FEBS Letters 584 (2010) 1073–1079

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FEBS Letters

journal homepage: www.FEBSLetters.org

PINK1 is recruited to mitochondria with parkin and associates with LC3 in mitophagy

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ARTICLE INFO

Article history:
Received 16 December 2009
Revised 29 January 2010
Accepted 2 February 2010
Available online 12 February 2010

Edited by Jesus Avila

Keywords:
PTEN-induced putative kinase 1
Parkin
Mitophagy
Autophagy
Parkinson's disease

ABSTRACT

Mutations in *PTEN-induced putative kinase 1 (PINK1)* cause recessive form of Parkinson's disease (PD). PINK1 acts upstream of parkin, regulating mitochondrial integrity and functions. Here, we show that PINK1 in combination with parkin results in the perinuclear mitochondrial aggregation followed by their elimination. This elimination is reduced in cells expressing PINK1 mutants with wild-type parkin. Although wild-type PINK1 localizes in aggregated mitochondria, PINK1 mutants localization remains diffuse and mitochondrial elimination is not observed. This phenomenon is not observed in autophagy-deficient cells. These results suggest that mitophagy controlled by the PINK1/parkin pathway might be associated with PD pathogenesis.

Structured summary:
MINT-7557195: *PINK1* (uniprotkb:Q9BXM7) *physically interacts* (MI:0915) with *LC3* (uniprotkb:Q9GZQ8) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7557109: *LC3* (uniprotkb:Q9GZQ8) and *PINK1* (uniprotkb:Q9BXM7) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7557121: *tom20* (uniprotkb:Q15388) and *PINK1* (uniprotkb:Q9BXM7) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7557138: *parkin* (uniprotkb:O60260), *PINK1* (uniprotkb:Q9BXM7) and *tom20* (uniprotkb:Q15388) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7557173: *LC3* (uniprotkb:Q9GZQ8) *physically interacts* (MI:0915) with *PINK1* (uniprotkb:Q9BXM7) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra. Mitochondrial dysfunction has been proposed as a major factor in the pathogenesis of sporadic and familial PD [1. In particular, the identification of mutations in *PTEN-induced putative kinase 1 (PINK1)* has strongly implicated mitochondrial dysfunction in the pathogenesis of PD [2]. PINK1 contains an N-terminal mitochondrial targeting sequence (MTS) and a serine/threonine kinase domain [2].

Several studies have shown that PINK1 acts upstream of parkin in the same genetic pathway [3,4]. Overexpression of PINK1 promotes mitochondrial fission [5]. Fission followed by selective fusion segregates dysfunctional mitochondria and permits their removal by autophagy [6]. Parkin is associated with mitochondrial elimination in cultured cells treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [7], but little is known about the biological function of PINK1 in this context. Likewise, although co-overexpressed both PINK1 and parkin colocalized with mitochondria [8] and are associated with mitochondrial autophagy (mitophagy) [9], the exact mechanism of the mitochondrial elimination via autophagy has not been examined.

Here, we describe the characterization of mitophagy induced by co-overexpressing both proteins and report that the phenomenon is dependent on PINK1 kinase activity and mitochondrial localization.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 3-MA, 3-methyladenine; MEFs, mouse embryonic fibroblasts; MTS, mitochondrial targeting sequence; PD, Parkinson's disease; PINK1, PTEN-induced putative kinase 1; UPS, ubiquitin–proteasome system

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.02.016

Furthermore, we found that PINK1 interacts with LC3–phospholipid conjugate (LC3-II), a well established marker for autophagosomes [10]. These results provide novel insights into the pathogenesis of PD.

2. Materials and methods

2.1. Antibodies

Anti-FLAG antibodies (M2, polyclonal) and anti-LC3B antibody (rabbit) were obtained from Sigma. Anti-actin antibody (mouse) was from Millipore. Anti-Tom20 antibody (rabbit) was from Santa Cruz Biotechnology. Anti-LDH antibody (goat) was from Abcam. Anti-LC3 antibody (rabbit) was from MBL. Secondary antibodies, conjugated to horseradish peroxidase, were from GE HealthCare Bio-Sciences and Alexa Fluor 488, 546, 594, and 647 conjugated secondary antibodies were from Invitrogen-Molecular Probes.

2.2. Plasmids

A cDNA encoding wild-type PINK1 was amplified with appropriate primers and ligated into the BamHI sites of the (C-terminal tagged) 3xFLAG pCMV-10™ expression plasmid (Sigma). Mutations in PINK1 were introduced by site-directed mutagenesis (Stratagene) according to manufacturer's instructions. GFP-parkin was subcloned into pcDNA3.1 (Invitrogen).

2.3. Cell culture

HeLa, HEK293 cells and Atg7$^{+/+}$ and $^{-/-}$ mouse embryonic fibroblasts (MEFs) (a gift from Dr. Komatsu) were grown in DMEM (Sigma) supplemented with 10% FBS (Sigma) and 1% penicillin–streptomycin (Invitrogen) at 37 °C and 5% CO_2. For pharmacological studies, E64d, pepstatin A, rapamycin, 3-MA, and CCCP (Sigma) were added at indicated times and concentrations.

