INTEGRATING IN SILICO AND IN VITRO APPROACHES TOWARDS GAINING MECHANISTIC INSIGHTS INTO GLUCOSE-STIMULATED INSULIN SECRETION

By

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

JULY 2026

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Submitted

In fulfilment of the requirements of the degree of

Doctor of Philosophy

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

JULY 2026

CERTIFICATE

This is to certify that the thesis titled "**Integrating *In silico* and *In vitro* Approaches Towards Gaining Mechanistic Insights into Glucose-Stimulated Insulin Secretion**" being submitted by Mr. Tapabrata Pramanik (Entry No. 2019BLZ8326) to the Indian Institute of Technology, Delhi, for the award of the degree of Doctor of Philosophy is a record of bonafide research work carried out by him, which has been prepared under my supervision and guidance in conformity with the rules and regulations of "Indian Institute of Technology, Delhi". The research reports and the results presented in this thesis have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

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ACKNOWLEDGEMENTS

Life gives us opportunities that encompass professional and personal growth. I am a staunch believer of both, since we are genetically encoded social beings. The most important attribute of growth is the ability to communicate with, listen to and understand others. By doing so, we dissipate of sense of comfort and positivity for the greater good of the society. Hence, as has been the scenario throughout my life, my doctoral thesis would not have been accomplished without the steadfast support of many individuals who made this journey possible.

First and foremost, I express my fervent gratitude to the omnipotent, omniscient, omnipresent and omnibenevolent, the Almighty – the holy trio of Sri Ramakrishna, Sri Ma Sarada Devi and Swami Vivekananda for bestowing upon me with blessings, strength, wisdom and determination towards the completion of this work!

After the Almighty, I am immensely grateful and forever indebted to my supervisor Prof. Aditya Mittal whose scientific as well as philosophical acumen and erudition have always inspired me to confront as well as resolve challenges and excel in various aspects of my life. He has inculcated in me the belief that work is the highest form of worship. His constant guidance, not only as my advisor, but also as my local guardian, and his resolute support through meetings or over the call discussions have always served me as a beacon of hope and faith. I will continue to follow his ideals and principles throughout my life.

I am also immensely grateful to my scientific research committee members, the chairperson, Prof. Chinmoy Sankar Dey, external expert Prof. Prashant Mishra, and internal expert Prof. Manoj Balakrishna Menon for their constant suggestions and guidance which have borne fruit in the betterment of my work. I am sincerely thankful to all the faculty members of my department, especially Prof. Vivekanandan Perumal, Prof. Archana Chugh, Prof. Tapan K. Chaudhuri, Prof. Ashok K. Patel, Prof. Tapan K. Nayak, Prof. James Gomes, Prof. Bishwajit Kundu, Prof. Manideepa Banerjee, Prof. Anita Roy and Prof. Shilpi Minocha for their encouragement, support and guidance, including providing access to their labs, instruments and other facilities. I am immensely grateful to Dr. Tanushree Banerjee, Prof. Sharmila Chakraborty, and Prof. Krishnakali Basu Niyogi for shaping me during my master's and bachelor's programs, and for their invaluable guidance in helping me become who I am today. I am also grateful to the departmental staff members, Mohan Rao sir, Mini madam, Pushplata

madam, Pooja madam, Praveen sir, Kirshan sir, Devender sir, Pradeep sir, Rakesh sir, and Gyan sir of the PG section, IITD who have helped me in various aspects of my administrative work.

I am also grateful to the GATE-MoE, GoI for awarding me with a monthly research fellowship during my doctoral program.

I am immensely grateful to Abhishek sir of the IITD security unit. My stay at Girnar hostel was made pleasant and comfortable by the continuous support of the hostel caretaker, Sanjeev sir, the mess manager, Santosh sir, and mess staff members Mangal bhaiya, Sonu bhaiya, Girish bhaiya, Sachidananda bhaiya and Rajkumar bhaiya. I also extend my sincere thanks to Manoj, Shyam and Pardeep for their dedicated services at the night canteen.

