Integrin linked kinase regulates endosomal recycling of N-cadherin in melanoma cells

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ABSTRACT

Malignant transformation is characterized by a phenotype “switch” from E- to N-cadherin – a major hallmark of epithelial to mesenchymal transition (EMT). The increased expression of N-cadherin is commonly followed by a growing capacity for migration as well as resistance to apoptosis. Integrin Linked Kinase (ILK) is a key molecule involved in EMT and progression of cancer cells. ILK is known as a major signaling mediator involved in cadherin switch, but the specific mechanism through which ILK modulates N-cadherin expression is still not clear.

Studies were carried out on human melanoma WM793 and 1205Lu cell lines. Expression of proteins was analyzed using PCR and Western Blot; siRNA transfection was done for ILK. Analysis of cell signaling pathways was monitored with phospho-specific antibodies. Subcellular localization of protein was studied using the ProteoExtract Subcellular Kit and Western blot analysis.

Our data show that ILK knockdown by siRNA did suppress N-cadherin expression in melanoma, but only at the protein level. The ILK silencing-induced decrease of N-cadherin membranous expression in melanoma highlights the likely crucial role of ILK in the coordination of membrane trafficking through alteration of Rab expression. It is essential to understand the molecular mechanism of increased N-cadherin expression in cancer to possibly use it in the search of new therapeutic targets.

1. Introduction

A reduction or loss in the expression of E-cadherin and induction of N-cadherin expression has been recognized as an important primary event in melanoma progression [1]. The process, also known as the cadherin switch, is a hallmark of the Epithelial Mesenchymal Transition (EMT) process, which is probably required for epithelial cells to adopt mesenchymal characteristics [2]. In spite of the fact, that melanoma develops from cells of neural crest cells - melanocytes – the hallmarks of EMT play an essential role in progression of melanoma. EMT is a phenomenon associated with downregulation of E-cadherin and upregulation of N-cadherin what contribute to the metastatic potential of melanocytes during early step in melanoma development. N-cadherin is involved in the process allowing for dissemination of melanoma cells from the original tissue to distant organs; the expression of N-cadherin is responsible for increased proliferation and invasion of melanoma cells [3,4]. We have previously showed that in melanoma cells, EMT markers are dependent on ILK activity [5]. ILK is commonly acknowledged as one of the important cellular signaling kinases [6]. Interacting with other elements of downstream signaling pathways, ILK regulates cell proliferation, cell-extracellular matrix interaction, apoptosis or cell migration. The role of ILK in the control of N-cadherin expression in melanoma is not well understood. We showed that silencing of ILK in melanoma cells resulted in the decrease of an important EMT marker – N-cadherin expression on the protein level. EMT transition involves genetic and epigenetic changes as well as an alteration in protein transcription, translation, and post-translational modification, and – unexpectedly – alteration in membrane trafficking [7]. A wealth of evidence points to endocytosis and recycling as important factors in the regulation of membranous expression of N-cadherin [8–10]. Endocytosis regulates a variety of cellular functions including proteins internalization from the surface, recycling of integrins, receptor and adhesion molecules, as well as protein degradation. The receptor clearance defines the specificity with which a cell can react to extra-cellular stimuli. This shows that endocytosis may govern many cellular signaling processes, as well as EMT. Though endocytosis and cellular signaling have earlier been described as distinct processes, endocytosis is currently seen as a phenomenon associated with many aspects of cellular signaling [11,12]. Ras-associated binding (Rab)-GTPases are members of the Ras family of small GTPases and they are responsible...
for regulation of the expression of surface receptors [13–15]. Rab proteins (5, 4, 11 and 7), which are engaged in endocytosis, recycling, and lysosomal degradation, play an essential role in the regulation of membranous expression of N-cadherin [16,17]. Endosomal motility depends also on the interaction with microtubules and actin cytoskeleton. An important scaffold protein that modulates both actin and microtubule dynamics is ILK [18]. We previously showed that ILK can regulate the N-cadherin expression through an unknown mechanism only on the protein level in melanoma [5], and can also instigate cadherin switch of bladder cancer through transcriptional regulation by Twist-1, Zeb-1, Snail and PARP-1 [19].

