CYTOSKELETON BIOLOGY IN NORMAL AND PATHOLOGICAL MEGAKARYOPOIESIS

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KUSUMA SCHOOL OF BIOLOGICAL SCIENCES

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CYTOSKELETON BIOLOGY IN NORMAL AND PATHOLOGICAL MEGAKARYOPOIESIS

By

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KUSUMA SCHOOL OF BIOLOGICAL SCIENCES

Submitted

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CERTIFICATE

This is to certify that the thesis titled, "**Cytoskeleton biology in normal and pathological megakaryopoiesis"**, being submitted by Mr Saurabh Shrivastva to the Indian Institute of Technology, Delhi for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research carried out by him, which has been prepared under my supervision and guidance in conformity of the rules and regulations of the Indian Institute of Technology, Delhi, India. The results described in this study have not been submitted in part or in full to any other university for the award of any degree or diploma.

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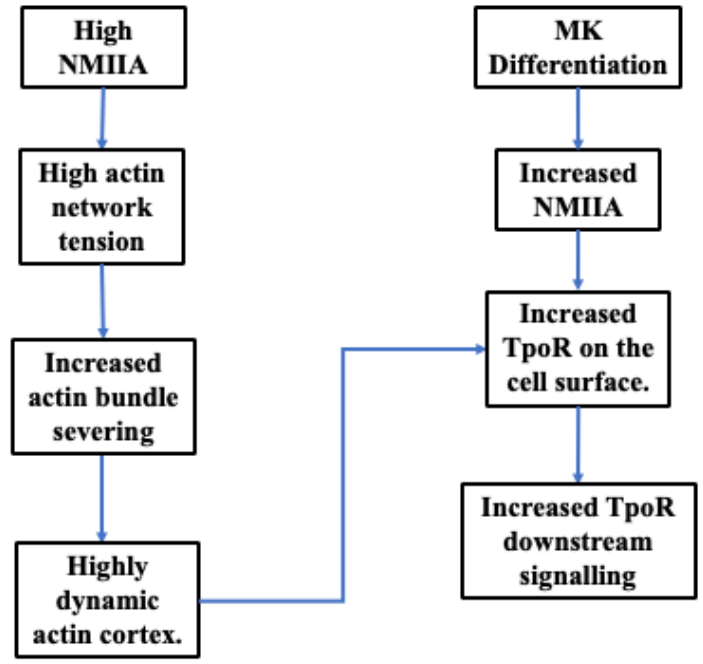
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ABSTRACT

Megakaryocytes (MK) are the cells that produce platelets, which are essential for hemostasis. MK differentiation takes place in three stages: 1. Rapid proliferation phase, 2. Endomitosis 3. Proplatelet formation. Cytoskeletal rearrangement plays crucial roles during all these stages. Specifically, during the last stage, MKs extend long protrusions, and platelets are formed from the ends of these protrusions. Microtubules play crucial roles in platelet extension, and the actin cytoskeleton is essential for branching proplatelets. The actin cytoskeleton is mainly regulated by actin-binding proteins, which help in polymerisation and depolymerisation. During the dynamic process of proplatelet branching motor proteins including Non-muscle myosin II (NMII) which bind to actin filaments generate the required forces. During megakaryocyte (MK) differentiation, the levels of NMII isoforms have been shown to change, with NMIIA levels increasing and NMIIB levels decreasing.

Along with differential expression of NMII isoforms, their distribution also changes. NMIIA and NMIIB both localise to the cleavage furrow of the MKs during the proliferation phase. However, at the commencement of the endomitosis, NMIIA does not localise to the cleavage furrow, and NMIIB is knocked down via RUNX1-mediated silencing. This lack of NMIIA localisation results in cytokinesis failure. We explored the roles of NMII isoforms during MK development. One such aspect is the surface expression of the thrombopoietin receptor (TpoR). TpoR is crucial for platelet production, and its surface expression is regulated by its ligand, thrombopoietin (TPO). In our study, we found that the traffic, surface expression, and signalling of TpoR are augmented by NMIIA expression, which was attributed to NMIIA-dependent remodelling of the cortical actin network. Cell-scale actin remodelling requires rapid depolymerisation beyond that generated by actin severing proteins such as cofilin and gelsolin. Myosin activity restricts the length of actin filaments, but it was unknown if NMII isoforms (NMIIA and IIB) could generate cell-scale actin dynamics. We found higher network tension and faster network rupture with NMIIA than NMIIB in linear actomyosin network simulation. Live cell imaging in COS7 cells showed many actin bundle severing events in the presence of NMIIA, while NMIIB produced fewer actin bundle severing events. NMIIA overexpression resulted in the formation of peripheral actin arcs and long actin fibres, which were absent in the cells expressing NMIIB. The

actin network dynamics increased with the increasing expression of NMIIA but not NMIIB, establishing NMIIA as the predominant generator of cell-scale actin dynamics. ROCK inhibition, which alters the cortical actin network, reduced TpoR surface expression and signalling. This indicated that megakaryopoiesis-dependent alteration in NMIIA expression contributed to enhanced actin dynamics and severing, resulting in enhanced TpoR surface expression and signaling.