I express my gratitude to all my lab members and friends, Dr. Prachi, Dr. Firdos, Dr. Pragya, Dr. Dinesh, Arinjoy, Akanksha, Abin, Deepak, Moumita, Arsalan, Dheeraj, Danish, Aman, Madhvi and Sandeep, for their constant support and guidance throughout various aspects of my doctoral journey. I am also thankful to my friends Dr. Ashish, Dr. Bibaswan, Dr. Debajit, Dr. Debashruti, Dr. Rajarshi (MD), Dr. Saurabh, Dr. Shankari, Dr. Sourav, Dr. Suvom, Dr. Subham (MD), Dr. Subho (MD), Dr. Sudeep, Dr. Stanzin, Dr. Tathagata, Dr. Upasak (MBBS), Dr. Vipul, Dr. Yogesh, Anurag, Arkaprovo, Deepali, Kaushik, Mayank, Manoj, Neha, Pankaj, Praveen, Ritesh, Rupam, Sanket, Shabih, Shraddha, Sibojyoti and Sushanth, for their constant company and unconditional support throughout my doctoral journey.

My deepest thanks to my beloved family, Roshni Pramanik, Jishnu Pramanik, Ragnvald Pramanik (Gablu), Vilgot Pramanik (Gogol) and Swarupa Mandal for their constant love, guidance, encouragement, understanding, reassurance and unconditional support. Although thanking them can never suffice, I express my deepest love and gratitude to them from the bottom of my heart. They have always believed in me and stood by me, come what may, irrespective of the geographical distance.

I also extend my sincere thanks and regards to my extended family, Chandrasekhar Mitra, Pratap Mitra, Benu Gopal Mitra, Indrani Debnath, Ranjika Debnath and their families, and Prof. Dr. Amita Giri (MD) for their constant love and support.

Lastly, I am forever indebted to my mother, Sarbani Pramanik, who has always been my beacon of strength, indomitable spirit and inspiration. I deeply miss my father Late Prof. Dr. Raghunath Pramanik. If tears could build a staircase and memories a lane, I would walk right up to you to bring you back again. You live within me, father!

ABSTRACT

Despite ~100 years of literature on diabetes mellitus, and substantial reports on glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells, cellular-level understanding of many fundamental aspects remains elusive. One such unknown aspect is the reason(s) behind the glucose specificity of GSIS, which was/is believed that ATP generated from glucose metabolism is primarily responsible for GSIS. However, this ignores two well-established facts: (i) ATP molecules produced from other metabolic sources are indistinguishable from those produced via glucose metabolism, while ATP production saturates beyond specific levels of extracellular glucose concentrations, and (ii) the rate of intracellular glucose transport by glucose transporter proteins (GLUTs) surpasses glucose phosphorylation rate by 100 times. Additionally, previous studies have primarily focused on the role of extracellular glucose towards inciting GSIS. Thus, considering this particularly fascinating yet unaddressed possibility, we wondered whether intracellular, but unphosphorylated glucose molecules somehow contribute towards the glucose specificity of GSIS. Through differential gene expression analyses of the well-established cell culture model system of Mouse Insulinoma 6 (MIN6) cells exposed to high extracellular glucose concentration (high EGC), our lab had recently identified the upregulation of 3 secretion-specific genes corresponding to the proteins KIF11, ATP6V0A4, and CACNB4, from whole transcriptome data. Using glucose as a ligand, we carried out rigorous computational investigations with the 3 test proteins, which will require further wet experimental validation. The glucose binding scores (in kcal/mol) obtained were also compared with binding scores for positive controls (GCK and GLUT2), along with negative controls (RPA1, KU70-80, POLA1, ACAA1A, POLR1A), respectively. Binding affinity scores of glucose molecules for the 3 proteins were found to be closer to positive controls. Therefore, we report glucose binding ability of 3 secretion-related proteins, and a possible direct role of intracellular glucose molecules in GSIS.

