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Overexpression of Plk3 causes Morphological Change and Cell Growth Suppression in Ras Pathway-activated Cells

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To unravel the growth inhibition mechanism of Polo-like kinase 3 (Plk3), the effect of overexpression of Plk3 was examined in 293T cells. Cell rounding, changes in actin organization and cellular detachment were induced by Plk3 transfection in a kinase activity-dependent manner. Although apoptosis was not observed, Plk3 overexpression suppressed cellular growth in a long-term colony-forming assay. Because both Plk3 and Ras affect F-actin organization, the effect of co-transfection of Plk3 and Ras was evaluated. Adhesion was synergistically lost by co-transfection of these two genes, compared with transfection of Plk3 alone. Furthermore, overexpression of Plk3 caused long-term growth suppression in Ras-transformed NIH3T3. Collectively, Plk3 activation might cause cytoskeleton re-organization and result in growth suppression more pronouncedly in Ras pathway-activated cells.

Key words: growth suppression, H-Ras, morphology, overexpression, Plk3.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; PCR, polymerase chain reaction; Plk, polo-like kinase; PVDF, polyvinylidene difluoride; WT, wild-type.

Cytoskeletal change is a hallmark of cellular transformation (*1*). The transformation is often associated with the down-regulation of cytoskeletal proteins (*2*); the expression of these proteins suppresses growth on soft agar and tumourigenicity in nude mice. Thus, cytoskeletal change is closely connected to anchorage-independent growth and invasion.

Ras is a key driver of cellular transformation. Ras signalling regulates actin filament proteins via several pathways; one pathway is the down-regulation of tropomyosin that contributes to the transformed phenotype (*3*) and the other pathway is the suppression of the ROCK-LIM kinase pathway, which regulates actin dynamics through the Ras–MAPK pathway (*4–6*). The oncogene Ras–MAPK pathway regulates actin cytoskeleton dynamics and cell adhesion, and these processes are important for cellular transformation by Ras.

Polo-like kinase 3 (Plk3) is a member of the Plk family, which mainly functions as a cell- cycle regulator. However, unlike other members of the Plk family, Plk3 has also been implicated in cytoskeletal changes. Overexpression of Plk3 induces rounded morphology with the disruption of the cellular F-actin network (*7*). Additionally, Plk3 appears to be a downstream mediator of MEK1 in the signalling pathway leading to Golgi fragmentation (*8*). Thus, it is possible that Plk3 is involved in Ras–MAP kinase-meditated cytoskeletal signalling. In addition to its role in cytoskeletal machinery, Plk3 may also function in apoptosis induction. Plk3 physically interacts with and phosphorylates p53 (*9*). Overexpression of Plk3 induces apoptosis in mammalian cells that is mediated in part via p53 activation (*10*), although the underlying mechanism of cell death remains unclear.

In the present study, we investigated the growth inhibition mechanism of Plk3. Overexpression of Plk3-induced cell rounding, changes in actin organization and cellular detachment in a kinase activity-dependent manner. Although apoptosis was not observed, Plk3 overexpression suppressed cellular growth in long-term colony-forming assay. Furthermore, we found that these morphological effects and long-term growth suppression by Plk3 expression are more pronounced in the presence of activated H-Ras, implying a potential therapeutic approach for cancer with the activated Ras pathway.

MATERIALS AND METHODS

Antibodies—Anti-Flag M2-peroxidase conjugate was purchased from Sigma (St Louis, MO, USA).

Plasmid Construction—The human Plk3 genes were amplified by PCR from human testis cDNA (Clontech, Palo Alto, CA, USA). The resultant PCR products were cloned into pBlueScript SKII(+). For expression in mammalian cells, Plk3 cDNA was cloned into pCMVTag2B (Stratagene, La Jolla, CA, USA) or pcDNA3.1/Hygro(+) (Invitrogen, Carlsbad, CA, USA), and H-RasV12 cDNA was cloned into pEF1/Myc-HisA (Invitrogen). To generate kinase-inactive Plk3, the lysine residue at position 91 was replaced with methionine by using the QuickChange site-directed mutagenesis kit (Stratagene).