This article presents the results of our recent study on the mechanism of the ILK silencing-induced decrease of N-cadherin membranous expression in melanoma, highlighting the possibly crucial role of ILK in the coordination of endocytosis by regulation of Rab expression.

2. Materials and methods

2.1. Cell culture

Human melanoma cell lines: WM793 (vertical growth phase – VGP) and 1205Lu (metastatic) cell line was derived from lung metastases of WM793 after subcutaneous injection into immunodeficient mice [20,21]. 1205Lu cells are highly invasive and exhibit spontaneous metastasis to lung and liver. Cell lines are used in the search for understanding the molecular mechanism of cancer signaling despite the fact that cultured cells show instability of their metastatic phenotype during loss of in vivo microenvironment. Spontaneous transformation towards a more malignant phenotype may occur in RGP primary melanoma cells but cells selected in animal models (like 1205Lu) have a relatively stable metastatic phenotype. The comparison of melanoma cell lines to melanoma tumors by Vincent and Postovita showed that melanoma cell lines correspond transcriptionally to their respective tumors. The majority of genes have a similar expression in cell lines and tumors, but the biggest differences were observed in respect to immune genes [22]. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were obtained from the ESTDAB Melanoma Cell Bank (Tubingen, Germany). Chloroquine was purchased from Sigma and was used in final concentration 50 μM. Human endothelial aortic cells (HEAC) obtained from Małgorzata Lasota (UJ CM, Faculty of Medicine, Department of Transplantation, Poland) were cultured in endothelial medium EBM-2 (CC-3156; Lonza). For siRNA transfection and endothelial adhesion or transendothelial migration assays serum-free medium Opti-MEM (Gibco) was used. All cell lines are examined for mycoplasma infection as per the producer’s protocol; afterwards, preparation of cDNA was subsequently transferred onto a PVDF membrane. Antibodies against: ILK #611803, AKT #610861, GSK-3β #610201, phospho-GSK-3β(Y216)#612,312, vimentin #550513 (all Transduction Laboratories, BD), phospho-AKT(S473) #9271, phospho-GSK-3β(S9) #9336, Src #2109, phosphor-Src (Y416) #6943, phosphor-p44/42 (Erk1/2) (T202/Y204) #4370, Rabo4 #2167, Rab5 #3547, Rab9 #5118, Rab11 #5589, Rab11FIP1 #12849 (all Cell Signaling Technology), N-cadherin #BTA7 (R&D) and β-actin #A1978, ZEB1 #SAB35005144 (Sigma), SNAIL #AP2054a (ABGENT), Calnexin #20883, HSP-90 #CA1023 (all Calbiochem) were used to detect indicated proteins in dilution 1:1000. The presence of the primary antibody was revealed with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 (Cell Signaling Technology) and visualization with an enhanced chemiluminescence detection system (Bio-Rad), as previously described. β-actin served as a loading control. All immunoblots were stripped with stripping buffer containing 25 mM glycine-HCl, pH 2, 1% (wt / v) SDS for 30 min, and incubated in antibody against β-actin (Sigma), which served as a loading control. Molecular masses were estimated by reference to standard proteins (Fermentas). To obtain quantitative results, immunoblots were scanned using the public domain ImageJ software (National Institute of Health). Each data point was normalized against its corresponding β-actin data point.

2.2. siRNA transfection

Melanoma cells were grown until 60% confluency was reached and then transfected using INTERFERin™ as per the manufacturer’s protocol (Polyplus Transfection) with three different 21 bp double-stranded siRNA molecules specifically targeting the ILK (Ambion ID#288570; ID#145116; ID#145117) or a control nonsilencing sequence (Ambion ID#4611). WM793 cells were in each case transfected with 60 nM and 1205Lu cells with 80 nM siRNA, respectively. As transfection control, melanoma cells were transfected 19 bp scrambled sequence with 3′dT overhangs. The sequences have no significant homology to any known human gene sequences. Since there were no differences between untransfected cells and silenced negative control cells (not shown), the latter were selected as control cells in other experiments. After 24 h, the medium was replaced with a fresh one and cells were grown for an additional 24 h period (48 h post-transfection) prior to analysis.

2.3. Preparation of cytoplasmic, membrane, nuclear and cytoskeletal cell lysates

Cytoplasmic, membrane, nuclear, and cytoskeletal extracts were prepared using the ProteoExtract® Subcellular Proteome Extraction Kit (MERC Millipore) as per the producer’s protocol for frozen cell pellet in amount 10^6 cells and afterwards equal amounts of protein were used for immunoblots analysis.