Molecular mechanisms leading to onset of Type 1 diabetes mellitus (T1DM) through viral infections are limited, although clinical correlations between the onset of T1DM and viral infections are frequently reported. Hence, another important aspect was to obtain mechanistic insights into virus-induced T1DM at a cellular level. Thus, we extended our investigations towards obtaining mechanistic interactions of a Chikungunya viral peptide (we have termed it as CV1) towards PAC1R (PAC1 receptor), the receptor of an established GSIS regulator, Pituitary Adenylate Cyclase-Activating Polypeptide 38 (PACAP38 or P38). Using the recently reported Cryo-EM structure (PDB ID: 6M1I) of P38-bound PAC1R, as a positive control through AlphaFold 3 and through additional computational investigations, we found that CV1-PAC1R structure resembles P38-PAC1R, but with distinct binding in a nearby region. This observed conformational plasticity in binding explains and validates synergistic, but nonlinear modulations of GSIS by β-cells in presence of CV1 and P38, as obtained through previous wet experimental studies in our lab. We also calculated the equilibrium dissociation constant (K_d value) for the CV1-PAC1R complex using P38-PAC1R complex.

Having obtained mechanistic insights into 2 important aspects of GSIS through computational studies, we extended the obtained relationship of 'glucose-KIF11' towards understanding a cell biological aspect of GSIS, particularly the role of cell cycle in regulating GSIS, especially since KIF11 is an established cell cycle regulator. Using the cell culture model system of MIN6 cells, we found that cell cycle synchronization at the G2/M phase results in an enhanced GSIS, while total cellular RNA, cellular protein and supernatant protein remain constant, respectively. Interestingly, glucose interacts with the established residues located in an allosteric site of KIF11 which are known drug targets towards KIF11 inhibition. Thus, the binding of glucose to KIF11 might have potential functional implications towards KIF11 activity that remain to be explored, especially on the context of enhanced GSIS, underscoring the glucose specificity of GSIS. This also highlights the differential regulation of cellular secretory systems through

the cell cycle. Additionally, the observed increase in GSIS during G2/M phase arrest might provide first-of-its-kind cellular-level link to the clinically observed ‘honeymoon phase’ in T1DM and its variants.

सारांश

डायबिटीज मेलिटस पर लगभग 100 साल के लिटरेचर और पैंक्रियाटिक β-सेल्स में ग्लूकोज-स्टिम्युलेटेड इंसुलिन सिक्रीशन (GSIS) पर कई रिपोर्ट होने के बावजूद, कई बुनियादी पहलुओं की सेलुलर-लेवल पर समझ अभी भी अधूरी है। ऐसा ही एक अज्ञात पहलू GSIS की ग्लूकोज स्पेसिफिसिटी (विशिष्टता) का कारण है; माना जाता रहा है कि ग्लूकोज मेटाबॉलिज्म से बनने वाला ATP मुख्य रूप से GSIS के लिए जिम्मेदार है। हालाँकि, यह दो स्थापित तथ्यों को नजरअंदाज करता है: (i) अन्य मेटाबोलिक स्रोतों से बनने वाले ATP मॉलिक्यूल ग्लूकोज मेटाबॉलिज्म से बनने वाले ATP से अलग नहीं होते, जबकि एक्स्ट्रासेलुलर ग्लूकोज कंसंट्रेशन के एक निश्चित स्तर से ऊपर ATP का उत्पादन सैचुरेट हो जाता है, और (ii) ग्लूकोज ट्रांसपोर्टर प्रोटीन (GLUTs) द्वारा इंट्रासेलुलर ग्लूकोज ट्रांसपोर्ट की दर ग्लूकोज फॉस्फोराइलेशन दर से 100 गुना अधिक होती है। इसके अलावा, पिछली स्टडीज़ मुख्य रूप से GSIS को शुरू करने में एक्स्ट्रासेलुलर ग्लूकोज की भूमिका पर केंद्रित रही हैं। इसलिए, इस दिलचस्प लेकिन अनसुलझी संभावना को देखते हुए, हमने सोचा कि क्या इंट्रासेलुलर, लेकिन नॉन-फॉस्फोराइलेटेड ग्लूकोज मॉलिक्यूल किसी तरह GSIS की ग्लूकोज स्पेसिफिसिटी में योगदान करते हैं। हाई एक्स्ट्रासेलुलर ग्लूकोज कंसंट्रेशन (high EGC) के संपर्क में आने वाले माउस इंसुलिनोमा 6 (MIN6) सेल्स के अच्छी तरह से स्थापित सेल कल्चर मॉडल सिस्टम के डिफरेंशियल जीन एक्सप्रेशन एनालिसिस के माध्यम से, हमारी लैब ने हाल ही में पूरे ट्रांसक्रिप्टोम डेटा से KIF11, ATP6V0A4 और CACNB4 प्रोटीन से संबंधित 3 सिक्रीशन-स्पेसिफिक जीनों के अपरेगुलेशन की पहचान की थी। ग्लूकोज को लिगैंड के रूप में इस्तेमाल करते हुए, हमने 3 टेस्ट प्रोटीन के साथ गहन कम्प्यूटेशनल जांच की, जिसके लिए आगे वेट एक्सपेरिमेंटल वैलिडेशन की आवश्यकता होगी। प्राप्त ग्लूकोज बाइंडिंग स्कोर (kcal/mol में) की तुलना पॉजिटिव कंट्रोल (GCK और GLUT2) और नेगेटिव कंट्रोल (RPA1, KU70-80, POLA1, ACAA1A, POLR1A) के बाइंडिंग स्कोर से भी की गई। 3 प्रोटीन के लिए ग्लूकोज मॉलिक्यूल की बाइंडिंग एफिनिटी स्कोर पॉजिटिव कंट्रोल के