Cell Culture and Transfection—Human embryonic kidney transformed 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented

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with 10% heat-inactivated fetal bovine serum (Moregate BioTech, Hamilton, Australia). The 293T cells were transfected with the indicated expression plasmids by using FuGENE6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) in six-well dishes according to the manufacturer's instructions. To establish human H-RasV12-transfected mouse fibroblast NIH3T3 clones, cells were transfected with pEF-H-RasV12. Transfected cells were cultured in the presence of G418 (700 μg/ml) for 2 weeks and cloned. Overexpression of exogenous human H-RasV12 gene products was confirmed by western blot analysis.

Western Blot Analysis—Cells were lysed by cell lytic extraction buffer (Sigma), and protein concentrations were determined with BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein were resolved by SDS–PAGE following transfer to PVDF membrane. Western blots were visualized by enhanced chemiluminescence plus (GE Healthcare, Little Chalfont, UK).

Flow Cytometric Analysis—Sample cells were harvested, washed once with phosphate buffered saline (PBS) and fixed in 70% ethanol over 24 h at –20°C. Fixed cells were washed once with PBS, treated with RNase (1 mg/ml) and stained by propidium iodide (10 μg/ml). Flow cytometric analysis was performed with a FACSCalibur device (Becton Dickinson, Mountain View, CA, USA), and the cell cycle was analysed with CellQuest.

Long-term Colony-forming Assay—Vector control plasmid, wild-type (WT) and kinase dead Plk3 expression plasmids were transfected into 293T cells by Fugene6 (Roche Molecular Biochemicals). After a 24-h transfection, cells were selected with 1600 μg/ml G418 for colony formation. Colonies were washed with PBS and stained with Coomassie Blue G-250 (Bio-Rad Laboratories, Munich, Germany). The number of colonies was counted. The same transfection method was used for parental and H-Ras-transformed NIH3T3. Transfected cells were selected with 400 μg/ml hygromycin for colony formation.

Immunofluorescence and Microscopy—The 293T cells on the cover glass in six-well plates were transfected with pCMVTag2B Plk3. After 48 h, the cells were fixed with 3.7% formaldehyde, then permeabilized with 0.05% Triton X-100. The Flag Plk3 and F-actin were stained with fluorescein isothiocyanate (FITC)-conjugated anti-Flag antibody (Sigma) and Alexa Fluor 633 phalloidin (Invitrogen), respectively. The localization of Plk3 and actin was microscopically observed with LSM510 META (Carl Zeiss, Oberkochen, Germany). Similar images were observed in over 50 cells.

Immuno-complex Kinase Assay—For immunoprecipitation (IP) in the transient transfection, 293T cells transfected with expression plasmid were lysed by Cell Lytic extraction buffer (Sigma) with protease inhibitors on ice for 30 min. Soluble cell extracts were obtained by centrifugation (12 000*g*) at 4°C 30 min. Cell extracts were incubated with antibodies (40 μl of anti-FLAG M2 Affinity Gel Freezer-Safe (Sigma) for 10^6 cells) for 2 h at 4°C on a rotating wheel. Samples were washed three times with Cell Lytic extraction buffer. α-Casein was incubated as a substrate with precipitated gel at 30° for 30 min in R buffer (20 mM Tris–HCl, pH 7.4,10 mM $MgCl_2$, 4.5 mM 2-mercaptoethanol, 1 mM EGTA) that contained 50 μM ATP and 10 μCi[γ-^{33}P]ATP(3000 Ci/mmol; GE Healthcare). Phosphorylated proteins were analysed by SDS–PAGE with subsequent autoradiography.

Mitotic Index—Exponentially growing cells seeded at 1.5×10^3 cells into a 96-well poly-D-lysine-coated tissue culture plate were transfected with control, WT and mutant Plk3 expression vectors as indicated. After 48 h, mitotic cells were immuno-stained with rabbit anti-phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY, USA). Mitotic index was calculated with IN Cell Analyzer 1000 (GE Healthcare).

