSION

CONCLUSION

This thesis is a preliminary study towards a new direction of research. INPP4A has been explored extensively for its role in neurodegeneration and its role in maintaining endocytic pathways. But the role of INPP4A in regulating cellular proliferation, cell cycle and differentiation is still in its infancy. INPP4A being present in the nucleus and in cell's secretions are completely novel findings. Although, its association with the cell proliferation shown in this thesis should not come as a surprise considering the role of INPP4A in PI3K/Akt pathway and its counterpart INPP4B which has already been declared as a tumour suppressor.

Through this thesis, one can infer that INPP4A has a lot more to its function then just being lipid phosphatase function. The nuclear localization and secretion of INPP4A as well as PTEN in exosomes hints towards a close relationship of both localizations in maintaining the homeostasis of the body. This discovery opens up a new area to explore for various biomedical companies engaged in developing potential therapeutic aids for cancer and lung diseases. All the key mediators of PI3K pathway have been declared or on the verge of being declared as tumour suppressors or oncogenes. This makes this one pathway a major target to consider driving the normal cell cycle to become dysregulated towards tumor growth.

The small discoveries of this thesis may prove to start a new era for upcoming medical research works exploring the potential of INPP4A as its absence is a cause of pathogenesis of various diseases.

CHAPTER 6

FUTURE PROSPECTIVES

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This thesis is a prelim study towards a new direction of research. I have been able to show only a glimpse of a big INPP4A story though my thesis. Following can be the lead to further explore about nuclear INPP4A and the secreted INPP4A mystery.

NUCLEAR INPP4A-

- We will probe whether nuclear INPP4A action is independent of PI3K pathway or dependent by creating phosphatase mutants.
- We will determine presence on sumoylation on predicted sites on INPP4A via using Anti-SUMO antibodies and desumoylation agents.
- Mutants will be created of C2A, C2B, PEST and IP4P domains in INPP4A to determine which domain has a role to play in nuclear localization and function.
- We will also investigate other conditions in which nuclear INPP4A translocates into the nucleus such as IR radiations.
- We will search for other proteins working in close association with nuclear INPP4A via mass spectrometery techniques and deducing a mechanism of its action.

SECRETED INPP4A-

- We will investigate if INPP4A is present inside the exosomes or is membrane spanning via Immunoblotting techniques.
- The role of secreted INPP4A will be investigated by us via exosomes uptake studies in fibroblasts and cells having very low expression of INPP4A such as PBMC.
- Specific conditions under which INPP4A exosomes are secreted and what are the natural recipient of these INPP4A bearing exosomes will be investigated.
- Animal-based studies will be done probing the in vivo role of secreted INPP4A by administering anti-INPP4A antibodies in lungs.
- INPP4A tracking studies will be done to investigate the non-classical pathway taken by secreted INPP4A.

It's a long road ahead.

CHAPTER 7

REFERNCES

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