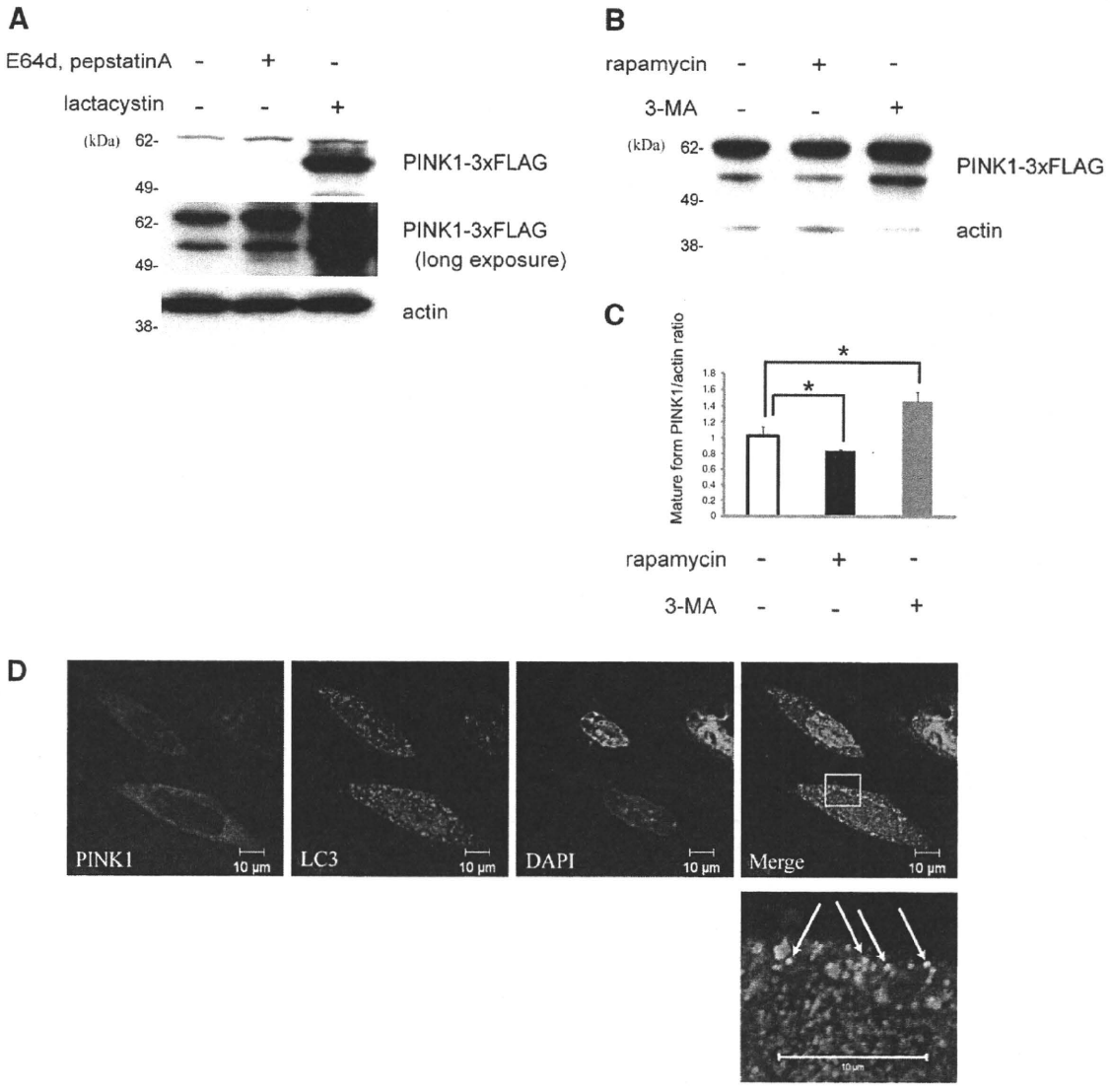

Fig. 1. PINK1 is degraded by autophagy as well as by the ubiquitin–proteasome system. (A and B) PINK1 levels in HEK293 cells stably expressing PINK1-3xFLAG treated or untreated with E64d (10 μg/ml) + pepstatin A (10 μg/ml) or lactacystin (10 μM) (A), and 3-MA (10 mM) or rapamycin (200 nM) (B), for 24 h were analyzed by immunoblotting with anti-FLAG antibodies (M2). The bottom panels show actin as loading control. (C) Quantification of (B); *$P < 0.05$. Error bars indicate standard deviation of at least three experiments. (D) Immunocytochemistry of HeLa cells transiently overexpressing PINK1-3xFLAG, 24 h after transfection. PINK1 or LC3 are in red or green, respectively. The boxed area is shown in the bottom image at a higher magnification. Bars, 10 μM.

2.4. Cell transfection and establishment of stable cell lines

Cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) and Lipofectamine LTX with PLUS Reagent (Invitrogen) according to manufacturer's instructions. For stable overexpression of PINK1, HEK293 cells were transfected with PINK1 plasmids and then selected using G418.

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with 1× PBS containing 0.5% Triton X-100, and incubated in PBS containing 10% FBS and 1% BSA. Cells were then incubated overnight with primary antibodies, followed by incubation with secondary antibodies for 1 h. Cells were then mounted with Vectashield containing DAPI (Vector Laboratories). Cells were visualized using a ZEISS LSM510 confocal microscope.

2.6. Cell fractionation

Cells were fractionated using the mitochondrial isolation kit for cultured cells (Pierce) according to manufacturer's instructions.

2.7. Immunoprecipitation and immunoblotting

Cells were lysed on ice in lysis buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitors (complete, Mini, EDTA-free, (Roche Applied Science))]. Cell lysates were immunoprecipitated using Dynabeads protein G (Invitrogen) according to manufacturer's instructions and immunoblotting was performed previously described elsewhere [11].

3. Results and discussion

3.1. PINK1 is degraded by autophagy as well as by the ubiquitin–proteasome system (UPS)

Previous studies have shown that PINK1 is degraded by the UPS [12], but it remains unclear whether autophagy also affects its degradation. We, therefore, tested whether PINK1 could be degraded via the UPS and/or autophagy, using HEK293 cells stably expressing PINK1-3xFLAG. Immunoblot analysis identifies PINK1 in two bands. The upper band represents full-length PINK1 (~66 kDa), whereas the lower band represents the mature form of PINK1 (~55 kDa), in which the MTS has been removed [13,14]. As others

