Integrin-linked kinase controls neurite outgrowth in N1E-115 neuroblastoma cells

Ishii et al. (2001) Integrin-linked Kinase Controls Neurite Outgrowth in N1E-115 Neuroblastoma Cells., J. Biol. Chem. 276: 42994-43003.

Integrin-linked Kinase Controls Neurite Outgrowth in N1E-115 Neuroblastoma Cells

Toshiaki Ishii, Eiki Satoh, and Masakazu Nishimura

Department of Pharmacology, University of Obihiro School of Veterinary Medicine,

Obihiro Hokkaido 080-8555, Japan.

Correspondence to: Dr. Masakazu Nishimura
Department of Pharmacology,
University of Obihiro School of Veterinary Medicine,
Obihiro Hokkaido 080-8555, Japan.
(Phone) 81-155-49-5365
(Fax) 81-155-49-5369
(E-mail) ishii@obihiro.ac.jp

Running title: The role of ILK in neuronal differentiation

Mouse N1E-115 cells grown on a laminin matrix exhibit neurite outgrowth in response to serum deprivation. Treatment of cells with an antibody against β1 integrin inhibits neurite outgrowth. Thus, β1 integrin is involved in the neuritogenesis of N1E-115 cells on a laminin matrix. Integrin-linked kinase (ILK), a recently identified cytoplasmic serine/threonine protein kinase that binds to the cytoplasmic domain of β1 integrin, has an important role in transmembrane signal transduction *via* integrins. We report that ILK is expressed in N1E-115 cells, the expression levels of which are constant under both normal and differentiating conditions. A stable transfection of a kinase-deficient mutant of ILK (DN-ILK) results in inhibition of neurite outgrowth in serum-starved N1E-115 cells grown on laminin. On the other hand, a transient expression of wild-type ILK stimulated neurite outgrowth. The ILK activity in the parental cells was transiently activated after seeding on the laminin matrix, whereas that in the DN-ILK transfected cells was not. These results suggest that transient activation of ILK is required for neurite outgrowth in serum-starved N1E-115 cells on laminin. Under the same conditions, p38 mitogen-activated protein (MAP) kinase, but neither MAP kinase / extracellular signal-regulated kinases kinase (MEK) nor extracellular signal-regulated kinases (ERK), was transiently activated after N1E-115 cell attachment to laminin, but not in the DN-ILK expressed cells. The time course of p38 MAP kinase activation was very similar to that of ILK activation. Furthermore, a p38 MAP kinase inhibitor, SB203580, significantly blocked neurite outgrowth. Thus, activation of p38 MAP kinase is involved in ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth in N1E-115 cells.

Introduction

Cell interactions with extracellular matrix (ECM) proteins are mediated primarily by integrins that function as cell-surface receptors composed of heterodimers of the α and β subunits. Such interactions are important in the regulation of cell proliferation and differentiation (1). Clustering of integrins on the cell surface in contact with the ECM induces focal adhesion that recruits numerous mitogenic signaling proteins, such as growth factor receptors (2), mitogen-activated protein (MAP) kinase (3), and small GTP-binding proteins (4) to integrin receptors. Thus, integrin-associated focal adhesions serve as signaling centers where adhesive and mitogenic pathways can integrate. Numerous physical interactions between integrins or focal adhesion components and mitogenic signaling proteins have been demonstrated (5). These studies indicate a biochemical coupling between integrin and growth factor signaling pathways, however, the functional significance of these interactions in the context of the regulation of proliferation and differentiation is not well understood.

The intracellular signaling cascades that are activated when integrins bind to their ECM ligands are varied in many different cell types (1). The specific intracellular pathways differ depending on the specific integrin / ECM ligand interaction and/or on the specific integrin / intracellular signal proteins coupling in different cell types. In neuronal cells, neurite formation in response to differentiation signals is strongly promoted by ECM ligands such as laminin, fibronectin, or collagen (6,7). Mouse N1E-115 neuronal cells exhibit neurite outgrowth in response to serum deprivation (8,9). Neurite outgrowth in serum-starved N1E-115 cells is enhanced when the cells are grown on a laminin matrix

(10). On the other hand, neurite outgrowth of PC12 cells, which are induced to differentiate into sympathetic neuron-like cells by growth factors such as nerve growth factor (NGF), is also affected by cell adhesion to ECM (11,12). The mechanisms by which the ECM regulates neuronal differentiation, however, are currently poorly understood.

Integrin-linked kinase (ILK) is a cytoplasmic protein serine/threonine kinase that interacts with β1 integrin (13). Recent biochemical and functional studies indicate that ILK serves as a mediator in integrin- and growth factor-mediated signal transduction (14,15). Overexpression of ILK in epithelial cells results in an altered cellular morphology, a reduction in cell adhesion to ECM, and also stimulation of anchorage- (13), but not serum-independent growth (16). The present study investigated the roles and potential mechanisms of ILK in the control of neuronal differentiation of N1E-115 neuroblastoma cells. We demonstrated that ILK activation is an early and important event in the integrin-mediated signal pathway and is necessary for neurite outgrowth in serum-starved N1E-115 cells on laminin. We also report that activation of p38 MAP kinase, but neither MEK nor ERK, is critically involved in the ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth.

Experimental procedures

Reagents - LY294002 was obtained from Sigma Chemical Co. (St. Louis, MO). The rabbit polyclonal anti-ILK IgG (UB 06-550 and UB 06-592) and myelin basic protein (MBP) were

obtained from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-p38 MAP kinase, anti-p38 MAP kinase, anti-phospho-MEK, and anti-MEK antibodies were obtained from New England Biolabs (Beverly, MA). Anti-phospho-p44/42 MAP Kinase (Anti-phospho-ERK1/ERK2) and anti-p44/42 MAP Kinase (anti-ERK1/ERK2) antibodies were obtained from Promega (Madison, WI). All other chemicals were of analytical grade and were obtained from Sigma Chemical Co. or Wako Pure Chemical Co. (Osaka, Japan) unless otherwise specified.

Kinase assays - Kinase assays were performed as described by Delcommenne et al. (17). Cells were lysed in 50 mM Hepes buffer (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 µg/ml leupeptin, 2.5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM sodium fluoride, and 1 mM sodium orthovanadate. The lysates were incubated with anti-ILK antibody (UB 06-592) at 4^{o}C for 12 h. After incubation, the lysates were precleared and immune complexes were collected with Protein A-sepharose. The immunoprecipitated ILK was incubated for 20 min at 30^{o}C in the presence or absence of 10 µg of the exogenous substrate MBP in a total volume of 50 µl kinase reaction buffer (50 mM HEPES pH 7.0, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM NaF, 1 mM Na_3VO_4) containing 10 µCi [γ-^{32}P]ATP (6000Ci/mmol, NEG-502Z, Dupont NEN, Wilmington, DE). The reaction was stopped by the addition of an equal volume of 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The kinase reaction products were analyzed using

SDS-PAGE (5-20% polyacrylamide) and autoradiography. For detection of the immunoprecipitated ILK and DN-ILK proteins, the precipitated proteins were released from the immunobeads by boiling in 80 µl of SDS-PAGE sample buffer for 5 min. Equal volumes of the samples were loaded onto SDS-PAGE. Total ILK and DN-ILK proteins were detected by immunoblotting with an anti-ILK antibody (UB 06-550) that recognizes both ILK and DN-ILK proteins.

cDNA cloning - A cDNA library was constructed in Uni-ZAP XR using cDNA synthesis kits (No.200400-200402, Stratagene, La Jolla, CA). Poly(A)$^+$RNA prepared from the whole brain of an adult guinea pig was converted to cDNA by oligo(dT)-primed reverse transcription. The obtained cDNAs were ligated between *Eco*RI and *Xho*I sites of Uni-ZAP XR as described by the manufacturer. This cDNA library was screened with ^{32}P-labeled anti-sense oligonucleotides, 3'-ATACGTGGACGGACCCAT-5' and 3'-GGACTTCTGTGTTTGTCT-5', both of which were designed to bind to the encoding sequences for the amino acids sequences, $_{351}$YAPAWV$_{356}$ and $_{365}$PEDTNR$_{370}$, respectively, within the catalytic domain of human ILK (13). One positive clone containing a 1.8 - kbp insert, which binds both oligonucleotide-probes, was isolated from 2x10^5 plaques. After excision of the pBluescript phagemid vector from the Uni-ZAP XR, the cDNA insert was cloned into the pBluescript SK plasmid as described by the manufacturer, and sequenced. The nucleotide sequence of guinea pig ILK has been submitted to the GenBank data base with accession number AF256520. The encoded

protein has a 98.9% amino acid homology with the human ILK.

Construction of cDNA- The kinase deficient ILK (DN-ILK) was generated by site-directed mutagenesis (Glu to Lys) at amino acid residue 359 within the kinase domain using the polymerase chain reaction (PCR) as follows. A 1153 - kbp cDNA containing the 5'-untranslated sequence and the coding region for Met_{1}-Glu_{366} of guinea pig ILK, in which Glu_{359} was mutated to Lys, was synthesized using PCR with two oligonucleotide primers, 5'-TGGCGGCCGCTCTAGAACTAGTGGAT-3' (pBluescript vector primer, the *Not* I site is underlined) and 5'-TTCAGGTTTCTTCTGCAAAGCTT(T)AGGGGCTA-3' [a unique *Hind* III site of ILK cDNA is underlined, and a single mutation site (C to T) is shown in parentheses], and ILK cDNA in pBluescript vector as a template. The PCR fragment was digested with *Not* I/*Hind* III restriction enzymes, and was then placed back into a full-length ILK cDNA in a pBluescript vector. The nucleotide sequence of the PCR fragment was determined using a standard DNA sequencing technique (18). Wild type ILK and DN ILK cDNAs were ligated into the polylinkers in a mammalian expression vector, pTracerTM-CMV2 (V885-01, Invitrogen Corp., Carlsbad, CA), and were introduced into N1E-115 cells.

