Biochemical and Biophysical Research Communications 422 (2012) 80–84

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Invasion inhibition by a MEK inhibitor correlates with the actin-based cytoskeleton in lung cancer A549 cells

Junmei Niu [a], Qingjiang Mo [b], Helin Wang [c], Mingying Li [d], Junwei Cui [a], Zhenyun Li [a], Zhenkui Li [c,*]

[a] Department of First Internal Medicine, Tuberculosis Division, The First Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China
[b] Department of Laboratory Medicine, Tuberculosis Division, The First Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China
[c] Department of Second Internal Medicine, Tuberculosis Division, The First Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China
[d] Department of Fourth Internal Medicine, Tuberculosis Division, The First Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China

ARTICLE INFO

Article history:
Received 15 April 2012
Available online 27 April 2012

Keywords:
MEK inhibitor
Lung cancer
Actin cytoskeleton
Invasion

ABSTRACT

Metastasis remains the primary cause of lung cancer. The molecules involved in metastasis may be candidates for new targets in the therapy of lung cancer. The MEK/ERK signaling pathway has been highlighted in a number of studies on invasiveness and metastasis. In this paper, we show that the MEK inhibitor U0126 induces flattened morphology, remodels the actin-based cytoskeleton, and potently inhibits chemotaxis and Matrigel invasion in the human lung cancer A549 cell line. Furthermore, down-regulation of ERK by small interfering RNA significantly inhibits the invasion of A549 cells and induces stress fiber formation. Taken together, our findings provide the first evidence that the inhibition of invasion of lung cancer A549 cells by inhibiting MEK/ERK signaling activity is associated with remodeling of the actin cytoskeleton, suggesting a novel link between MEK/ERK signaling-mediated cell invasion and the actin-based cytoskeleton.

1. Introduction

Lung cancer is the most commonly diagnosed malignancy and the main cause of cancer-related deaths in males in Asian and Western populations [1]. Metastasis remains the primary cause of lung cancer. The inhibition of lung cancer metastasis is thought to be an important therapeutic strategy. The molecules involved in metastasis may be candidates for new targets in the therapy of lung cancer [2].

The MEK/ERK signaling pathway is likely one of the most commonly activated pathways in malignant human cancers [3]. Its role in the control of proliferation and survival during tumorigenesis has been extensively studied [4,5]. In addition to its role in regulating cell proliferation and survival, MEK/ERK signaling has been highlighted in a number of studies of invasiveness and metastasis. For example, using effector domain mutants of the Ras oncoprotein designed for the activation of specific downstream effectors, Webb et al. [6] showed that although all of the mutants were tumorigenic in nude mice when overexpressed in NIH3T3 fibroblasts, only a mutant that activated the ERK MAPK pathway (V12 S35) induced lung metastasis. In epithelial cells, other studies have shown that MEK/ERK signaling, in cooperation with TGFβ, induces invasiveness *in vitro* and metastasis *in vivo* [7,8].

A recent group of studies has shown that ERK plays a critical role in the metastasis of lung cancer. In orthotopic human lung cancer models, combined MEK and VEGFR inhibition results in enhanced inhibition of tumor angiogenesis, growth, and metastasis [9]. Ueno et al. [10] reported that asialoglycoprotein receptor promotes cancer metastasis by activating the EGFR–ERK pathway.

In this paper, we provide the first evidence that the inhibition of invasion of lung cancer A549 cells by inhibiting MEK/ERK signaling activity is associated with remodeling of the actin cytoskeleton. We have identified a novel link between MAPK-mediated cell invasion and the actin-based cytoskeleton.

2. Materials and methods

2.1. Antibodies and reagents

The following primary antibodies (Abs) were used: rabbit polyclonal antibodies against MEK1 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) and antibodies against phospho-MEK1/2$^{S217/221}$, phospho-ERK1/2$^{Thr202/Tyr204}$ and ERK1/2 (Cell Signaling Technology, Beverly, MA). The MEK1/2 inhibitor (U0126) and actin polymerization inhibitor jasplakinolide were purchased from Calbiochem (San Diego, CA).

2.2. Cell culture

Human lung cancer A549 cells were maintained in F12K medium supplemented with 10% FBS, 1% penicillin–streptomycin

* Corresponding author. Fax: +86 373 4402534.
E-mail address: lzk75@yahoo.cn (Z. Li).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.04.109

(100 units/ml penicillin and 100 μg/ml streptomycin) and 1% glutamine and grown at 37 °C and 5% CO_2.

2.3. Cell morphology

A549 cells plated overnight in 6-well dishes in the presence or absence of U0126 (10 μM) or jasplakinolide (200 nM) were photographed under a phase-contrast microscope.