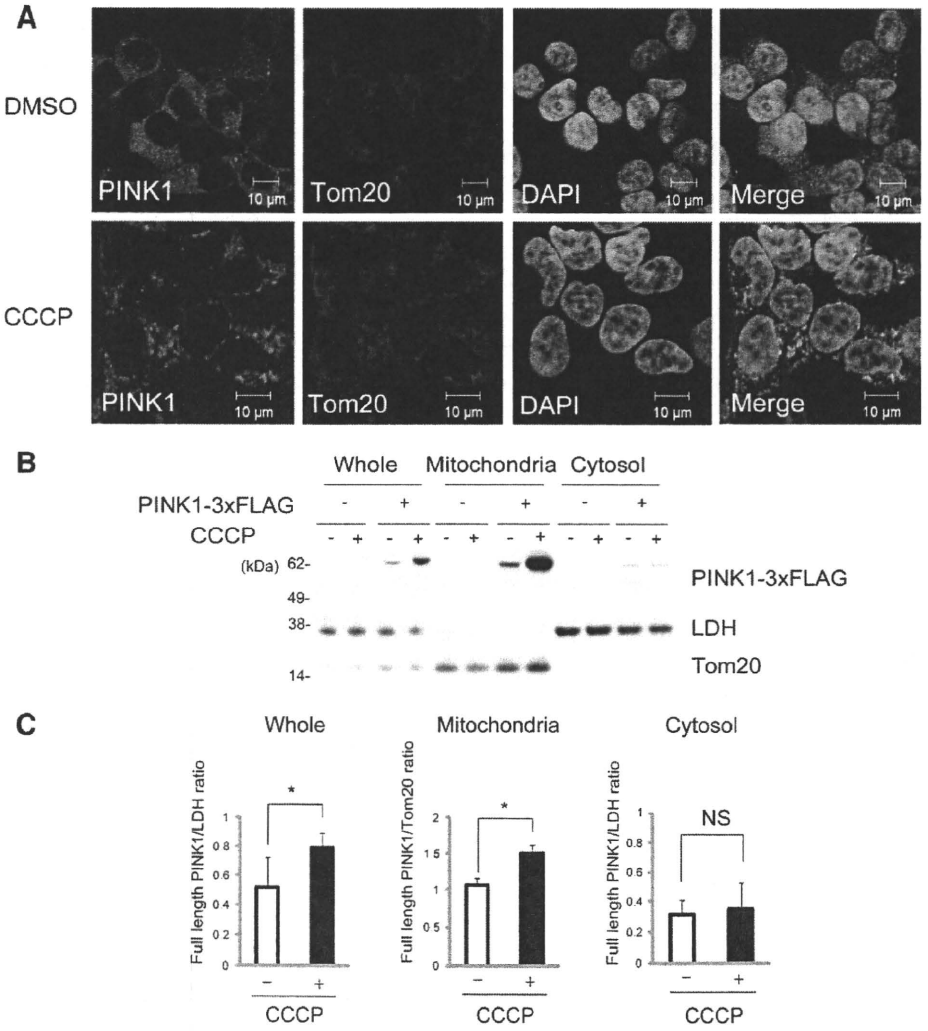

Fig. 2. PINK1 is recruited to depolarized mitochondria. (A) Immunocytochemistry of HEK293 cells stably expressing PINK1-3xFLAG treated with DMSO or CCCP (10 μM) for 3 h. PINK1 or Tom20 are in green or red, respectively. Bars, 10 μM. (B) HEK293 cells stably expressing 3xFLAG-empty vector or PINK1-3xFLAG treated with DMSO or CCCP (10 μM) for 3 h were fractionated and immunoblotted for FLAG, Tom20, and LDH. (C) Quantification of (B); $^*P < 0.05$. NS, non-significant. Error bars indicate standard deviation of at least three experiments.

have shown [12], levels of mature form PINK1 were increased by treatment with a proteasome inhibitor (lactacystin) (Fig. 1A). Also, they were increased by lysosome protease inhibitors (E64d + pepstatin A), which block autophagic flux (Fig. 1A). Next, we investigated the effects of other autophagy modulators (rapamycin and 3-methyladenine (3-MA)) on levels of PINK1. As expected, rapamycin treatment decreased mature form PINK1 levels, although 3-MA treatment increased them (Fig. 1B and C). Immunocytochemistry of HeLa cells transiently overexpressing PINK1-3xFLAG demonstrated partial colocalization of PINK1 with endogenous LC3 (an autophagosomal marker) (Fig. 1D). These data suggested that PINK1 is degraded via autophagy as well as by the UPS.

3.2. PINK1 is recruited to depolarized mitochondria

In cultured cells treated with CCCP, parkin is selectively recruited to degraded mitochondria and promotes mitophagy [7]. PINK1 and parkin function in the same genetic pathway to regulate mitochondrial integrity [3,4]. To investigate the interaction between depolarized mitochondria and PINK1, we tested for a change of PINK1 localization following treatment with CCCP. In HEK293 cells stably expressing PINK1, mitochondria stained with antibody against Tom20, a receptor protein of the mitochondrial outer membrane, were aggregated around the nucleus and fragmented and showed marked accumulation of PINK1 (Fig. 2A). The mitochondrial translocation of PINK1, caused by CCCP, was also assayed by immunoblotting. Levels of full-length PINK1 in the mitochondrial fraction were increased by CCCP treatment (Fig. 2B and C).

3.3. Mitochondrial elimination is accomplished by overexpression of wild-type PINK1 in combination with parkin

PINK1 has been reported to promote parkin translocation to mitochondria [8]. To further investigate the molecular interaction between PINK1 and parkin, the following experiments with HeLa cells overexpressing both PINK1 and parkin were performed. Twenty-four hours after transfection, mitochondrial aggregation and recruitment of PINK1 and parkin to the aggregated mitochondria were observed only in cells positive for GFP-parkin and PINK1-3xFLAG. Moreover, parkin completely colocalized with aggregated mitochondria (Fig. 3A and B). Partial colocalization of wild-type PINK1 with parkin has been previously reported in cells overexpressing both PINK1 and parkin; however, the association with mitochondria was not examined [15]. Also, parkin colocalization with PINK1 in aggregated mitochondria has been previously reported [8]. To confirm the exact effect of both PINK1 and parkin on mitochondria, we examined the fate of the degraded mitochondria, 48 h after transfection. In cells overexpressing both PINK1 and parkin, the mitochondria were completely absent, while mitochondria were still present in cells overexpressing either PINK1 or parkin (Fig. 3C). These data indicate that both PINK1 and parkin might be indispensable for mitochondrial elimination.