Stable expression and cloning of cell lines- DN-ILK cDNA was transfected into N1E-115 cells (5 x 10^5 cells/100 mm culture dish) using the calcium/phosphate precipitation method as described by Graham and van der Eb (19), and 48 individual Zeocin -

resistant cell lines were isolated over the next 4 to 5 weeks. Among them, three different cell lines were selected on the basis of detection of the GFP fluorescence and confirmation of gene transcription using RT-PCR. The cloned cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) containing 20% fetal bovine serum (FBS; Hyclone, Logan, UT) and Zeocin (0.5 mg/ml).

Transient expression- N1E-115 cells were seeded at a density of 5 X 10^4 per dish onto 35 mm non-coated culture dishes and grown in DMEM containing 10% FBS. At 20 h following plating, cells were transfected with the plasmid containing the wild-type ILK cDNA using Lipofectamine transfection reagent (GibcoBRL). Control cells were transfected with the empty plasmid. Briefly, cells were washed in serum-free medium, and incubated in serum-free medium for 1 h. During this time, the plasmid DNAs were mixed with the Lipofectamine reagent and incubated for 30 min at room temperature, and then added to the cells. Ten percent FBS was added to the cells 6 h after addition of the transfection mix. At 18 h and 36 h following the transfection, the morphological changes produced by the transfection of the plasmid of interest were examined using fluorescence microscopy. Cells that had positively expressed green fluorescent protein (GFP) were assumed to be expressing the protein of interest cDNA. Cells that have a process longer than 2-fold the length of a cell body were categorized as being neurite bearing cells, and neurite bearing cell was assessed as the percentage of the total number of GFP-expressing cells

Western blot analysis- Cells were solubilized in 5 volumes of buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EGTA, and 2 mM PMSF at 4^{o}C. The solubilized materials were subjected to SDS-PAGE (5-20% gradient, 6.5% or 10% polyacrylamide) and transferred onto nitrocellulose membranes at 4^{o}C in 25 mM Tris-HCl (pH 8.4), 192 mM glycine, 20% methanol, and 0.025% SDS. After blocking, the blots were probed with anti-ILK polyclonal antibody (UB 06-550) for the detection of endogenous ILK in PBS containing 0.05% Tween20, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. The final protein/IgG complexes were visualized following the reaction to 3,3'-diaminobenzidine tetrahydrochloride. For detection of the phosphorylation status of p38 MAP kinase and MEK, polyclonal antibodies reactive with the phosphorylated form of p38 MAP kinase and MEK were purchased from New England Biolabs. For detection of the phosphorylation status of p44/42 MAP Kinase (ERK1/ERK2), polyclonal antibodies reactive with the phosphorylated form of p44/42 MAP Kinase (ERK1/ERK2) was purchased from Promega. Preparation of cell lysate and protein blotting for detection of these phosphorylated MAP kinases was conducted according to the manufacturer's instructions.

Results

Stimulation of neurite outgrowth in serum-starved N1E-115 cells grown on a laminin matrix requires cell adhesion *via* β1 integrin. Mouse N1E-115 neuroblastoma cells exhibit neurite outgrowth in response to serum deprivation (8,9), which is strongly

affected by the substrates that the cells adhere to in the extracellular matrix (10,20). We observed neurite outgrowth of cells grown on laminin-coated plates under serum-free conditions. In this condition, cells became flattened and then gradually extended neurites within 8 h, and approximately 88% of the cells possessed neurites after 16 h (Fig. 1B and see also Fig. 3). The number of neurite-bearing cells grown on non-coated plates, however, was quite low (8.6 ± 3.0% at 8 h and 18.3 ± 5.2% at 16 h). Sarner et al. (10) found that pre-treatment of N1E-115 cells with β1 integrin antibody blocks adhesion and neurite outgrowth of the cells plated on laminin-coated glass slide in serum-free condition, suggesting that β1 integrin is involved in both adhesion and neuritogenesis of N1E-115 cells grown on a laminin matrix. We also examined the effect of β1 integrin antibody on neurite outgrowth in serum-starved N1E-115 cells grown on the laminin-coated plastic plate. Cells were briefly pretreated for 7 min with varying concentrations of an antibody directed against β1 integrin and then seeded on laminin-coated plates under serum-free conditions. As shown in Fig. 1, higher concentrations of antibody significantly blocked adhesion. On the other hand, lower concentrations of antibody (0.3 and 1.0 μg/ml) did not affect cell adhesion but significantly inhibited neurite outgrowth 8 h and 16 h after antibody-treatment. Our observations are similar to those of Sarner et al. (10). Thus, β1 integrin is involved in both adhesion and neuritogenesis of N1E-115 cells grown on a laminin matrix under serum-free conditions.

ILK is highly expressed in N1E-115 cells and its expression level remains constant

during neuronal differentiation. ILK is a serine/threonine protein kinase that interacts directly with the cytoplasmic domain of the β1 integrin subunit (13) and whose kinase activity is modulated by cell-extracellular matrix interactions and insulin in a phosphatidylinositol 3 (PI 3) - kinase dependent manner (17). ILK has important roles in integrin- and growth factor-mediated signal transduction in several different cells, leading to regulation of cell adhesion, growth, migration, survival, proliferation, and differentiation (13,16,21,22,23). Little is known, however, regarding the role of ILK in neuronal cells. Because β1 integrin is involved in both adhesion and neuritogenesis of N1E-115 cells grown on the laminin matrix (Fig.1), we further examined whether ILK is expressed in the cells. We found that ILK is highly expressed in N1E-115 cells and also that the expression level of ILK did not change during neuronal differentiation (Fig.2).

Transient activation of ILK activity after seeding on a laminin matrix is required for neurite outgrowth in serum-starved N1E-115 cells. We first examined whether endogenous ILK is involved in integrin-dependent neurite outgrowth. To inactivate the endogenous ILK, cells were stably transfected with a kinase-deficient mutant of ILK (DN-ILK) that behaves as a dominant negative (17). Based on the results obtained from immunoblotting (Fig.4 inset), the expression level of DN-ILK protein in DN-ILK transfected cells could be estimated to be at least twice more than that of endogenous ILK protein. As shown in Figs. 3 and 4, neurite outgrowth of the DN-ILK transfected cells was significantly inhibited compared with that of untransfected parental cells 8 h and 16 h

after seeding on the laminin matrix under serum-free conditions. This result suggests that the kinase activity of endogenous ILK is critical for neurite outgrowth in serum-starved N1E-115 cells grown on a laminin matrix. We also examined the effect of transient expression of the wild-type ILK on neurite outgrowth of the cells. Over-expression of the wild-type ILK stimulated neurite outgrowth in the cells even grown on non-coated plate (Fig.5). We next examined whether ILK is activated after seeding on a laminin matrix under serum-free conditions. As shown in Fig. 6, the ILK activity in the parental cells was transiently activated after seeding on the laminin matrix, whereas that in the DN-ILK transfected cells did not change after cell attachment to the laminin. Maximal stimulation of ILK activity in the parental cells occurred 60 min after plating, and then rapidly disappeared. Moreover, ILK activation was prevented by treatment of the cells with LY294002, a specific inhibitor of PI 3-kinase (24). Thus, ILK activation following cell attachment to the laminin under serum-free conditions is PI 3-kinase dependent. These results suggest that transient activation of ILK activity after cell attachment to a laminin matrix is required for neurite outgrowth in serum starved N1E-115 cells.

p38 MAP kinase, but neither MEK nor ERK, is activated after seeding on a laminin matrix under serum-free conditions, and endogenous ILK is involved in this activation. Activation of the MEK/ ERK pathway is required for NGF-induced neuronal differentiation in PC12 cells (25,26). Unlike the NGF-induced neuronal differentiation in PC12 cells, however, activation of MEK and/or ERK was not detected in the

integrin-dependent neuronal differentiation of N1E-115 cells (Fig. 7A & B). We next examined the possible involvement of another member of the MAP kinase family, p38 MAP kinase, in the signaling pathway of integrin-dependent neuronal differentiation of N1E-115 cells. As shown in Fig. 7A&C, the p38 MAP kinase in the parental control cells was transiently activated after seeding on the laminin matrix under serum-free conditions. The activation of p38 MAP kinase in the parental cells reached a maximum level within 60 min and then declined rapidly. Weak activation was still detected 90 min after plating. Thus, the time-course of the activation of p38 MAP kinase was very similar to that of the ILK. In contrast, activation of p38 MAP kinase in the DN-ILK transfected cells was not detected (Fig.7A&C). Thus, endogenous ILK is somehow involved in the activation of p38 MAPK. These results suggest that the ILK activation after cell attachment to laminin is necessary for the activation of p38 MAP kinase in serum starved N1E-115 cells.

Activation of p38 MAP kinase is critical for integrin-dependent neurite outgrowth in serum-starved N1E-115 cells. To evaluate the specific role of the p38 MAP kinase signaling pathway in integrin-dependent neurite outgrowth of N1E-115 cells, the cells were treated for 3 h with varying concentrations of SB203580, a specific inhibitor of p38MAP kinase (27). SB203580 was applied after seeding the cells on laminin-coated plates under serum-free conditions. Treatment was terminated by changing the medium. As shown in Fig. 8, treatment of the cells with SB203580 markedly, but not completely, inhibited integrin-dependent neurite outgrowth in a dose-dependent manner with a

maximal inhibition obtained at 10 μM. PD98059, a specific inhibitor of MEK (26), did not affect neurite outgrowth (Fig. 8). On the other hand, the same treatment of DN-ILK transfected cells with SB203580 did not affect neurite outgrowth (Fig. 9), suggesting that p38 MAP kinase is not involved in ILK-independent neurite outgrowth. Moreover, SB203580 (10 μM) maximally blocked only 75% of the ILK-dependent neurite outgrowth (Fig. 8 and 9), suggesting that signaling pathways other than p38 MAP kinase, which can be activated via ILK activation, might also be involved in integrin-dependent neurite outgrowth. These results suggest that p38 MAPK activated via the PI 3-kinase-dependent activation of ILK, but neither MEK nor ERK, is required for integrin-dependent neurite outgrowth in serum-starved N1E-115 cells.