RESULTS

Plk3 Induces Apoptosis-independent Morphological Changes in 293T Cells—The overexpression of Plk3 induces apoptosis with DNA ladder formation (*10*, *11*). To unravel the pro-apoptotic mechanism of Plk3, the effect of overexpression of Plk3 on cellular phenotypes was analysed. First, the effect of the transient expression of Plk3 on cellular morphology was determined by co-expressing green fluorescence protein (GFP) to visualize the morphology. As a positive control, pro-caspase-9 was overexpressed to induce apoptosis (*12*). The 293T cells carrying inactive p53 were selected for transfection to exclude the effect of p53, because Plk3 appears to partially act upstream of p53 (*10*, *11*). Overexpression of Plk3 led to a dramatic morphological change, as evident by the transfected cells showing a round cell shape (Fig. 1A). However, no significant cell rounding could be detected in cells transfected with a kinase-defective mutant of Plk3 (K91M) or the vector control. These results suggest that the morphological change is kinase activity dependent. Although the cell rounding phenotype seemingly resembles apoptosis-like morphology, no other apoptotic morphological characters were detected, including membrane blebbing and apoptotic body formation, as compared with pro-caspase-9-overexpressing cells (Fig. 1A). To characterize the apoptotic phenotypes of nuclear condensation and fragmentation, fluorescence-activated cell sorting (FACS) analysis was performed. The transfection efficiency, as determined by counting the GFP-expressing cells, was >80%. As shown in Fig. 1B, overexpression of WT Plk3 did not induce an increase in the subG1 fractions in 293T cells, whereas the overexpression of Caspase9 did. Thus, the kinase activity of Plk3 triggers morphological changes in 293T cells, and this effect is not a consequence of apoptosis. Additionally, because the cell rounding phenotype is also observed when cells are arrested in the mitotic phase, the mitotic index was examined by using phospho-histone H3 antibody. However, the mitotic index was not increased in Plk3-overexpressing cells, whereas an increase in the mitotic index was observed in nocodazole-treated cells (Fig. 2). Therefore, the cell rounding phenotype induced by the overexpression of Plk3 is not a consequence of mitotic arrest.

Plk3 Suppresses Cell Growth in a Kinase Activity-dependent Manner—The overexpression of Plk3 induced morphological changes but did not induce apoptosis in 2 days of culture. However, cell detachment was