3.4. PINK1 mutants remain diffusely distributed and are not recruited to mitochondria, resulting in reduced mitochondrial elimination

PD-associated mutations in PINK1 have been found in both the kinase and C-terminal domains [2,16]. Among these mutations, G309D, L347P, and G409V are expected to cause a reduction in the kinase activity of PINK1 [13,14,17]. Therefore, we generated PINK1 mutants with G309D, L347P and G409V and two deletion mutants without the MTS (ΔN: deleted amino acids 156–581 and KD: deleted amino acids 156–509) and performed similar transfection experiments to those described above. Neither recruitment of parkin and/or mutant PINK1 to the mitochondria nor mitochondrial aggregation was detected 24 h after transfection (data not shown). Forty-eight hours after transfection, the G309D/L347P/G409V mutants preserved their mitochondrial localization, whilst less mitochondrial elimination was detected compared with those cells expressing both wild-type PINK1 and parkin. The ΔN/KD mutants were diffusely distributed without apparent colocalization to the mitochondria and no mitochondrial elimination was observed (Fig. 4A–C). Also, we confirmed mitochondrial elimination by immunoblotting. Levels of Tom20 were decreased in cells expressing both parkin and wild-type PINK1 but not in the cells expressing PINK1 mutants (Fig. 4D and E). These findings suggested that the

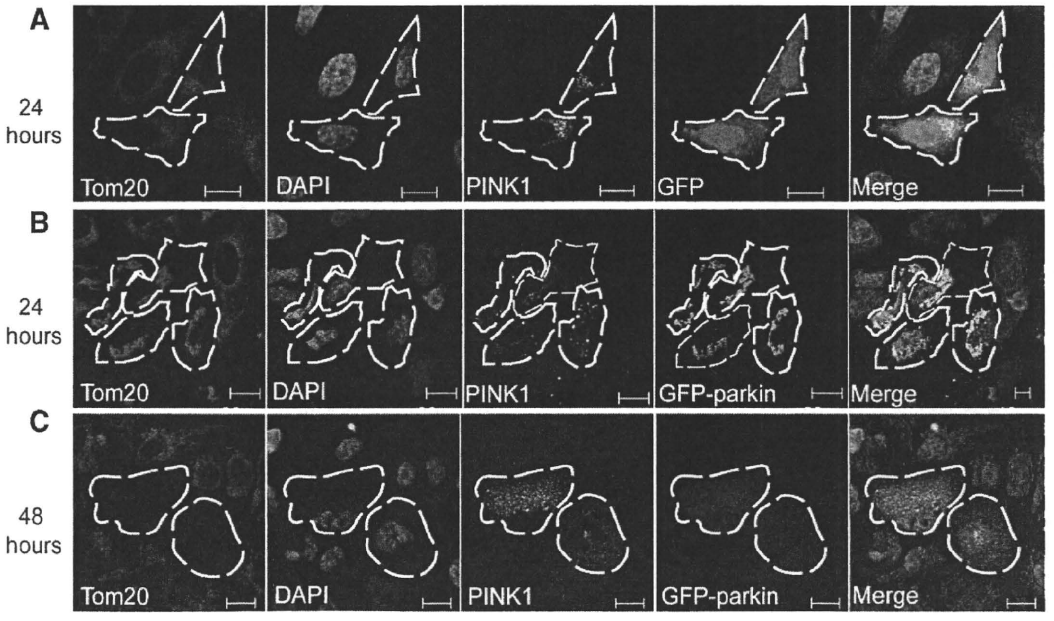

Fig. 3. Mitochondrial elimination is accomplished by overexpression of wild-type PINK1 in combination with parkin. (A and B) Immunocytochemistry of HeLa cells 24 h after transient co-overexpression of wild-type PINK1-3xFLAG (PINK1-WT) and GFP-empty vector (GFP) (A) or GFP-parkin (B) and 48 h after transient co-overexpression of PINK1-WT and GFP-parkin (C). Tom20, wild-type PINK1, or GFP are red, white, or green, respectively. Bars, 10 μm.