PI 3-kinase is involved in both ILK-dependent and -independent signaling pathways in neurite outgrowth of serum-starved N1E-115 cell on laminin. Although neurite outgrowth in the DN-ILK transfected cells was markedly inhibited in comparison with that in the parental cells, approximately 30% of the DN-ILK transfected cells, in which the kinase activity of ILK is negligible, extended neurites 16 h after plating (Fig.4). Indeed, ILK and p38 MAP kinase activity in the DN-ILK transfected cells was not stimulated at all after plating the cells on laminin-coated plates under serum-free conditions (Fig.6 and 7). In this condition, neurite outgrowth in the DN-ILK transfected cells was not affected by SB203580, but was significantly inhibited by LY294002 (Fig.9). These results suggest that PI 3-kinase, but not p38 MAP kinase, is also involved in

ILK-independent neurite outgrowth. Thus, it seems that PI-3 kinase activates not only ILK-dependent but also ILK-independent signaling pathways in neurite outgrowth of serum-starved N1E-115 cells on laminin.

Discussion

The results of the present study demonstrate that ILK participates in integrin-dependent neuritogenesis in serum-starved N1E-115 cells grown on a laminin matrix, and also that activation of p38 MAP kinase is critical to the effect of ILK. These results are based on the following observations: 1) Both ILK and p38 MAP kinase were activated in a similar time course after seeding on a laminin matrix under serum-free conditions, 2) neurite outgrowth and p38 MAP kinase activation were inhibited in N1E-115 cells expressing the dominant negative ILK, and 3) treatment of cells with SB203580, a specific inhibitor of p38 MAP kinase, significantly blocked neurite outgrowth.

Integrin signaling is required for neuritogenesis in serum-starved N1E-115 cells (10,20). We demonstrated that neurite outgrowth in serum-starved N1E-115 cells on laminin depends on an integrin-dependent signal pathway, because the anti-β1 integrin antibody inhibited neurite outgrowth (Fig.1). How signals are transduced into the cell via integrin and what intracellular events occur after the cell-extracellular matrix interaction, however, are not fully understood in N1E-115 cells. Although higher concentrations of the antibody blocked cell adhesion, lower concentrations of the antibody inhibited neurite outgrowth without affecting cell adhesion (Fig.1). These results imply that mechanical

stresses brought by the direct interaction of integrin with the antibody have influences on the formation of focal adhesion complexes or the integrin / ECM-mediated intracellular signaling without affecting cell adhesion *via* integrin / ECM ligand interaction. Indeed, previous study using beads coated with anti-β1 antibodies demonstrated that the binding of anti-β1 antibody to integrin induces a rapid recruitment of mRNA and ribosomes to focal adhesion (28). Thus, such an Integrin-mediated unknown signaling might be involved in the inhibition of neurite outgrowth by lower concentrations of antibody.

In the present study, we determined that ILK transduces signals derived from the cell-laminin matrix interaction into the cell and functions as an important regulator in the initial process of neuronal differentiation in N1E-115 cells. ILK is located in the cell-matrix adhesion site (29). The kinase activity of ILK can be activated by integrin-mediated cell adhesion to the extracellular matrix and insulin in a PI 3-kinase-dependent manner (17). ILK is expressed in N1E-115 cells, and also adhesion of the cells to laminin stimulates ILK activity under serum-free conditions (Fig.2 and 6). The stimulation was maximal 60 min after plating and then declined rapidly (Fig.6). The activation of ILK was prevented by treatment with the PI 3-kinase inhibitor, LY294002 (Fig.6). These results suggest that activation of ILK after cell adhesion to laminin is mediated through a PI 3-kinase-dependent mechanism, and not likely through direct interaction between ILK and integrin, even though ILK interacts directly with the β1 integrin cytoplasmic domain (13). To investigate the role of endogenous ILK in the neuronal differentiation of N1E-115 cells, we cloned cell lines that highly express a

kinase-deficient mutant of ILK (DN-ILK), which behaves as a dominant negative. Indeed, activation of ILK was completely blocked in DN-ILK expressed cells (Fig.6). The overexpression of DN-ILK results in a significant inhibition of neurite outgrowth (Fig.3&4), suggesting that endogenous ILK is an important mediator for integrin-dependent neuritogenesis in N1E-115 cells.

The pheochromocytoma cell line PC12 has been well studied for neuronal differentiation induced by neurotrophic factors such as NGF. These studies suggest that growth factor-induced activation of MAP kinase pathways is critical for neuronal differentiation of the cells (26,30-33). On the other hand, integrin-dependent adhesion induces MAP kinase activation (34-36) and also cooperates with growth factors to efficiently activate MAP kinase pathways (3). A recent study of myogenic differentiation demonstrated that overexpression of ILK induces sustained activation of MAP kinase (21). Cross-talk between ILK and MAP kinase has also been reported in studies on anchoring-independent cell cycle progression (37). Because MAP kinase serves as a downstream effector of β1 integrin (38), integrin clustered in focal adhesions on EMC is considered to recruit ILK *via* β1 integrin and activate its enzyme activity, which in turn regulates MAP kinase activity. In the present study, both β1 integrin and ILK were necessary for integrin-dependent neurite outgrowth in serum-starved N1E-115 cells. Therefore, we further examined the possible involvement of MAP kinase in the neuronal differentiation of N1E-115 cells. Two different MAP kinase inhibitors were used, a specific inhibitor of p38 MAP kinase, SB203580, and a specific inhibitor of MEK, PD98059, to

examine whether p38 MAP kinase or the ERK pathway is involved in integrin-dependent neuronal differentiation of N1E-115 cells. SB203580 (10 μM) significantly inhibited neurite outgrowth of N1E-115 cells with a maximal inhibition of 50.3%, which compareds to 75% inhibition in ILK-dependent neurite outgrowth. PD98059 had no effect on neurite outgrowth (Fig.8). These results suggest that p38 MAP kinase, but not the ERK pathway, is necessary for integrin-dependent neurite outgrowth. Indeed, we demonstrated that p38 MAP kinase, but neither MEK nor ERK, was activated after cell attachment to laminin under serum-free conditions (Fig.7). The results also suggest, however, that activation of p38 MAP kinase alone is not sufficient for neurite outgrowth in serum-starved N1E-115 cells on laminin because SB203580 did not completely inhibit neurite outgrowth. On the other hand, LY294002 completely blocked ILK-dependent neurite outgrowth and also significantly inhibited ILK-independent neurite outgrowth (Fig. 9). Thus, PI 3-kinase is critical for the activation of not only the ILK-p38 MAP kinase pathway but also other pathways involved in the neuronal differentiation of N1E-115 cells. Recently, Sarner et al. (10) suggested that PI 3-kinase-dependent activation of Cdc42 and Rac1 *via* Ras signals, but not ERK or JNK, is critically involved in neurite outgrowth in N1E-115 cells. This report supports the present results in which activation of ERK is not required for neurite outgrowth in N1E-115 cells. Thus, it seems that both p38 MAP kinase and Ras signal pathways might be necessary for full induction of integrin-dependent neurite outgrowth in serum-starved N1E-115 cells, although the interaction and/or the cross-talk between ILK and Ras signaling pathways remains unknown.

To more directly examine the role of ILK in integrin-dependent neuronal differentiation, we attempted to express the wild type ILK in either transient or stable overexpression systems. We clearly showed that transient over-expression of the wild-type ILK results in significant stimulation of neurite outgrowth in the cells even grown on non-coated plate (Fig. 5). Transient ILK-expression in the cells grown on laminin-coated plate, however, strongly inhibited cell adhesion on a laminin matrix, and all ILK-expressed cells, but not vector-expressed control cells, were detached from the plate 24 h after transfection but still survived in suspension for a while (data not shown). Furthermore, we failed to generate stable cell lines that highly expressed the wild type ILK, while a kinase-deficient mutant readily yielded such cell lines. Therefore, we could not exactly determine the effect of exogenous overexpression of wild type ILK on the laminin / integrin-dependent neuronal differentiation of N1E-115 cells. Previous studies demonstrated that overexpression of ILK in epithelial cells suppresses suspension-induced apoptosis and stimulates anchorage-independent cell cycle progression (16). Thus, ILK in N1E-115 cells might have an important roles in bi-directional transmembrane signaling pathways *via* integrin to regulate a variety of functions, such as cell adhesion, growth, and survival besides neuronal differentiation.

Over-expression of DN-ILK completely prohibited the activation of p38 MAP kinase (Fig. 7A&C). Furthermore, the time course of p38 MAP kinase activation was almost identical to that of ILK activation after cell attachment to laminin (Fig. 6 and 7). These results strongly suggest that ILK activation is necessary for p38 MAP kinase

activation in N1E-115 cells. The mechanism by which ILK might trigger p38 MAP kinase activation after adhesion to laminin under serum-free conditions, however, remains unknown. ILK interacts with β1 integrin through the carboxy-terminal domain (13) and also with PINCH, an adaptor protein comprising five LIM domains, through the amino-terminal ankyrin repeat domain (39). PINCH can interact with the SH2/SH3 domain-containing adaptor protein Nck-2, which associates with ligand-activated growth factor receptor kinases or intracellular components of growth factor signaling pathways such as insulin receptor substrate (40,41). Thus, ILK-PINCH interaction is critical not only for proper subcellular localization of ILK but also formation of a signaling complex coupling integrins and growth factor receptors. On the other hand, Pak (p21-activated kinase), a family of Ser / Thr kinases activates p38 MAP kinase (42). Nck-2 can recognize and activate Pak, the activation process of which has been suggested to involve membrane localization mediated by the SH2 / SH3 domain from Nck (43). Pak is also a potential mediator of Rac / Cdc42 signaling, which leads to activation of p38 MAP kinase via Pak (44). Therefore, one possible mechanism by which ILK activates p38 MAP kinase is that the Pak is activated by direct interaction with Nck-2, which probably occurs following the ILK-PINCH interaction. The present results reveal a new aspect of ILK function, *i.e.*, control of neuronal differentiation in neuroblastoma cells. The challenge will be to unravel the molecular mechanisms underlying this phenomenon.