2.4. Western blot analysis

Cells were lysed in sample buffer (0.0625 M Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercapto-ethanol). Cell lysates containing equal amounts of protein were separated on 10% SDS-PAGE gels, and subsequently transferred onto a PVDF membrane. Antibodies against: ILK #611803, AKT #610861, GSK-3β #610201, phospho-GSK-3β(Y216)#612,312, vimentin #550513 (all Transduction Laboratories, BD), phospho-AKT(S473) #9271, phospho-GSK-3β(S9) #9336, Src #2109, phosphor-Src (Y416) #6943, phosphor-p44/42 (Erk1/2) (T202/Y204) #4370, Rabo4 #2167, Rab5 #3547, Rab9 #5118, Rab11 #5589, Rab11FIP1 #12849 (all Cell Signaling Technology), N-cadherin #BTA7 (R&D) and β-actin #A1978, ZEB1 #SAB35005144 (Sigma), SNAIL #AP2054a (ABGENT), Calnexin #20883, HSP-90 #CA1023 (all Calbiochem) were used to detect indicated proteins in dilution 1:1000. The presence of the primary antibody was revealed with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 (Cell Signaling Technology) and visualization with an enhanced chemiluminescence detection system (Bio-Rad), as previously described. β-actin served as a loading control. All immunoblots were stripped with stripping buffer containing 25 mM glycine-HCl, pH 2, 1% (wt / v) SDS for 30 min, and incubated in antibody against β-actin (Sigma), which served as a loading control. Molecular masses were estimated by reference to standard proteins (Fermentas). To obtain quantitative results, immunoblots were scanned using the public domain ImageJ software (National Institute of Health). Each data point was normalized against its corresponding β-actin data point.

2.5. RNA extraction, cDNA synthesis and RT-PCR analysis

RNA extraction was prepared using the RNAqueus-Micro (Ambion) as per the producer’s protocol; afterwards, preparation of cDNA was prepared using NqDART RT kit as per the manufacturer’s protocol (EURx). RT-PCR reaction was carried out with Color OpyiTaq PCR Master Mix (EURx). The temperature profile of RT-PCR amplification consisted of activation of Taq polymerase at 95 °C for 4 min, denaturation of cDNA at 95 °C for 30–45 s, applying primer annealing temperature for 30–40 s, elongation at 72 °C for 40–60 s for the following 30 cycles and completion of the process by the extension step for 10 min. The following specific primers were used: GAPDH; (59 °C, 30 s): 5′CACGCGCTCAGGCTTGTCACAT 3′ and 5′CTGCTGTTCTGGTTGGA TGGC3′; N-CADHERIN; (60 °C, 40 s): 5′GTGCCATTAGGCAAGGGAATTCGACG3′ and. 5′CGAGGATACCTACCTTGCCTGGC 3′; E-CADHERIN; (60 °C, 40 s): 5′GCCAAGCAGCAGTACATTTCTACAGG3′ and. 5′GTCGTTCTCAAGTCTGCTCAAATCC3′;

The PCR reaction products were separated electrophoretically on 2% agarose gel and visualized with ethidium bromide.

2.6. Endothelial adhesion assay

For in vitro endothelial adhesion assay HEAC cells (1,5 × 10^5) passages (3–5) were seeded in black-walled 24–well plates for 48 h before adhesion assay. Melanoma cells dissociated with 0,02% EDTA
solution (1 × 10^5) labeling with 5 μM Calcein AM (BD Pharmingen cat.No 564061) for 30 min at 37 °C, after washing, were added to HEAC monolayer for 0.5 h at 37 °C. After incubation, nonadherent melanoma cells were removed from monolayer of HEAC and after washing with PBS samples were measured on a plate reader (BIO-TEK). Quantitative analysis of adherent cells was carried out by measuring the fluorescence of samples at excitation/emission wavelengths of 495/515 nm sensitivity 50.