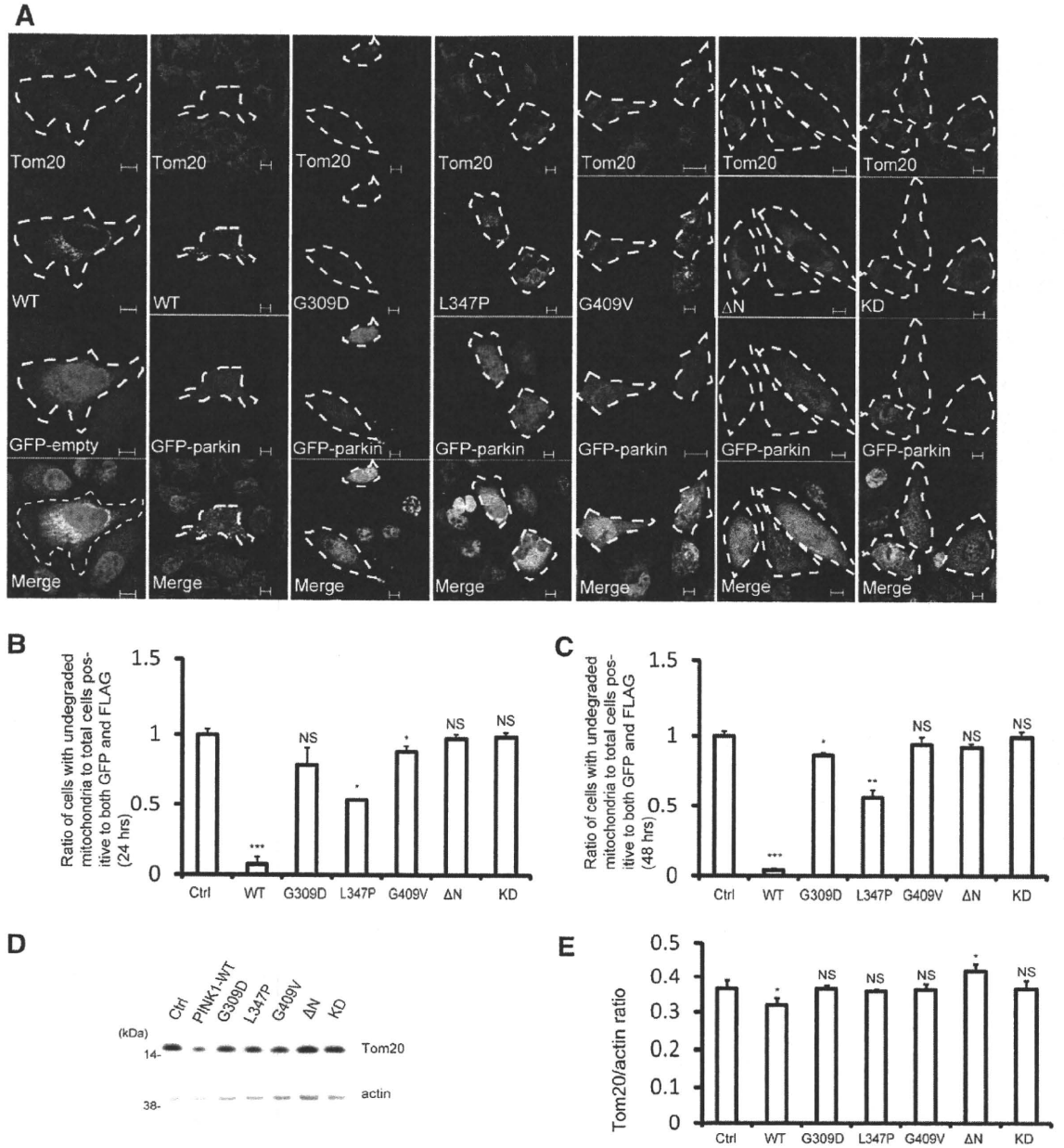

Fig. 4. PINK1 mutants remain diffusely distributed and are not recruited to mitochondria, resulting in reduced mitochondrial elimination. (A) Immunocytochemistry of HeLa cells transiently overexpressing GFP-empty vector and PINK1-3xFLAG (WT) as control, and GFP-parkin and PINK1-3xFLAG (WT or various mutants), 48 h after transfection. Tom20, WT and PINK1 mutants, or GFP are in red, white, or green, respectively. Bars, 10 μm. (B and C) Ratio of cells with undegraded mitochondria to total cells positive for both GFP and FLAG, 24 h (B) and 48 h (C) after transfection are shown in the graph. (D) Levels of Tom20 in HeLa cells transiently overexpressing GFP-empty vector and PINK1-3xFLAG (WT), and GFP-parkin and PINK1-3xFLAG (WT or various mutants) were analyzed with immunoblotting. (E) Quantification of (D); error bars indicate standard deviation of at least three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, non-significant.

kinase activity and mitochondrial localization of PINK1 are indispensable for mitochondrial elimination.

3.5. Overexpression of wild-type PINK1 in combination with parkin induces mitophagy

Very recently, it has been reported aggregated mitochondria in cells overexpressing both PINK1 and parkin colocalize with lysosomes as well as autophagosomes [9]. However, the fate of perinuclear aggregated mitochondria has not been examined. Therefore, we checked whether PINK1–parkin dependent mitochondrial elimination was dependent on mitophagy. Mitochondrial elimination was enhanced by overexpression of PINK1 with parkin in wild-type MEFs. On the other hand, Atg7$^{-/-}$ MEFs, which lack a key component of the autophagy system, retain expression of Tom20 (Fig. 5A and B). To further analyze this hypothesis, we examined the change of endogenous LC3 distribution following both PINK1 and parkin overexpression. We confirmed accumulation of parkin, which overlaps with aggregated mitochondria (refer to Fig. 3), in cells expressing both wild-type proteins. Likewise, endogenous LC3 mainly colocalized with wild-type PINK1, adjoined to the outer mitochondrial membrane, but did not colocalize with G409V or ΔN