References

1. Hynes, R. O. (1992) *Cell* **69**, 11-25

2. Plopper, G. E., McNamee, H. P., Dike, L. E,, Bojanowski, K., and Ingber, D. E. (1995) *Mol. Biol. Cell* **6**, 1349-1365

3. Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M. (1996) *J. Cell Biol.* **135**, 1633-1642

4. Hotchin, N. A. and Hall, A. (1995) *J. Cell Biol.* **131**, 1857-1865

5. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) *J. Cell Sci.* **113**, 3563-3571

6. Turner, D. C., and Flier, L. A. (1989) *Dev. Neurosci.* **11**, 300-312

7. Turner, D. C., Flier, L. A., and Carbonetto, S. (1989) *J. Neurosci.* **9**, 3287-3296

8. Jalink, K., van Corven, E. J., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W. H. (1994) *J cell Biol* **126**, 801-810

9. Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997) *Mol Cell Biol* **17**,1201-1211

10. Sarner, S., Kozma, R., Ahmed, S., and Lim, L. (2000) *Mol Cell Biol* **20**,158-172

11. Rossino, P., Gavazzi, I., Timpl, R., Aumailley, M., Abbadini, M., Giancotti, F., Silengo, L., Marchisio, P. C., and Tarone, G. (1990) *Exp. Cell Res.* **189**, 100-108

12. Zhang, Z., Tarone, G., and Turner, D. C. (1993) *J. Biol. Chem.* **268**, 5557-5565

13. Hannigan, G. H., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996) *Nature* **379**, 91-96

14. Dedhar, S., Williams, B., and Hannigan, G. (1999) *Trends Cell Biol.* **9**, 319-323

15. Huang, Y., and Wu, C. (1999) *Int. J. Mol. Med.* **3**, 563-572

16. Radeva, G., Petrocelli, T., Behrend, E., Leung-Hagesteijn, C., Filmus, J., Slingerland, J., and Dedhar, S. (1997) *J. Biol. Chem.* **272**, 13937-13944

17. Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 11211-11216

18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467

19. Graham, F. L., and van der Eb, A. J. (1973) *Virology* **52**, 456-467

20. van Leeuwen, F. N., Kain, H. E. T., van der Kammen, R. A., Michiels, F., Kranenburg, O. W., and Collard, J. G. (1997) *J cell Biol* **139**, 797-807

21. Huang, Y., Li, J., Zhang, Y., and Wu, C. (2000) *J. Cell Biol.* **150**, 861-871

22. Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J., and Dedhar, S. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 3207-3212

23. Wu, C., Keightley, S. Y., Leung-Hagesteijn, C., Radeva, G., Coppolino, M., Goicoechea, S., McDonald, J. A., and Dedhar, S. (1998) *J. Biol. Chem.* **273**, 528-536

24. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) *J. Biol. Chem.* **269**, 5241-5248

25. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) *Cell* **77**, 841-852

26. Pang, L., Sawada, T., Decker, S. J., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 13585-13588

27. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) *FEBS Lett.* **364**, 229-233

28. Chicurel, M.E., Singer, R.H., Meyer, C.J., and Ingber, D.E. (1998) *Nature* **392**, 730-733

29. Li, F., Zhang, Y., and Wu, C. (1999) *J. Cell Sci.* **112**, 4589-4599

30. Lange-Carter, C. A., and Johnson, G. L. (1994) *Science* **265**, 1458-1464

31. Leppa, S., Saffrich, R., Ansorge, W., and Bohmann, D. (1998) *EMBO J.* **17**, 4404-4413

32. Morooka, T., and Nishida, E. (1998) *J. Biol. Chem.* **273**, 24285-24288

33. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. S. (1998) *Nature* **392**, 622-626

34. Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K., and Juliano, R. L. (1994) *J. Biol. Chem.* **269**, 26602-26605

35. Clark, E. A., and Brugge, J. S. (1995) *Science* **268**, 233-239

36. Zhu, X., and Assoian, R. K. (1995) *Mol. Biol. Cell* **6**, 273-282

37. Wang, F., Weaver, V. M., Petersen, O. W., Larabell, C. A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M. J. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14821-14826

38. Sastry, S. K., Lakonishok, M., Wu, S., Truong, T. Q., Huttenlocher, A., Turner, C. E., and Horwitz, A. F. (1999) *J. Cell Biol.* **144**, 1295-1309

39. Tu, Y., Li, F., Goicoechea, S., and Wu, C. (1999) *Mol. Cell. Biol.* **19**, 2425-2434

40. Tu, Y., Li, F., and Wu, C. (1998) *Mol. Biol. Cell* **9**, 3367-3382

41. Wu, C. (1999) *J. Cell Sci.* **112**, 4485-4489

42. Manser, E., Leung, T., Salihuddin, H., Zhao, Z., and Lim, L. (1994) *Nature* **367**, 40-46

43. Lu, W., Katz, S., Gupta, R., and Mayer, B. J. (1997) *Curr. Biol.* **7**, 85-94

44. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995) *J. Biol. Chem.* **270**, 23934-23936

Footnotes

The abbreviations used are: ILK, integrin-linked kinase; NGF, nerve growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK kinase; ECM, extracellular matrix; GFP, green fluorescent protein; PI 3, phosphatidylinositol 3; Pak, p21-activated kinase.

Key words: Integrin-linked kinase, N1E-115 cell, Neurite outgrowth, p38 MAP kinase, Neuronal differentiation, Laminin, Integrin, Cell adhesion

Acknowledgements. We thank Dr. H. Niinobe (Institute for Protein Research, Osaka University, Japan) for providing N1E-115 cells. This work was supported by grants from the Japanese Ministry of Education, Science and Culture; and a Grant-in-Aid for Encouragement of Young Scientists (T.I.)

Figure legends

Figure 1. Inhibition of cell adhesion (A) and neurite outgrowth (B) by treatment of

N1E-115 cells with the anti-β1 integrin antibody. (A) Cells were treated with the anti-β1 integrin antibody for 7 min prior to plating and then seeded onto laminin-coated plates. The number of cells adhering to the 35 mm plates was counted 8 h and 16 h following pretreatment with the anti-β1 integrin antibody. (B) The number of cells possessing neurites greater than twice the length of a cell body was assessed 8 h and 16 h following pretreatment with the anti-β1 integrin antibody. Numbers of neurite bearing cells are shown as a percentage of the 400 cells counted for each plate. A total of five independent plates were examined. Values are the means ± SD of five separate experiments.

Figure 2. Expression level of endogenous ILK in N1E-115 cells. The expression level of ILK was examined after culturing cells in either normal or differentiating conditions. Cells were seeded on laminin-coated plates and cultured in serum-free conditions for the indicated time period, and then lysed. ILK expression levels in cell lysates (20 μg) were analyzed using Western blotting methods with an affinity purified polyclonal anti-ILK antibody as described under " *Experimental procedures* ".

Figure 3. Time course of neurite outgrowth in parental and DN-ILK transfected N1E-115 cells after seeding on laminin-coated plates in the presence and absence of serum. Photomicrographs were taken 8 h (b and e) and 16 h (a, c, d, and f) after plating. Parental (a-c) and DN-ILK transfected (d-f) cells were seeded on laminin-coated plates and cultured in the presence of 20% FBS (a and d) and in the absence of FBS (b, c, e, and f).

Parental cells cultured in serum-free conditions exhibited extensive neurite outgrowth. In contrast, DN-ILK transfected cells cultured in serum-free medium exhibited less neurite outgrowth.

Figure 4. Effect of stable expression of DN-ILK on neurite outgrowth of serum-starved N1E-115 cells grown on the laminin matrix. The number of cells possessing neurites greater than twice the length of a cell body was assessed 8 h and 16 h after seeding on laminin-coated plates in serum-free medium. The number of neurite bearing cells is shown as a percentage of 400 cells counted for each plate. A total of four independent plates were examined. Values are the means ± SD of four separate experiments. Inset shows the expression level of endogenous ILK and exogenous DN-ILK proteins in parental cells (P) and DN-ILK transfected cells (D). ILK and DN-ILK proteins were detected by immunoblotting with an anti-ILK antibody that recognizes both ILK and DN-ILK proteins. The expression level of DN-ILK protein in DN-ILK transfected cells could be estimated to be at least twice more than that of endogenous ILK protein.

Figure 5. Effect of transient expression of wild-type ILK on neurite outgrowth in N1E-115 cells grown on the non-coated plate. N1E-115 cells were seeded at a density of 5 X 10^4 per dish onto 35 mm non-coated culture dishes and grown in DMEM containing 10% FBS. At 20 h following plating, cells were transfected with the plasmid containing the wild-type ILK cDNA (Wild-type ILK) using Lipofectamine transfection reagent, as described under

"*Materials and Methods*". Control cells were transfected with the empty plasmid (Vector). At 18 h and 36 h following the transfection, the morphological changes produced by the transfection of the plasmid were examined using fluorescence microscopy. At least 100 randomly selected GFP-positive cells were assessed, and the cells that have a process longer than 2-fold the length of a cell body were categorized as being neurite bearing cells. The neurite bearing cell was assessed as the percentage of the total number of GFP-expressing cells. Each experiment was repeated at least three times. Values are the means ± SD.

Figure 6. Stimulation of ILK activity after cell adhesion on laminin in serum-free condition. Cells were seeded on laminin-coated plates in serum-free medium and cultured for the indicated time periods. The cells were lysed, and ILK was immunoprecipitated from cell extracts. ILK activity was determined using MBP as an exogenous substrate, as described under " *Experimental procedures* "(Top). To examine the involvement of PI 3-kinase in the stimulation of ILK activity, cells were treated with 40 μM LY294002, a specific inhibitor of PI 3-kinase, for 1 h by direct addition to the culture medium. Total ILK and DN-ILK proteins in the immunoprecipitates were detected by immunoblotting with an anti-ILK antibody that recognizes both ILK and DN-ILK proteins , as described under " *Experimental procedures* "(Bottom).