2.7. Transendothelial migration assay

The in vitro transendothelial migration assay were study using Millipore QCM Tumor Cell Transendothelial Cell migration Assay (MERC Millipore cat.No. ECM558) according to producer's protocol. The assay allows quantitative analysis of tumor cell migration. HEAC cell passages (3–5) were used, 0.02% EDTA solution (Sigma) was used as harvesting buffer for cancer cells detachment. After 20 h migrated cells were stained and measured by OD 570 nm accordingly to the assay instruction. Results were described as the value of tumor transendothelial migration minus the amount of negative control chambers contained HEACs only. The cells which migrated towards the opposite side of the membrane were viewed under invert microscope, the images of the migrated cells were taken using digital camera (Nikon, Tokyo, Japan) as additional data.

2.8. Statistical analysis

Shapiro–Wilk W-test was used to check the normality of each variable. Levene's test was used for assessed homogeneity of variance. Statistical analyses of data from in vitro studies were performed by one-way analysis of variance (ANOVA), followed by Dunnett's post hoc comparison test to determine which values differed significantly from the controls. All analyses were made using Statistica 13 software (StatSoft Inc., Tulsa, OK, USA). Data were presented as mean ± SD and considered statistically significant at *p < .05, **p < .01, and ***p < .001.

3. Results

3.1. siRNA-mediated knockdown of ILK results in the decrease of prosurvival signaling

The expression and activity of ILK were also elevated in melanoma, especially in cells sourced from advanced stages [23]. We received comparable results; a higher level of ILK was observed in the metastatic melanoma cell line compared with the one derived from VGP site [5]. Knockdown of ILK expression caused a significant reduction of Akt activation with a negligible difference in its total expression. We used phospho-specific antibodies against phospho-Akt (S473) for monitoring expression of activated Akt. siRNA silencing of ILK resulted in the activation of GSK-3β seeing as the phosphorylation on Ser 9 was markedly lower, while the phosphorylation of GSK-3β on Tyr 216 was higher in comparison to control melanoma cells (Fig. 1).We noticed slight difference in the expression of total GSK-3β. Signaling molecules activated by ILK also include Src family and Erk kinase. We observed a visible decrease of activity of tested kinase Src, especially in WM793 cell line upon ILK silencing in comparison to control melanoma cells (Fig. 1), but we did not notice changes in activation of ERK1/2 kinase. siRNA for ILK effectively reduced the pro-survival pathway in melanoma cells.

3.2. ILK regulates N-cadherin protein levels

Loss of E-cadherin expression and induction of N-cadherin expression are hallmarks of the EMT process, and ILK signaling is known to induce EMT. The studied melanoma cell lines – WM793 from VGP, and 1205Lu from metastasis – both express N-cadherin but no E-cadherin on mRNA and protein levels (Fig. 2). In our previous work, we showed that ILK knockdown was sufficient to down-regulate the expression of N-cadherin protein as EMT marker in both cell lines [5]. In this study, despite the decreased N-cadherin protein expression upon ILK silencing, no difference on mRNA level was observed. However, melanoma cells showed significantly decreased expression of EMT-inducing transcriptional factors Zeb and Snail. Also, loss of nuclear translocation of Snail was visible in WM793 cell line (Fig. 3). ILK silencing was insufficient to re-express E-cadherin on protein or mRNA level. These results suggest that ILK in studied melanoma cells does not affect the transcription of cadherins.

3.3. Silencing of ILK affects N-cadherin plasma membrane (surface) expression

N-cadherin favors cell migration and invasion in many types of cancers; hence, N-cadherin surface expression in cancer is an important factor in cancer progression. In previous works, we showed that N-cadherin expression contributes to increased proliferation and invasive potential of studied melanoma cells [3,4]. We studied whether the ILK signaling pathway is necessary for the reduction of N-cadherin protein level, and also if it may influence endosomal recycling of N-cadherin to the membrane surfaces. We checked intracellular localization of N-cadherin after silencing of ILK in comparison to control non-silencing RNA-treated melanoma cells. Western blot analysis of the subcellular fraction showed that in melanoma cells N-cadherin is located in the cytoplasm and membrane fraction, but after silencing of ILK, N-cadherin was reduced in the cytoplasm fraction and completely abolished in the membrane fraction (Fig. 3). These results suggest that ILK silencing is involved in directing N-cadherin to the cell surface.