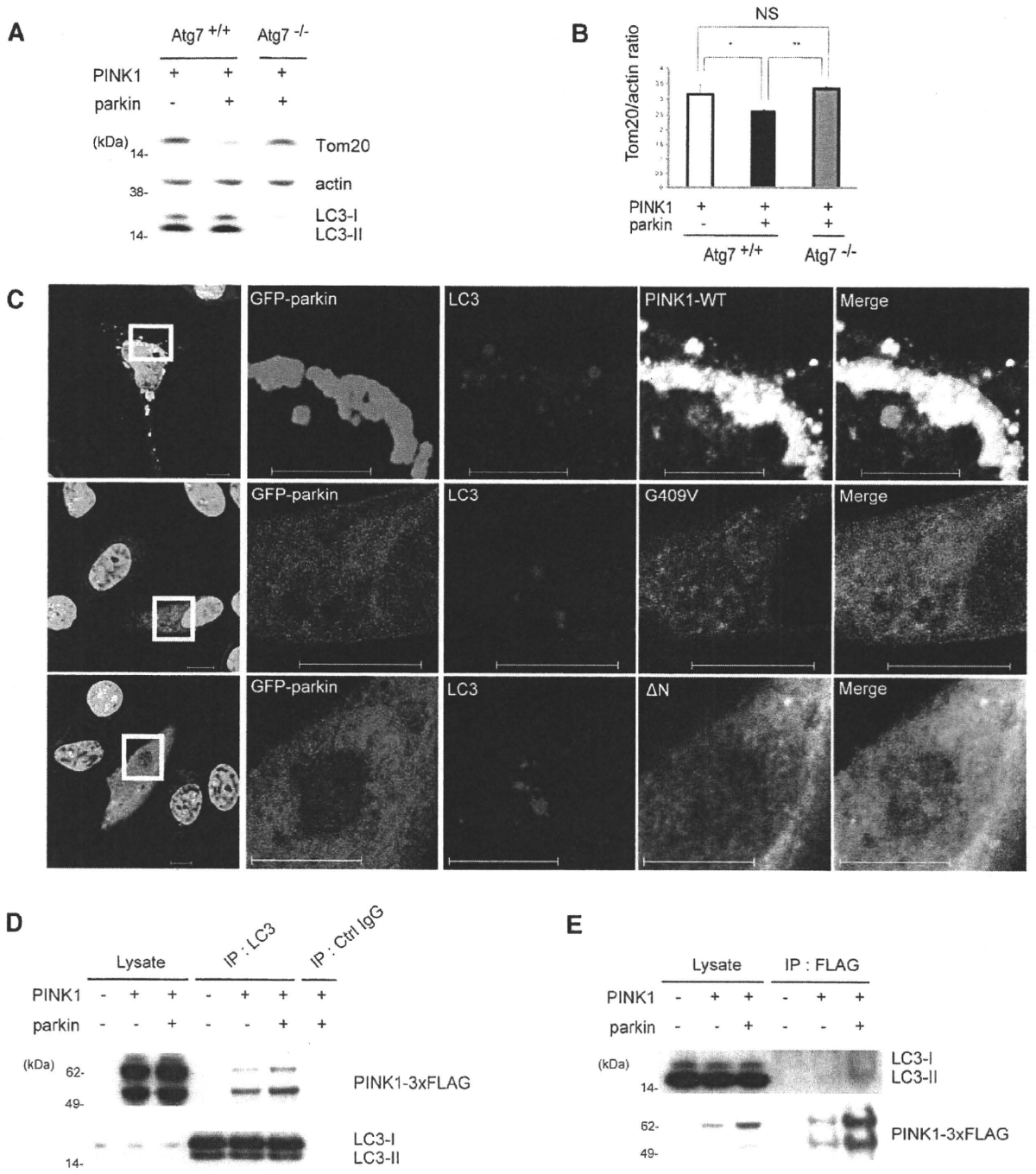

Fig. 5. Overexpression of wild-type PINK1 in combination with parkin induces mitophagy. (A) Levels of Tom20 in Atg7$^{+/+}$ MEFs overexpressing GFP-empty vector and PINK1-3xFLAG and those in Atg7$^{+/+}$ and Atg7$^{-/-}$ MEFs overexpressing GFP-parkin and PINK1-3xFLAG, 48 h after transfection were analyzed by immunoblotting. (B) Quantification of (A); error bars indicate standard deviation of at least three experiments. *$P < 0.05$, **$P < 0.01$, NS, non-significant. (C) Immunocytochemistry of HeLa cells transiently overexpressing GFP-parkin and PINK1-3xFLAG (wild-type, G409V, and ΔN), 24 h after transfection. PINK1 (WT and mutants), LC3, or GFP-parkin are in white, red, or green, respectively. The boxed areas are shown in the three right-hand images at a higher magnification. Bars, 10 μm. (D and E) HeLa cells overexpressing 3xFLAG-empty vector and GFP-empty vector, PINK1-3xFLAG and GFP-empty vector, or PINK1-3xFLAG and GFP-parkin, 24 h after transfection were immunoprecipitated with anti-LC3 antibodies (D) or anti-FLAG antibodies (E) and immunoblotted for FLAG or LC3. Immunoblotting of total lysates was performed to test the expression levels. IP, immunoprecipitation.

(Fig. 5C), nor with G309D, L347P and KD (data not shown). LC3 also partially colocalized with parkin. Compared with Fig. 1D, the colocalization of endogenous LC3 with PINK1 was markedly enhanced. Next, to investigate direct interaction, cell lysates were immunoprecipitated using anti-LC3 antibodies and immunoblotted with anti-FLAG antibodies (Fig. 5D). The reverse immunoprecipitation was also performed (Fig. 5E). These results allowed us to conclude that PINK1 binds with LC3-II. Taken together, we concluded that mitochondrial elimination by the PINK1–parkin pathway is dependent on mitochondrial autophagic activity.

In this study, we have found that overexpression of both proteins enhances mitochondrial elimination via autophagy. In

contrast to the results of a *Drosophila* study, a PINK1–parkin pathway promotes mitochondrial enlargement or aggregation in mammalian cellular models [8,18]. However, only PINK1 overexpressing cells exhibit longer mitochondria with increased interconnectivity, but the abnormal mitochondria do not elicit an autophagic response [12]. Consistent with this, wild-type PINK1 overexpression did not change the level of endogenous LC3-II (data not shown). Therefore, coordinating activation of parkin and PINK1 contributes to mitophagy.

PINK1 localization in mitochondria is dependent on its MTS region and it exhibits autophosphorylation activity in vitro [2,13,14]. Parkin is recognized as an in vivo substrate of PINK1 and parkin site-direct phosphorylation by wild-type PINK1 is critical for the translocation of parkin into the mitochondria in cellular and *Drosophila* models [8]. In agreement with this report, our study showed that parkin recruitment to the mitochondria by PINK1 was dependent on PINK1 kinase activity.

Silencing of PINK1 with shRNA increased mitochondrial fission and induced mitophagy [12]. Although PINK1 overexpression is protective against oxidative stress-induced apoptotic cell death [2,19,20], excess wild-type PINK1 without parkin overexpression does not elicit mitochondrial autophagy [12]. Our immunocytochemical experiments revealed that PINK1 co-overexpressed with parkin colocalized mainly with LC3-positive vesicles and partially with perinuclear aggregated mitochondria, which were expected to colocalize with aggregated parkin. In addition, molecular binding between PINK1 and LC3-II was confirmed by immunoprecipitation, which suggests that association between PINK1 and LC3 contribute to mitophagy. Combined with the observation by Vives-Bausa et al., although it is not clear whether an excess of mitophagy would be harmful to cells, a PINK1–parkin pathway, which is regulated by kinase activity and/or the mitochondrial localization of PINK1, would control mitochondrial maintenance via the autophagic machinery.

Acknowledgments

We thank Drs. Masaaki Komatsu and Yu-shin Sou (Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science) for providing Atg7$^{-/-}$ MEFs and Hattori's laboratory members for helpful discussions. We are grateful to Drs. Junichi Nakamoto, Yoko Imamichi, and Akiko Egashira for technical assistance. This study was supported by a Young Scientist Grant (F.S. and S. Saiki), an All Japan Coffee Association Grant (S. Saiki), a Takeda Scientific Association Grant (S. Saiki) and a Grant from Nagao Memorial Fund (S. Saiki).

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Elsevier Editorial System(tm) for Bioorganic & Medicinal Chemistry Letters
Manuscript Draft

Manuscript Number: BMCL-D-10-02214R1

Title: Synthesis and anti-migrative evaluation of moverastin derivatives

Article Type: Article

Keywords: cell migration; chemical synthesis; UTKO1

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Abstract: Cell migration of tumor cells is essential for invasion of the extracellular matrix and for cell dissemination. Inhibition of the cell migration involved in the invasion process represents a potential therapeutic approach to the treatment of tumor metastasis; therefore, a novel series of derivatives of moverastins (moverastins A and B), an inhibitor of tumor cell migration, was designed and chemically synthesized. Among these moverastin derivatives, several compounds showed stronger cell migration inhibitory activity than parental moverastins, and UTKO1 was found to have the most potent inhibitory activity against the migration of human esophageal tumor EC17 cells in a chemotaxis cell chamber assay. Interestingly, although moverastins are considered to inhibit tumor cell migration by inhibiting farnesyltransferase (FTase), UTKO1 did not inhibit FTase, indicating that UTKO1 inhibited tumor cell migration by a mechanism other than the inhibition of FTase.

Cover Letter

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january 6, 2011

Bioorganic Medicinal Chemistry Letters
Regional Editor

Dear Prof. Masakatsu Shibasaki

Thank you very much for your letter, with regard to our manuscript (BMCL-D-10-02214) together with the comments from reviewers.

We are resubmitting this manuscript to *Bioorganic Medicinal Chemistry Letters* after carefully considering the suggestion made by the reviewer.