Figure 7. Activation of p38 MAPK, MEK and ERK after seeding on laminin-coated plates

in serum-free conditions. (A) Parental N1E-115 cells and DN-ILK transfected cells were seeded on laminin-coated plates in serum-free conditions and cultured for the indicated time periods. The cells were lysed, and the lysates were used for the following immunoblotting analysis. The active forms of p38 MAPK, MEK and ERK were detected by immunoblotting with an anti-phospho p38 MAPK, an anti-phospho MEK, and an anti-phospho ERK antibody, respectively. Total proteins of p38 MAPK, MEK and ERK were detected by immunoblotting with an anti-p38 MAPK, an anti- MEK, and an anti-ERK antibody, respectively. Data shown are representative of three separate experiments that gave essentially the same results. (B) PC12 cells were treated with 50 ng/ml NGF for the indicated time periods, and then cells were lysed. The active forms of ERK and total proteins of ERK were detected by immunoblotting with an anti-phospho ERK antibody and anti-ERK antibody, respectively. (C) Time-course of the activation of p38 MAP kinase. The visualized bands on the membranes were analyzed by Image Scanner (EPSON, GT8000), and the band densities were quantified with image analysis software (NIH, Image).

Figure 8. Inhibition of p38 MAPK, but not MEK, prevented integrin-dependent neurite outgrowth of N1E-115 cells. The cells were seeded on laminin-coated plates in serum-free medium in the presence of varying concentrations of SB203580 or 10 μM PD98059, and cultured. The inhibitors were removed by changing the culture medium 3 h after seeding the cells. The number of cells possessing neurites greater than twice the

length of a cell body was assessed 8 h and 16 h after plating the cells. Values are the means ± SD of four separate experiments.

Figure 9. Effects of SB203580 and LY294002 on neurite outgrowth of parental cells and DN-ILK transfected cells. The cells were seeded on laminin-coated plates in serum-free medium in the presence of 10 μM SB203580 or 10 μM PD98059, and cultured. The inhibitors were removed by changing the culture medium 3 h after seeding the cells. The number of cells possessing neurites greater than twice the length of a cell body was assessed 8 h and 16 h after plating the cells. Values are the means ± SD of four separate experiments.

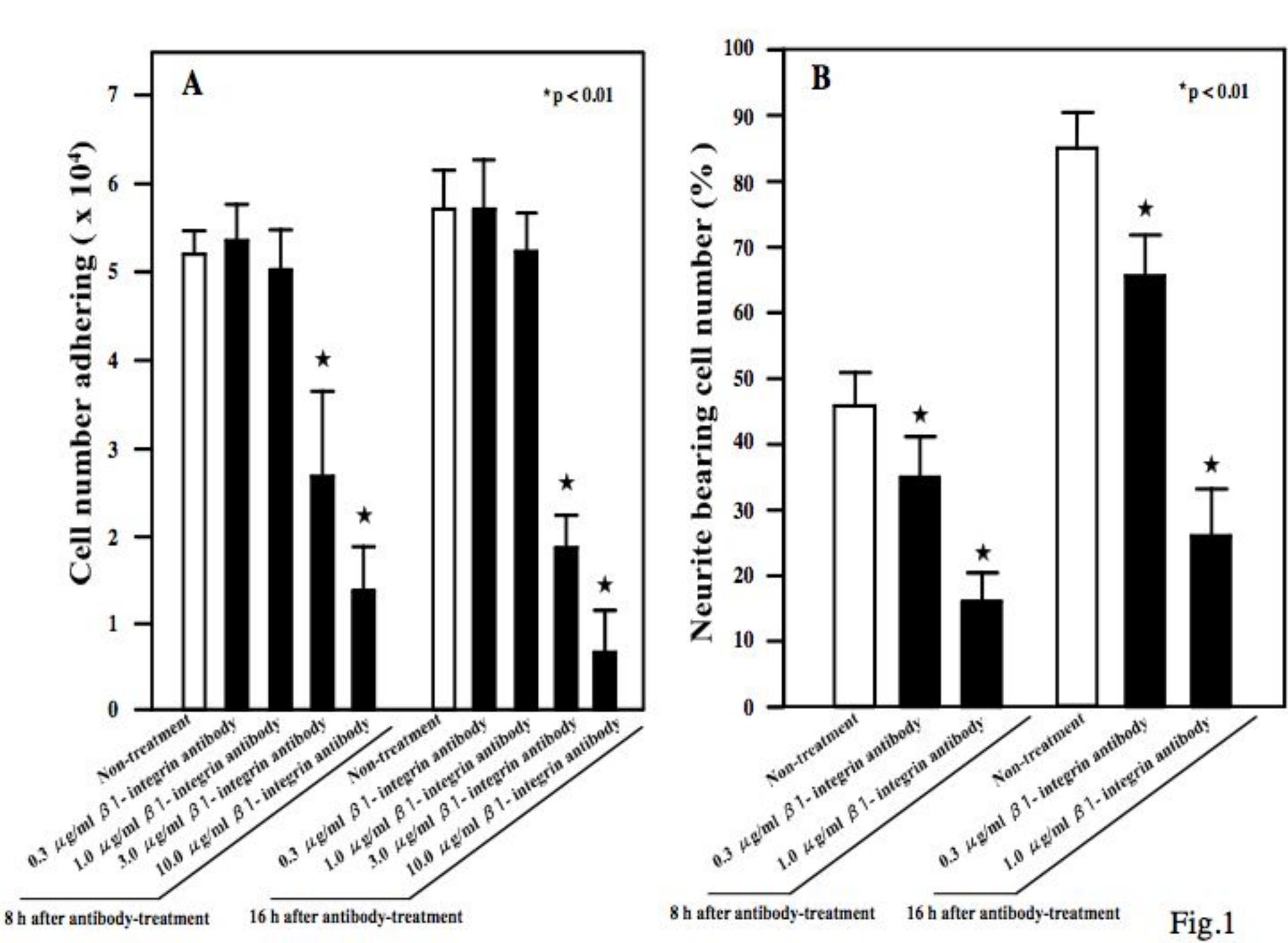

A
*p < 0.01
Cell number adhering (x 10⁴)
0
1
2
3
4
5
6
7
Non-treatment
0.3 μg/ml β1- integrin antibody
1.0 μg/ml β1- integrin antibody
3.0 μg/ml β1- integrin antibody
10.0 μg/ml β1- integrin antibody
8 h after antibody-treatment
16 h after antibody-treatment
B
*p < 0.01
Neurite bearing cell number (%)
0
10
20
30
40
50
60
70
80
90
100
Non-treatment
0.3 μg/ml β1- integrin antibody
1.0 μg/ml β1- integrin antibody
8 h after antibody-treatment
16 h after antibody-treatment

Fig.1

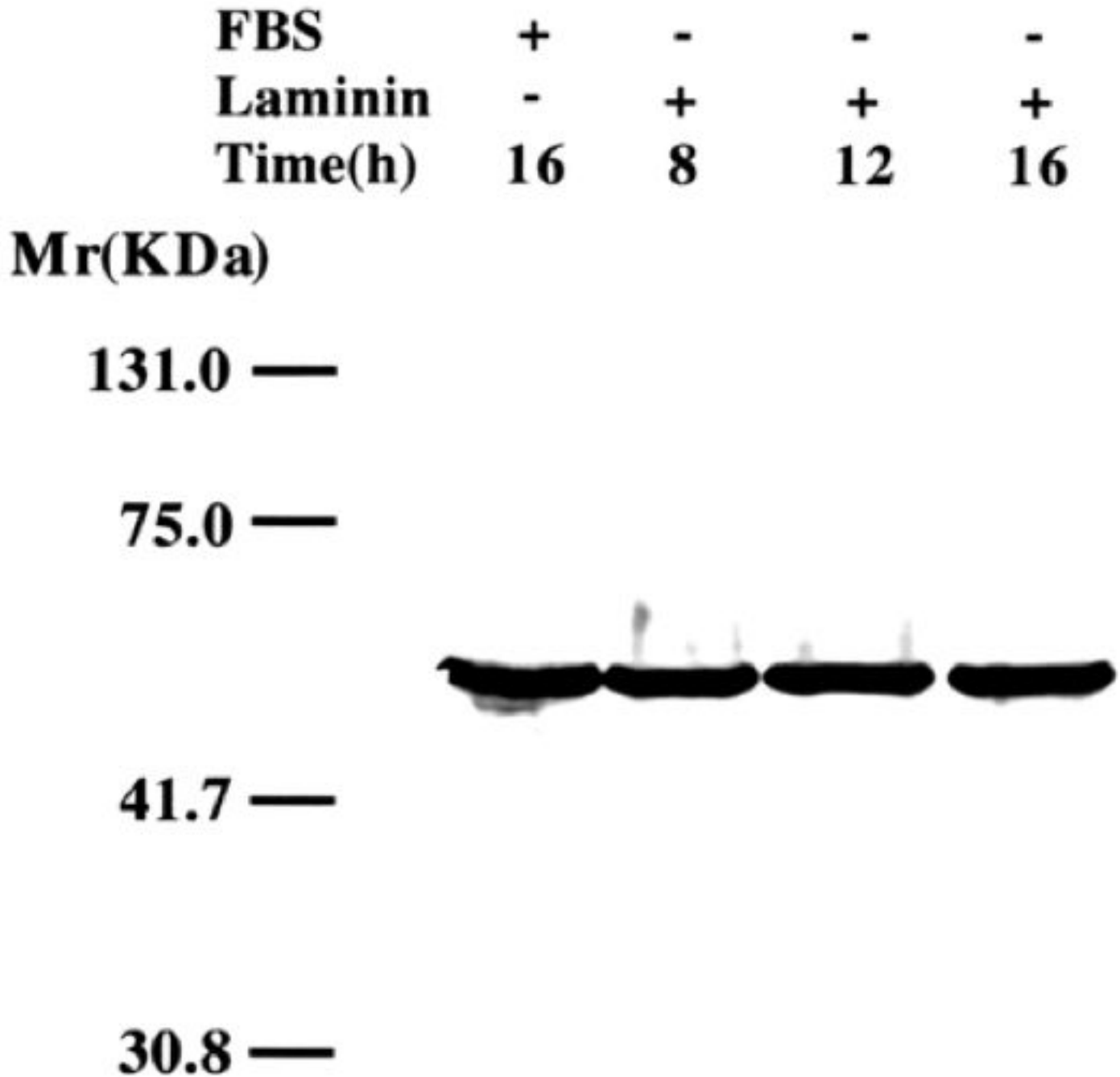
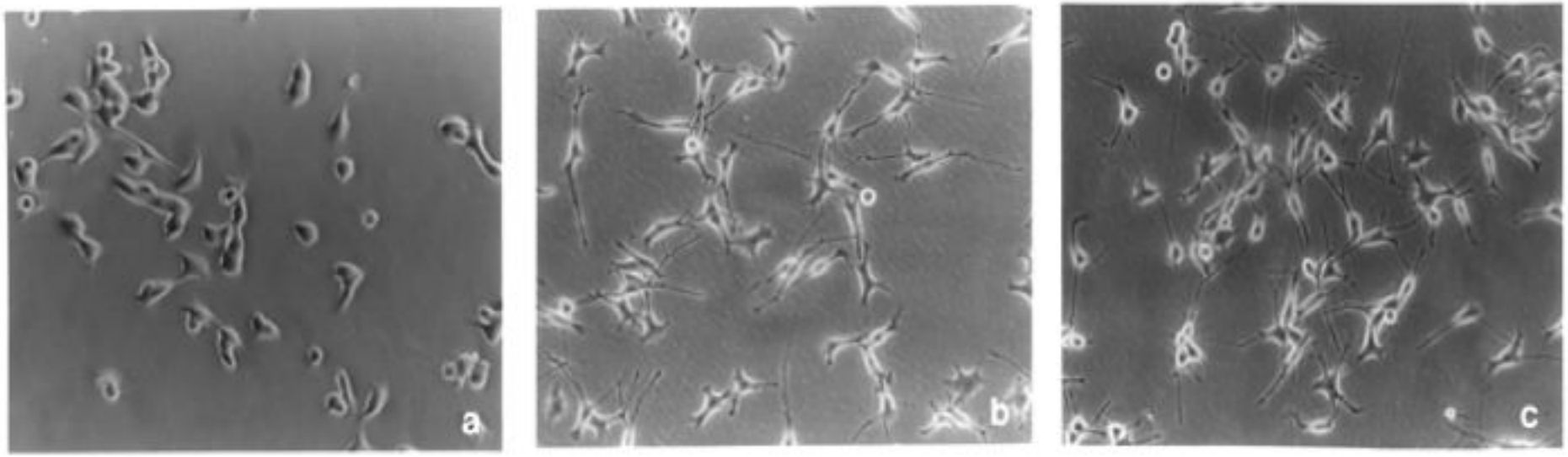