Schematic diagram depicting the effects of actomyosin network regulation on thrombopoietin receptor surface expression and signalling.

सार

मेगाकेरियोसाइट्स (एमके) वे कोशिकाएँ हैं जो प्लेटलेट्स का उत्पादन करती हैं, जो रक्तस्तम्भन के लिए आवश्यक हैं। एमके विभेदन तीन चरणों में होता है: 1. तीव्र प्रसार चरण, 2. एंडोमाइटोसिस 3. प्रोप्लेटलेट निर्माण। इन सभी चरणों के दौरान साइटोस्केलेटल पुनर्व्यवस्था महत्वपूर्ण भूमिका निभाती है। विशेष रूप से, अंतिम चरण के दौरान, एमके लंबे उभारों का विस्तार करते हैं, और इन उभारों के सिरों से प्लेटलेट्स बनते हैं। प्लेटलेट्स के विस्तार के दौरान सूक्ष्मनलिकाएँ महत्वपूर्ण भूमिका निभाती हैं, और एक्टिन साइटोस्केलेटन इन प्रोप्लेटलेट्स की शाखाओं के लिए आवश्यक है। एक्टिन साइटोस्केलेटन मुख्य रूप से एक्टिन-बाइंडिंग प्रोटीन द्वारा नियंत्रित होता है, जो पोलीमराइजेशन और डीपोलीमराइजेशन में मदद करते हैं। प्रोप्लेटलेट शाखाओं के गतिशील प्रक्रिया के लिए, मोटर प्रोटीन और गैर-मांसपेशी मायोसिन II, जो एक्टिन तंतुओं से बंधते हैं, आवश्यक बल उत्पन्न करते हैं। मेगाकैरियोसाइट्स (एमके) के विभेदन के दौरान, गैर-मांसपेशी मायोसिन II (एनएमआईआई) आइसोफॉर्म के स्तर में परिवर्तन देखा गया है, जहां एनएमआईआईबी का स्तर घटता है और एनएमआईआईए का स्तर बढ़ता है।

एनएमआईआई आइसोफॉर्म की विभेदक अभिव्यक्ति के साथ-साथ, उनके वितरण में भी परिवर्तन देखा गया है। एनएमआईआईए और एनएमआईआईबी दोनों प्रसार चरण के दौरान एमके के विदलन खांचे में स्थानीयकृत होते हैं; हालांकि, एंडोमाइटोसिस की शुरुआत में, एनएमआईआईए विदलन खांचे में स्थानीयकृत नहीं होता है, और एनएमआईआईबी को आरयूएनएक्स1-मध्यस्थ मौन के माध्यम से नष्ट कर दिया जाता है। एनएमआईआई स्थानीयकरण की इस कमी के परिणामस्वरूप साइटोकाइनेसिस विफल हो जाता है। हमने एमके विकास के दौरान एनएमआईआई आइसोफॉर्म की भूमिका का पता लगाया। ऐसा ही एक पहलू थ्रोम्बोपोइटिन रिसेप्टर (टीपीओआर) की सतही अभिव्यक्ति है। टीपीओआर प्लेटलेट उत्पादन के लिए महत्वपूर्ण है, और इसकी सतही अभिव्यक्ति इसके लिगैंड, थ्रोम्बोपोइटिन (टीपीओ) द्वारा नियंत्रित होती है। हमारे अध्ययन में, हमने पाया कि TpoR का ट्रैफ़िक, सतही अभिव्यक्ति और सिग्नलिंग NMIIA की अभिव्यक्ति द्वारा संवर्धित होते हैं, जिसका श्रेय कॉर्टिकल एक्टिन नेटवर्क के NMIIA-निर्भर रीमॉडलिंग को दिया गया। कोशिका-स्तरीय एक्टिन रीमॉडलिंग के लिए कोफिलिन और जेल्सोलिन जैसे एक्टिन विच्छेदन प्रोटीन द्वारा उत्पन्न डीपॉलीमराइजेशन से परे तीव्र डीपॉलीमराइजेशन की आवश्यकता होती है। मायोसिन गतिविधि एक्टिन तंतुओं की लंबाई को सीमित करती है, लेकिन यह अज्ञात था कि क्या गैर-मांसपेशी मायोसिन II आइसोफॉर्म (NMIIA और IIB) कोशिका-स्तरीय एक्टिन गतिशीलता उत्पन्न कर सकते हैं। हमने रैखिक एक्टोमायोसिन नेटवर्क सिमुलेशन में NMIIB की तुलना में NMIIA के साथ उच्च नेटवर्क तनाव और तेज़ नेटवर्क टूटना पाया। COS7 कोशिकाओं में लाइव सेल इमेजिंग ने NMIIA की उपस्थिति में कई एक्टिन बंडल विच्छेदन घटनाओं को दिखाया। NMIIA की अभिव्यक्ति बढ़ने के साथ एक्टिन नेटवर्क की गतिशीलता बढ़ी, लेकिन NMIIB की नहीं, जिससे NMIIA कोशिका-स्तरीय एक्टिन गतिशीलता का प्रमुख जनरेटर बन गया। ROCK अवरोध, जो कॉर्टिकल एक्टिन नेटवर्क को बदल देता है, TpoR सतह अभिव्यक्ति और संकेतन को कम कर देता है। इससे पता चलता है कि NMIIA अभिव्यक्ति में मेगाकैरियोपोइजिस-निर्भर परिवर्तन ने एक्टिन गतिशीलता और पृथक्करण को बढ़ाया, जिसके परिणामस्वरूप TpoR सतह अभिव्यक्ति और संकेतन में वृद्धि हुई।

