<u>The Novel Presence and Role of Inositol</u> <u>Polyphosphate 4-Phosphatase Type I in the Nucleus</u> <u>and Extracellular Milieu</u>



to be submitted as Major <u>Project</u> in partial fulfilment of the requirement for the degree of

M.Tech. (Biomedical Engineering)

Submitted by

DAMINI VATSA

(2K14/BME/06) Delhi Technological University, Delhi, India

Under the supervision of

DR. B.D. MALHOTRA

Department of Bio-Technology, Delhi Technological University, Delhi-110042

DECLARATION



I, Damini Vatsa, hereby declare that the dissertation entitled 'The Novel Presence and Role of Inositol Polyphosphate 4-phosphatase Type I in the Nucleus and Extracellular Milieu' submitted is in the partial fulfilment of the requirements for the reward of the degree of Master of Technology in Biomedical Engineering, Delhi Technological University. It is a record of original and independent research work done by me under the supervision and guidance of Dr. Anurag Agrawal, Principal Scientist, CSIR-IGIB and Prof. Bansi D. Malhotra, Department of biotechnology, DTU. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

Date

DAMINI VATSA 2K14/BME/06 M. Tech. (Biomedical Engineering) Delhi Technological University

CERTIFICATE



This is to certify that the dissertation entitled **'The Novel Presence and Role of Inositol Polyphosphate 4-phosphatase Type I in the Nucleus and Extracellular Milieu'** submitted by **Damini Vatsa (2K14/BME/06)** is in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

Prof. D. Kumar Head Department of Biotechnology Delhi Technological University **Prof. Bansi D. Malhotra** Project Mentor Department of Biotechnology Delhi Technological University

ACKNOWLEDGEMENT

The writing of this project work has been one of the most significant academic challenges I have ever had to face. Without the support, patience and guidance of the following people, this study would not have been completed. It is to them I owe my deepest gratitude.

I take this opportunity to express my profound gratitude and deep regards to my mentor Prof. **B.D.Malhotra**, Department of Biotechnology, DTU for his exemplary guidance, monitoring and constant encouragement throughout the course of this project. The blessing, help and guidance given by him shall carry me a long way in the journey of life. I also acknowledge Prof. D. Kumar, Head, Department of Biotechnology, DTU for supporting me and allowing me to carry out these studies outside the premises of DTU.

I am highly indebted to **Dr.Anurag Agarwal**, Scientist, CSIR-IGIB, Mall Road, Delhi for the confidence he showed in me and for providing me with an opportunity to carry out my project in his venerated laboratory under his much needed guidance.

I am extremely thankful to **Ms. Kritika Khanna** and **Ms. Rituparna Chaudhary**, (Research Scholars) who not only offered me insight into molecular biology techniques but also gave me useful suggestions along with a critical evaluation of this report. Their kind cooperation and their support were crucial for the outcome of this project.

I am also thankful to **Mr. Bijay Pattnaik** and **Ms. Lipsa Panda** who rendered their knowledge and expertise during the period of this project. Their timely suggestions and much needed technical advices helped me in carrying out experiments with precision and accuracy.

Last, but not the least, I pay my sincere sense of gratitude to **my family and friends**, without whose encouragement and moral support, I would not have seen this day of brim. Though, mere words could not convey it all, it is an honest attempt to express my heartfelt gratitude to everybody who made this project successful.

Damini Vatsa

(2K14/BME/06)

CONTENTS

TOPIC	PAGE NO.
Abstract	1
CHAPTER 1: Introduction	4
CHAPTER 2: Literature review	8
2.1 PI3Kinase Signalling Pathway	9
2.2 Significance of PtdIns(3,4)P2	12
2.3 Inositol Polyphosphate 4-phosphatase: Structure	13
2.4 Inositol Polyphosphate 4-phosphatase: localization and function	15
2.5 PI3K Signalling mediators in Cell Nucleus	18
CHAPTER 3: Materials and Methods	23
Objective I	24
3.1 Immunoblotting for detection of INPP4A in the nucleus of cells	24
3.2 Immunoprecipitation done to ensure the presence of INPP4A in nuclear extracts	32
3.3 Immunocytochemistry(icc) of cells to visualize INPP4A in nucleus of cells	33
3.4 Predicting sumoylation sites	34
3.5 Proliferation rate analysis by overexpressing INPP4A	34
3.6 To investigate the conditions under which inpp4a translocates to the nucleus & its co-relation with cell cycle	36

Objective II

3.7 Protein isolation and purification from cell cu	ltured media and
immunoblotting for INPP4A	38
minunoolounig for myr4A	
3.8 Predicting n-/0- glycosylation in INPP4A iso	forms 40
3.9 Determining if secreted INPP4A was glycosy	vlated 40
3.10 Predicting signal peptide in INPP4A isoform	as 42
3.11 Isolating exosomes from cell culture superna	
identifying the association of INPP4A with secret	ted exosomes 42
CHAPTER 4: Results and Discussion	45
Objective I	
4.1 Immunoblotting for detection of INPP4A in n	auclear extracts 47
4.2 Immunoprecipitation of INPP4A in nuclear ex	xtracts 48
4.3 Immunocytochemistry (icc) of cells to visuali nucleus	ze INPP4A in 49
4.4 Predicting sumoylation sites on INPP4A	50
4.5 Effect of INPP4A overexpression on prolifera	
4.6 Difference in levels of nuclear INPP4A expre	
cancerous and normal cell types	55
4.7 Nuclear INPP4A expression with cell cycle	57
Objective II	
4.8 Detection of INPP4A in cell's extracellular m	ilieu by tca
precipitation method and molecular weight cut-of	f filter based 60
concentration method.	00
4.9 Predicting glycosylation sites in INPP4A	62
4.10 Demonstrating the presence of glycosylation	
INPP4A	65
4.11 Absence of signal peptide in INPP4A	66