We believe that our revised manuscript adequately addresses all of the comments and hope that it is now acceptable for publication in *Bioorganic Medicinal Chemistry Letters*.

Sincerely,

Imoto Masaya, Ph. D

Responses to Reviewers

In the revised manuscript, corrected points were marked in highlight.

Reviewer #1

References have been corrected as suggested.

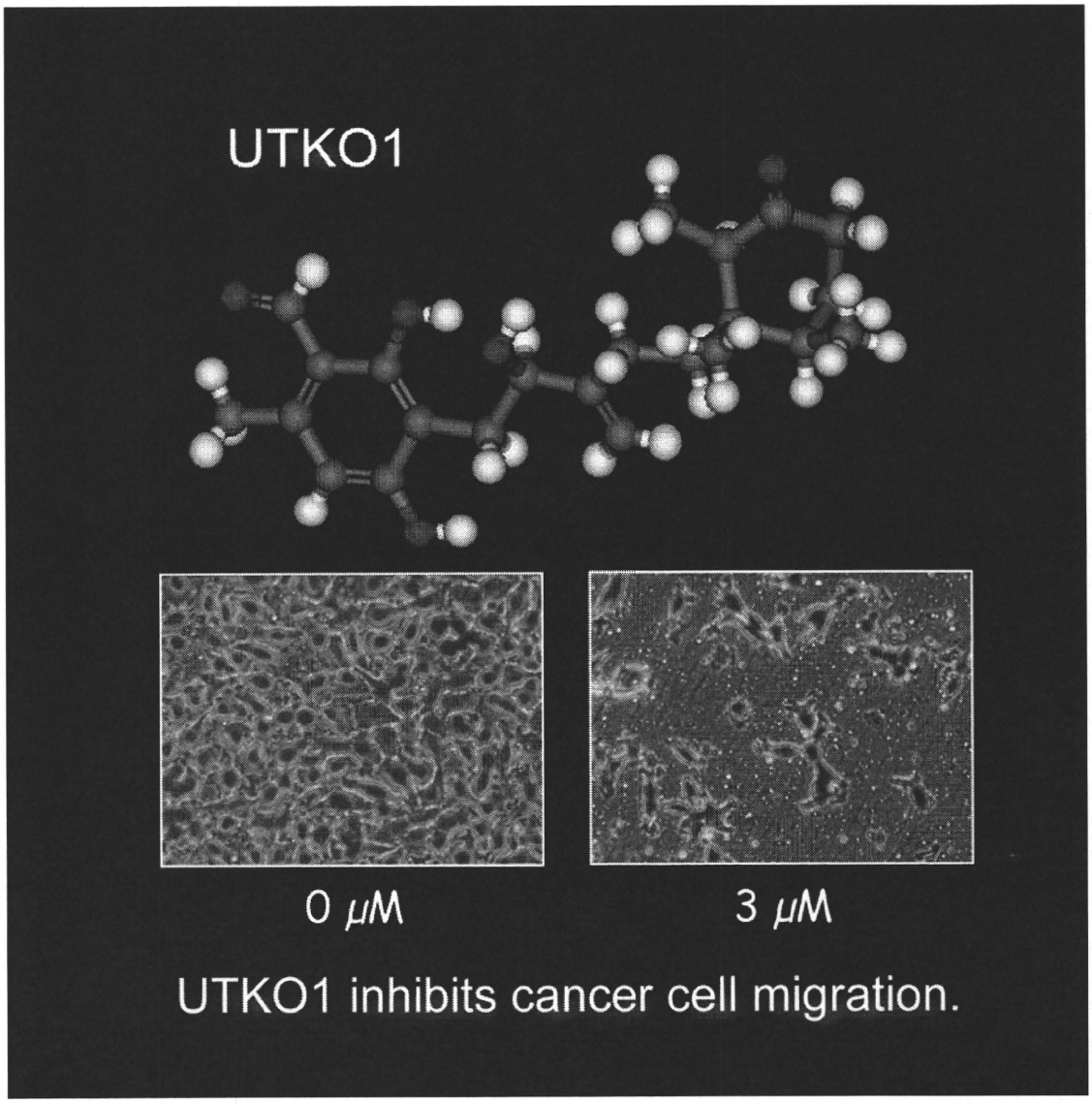
Reviewer #2

1) According to reviewer's suggestion, experimental details of the bioassays have been described in Table legends, and related-references have been cited.

2) In our chemotaxis cell chamber assay, filters were not coated by matrix. Therefore, using this assay system we have evaluated the inhibitory activity of UTKO compounds against cell migration quantitatively.

3) Not only UTKO1 but also other active UTKO compounds showed cell migration inhibitory activity in the wound-healing assay.

4) According to reviewer's suggestion, the sentences have been modified. (Page 7, Lines 6-11)


UTKO1
0 μM
3 μM
UTKO1 inhibits cancer cell migration.

Synthesis and anti-migrative evaluation of moverastin derivatives

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Keywords: cell migration / chemical synthesis / UTKO1

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Abstract

Cell migration of tumor cells is essential for invasion of the extracellular matrix and for cell dissemination. Inhibition of the cell migration involved in the invasion process represents a potential therapeutic approach to the treatment of tumor metastasis; therefore, a novel series of derivatives of moverastins (moverastins A and B), an inhibitor of tumor cell migration, was designed and chemically synthesized. Among these moverastin derivatives, several compounds showed stronger cell migration inhibitory activity than parental moverastins, and UTKO1 was found to have the most potent inhibitory activity against the migration of human esophageal tumor EC17 cells in a chemotaxis cell chamber assay. Interestingly, although moverastins are considered to inhibit tumor cell migration by inhibiting farnesyltransferase (FTase), UTKO1 did not inhibit FTase, indicating that UTKO1 inhibited tumor cell migration by a mechanism other than the inhibition of FTase.

MAIN TEXT

Despite significant advances in understanding the fundamental aspects of cancer, the development of metastatic lesions remains the predominant cause of death for most cancer patients[1, 2]. Cell migration is a crucial event in the spread of cancer and, consequently, the metastatic process[3, 4]. This prompted us to develop inhibitors of tumor cell migration as novel anti-metastatic drugs.