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
µg	Microgram
mg	Milligram
g	Gram
ng	Nanogram
µL	Microliter
mL	Milliliter
L	Liter
µM	Micromolar
mM	Millimolar
M	Molar
nM	Nanomolar
%	Percentage
°C	Degree Celsius
rpm	Revolutions per minute
×g	Relative centrifugal force
pH	Potential of hydrogen
t½	Half-life of recovery
h	Hour
min	Minute
s	Second
kDa	Kilodalton
nm	Nanometer
λ	Wavelength
RT	Room temperature
v/v	Volume per volume
w/v	Weight per volume
AKT	Protein kinase B
CALR	Calreticulin
CFU-GEMM	Colony-forming unit–granulocyte, erythroid, monocyte, megakaryocyte
CFU-MK	Colony-forming unit–megakaryocyte
CMK	Human megakaryoblastic leukemia cell line
COS7	African green monkey kidney fibroblast-like cell line
CR	Cortical rigidity
DAB2	Disabled homolog 2
DCB	dihydrocytochalasin B
DMEM	Dulbecco's Modified Eagle Medium
ELC	Essential light chain
EGFP	Enhanced green fluorescent protein
ERK	Extracellular signal-regulated kinase
F-actin	Filamentous actin
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FRAP	Fluorescence recovery after photobleaching

GFP	Green fluorescent protein
GMP	Granulocyte–monocyte progenitor
HBSS	Hanks' balanced salt solution
HEL	Human erythroleukemia cell line
HMM	Heavy meromyosin
HSC	Hematopoietic stem cell
IHM	Interacting-heads motif
IMS	Imaging medium solution
INCENP	Inner centromere protein
JAK2	Janus kinase 2
LSK	Lin^- Sca^+ c-Kit^+ cells
LT-HSC	Long-term hematopoietic stem cell
MAPK	Mitogen-activated protein kinase
MEP	Megakaryocyte–erythroid progenitor
MK	Megakaryocyte
MKB	Megakaryoblast
MKLP1	Mitotic kinesin-like protein 1
MKLP2	Mitotic kinesin-like protein 2
MK-P	Megakaryocyte progenitor
MLCK	Myosin light chain kinase
MPF	M-phase promoting factor
MPP	Multipotent progenitor
MYH14	Myosin heavy chain 14
NMHC	Non-muscle myosin heavy chain
NMII	Non-muscle myosin II
NMIIA	Non-muscle myosin II A
NMIIB	Non-muscle myosin II B
NMIIC	Non-muscle myosin II C
NR1	Nuclear receptor subfamily 1
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PRC1	Protein regulator of cytokinesis 1
RHOA	Ras homolog family member A
RLC	Regulatory light chain
ROCK	Rho-associated protein kinase
RPMI	Roswell Park Memorial Institute medium
RUNX1	Runt-related transcription factor 1
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
ST-HSC	Short-term hematopoietic stem cell
TGN	Trans-Golgi network
TPO	Thrombopoietin
TpoR	Thrombopoietin receptor (c-MPL)

vMPL	Viral myeloproliferative leukemia virus oncogene
vWF	von Willebrand factor
WASP	Wiskott–Aldrich syndrome protein
WT	Wild type