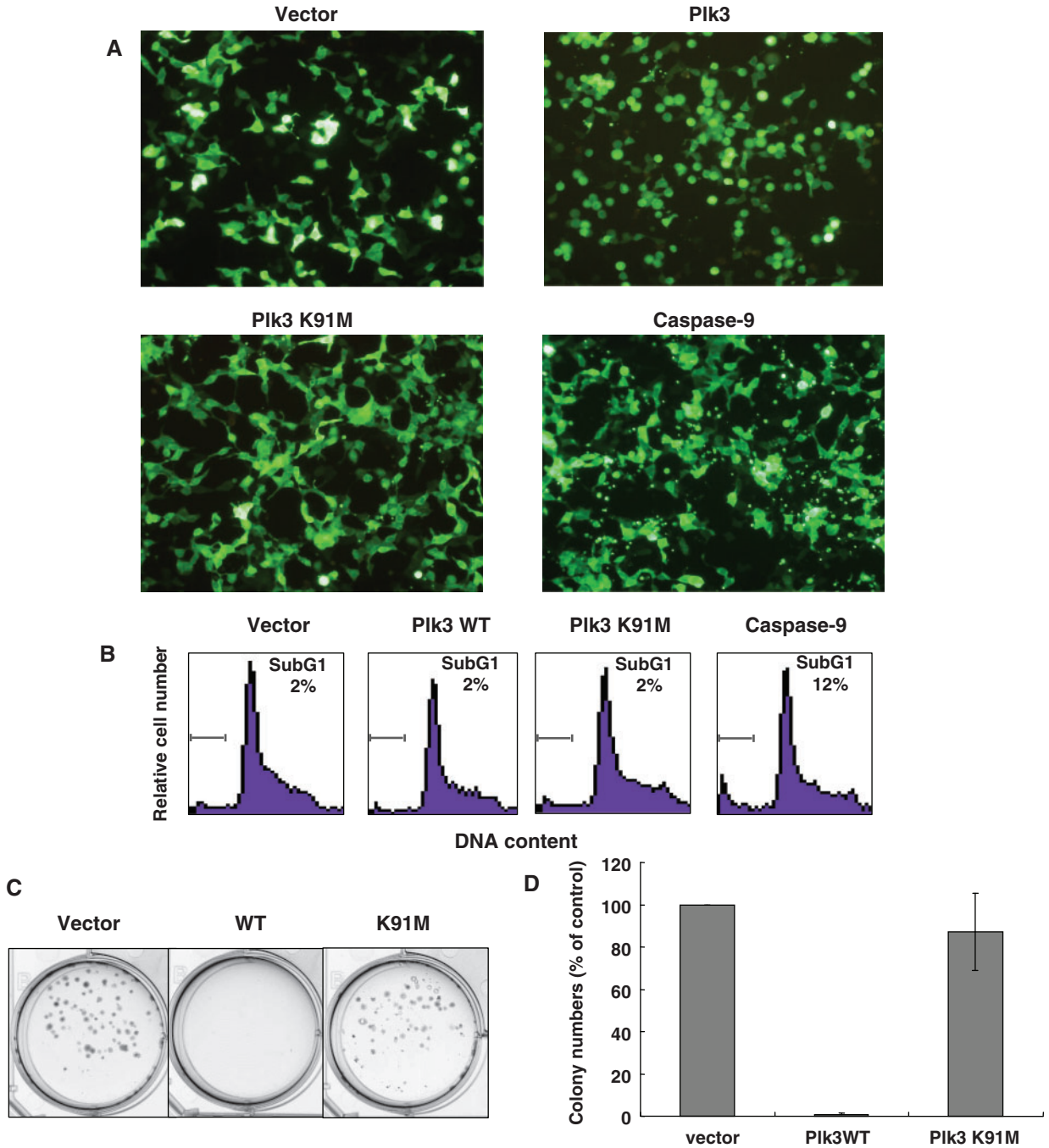

Fig. 1. **Analysis of the morphology, subG1 induction and colony formation by transfection of Plk3.** (A) Vector, Plk3WT, Plk3K91M or pro-caspase9 was transfected with EGFP into 293T cells. After 48 h, cell morphology expressing EGFP was photographed. (B) FACS analysis of transfected cells. Vector, Plk3WT, Plk3K91M or pro-caspase9 was transfected in 293T cells, respectively. After 48 h, the subG1 fraction was examined by FACS analysis. (C) Long-term cell colony-forming assay of Plk3-transfected cells. The 293T cells transfected with control expression vector, Plk3WT and Plk3K91M were cultured in medium containing G418 for 2 weeks. (D) Summary of the colony formation counts as shown in (C). Colonies were stained and counted. The number of colonies in the vector control was assumed to be 100%.

observed in WT Plk3-overexpressing cells, but not in a kinase-defective mutant of Plk3 (K91M), in the short-term assay system (Fig. 4A). These observations suggest that Plk3 suppresses cell adhesion in a kinase activity-dependent manner. Thus, to examine whether growth is suppressed as a consequence of morphological changes, a long-term colony-forming assay was conducted (Fig. 1C). Transfected cells were cultured in neomycin-containing medium for 2 weeks, and then the colonies were counted. As shown in Fig. 1C, the WT Plk3 clearly suppressed colony formation, while a kinase-defective mutant K91M and the vector control did not. Thus, the

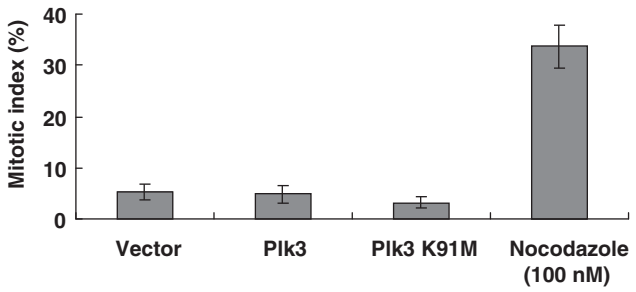

Fig. 2. **Analysis of the mitotic index by transfection of Plk3.** Vector, Plk3WT or Plk3K91M was transfected into 293T cells. After 48 h, mitotic index was analysed by IN Cell Analyzer 1000. Nocodazole-treated cells were used as positive controls.

cell rounding phenotype caused by overexpression of the Plk3 kinase appears to cause a loss of long-term viability. Collectively, these data strongly suggest that the overexpression of Plk3 induces morphological changes, cell detachment and eventually cell death.