Fig.2

Parental cell



DN-ILK

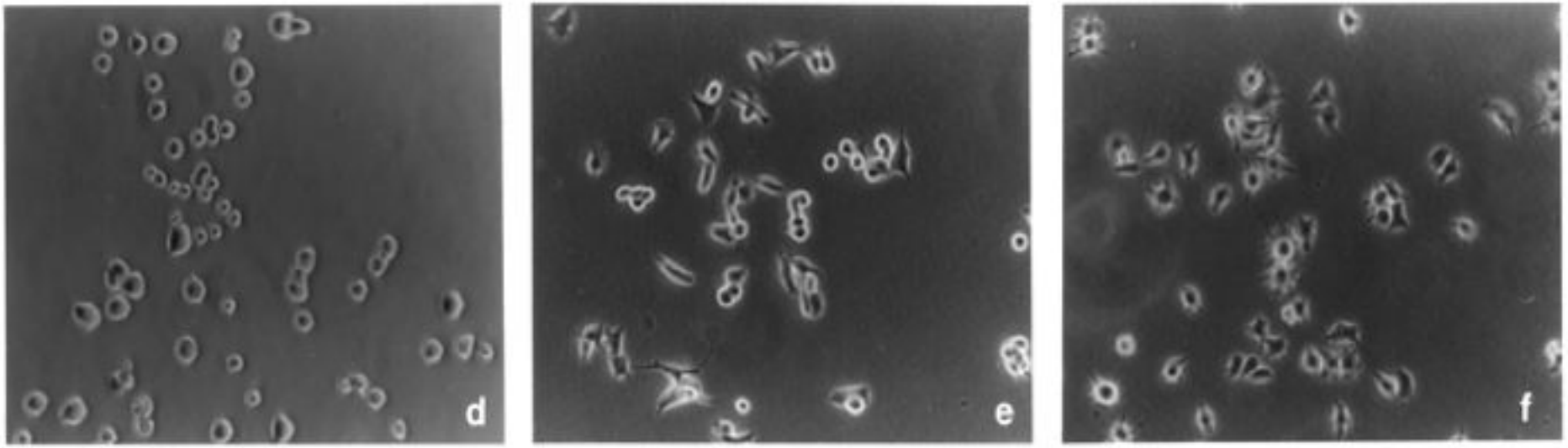


Fig.3

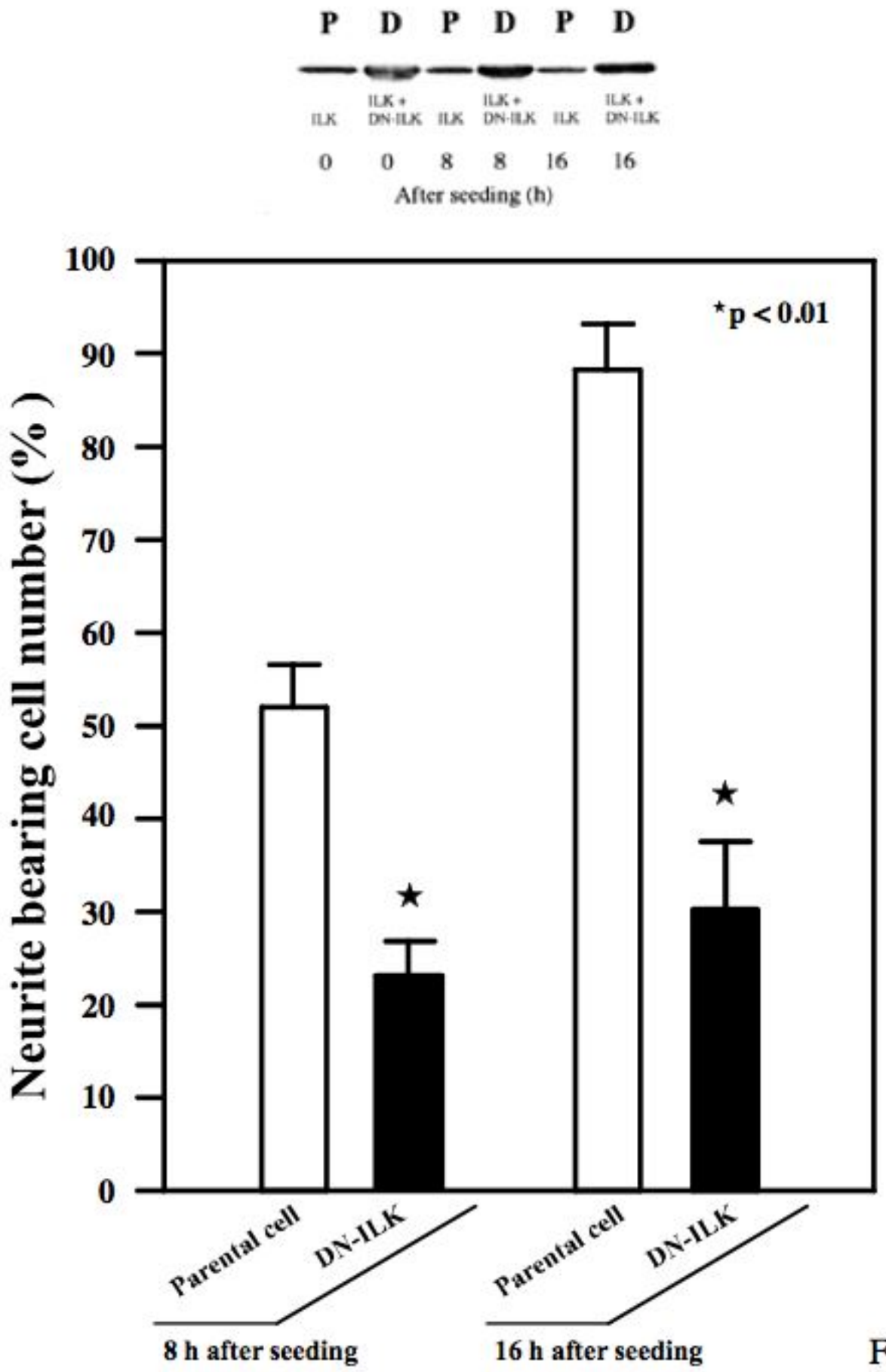
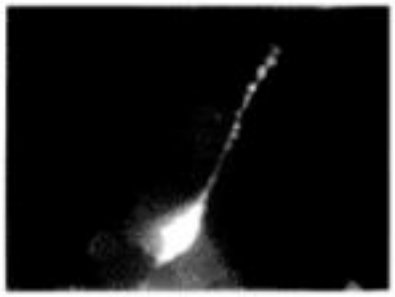

P D P D P D
ILK
ILK + DN-ILK
ILK
ILK + DN-ILK
ILK
ILK + DN-ILK
0 0 8 8 16 16
After seeding (h)
100
90
80
70
60
50
40
30
20
10
0
Neurite bearing cell number (%)
*p < 0.01
★
★
Parental cell
DN-ILK
Parental cell
DN-ILK
8 h after seeding
16 h after seeding