3.4. ILK knockdown is sufficient for N-cadherin endocytosis

Endocytosis is a mechanism well known to terminate receptor signaling, and required for the initiation of some signaling cascades [12]. Rab5 small G proteins function in endocytic processes at the plasma membrane and are engaged in N-cadherin endocytosis. The siRNA reduction of ILK expression had significant effect on Rab5 protein levels in both melanoma cell lines (Fig. 4). Rab5 expression was up-regulated 2.3-fold in WM793 and 1.7-fold in 1205Lu in comparison to control cells. Our observations show that ILK can regulate N-cadherin endocytosis. Rab5 is vital for the early endocytic pathway but Rab7 regulates late endosomal transport, and Rab9 controls transport from the late endosomes to trans-Golgi. Rab7 is involved in the degradation of cadherins in epithelia and neurons because it is associated with early sorting endosomes to a degradative compartment and is engaged in autophagosome maturation. Rab9 is required in non-canonical autophagy. We observed a stable level of Rab7 and an elevated level of Rab9 (1.5-fold in WM793 and 1.85 in 1205Lu) when ILK was knocked-down. Taken together, these results demonstrate that ILK plays an important role in the regulation of the endocytic pathway.

3.5. ILK contribute to the recycling of N-cadherin to the cell surface

Two distinct pathways are described for membrane receptors' internalization to early endosomes and recycling to the plasma membrane. Rab4 regulates delivery of early endosomes to the membrane in the short-range recycling pathway, but Rab11 controls membrane trafficking through long-range pathway. Both proteins are essential for regular N-cadherin supply to the plasma membrane [9]. We detected a significant decrease of expression of both Rab4 and Rab11 proteins, as well as Rab11 interacting protein (Rab11FIP1) after silencing of ILK, which is associated with inhibition of recycling of N-cadherin to the cell surface (Fig. 4). We used chloroquine to study the effect of inhibition of endosomes recycling to the plasma membrane on N-cadherin expression.
expression. Chloroquine and primaquine effectively inhibit recycling of endocytosed integral membrane proteins such as TfR (Transferrin receptor) so they may be used as potent inhibitors for membrane transport from endosomes to the plasma membrane [24]. As shown in Fig. 5, the inhibition by chloroquine was sufficient to downregulate the expression of N-cadherin on protein level in both cell lines.

3.6. Reduction of N-cadherin expression by ILK pathway inhibits invasive potential of melanoma cells

Cancer metastasis is a multistep process. The adhesion to the endothelium and the penetration of circulating cancer cells into the endothelium is a crucial step for cancer metastasis which involve N-cadherin. We checked whether the observed decreased of surface expression of N-cadherin after silencing of ILK was sufficient to down
regulate metastatic potential. The invasive potential of melanoma cells were studied as endothelial adhesion and transendothelial migration assays. Attachment of melanoma cells on the endothelial monolayer was significantly decreased after ILK knockdown in both cell lines (Fig. 6). Also reduction of surface N-cadherin expression decreased the amount of cells which were able to migrate across endothelial cells monolayer (Fig. 7). Transendothelial migration was inhibited by silencing of ILK in both WM793 and 1205Lu cells as a direct consequence of downregulation of N-cadherin. The data support an important role of ILK in regulation of N-cadherin surface exposition in melanoma cells.

4. Discussion

Evidence indicates that endocytosis controls cell adhesion and thereby cell migration, growth, survival, and development [11,12]. Endocytosis is also harnessed by the EMT process to promote dynamic changes in cellular identity to increase cancer progression. The key regulators of vesicular membrane transport on both the endocytosis and exocytosis are Rab proteins. Alteration in Rab protein expression has been revealed in several human diseases and in a variety of cancers [17,25]. Therefore, the precise molecular detail of the upstream regulation of Rab proteins is an important point requiring clarification. Our study underscores ILK as a critically important regulator of endosomal recycling of N-cadherin in melanoma cells. Caswell and colleagues have previously suggested that integrins can regulate the expression of other receptors by regulating endocytosis [26]. We found that ILK modulates the expression of Rab proteins associated with N-cadherin endocytosis, degradation, or recycling. We observed a very significant increase in Rab5 protein level after knockdown of ILK in the studied melanoma cells, which may explain the observed decrease in membrane expression of N-cadherin. Rab5 is important for sequestering receptors and formation of early endosomes. Rab5-mediated endocytosis is responsible for N-cadherin internalization in neuronal cells [16]. Additionally, silencing of Rab5 expression resulted with an increased level of N-cadherin on the cell surface [27]. The endocytosis mediated by Rab5 and Dynamin is a fundamental process in neuronal migration, and the main loading molecule of Rab5 in the neuronal migration is N-cadherin. Additionally, an increase in the amount of Rab5 promotes basal autophagy [28]. Another protein involved in autophagosome maturation is Rab7. Although we did not notice any significant changes in the level of Rab7 after silencing of ILK in melanoma, we observed a meaningful increase in the expression of Rab9.
which is required in non-canonical autophagy [29].