Plk3 Affects the Distribution of F-actin in a Kinase Activity-dependent Manner—To investigate the molecular mechanism of morphological changes induced by the overexpression of Plk3, the distribution of Flag-Plk3 and F-actin were examined in 293T cells. Flag-WT Plk3 was mainly localized to the cellular cortex (Fig. 3A). In contrast, the expression of a kinase-defective mutant, K91M, resulted in a diffuse cytoplasmic and nuclear distribution (Fig. 3E). In parallel, F-actin was co-localized with Plk3 in both cells (Fig. 3C and G). These results suggest that the distribution of Plk3 is altered in a kinase activity-dependent manner and that the localization of WT Plk3 to the cellular cortex contributes to cytoskeletal changes.

Cell Adhesion is Synergistically Lost by Co-expression of Plk3 and H-RasV12—The oncogene Ras–MAPK pathway regulates actin cytoskeleton dynamics and cell adhesion, and these processes are important for cellular transformation by Ras. On the other hand, our results and others (*7*) suggest that Plk3 overexpression affects cellular adhesion and actin cytoskeleton organization. Moreover, Plk3 appears to be a downstream mediator of MEK1 in the signalling pathway leading to Golgi fragmentation (*8*). Thus, it is interesting to test the possibility that Plk3 is involved in Ras–MAP kinase-meditated cytoskeletal signalling. To examine whether Plk3 is involved in the Ras-related adhesion pathway, Plk3 protein was co-expressed with active H-Ras protein. Overexpression of WT Plk3 induces cell detachment (Fig. 4A). In contrast, fewer detached cells were found in H-RasV12-overexpressing cells. However, the co-expression of Plk3 and H-RasV12 induced a dramatic increase in the number of detached cells as compared with the overexpression of Plk3 alone or H-RasV12 alone (Fig. 4A). The number of detached cells was not increased by forced expression of the kinase-defective form, suggesting that this synergism is kinase activity dependent. To examine whether Plk3 is activated by the expression of H-RasV12, an immuno-complex kinase assay was performed with α-casein as the substrate. However, the kinase activity of Plk3 in the cells expressing H-RasV12 was not affected by co-expression of the activated Ras (Fig. 4B). This observation suggests that Plk3 and H-RasV12 might be involved in the different pathway.

Plk3 Suppressed Cell Growth in H-Ras-transformed NIH3T3 cells—The results that Plk3 overexpression strongly disrupts cell adhesion in the presence of Ras suggested that Plk3 overexpression induces growth inhibitory effects preferably in Ras-transformed cells. To examine the Ras dependency on cell growth suppression by overexpression of Plk3, long-term colony-forming assays were performed for parental and H-RasV12-transformed NIH3T3 cells. Both cell lines transfected with Plk3 were cultured in hygromycin-containing medium for 2 weeks and then the colonies were counted. The summary data shown in Fig. 5B indicate that Plk3 overexpression induces growth suppression of H-Ras-transformed NIH3T3 cells, but not parental NIH3T3 cells, in a kinase activity-dependent manner. These results suggest that Ras-dependent tumour cells are susceptible to the growth-inhibitory effect of Plk3 overexpression.

DISCUSSION

In the present study, we found that Plk3 overexpression in 293T cells induced morphological changes and cell detachment in a kinase activity-dependent manner but did not induce apoptosis. The disruption of adhesion was correlated with the distribution change of Plk3 and F-actin, suggesting that the change in cytoskeletal organization induced by the kinase activity of Plk3 resulted in the cell rounding morphology and cell detachment. This effect is likely to eventually lead to the loss of viability in the long-term colony-forming assay. Thus, our results suggest that the mechanism of growth suppression caused by Plk3 expression is not apoptosis, but it is mediated by the loss of adhesion through the perturbation of cytoskeletal organization.