4.12 INPP4Asecretion via non-classical pathway	
4.13 Isolating exosomes from cell culture supernatant media and probing for INPP4A	70
CHAPTER 5: Conclusion	73
CHAPTER 6: Future Perspectives	75
CHAPTER 7: References	77

LIST OF ABBREVIATIONS

ABBREVIATIONS FULL FORMS APS Ammonium persulfate BAD Bcl-2-associated death promoter BALF Broncho-alveolar lavage fluid BCA WR Bicinchoninic acid Working reagent BIM Bcl-2-like protein 11 DTT Dithiothreitol FBS Fetal Bovine Serum FOXO Forkhead family of transcription factor Green fluorescent Protein GFP GPCR G-protein-coupled receptors HRP Horseradish peroxidase INPP4A Inositol polyphosphate-4 phosphatase type I **INPP4B** Inositol polyphosphate-4 phosphatase type II mTOR Mammalian target of rapamycin NGF Nerve Growth Factor NLS Nuclear localizing Sequence NMDAR N-methyl-D-aspartate receptor PBMC Peripheral blood mononuclear cell PBS Phosphate-buffered saline PBST Phosphate-buffered saline with 0.1% Tween-20 PDK1 Phosphoinositide-dependent kinase 1

PI	Phosphoinositide
PI	Protease Inhibitor
РІЗК	Phosphoinositide 3-kinase
PM	Plasma membrane
PTEN	Phosphatase and tensin homolog
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(4,5)P ₂	Phosphatidylinositol 4,5-Bisphosphate
$PtdIns(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate
$PtdIns(3,4)P_2$	Phosphatidylinositol 3,4-Bisphosphate
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay buffer
RPMI	Roswell Park Memorial Institute medium
RTK	Receptor Tyrosine Kinase
SDS	Sodium dodecyl sulfate
SGK3	Glucocorticoid-regulated kinase 3
TBST	Tris-Buffered Saline with 0.1% Tween 20

LIST OF FIGURES

Fig. No	Figure caption	Page no.
1	Structure of PtdIns and its phosphoinositides	5
2	PI3K Signalling pathway (Jabbour et al., 2014)	11
3	PI3K signalling reaction	12
4	Structure of INPP4	14
5	The transfer stack	30
6	(a) Different isoforms of INPP4A can be seen in cytoplasmic extracts of cell lines. (b) Nuclear extracts of cells showing 120kDa nuclear INPP4A.	48
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.	49
8	ICC image showing co-localization of INPP4A with DAPI	50
9	Predicted SUMO sites on different isoforms of INPP4A.	51
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. 	53

	(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	
11	expressing transfected cells were considered. Arrow shows ki67	54
	expression in transfected cells. (b) Graph showing ki67 fluorescence	
	quantified using R-language algorithm. Cells transfected with NLS-	
	INPP4A-GFP plasmids show less fluorescence.	
	(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).	
12	Significant difference in the expression of nuclear INPP4A can be	56
	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.	
	(a) CFSE staining of serum starved cells versus cells grown in	
13	complete media. (b) Nuclear INPP4A expression of serum starved	58
	cells harvested at different time points.	
	(a) Immunoblot showing INPP4A detection in cells harvested at	
	different hours post serum starve release. (b) Densitometric analysis	
14	of Immunoblot. Nuclear INPP4A levels were highest at 0 hour and	59
14	lowest at 30 hour. (c) Graph showing percentage of cells in different	57
	cell cycle phases at different hours post serum starve release.	
	con cycle phases at unicient nouis post seruin starve release.	

15	Immunoblotting of INPP4A in TCA precipitated protein and Amicon Ultra-15 Centrifugal Filter precipitated protein	61
16	(a) Immunoblot of INPP4A staining of TCA precipitated proteins (b)Immunoblot of INPP4A staining of proteins concentrated by Mol.Wt. cut off filter	62
17	(a) . Predicted result for N-glycosylation in Isoform I & II of INPP4A(b). Predicted result for N-glycosylation in Isoform III & IV of INPP4A	63,64
18	Predicted result for O-glycosylation in Isoform I INPP4A	64,65
19	Decrease in Mol Wt. after PNGase treatment in centrifugal filter used filtered supernatant culture media.	67
20	Signal peptide prediction of Isoform I & II of INPP4A Signal peptide prediction of Isoform III & IV of INPP4A	68
21	Immunoblot showing INPP4A still being secreted after treatment of cells with BFA.	69
22	Immunoblot of INPP4A staining showing different pellet contents isolated during exosome isolation protocol.	71

OBJECTIVES OF THESIS

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

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