The presence of N-cadherin at the plasma membrane surface is also dependent on transition after its endocytic uptake via recycling from the early endosome back to plasma membrane. This is supported by Rab4 positive endosomes in the fast-range pathway or Rab11 positive recycling endosomes in the slow-range pathway. Ho and colleagues proved that ILK can regulate endosomes recycling by modulation of microtubule stability and interaction with Rab4 and Rab11 containing endosomes [18]. Rab11 enhances the trafficking of internalized N-cadherin to the plasma membrane via recycling endosomes which promotes neuronal migration [27]. Our data indicated that ILK signaling can regulate the expression of Rab4 and Rab11 proteins, well-known regulators of recycling processes and exocytosis. Moreover, Lindsay and McCaffrey showed that knockdown of Rab coupling
protein (a Rab11 effector protein) inhibits the return of endocytosed N-cadherin in lung adenocarcinoma cells [9]. We also noticed a decrease of Rab11 interacting protein (Rab11FIP1) after silencing of ILK. In our previous work, we observed a decrease in the expression of N-cadherin after ILK silencing in melanoma cells [5]. Presented results show that in melanoma, ILK pathway regulates expression of N-cadherin at the protein level by controlling their presence in the membrane fraction.

This phenomenon has also physiological consequences, because we observe reduced adhesion to endothelium and significantly reduced transendothelial migration of melanoma cells after ILK silencing in comparison to control cells. The results confirm previous reports, that N-cadherin silencing reduces transendothelial melanoma cell migration [30], and with our earlier observations that it significantly inhibits melanoma cell migration through matrigel-coated Boyden chamber [4].

Rab11 also functions as an oncoprotein; high expression of Rab11 is associated with multiple biological processes of cancer such as tumor formation, progression, and poor prognosis [31,32]. Considering how important a factor expression of Rab11 is in tumorigenesis, our result is not without significance.

Our observation that ILK regulates expression of Rab proteins indicates that ILK may function as an upstream regulator of endocytic circuitries and be a critical player in fundamental intracellular trafficking pathways, and thus participate in the EMT process.

ILK is a key regulator of several signaling pathways. Its kinase activity results in phosphorylation of Ser 473 and activation of Akt, which coordinates survival genes. ILK can also inactivate GSK-3 kinase by direct phosphorylation at Ser 9, what leads to activation of some transcription factors implicated in the regulation of EMT markers [5,19]. Moreover, Akt/GSK-3β signaling cascade was indicated as a regulator of Dynamin-1 dependent clathrin-mediated endocytosis [33]. On the other hand, many Rab proteins participate in the regulation of Akt/ GSK-3β signaling pathways [31,34]. Rab proteins might function as both oncogens and suppressors, so a strategy based on the regulation of Rab expression would be reasonable in cancer treatment [13,34]. In view of the fact that exocytosis pathway regulated by Rab proteins plays an essential role in human cancer development, it may also be a a good target in tumor therapy.

5. Conclusion

Our results suggest that ILK regulates N-cadherin expression at the post-transcriptional level by controlling endosomal recycling (Fig. 8). These data highlight an important mechanism of cadherin trafficking regulation during tumor progression by integrin mediated signaling pathway. This extends the area for development of novel therapeutic strategies in cancer treatment, especially in targeted therapy.

Author contributions

D.G; conceived the original idea, designed the study, performed the experiments and wrote the manuscript; M.Z; performed the graphical...
abstract and helped with the preparation of figures; D.C.-W; discussed the results and commented on the manuscript; P.L; reviewed and discussed the manuscript; All authors have read and approved the final version of the manuscript.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

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