2.4. Chemotaxis and invasion assays

Chemotaxis and invasion were measured with the Boyden chamber assay using BD BioCoat Control Inserts (BD Biosciences). A549 cells were plated in the insert chambers in serum-free F12K medium. The lower chambers were filled with medium supplemented with 10% FBS. The cells were allowed to migrate for 16 h (chemotaxis) and 24 h (invasion), and cells that had not penetrated the filters were removed with cotton swabs. Chambers were fixed and stained using a Diff-Quik Stain Set (Dade Behring Inc., Newark, DE) and examined under a bright-field microscope. Values for migration were obtained by counting six fields per membrane (20× objective) and represent the average of three independent experiments. For drug treatment, U0126 (10 μM) or jasplakinolide (200 nM) was added to A549 cells for 18 h in F12/10% FBS prior to and during the migration and invasion assays.

2.5. Western blotting

A549 cells grown in the presence or absence of U0126 were plated in 10 cm dishes and serum starved overnight by incubation with serum-free F-12K medium. The cells were stimulated with 10% FBS in medium for 10 min and immediately lysed in RIPA buffer. A total of 30 μg total protein per sample was separated through SDS–PAGE, blotted onto PVDF membranes, blocked for 30 min with 5% bovine serum albumin in 1× TBS/T (0.1% Tween-20 in Tris-buffered saline) and then probed with the indicated antibodies. The membranes were washed in TBS/Tween/milk and incubated with goat anti-rabbit (1:1000) secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Visualization of the protein bands was performed with the Enhanced Chemiluminescence Plus kit as recommended by the manufacturer (Perkin Elmer, Boston, MA).

2.6. siRNA transfection

Synthetic ERK1 and 2 siRNA ON-TARGETplus SMARTpool, ONTARGETplus nontargeting siRNA and DharmaFECT 1 transfection reagent were purchased from Dharmacon. A549 cells were plated at equal densities in 6-well plates (3.0×10^4 per well) overnight. Cells were transfected with 50 nM of siRNA-NS or 50 nM of siRNA against ERK for 24 h using DharmaFECT 1 transfection