The expression of activated Ras suppresses the ROCK-LIM kinase pathway responsible for cofilin phosphorylation (*4–6*) and then depolymerizes F-actin (*13*, *14*). Because both Plk3 and Ras were reported to affect F-actin organization, the effect of the co-transfection of Plk3 and Ras was evaluated. We found that cell adhesion was synergistically lost by co-transfection in a kinase activity-dependent manner. This result suggests that Plk3 overexpression strongly disrupts the cell adhesion in the presence of Ras. Furthermore, the overexpression of Plk3-induced growth suppression in H-RasV12-transformed NIH3T3 cell lines in the long-term colony-forming assay. Thus, the growth suppressive effects of Plk3 are accelerated in cells with an activated Ras signalling pathway.

The mechanism by which the co-expression of Plk3 and H-Ras synergistically disrupts cell adhesion remains unclear. One possibility is that Plk3 might be involved in Ras-MAP kinase-mediated cytoskeletal signalling. However, from the immuno-complex kinase assay experiment, activation of Plk3 was not shown by the co-transfection of Plk3 and Ras. Thus, Plk3 might not be directly involved in the Ras signalling pathway.

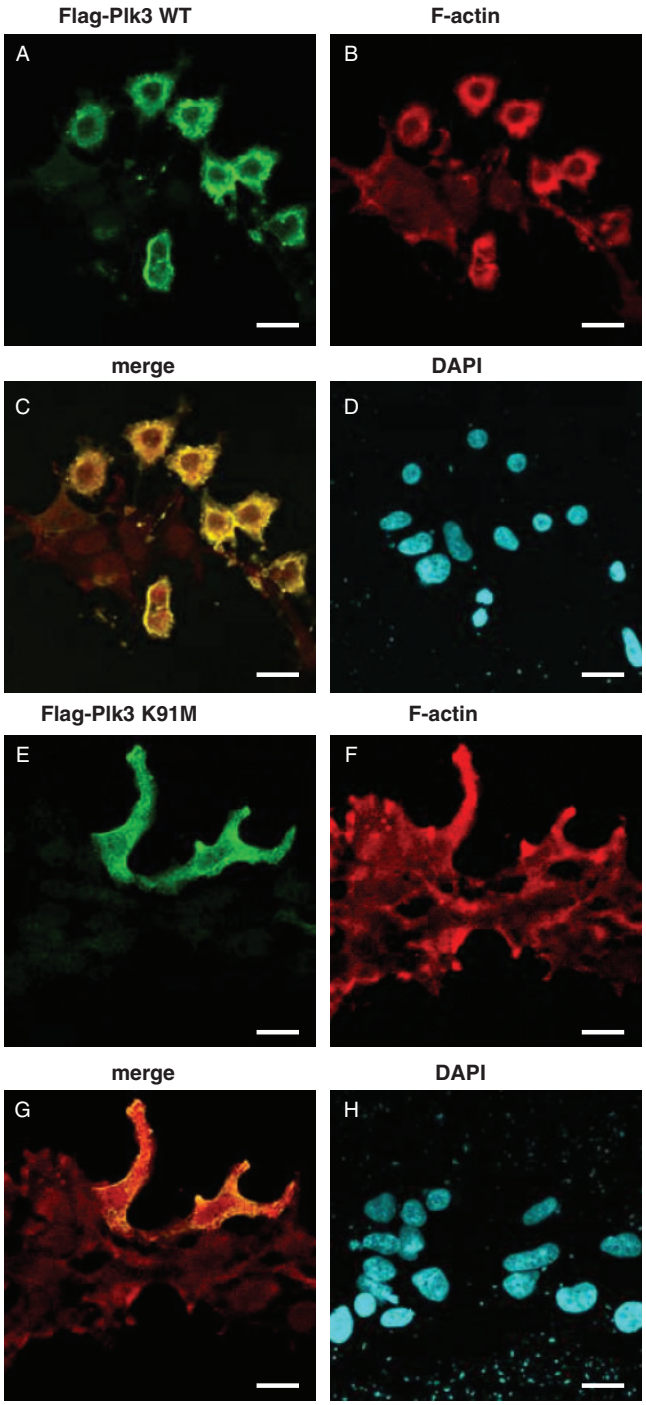

Fig. 3. **Cellular distribution of Plk3 and F-actin.** (A) Flag-Plk3WT was transfected into 293T cells. After 48 h, Flag Plk3 was stained with FITC-conjugated anti-Flag antibody. Localization of Plk3 was microscopically observed with LSM510 META. Bars: 20 μm. (B) Flag-Plk3WT was transfected in 293T cells. After 48 h, F-actin was stained with Alexa Fluor 633 