Previously, we screened for inhibitors of cancer cell migration derived from microbial origin, and obtained moverastin A and B (**1** and **2**, respectively), new members of the cylindrol family, from *Aspergillus* sp. F7720[5]. Their structures including the absolute stereochemistries were confirmed unambiguously by the synthesis as outlined in Scheme 1. Furthermore, moverastin A and B were found to inhibit FTase; therefore, moverastins were considered to inhibit the migration of tumor cells by inhibiting the farnesylation of H-Ras, and subsequent H-Ras-dependent activation of the PI3K/Akt pathway. However, because the inhibitory activity of moverastins for tumor cell migration was rather modest (IC_{50} value of 7 μM), we considered it an attractive lead compound in the search for other, more potent agents.

In this study, we chemically synthesized a series of moverastin derivatives and assessed

their potential as tumor cell migration inhibitors in several *in vitro* assays.

Structures of moverastin derivatives (UTKO1–12) synthesized in this study are shown in Figure 1. UTKO1–6 were synthesized by employing the same approach as that for our previous synthesis of moverastins[5]. The enol triflates (**7**, **10**, **12**, **15**, **18**) were prepared starting from readily available ketones or aldehydes (**5**, **9**, **11**, **13**, **16**, respectively) as shown in Scheme 2. Coupling reactions between the enol triflates (or 2-iodopropene for UTKO4) and aldehyde **4** were carried out successfully using the Nozaki-Hiyama-Kishi procedure[6, 7] and UTKO1–6 were obtained after acid hydrolysis of MOM ether. The dihydro analog (UTKO7) and etherified analogs (UTKO9 and 10) of UTKO1 were also synthesized by its hydrogenation, Mitsunobu reaction or methylation (Scheme 3). The unsaturated ketone analog of UTKO1 was also obtained by the PDC oxidation-deprotection of **19** which is the intermediate from **7** to UTKO1. UTKO11 and 12, deformylated analogs of moverastin and UTKO1, respectively, were also synthesized from aldehyde **21** instead of **4** (Scheme 4). Detailed synthetic procedure for UTKO compounds will be published elsewhere.

Next, the cell migration inhibitory activity of these moverastin derivatives was

examined by the chemotaxis cell chamber (BD Biosciences) assay using conditioned medium of human esophageal tumor EC17 cells as a source of chemoattractants as previously reported with some modifications[8]. In this assay, EC17-conditioned medium was initially placed in the lower compartment. EC17 cells were incubated in the upper chamber, where they were allowed to migrate and penetrate the filter separating the chambers in order to enter the lower chamber. After 24 hr of incubation, the number of cells attached to the lower side of the filter was counted. The IC_{50} values obtained in this study are listed in Table 1. Among moverastin derivatives, UTKO1 showed the most potent inhibitory activity of EC17 cell migration with an IC_{50} of 1.98 μM (Fig. 2). UTKO7, UTKO9 and UTKO12 are also significantly more active inhibitors of EC17 cell migration than the parental natural product, moverastin A, with IC_{50} values of 2.12, 2.00 and 2.17 μM, respectively (Table 1). These inhibitory effects are not due to the toxic effect of the drug because their 50 % inhibitory concentration for EC17 cell viability, as estimated by trypan blue dye exclusion assay, was at least 5-fold higher than that for cell migration.

Previously, we found that moverastin A showed inhibitory activity against FTase,

and demonstrated that moverastin A inhibited the migration of tumor cells by inhibiting the farnesylation of H-Ras[5]; therefore, next we examined the effect of moverastin derivatives on FTase *in vitro*. For this assay, FTase was partially purified from EC17 cells and recombinant GST-H-Ras and [^{3}H]-farnesylpyrophosphate were used as the substrates as described before[5]. As shown in Table 1, all moverastin derivatives tested, including UTKO1, UTKO7, UTKO9, and UTKO12, which showed strong inhibition of cell migration, did not inhibit FTase *in vitro* up to 100 μM. These results indicated that a mechanism other than the inhibition of FTase is responsible for UTKO-induced inhibition of EC17 cell migration. To examine this possibility, several cancer cell lines were investigated with respect to the inhibitory potential of moverastin A and the most potent moverastin derivative, UTKO1. The inhibitory effect of moverastin A depends on cell type, and there is a significant negative correlation between the sensitivity of each cell to moverastin A and the expression level of H-Ras, a substrate of FTase ($r = -0.86$, $p = 0.0013$) (Fig. 3). This result supported our previous conclusion that moverastins inhibited tumor cell migration due to the inhibition of FTase. On the other hand, the cell migration inhibitory activity of UTKO1 also depends on cell type, but there is no

correlation with the expression levels of H-Ras ($r = -0.38$, $p = 0.40$) (Fig. 3). These results suggested that the inhibitory mechanism of cell migration by UTKO1 is different from that of moverastin A. To understand the molecular basis by which UTKO1 inhibits tumor cell migration, biochemical identification of the protein target for UTKO1 is now under investigation.

Our preliminary structure-activity relationship study revealed that UTKO12 retained the same level of inhibitory activity toward EC17 cell migration as that of UTKO1, indicating that formyl group on benzene ring is not required for the inhibitory activity toward EC17 cell migration. On the other hand, the formyl groups of moverastins are essential for the FTase inhibition, because the inhibitory activity of UTKO11 toward FTase has been lost.

Although UTKO1 was initially synthesized as an analogous compound of moverastins, it possesses a different biological function from cylindrol family, and therefore, UTKO1 is expected to be a new lead compound in the search for more potent anti metastatic anti-cancer agents.

Acknowledgements

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology.

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Table

Table 1. Effects of UTKO compounds on cell migration, cell viability and *in vitro* FTase

activity in EC17 cells.

(a) For cell viability assay, a trypan blue dye (15250-061, Gibco, Invitrogen) exclusion assay were used to examine cell viability and performed according to previously reported protocols[9]. (b) For *in vitro* FTase assay, partially purified enzymes from EC17 cells were incubated with [^{3}H]-FPP plus recombinant GST-H-Ras in the presence or absence of test compound. The reaction was terminated by the addition of TCA. The radioactivity of the TCA insoluble fraction was measured.

Figure legends

Figure 1. Structures of moverastin derivatives

Figure 2. Effect of UTKO1 on migration of EC17 cells

EC17 cells were incubated with various concentrations of UTKO1 in the top chamber; the lower chamber contained EC17-conditioned medium, obtained from 24-hour cultures of EC17 cells maintained in RPMI1640 supplemented with 1 % FBS. After 24 hr, the number of cells that migrated through the filter to the lower surface was counted.