Fig.4

GFP positive cell / Vector



GFP positive cell / Wild-type ILK

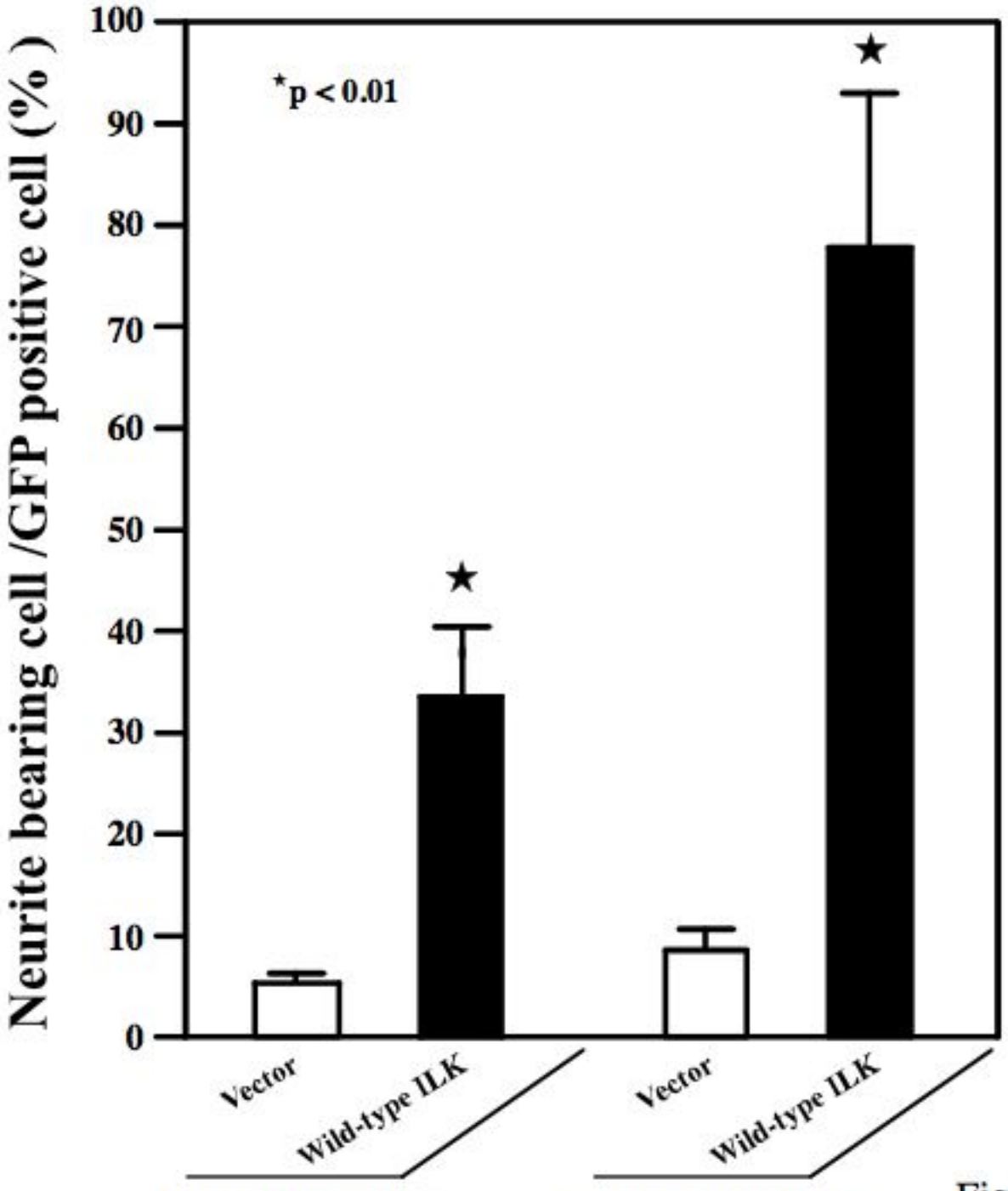


Fig.5

ILK activity

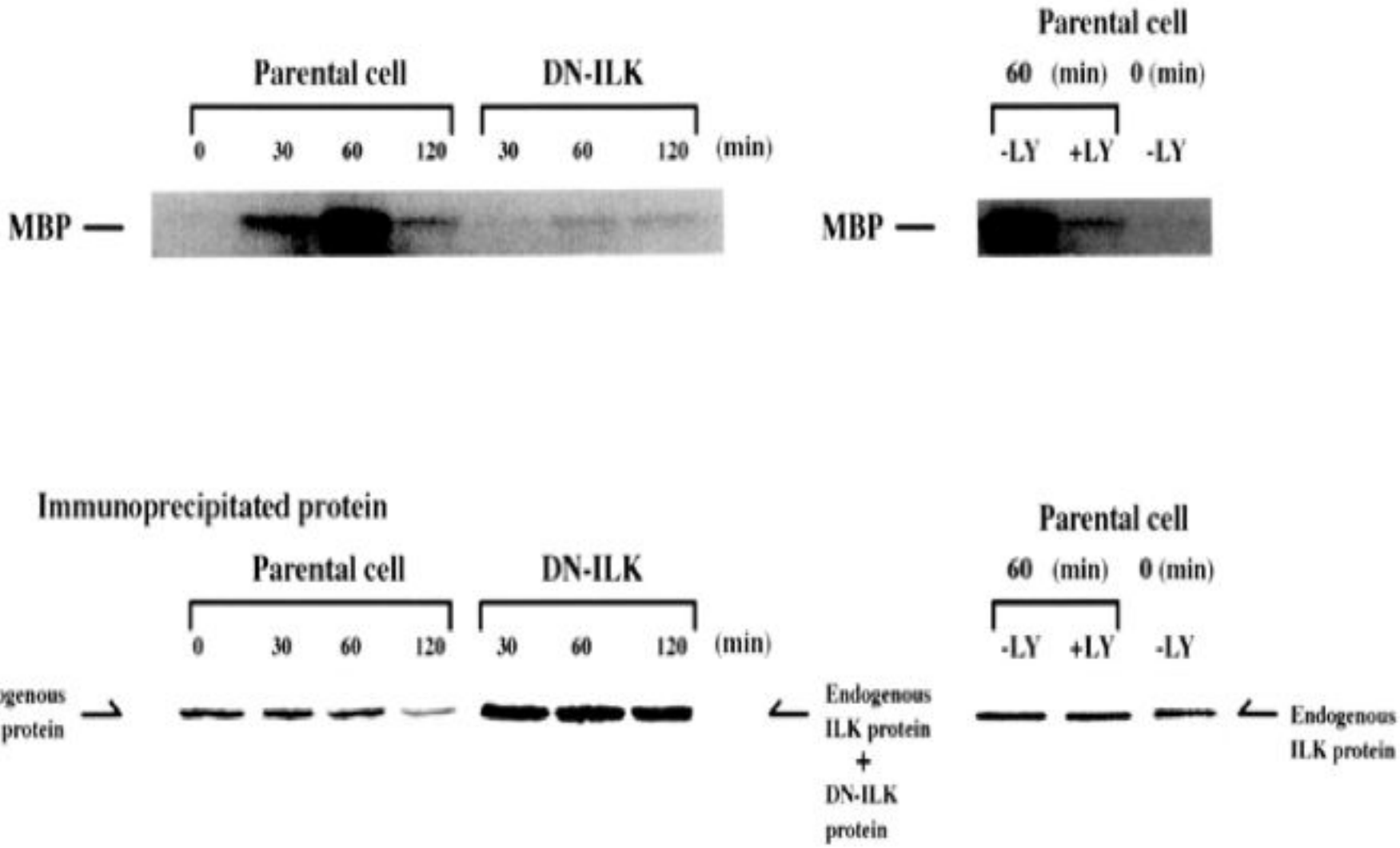


Fig.6

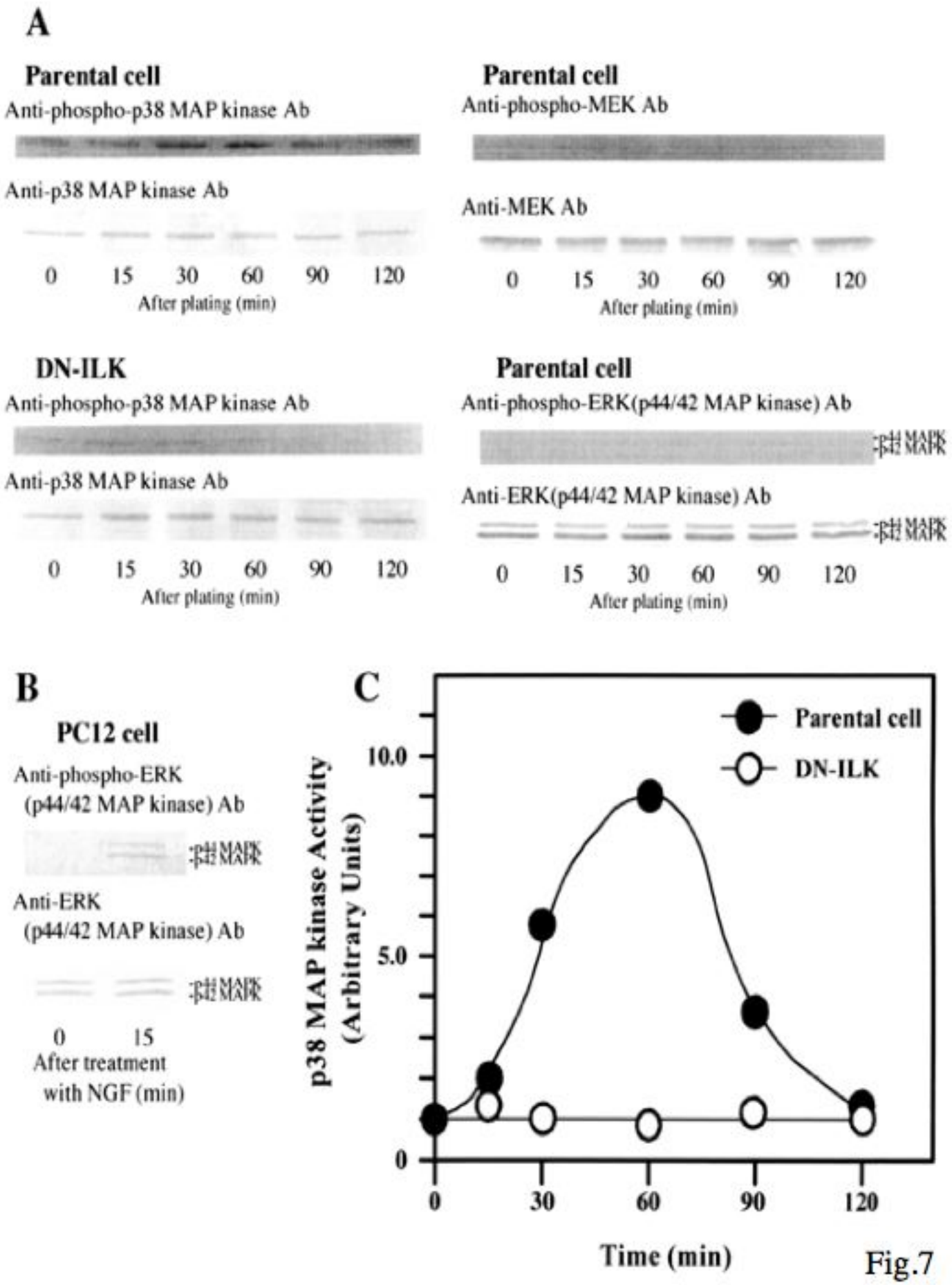

A
Parental cell
Anti-phospho-p38 MAP kinase Ab
Anti-p38 MAP kinase Ab
0 15 30 60 90 120
After plating (min)
Parental cell
Anti-phospho-MEK Ab
Anti-MEK Ab
0 15 30 60 90 120
After plating (min)
DN-ILK
Anti-phospho-p38 MAP kinase Ab
Anti-p38 MAP kinase Ab
0 15 30 60 90 120
After plating (min)
Parental cell
Anti-phospho-ERK(p44/42 MAP kinase) Ab
Anti-ERK(p44/42 MAP kinase) Ab
0 15 30 60 90 120
After plating (min)
B
PC12 cell
Anti-phospho-ERK
(p44/42 MAP kinase) Ab
Anti-ERK
(p44/42 MAP kinase) Ab
0 15