phalloidin. The localization of F-actin was microscopically observed with LSM510 META. Bars: 20 μm. (C) Merged image of (A) and (B). Bars: 20 μm. (D) Flag-Plk3WT was transfected into 293T cells. After 48 h, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The localization of nuclei was microscopically observed with LSM510 META. Bars: 20 μm. (E) Flag-Plk3K91M was transfected into 293T cells. After 48 h, Flag Plk3 was stained with FITC-conjugated anti-FLAG antibody. Localization of Plk3 was microscopically observed with LSM510 META. Bars: 20 μm. (F) Flag-Plk3K91M was transfected into 293T cells. After 48 h, F-actin was stained with Alexa Fluor 633 phalloidin. Localization of F-actin was microscopically observed with LSM510 META. Bars: 20 μm. (G) Merged image of (E) and (F). Bars: 20 μm. (H) Flag-Plk3 K91M was transfected into 293T cells. After 48 h, the nuclei were stained with DAPI. Localization of nuclei was microscopically observed with LSM510 META. Bars: 20 μm.

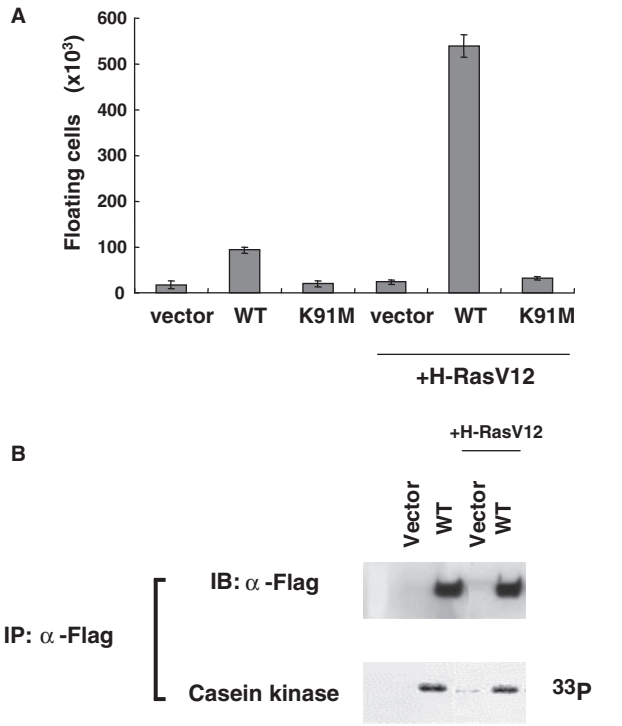

Fig. 4. **Co-transfection experiment of Plk3 and H-RasV12.** (A) The number of floating cells co-transfected with Plk3 and H-Ras. Vector, Plk3WT or Plk3 K91M was transfected with vector or H-RasV12 in 293T cells. After 48 h, the number of floating cells was counted. (B) Vector or Plk3WT was transfected with vector or H-RasV12 in 293T cells, respectively. After 48 h, cells were lysed and subjected to IP with antibodies to flag-M2. The resulting precipitates were then assayed for kinase activity with α-casein as the substrate and subjected to immunoblotting with anti-Flag-M2 antibody.