करीब पाए गए। इसलिए, हम 3 सिक्रीशन-संबंधित प्रोटीन की ग्लूकोज बाइंडिंग क्षमता और GSIS में इंट्रासेलुलर ग्लूकोज मॉलिक्यूल की संभावित प्रत्यक्ष भूमिका की रिपोर्ट करते हैं।

वायरल संक्रमण के माध्यम से टाइप 1 डायबिटीज मेलिटस (T1DM) की शुरुआत के आणविक तंत्र सीमित हैं, हालांकि T1DM की शुरुआत और वायरल संक्रमण के बीच नैदानिक संबंध अक्सर रिपोर्ट किए जाते हैं। इसलिए, एक और ज़रूरी पहलू सेलुलर लेवल पर वायरस से होने वाले T1DM के काम करने के तरीके (मैकेनिस्टिक जानकारी) को समझना था। इस तरह, हमने अपनी रिसर्च को आगे बढ़ाते हुए चिकनगुनिया वायरल पेप्टाइड (जिसे हमने CV1 नाम दिया है) और PAC1R (PAC1 रिसेप्टर) के बीच होने वाली इंटरैक्शन को समझने की कोशिश की। PAC1R एक स्थापित GSIS रेगुलेटर, पिट्यूटरी एडेनिलेट साइक्लेज-एक्टिवेटिंग पॉलीपेप्टाइड 38 (PACAP38 या P38) का रिसेप्टर है। AlphaFold 3 और अतिरिक्त कंप्यूटेशनल रिसर्च के ज़रिए, P38-बाउंड PAC1R के हाल ही में रिपोर्ट किए गए Cryo-EM स्ट्रक्चर (PDB ID: 6M1I) को पॉजिटिव कंट्रोल के तौर पर इस्तेमाल करके, हमने पाया कि CV1-PAC1R स्ट्रक्चर P38-PAC1R जैसा ही है, लेकिन इसमें पास के इलाके में अलग तरह की बाइंडिंग होती है। बाइंडिंग में देखी गई यह कन्फर्मेशनल प्लास्टिसिटी (आकार में बदलाव की क्षमता) CV1 और P38 की मौजूदगी में β-सेल्स द्वारा GSIS के सिनर्जिस्टिक (मिलकर काम करने वाले) लेकिन नॉन-लीनियर मॉड्यूलेशन को समझाती और सही साबित करती है, जैसा कि हमारी लैब में पहले किए गए वेट एक्सपेरिमेंटल स्टडीज़ में देखा गया था। हमने P38-PAC1R कॉम्प्लेक्स का इस्तेमाल करके CV1-PAC1R कॉम्प्लेक्स के लिए इक्विलिब्रियम डिसोसिएशन कांस्टेंट (Kd वैल्यू) की भी गणना की।