After treatment
with NGF (min)
C
Parental cell
DN-ILK
p38 MAP kinase Activity
(Arbitrary Units)
10.0
5.0
0
0 30 60 90 120
Time (min)

Fig.7

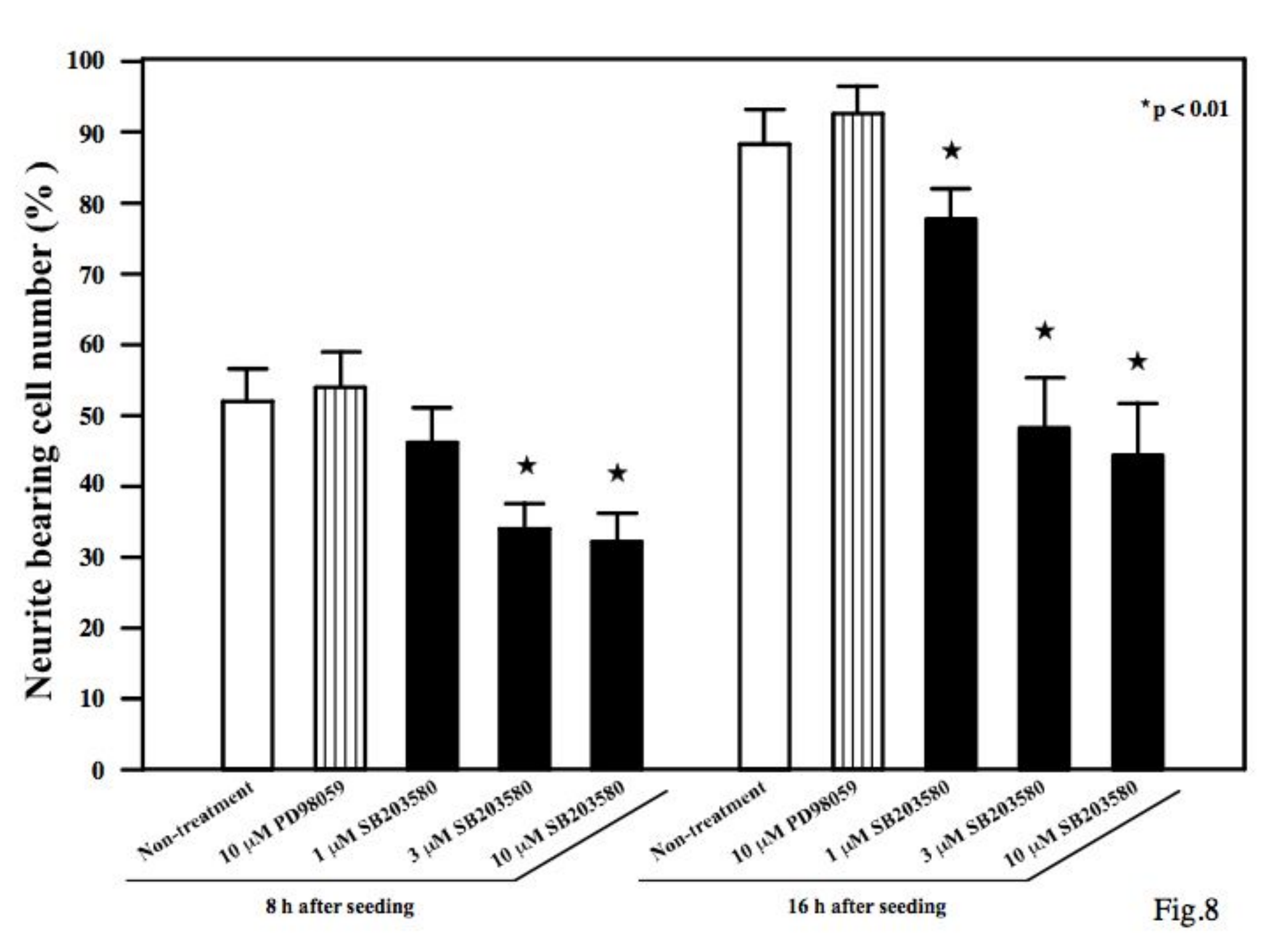

Neurite bearing cell number (%)
100
90
80
70
60
50
40
30
20
10
0
*p < 0.01
Non-treatment
10 μM PD98059
1 μM SB203580
3 μM SB203580
10 μM SB203580
Non-treatment
10 μM PD98059
1 μM SB203580
3 μM SB203580
10 μM SB203580
8 h after seeding
16 h after seeding

Fig.8

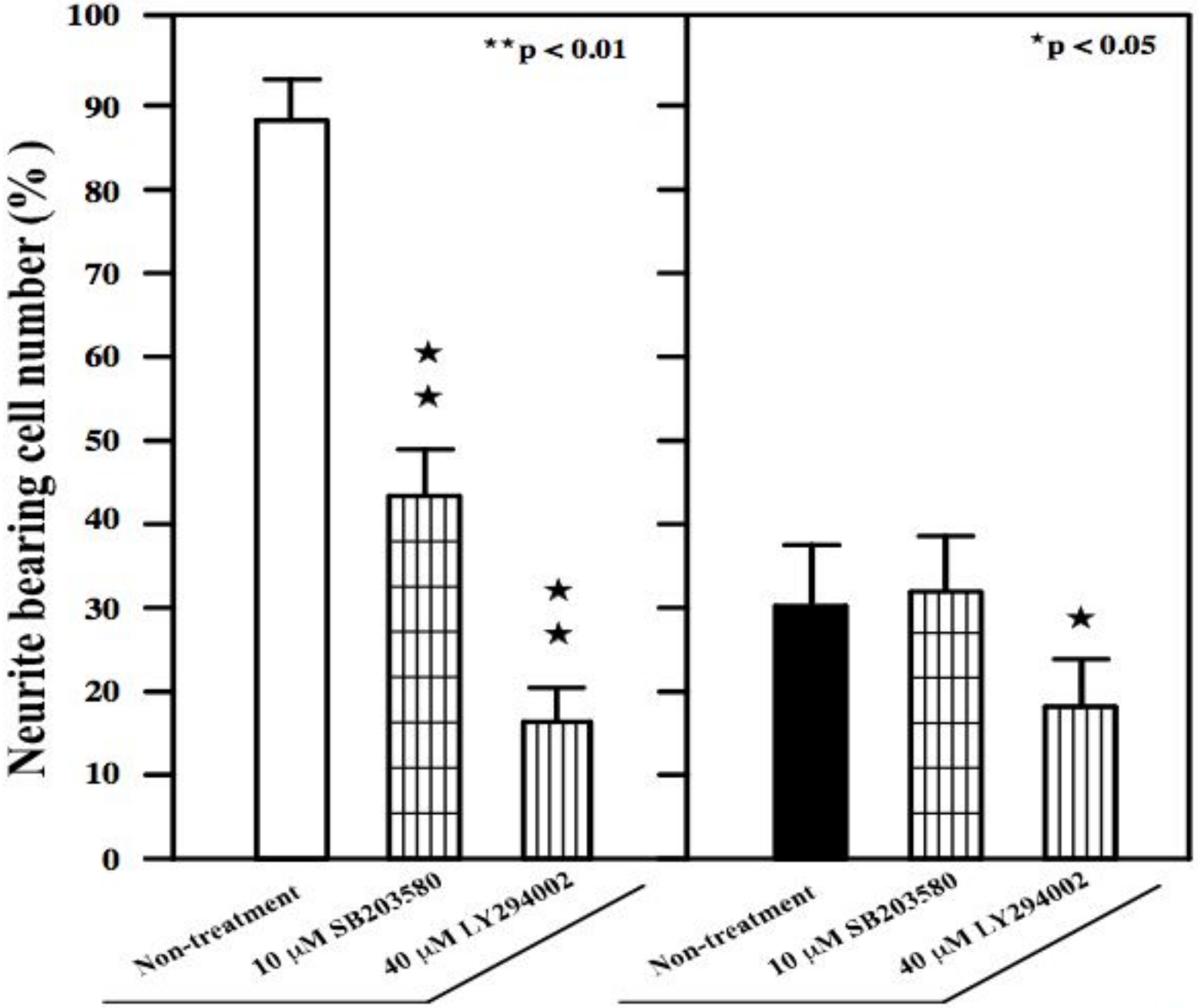

Neurite bearing cell number (%)
100
90
80
70
60
50
40
30
20
10
0
**p < 0.01
*p < 0.05
Non-treatment
10 μM SB203580
40 μM LY294002
Non-treatment
10 μM SB203580
40 μM LY294002
Parental Cells 16 h after seeding
DN-ILK transfected Cells 16 h after seeding

Fig.9