Another possibility is that Plk3 might affect the aberrant actin dynamics caused by activated Ras. De-regulated activation of Ras results in the aberration of actin dynamics followed by an increase in cell motility and invasion (*4–6*).

It is possible that there might be a surveillance system that maintains proper actin dynamics and that a tumour suppressor excludes the cancer cells with aberrant actin dynamics. For instance, death-associated protein kinase, which suppresses integrin-mediated cell adhesion (*15*), antagonizes Ras-mediated cellular transformation, and thus functions as a tumour suppressor (*16*). Plk3 has characteristics of a tumour suppressor, as evidenced by its down-regulation in tumour tissues (*17*, *18*) and its potential roles in checkpoint activation and apoptosis

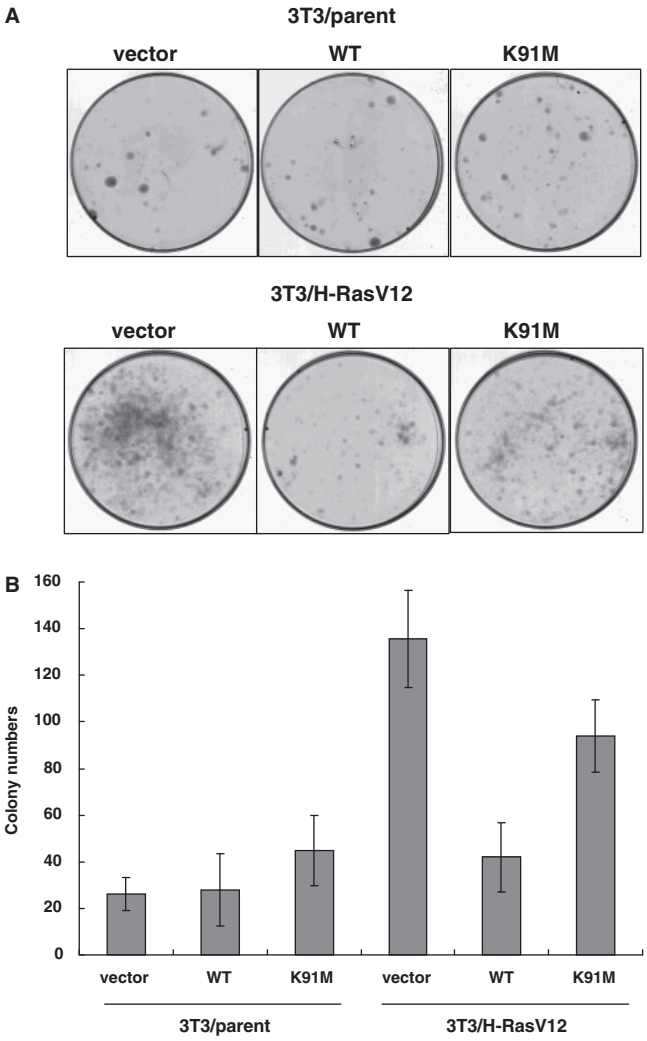

Fig. 5. **Long-term colony-forming assay of Ras-transformed NIH3T3 cells transfected with Plk3.** (A) Parental and Ras-transformed NIH3T3 cells transfected with control expression vector, Plk3WT and Plk3K91M were cultured in medium containing hygromycin for 2 weeks. (B) Summary of the colony formation counts. Colonies were stained and counted.

induction (*9*, *10*, *11*). Thus, we speculate that Plk3 acts as a tumour suppressor that excludes cancer cells with aberrant actin dynamics by accelerating the dysregulation of the adhesion-related pathway followed by the enhancement of cell detachment.

Our results demonstrate that the overexpression of Plk3 induces morphological change, disruption of cell adhesion, and cell growth inhibition in a kinase activity-dependent manner and that this inhibitory activity was amplified in cells in which the Ras signalling pathway is dominant. Therefore, a drug that affects the activity or expression of Plk3 might be effective on tumours dominated by Ras signalling. The clarification of the function of Plk3, such as the mechanism of morphological change, might lead to new cancer therapies.

CONFLICT OF INTEREST

None declared.

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