कंप्यूटेशनल स्टडीज़ के ज़रिए GSIS के 2 अहम पहलुओं के बारे में मैकेनिस्टिक जानकारी हासिल करने के बाद, हमने 'ग्लूकोज़-KIF11' के संबंध को GSIS के सेल बायोलॉजिकल पहलू को समझने की दिशा में आगे बढ़ाया, खासकर GSIS को रेगुलेट करने में सेल साइकिल की भूमिका को समझने के लिए, क्योंकि KIF11 एक स्थापित सेल साइकिल रेगुलेटर है। MIN6 सेल्स के सेल कल्चर मॉडल सिस्टम का इस्तेमाल

करके, हमने पाया कि G2/M फेज़ में सेल साइकिल सिंक्रोनाइज़ेशन से GSIS बढ़ जाता है, जबकि कुल सेलुलर RNA, सेलुलर प्रोटीन और सुपरनेटेंट प्रोटीन क्रमशः स्थिर रहते हैं। दिलचस्प बात यह है कि ग्लूकोज़ KIF11 की एलोस्टेरिक साइट पर मौजूद उन स्थापित रेसिड्यूज़ के साथ इंटरैक्ट करता है जिन्हें KIF11 इनहिबिशन के लिए ड्रग टारगेट के तौर पर जाना जाता है। इस तरह, KIF11 के साथ ग्लूकोज़ की बाइंडिंग का KIF11 की एक्टिविटी पर संभावित फंक्शनल असर हो सकता है, जिसे अभी और समझने की ज़रूरत है, खासकर बढ़े हुए GSIS के संदर्भ में, जो GSIS की ग्लूकोज़ स्पेसिफिसिटी को रेखांकित करता है। यह सेल साइकिल के ज़रिए सेलुलर सिक्रेटरी सिस्टम के अलग-अलग तरह के रेगुलेशन को भी उजागर करता है। इसके अलावा, G2/M फ़ेज़ अरेस्ट के दौरान GSIS में देखी गई बढ़ोतरी, T1DM और इसके प्रकारों में क्लिनिकली देखे जाने वाले 'हनीमून फ़ेज़' के साथ सेलुलर स्तर पर अपनी तरह का पहला संबंध बता सकती है।

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ABBREVIATIONS

GSIS: Glucose-stimulated insulin secretion

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

MIN6: Mouse Insulinoma 6

EGC: Extracellular glucose concentration

EGCs: Extracellular glucose concentrations

ADT: AutoDockTools 4.2.6

DS: Discovery Studio

CP: Cellular protein

SP: Supernatant protein

ELISA: Enzyme-Linked Immunosorbent Assay

KIF11: Kinesin family member 11

CACNB4: Voltage dependent L-type calcium channel subunit beta-4

ATP6V0A4: V-type proton ATPase 116 kDa subunit a isoform 4

GCK: Glucokinase

GLUT2: Solute carrier family 2, facilitated glucose transporter member 2

RPA1: Replication protein A 70 kDa DNA-binding subunit

POLA1: DNA polymerase alpha catalytic subunit

ACAA1A: 3-ketoacyl-CoA thiolase A, peroxisomal

POLR1A: DNA-directed RNA polymerase I subunit RPA1

PDB: Protein Data Bank

RMSD: Root Mean Squared Deviation

K_d: Equilibrium disassociation constant

$\Delta G°$: Gibb's free energy

CV1: a Chikungunya viral peptide

PACAP38: Pituitary Adenylate Cyclase-Activating Polypeptide 38 (PACAP38 or P38)

PAC1R: PAC1 receptor or PACAP receptor

NC: Negative control (scrambled peptide)

DMEM: Dulbecco's modified eagle's medium

FBS: Fetal bovine serum

KRBH: Kreb's-Ringer Bicarbonate buffer with HEPES

HEPES: N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

EDTA: Ethylenediaminetetraacetic acid

NaCl: Sodium chloride

Na_2HPO_4: Disodium phosphate

$MgCl_{2:}$ Magnesium chloride

$CaCl_{2:}$ Calcium chloride

KCl: Potassium chloride

HEPES: N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

KH_2PO_4: Monopotassium phosphate

$NaHCO_3$: Sodium bicarbonate

PFA: Paraformaldehyde

BSA: Bovine serum albumin

PBS: Phosphate buffer saline

DAPI: 4,6-diamidino-2-phenylindole dihydrochloride

PI: Propidium iodide

RNA: Ribonucleic acid

RPM: Revolutions per minute