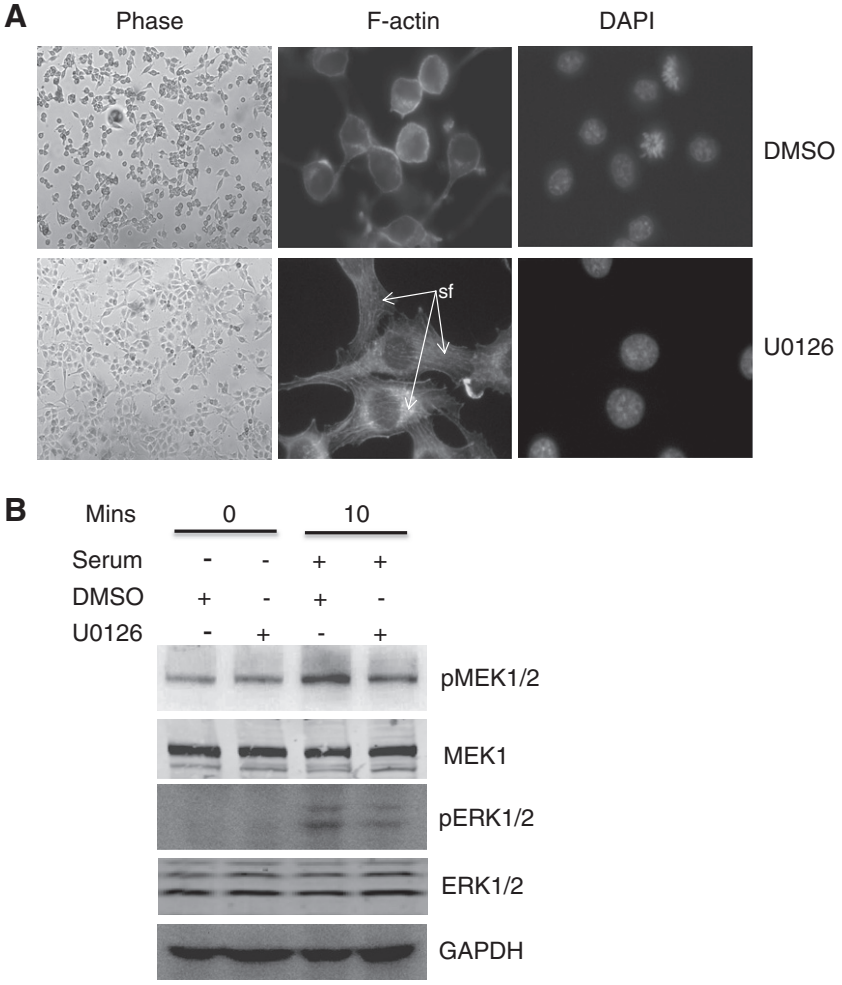

Fig. 1. Inhibition of ERK1/2 in A549 cells induced flattened morphology and remodeled the actin-based cytoskeleton. (A) Morphological changes induced in A549 cells treated with U0126. (B) U0126 induces actin stress fiber formation. A549 cells grown in the presence or absence of U0126 were imaged with phase-contrast microscopy (left panel) or fixed and stained for F-actin (rhodamine-phalloidin) (middle panel) and the nucleus (DAPI, right panel). sf: stress fibers. (C) Immunoblot analysis of p-MEK, MEK1, p-ERK1/2, ERK1/2 and GAPDH protein levels in A549 cells grown in the presence or absence of U0126.

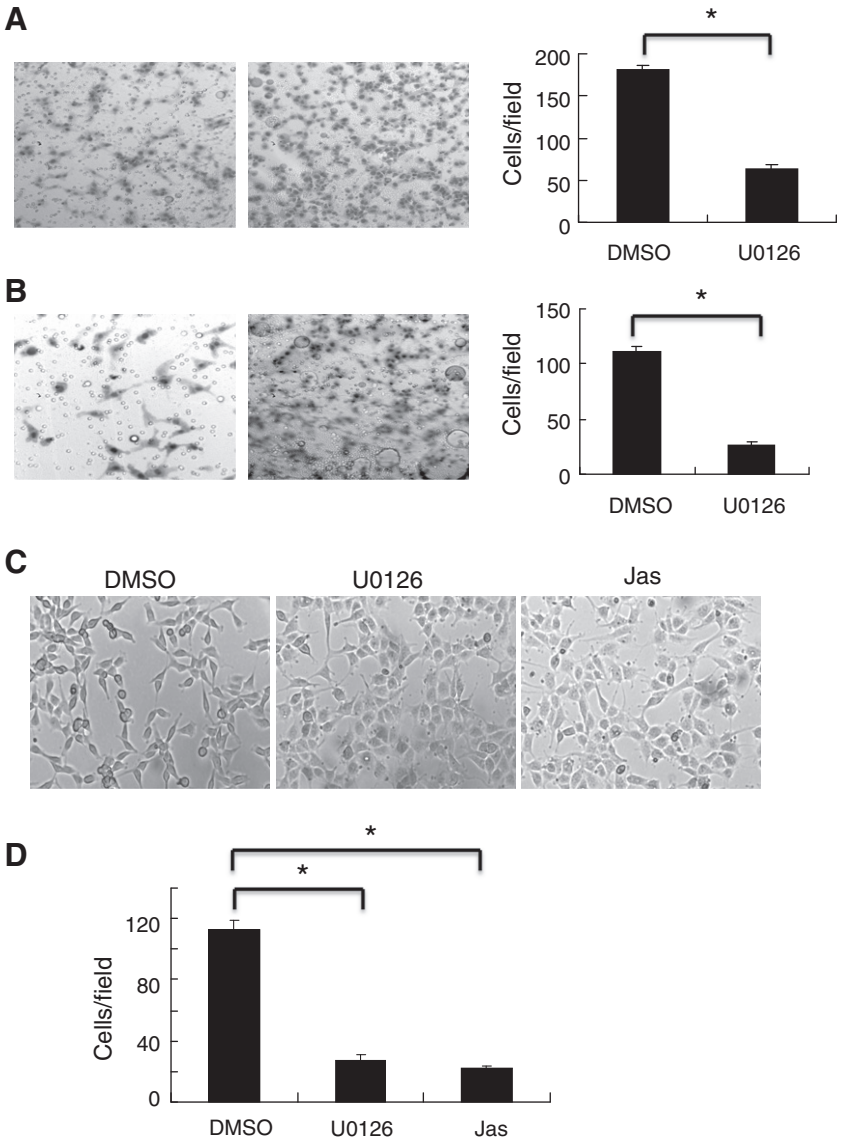

Fig. 2. U0126-mediated inhibition of chemotaxis and invasion correlates with the actin-based cytoskeleton. (A) U0126 inhibits chemotaxis. A549 cells treated with U0126 exhibit an approximately 3-fold reduction in chemotaxis (to 10% FBS) compared with the control. $^{*}p < 0.01$. (B) U0126 leads to an approximately 6-fold decrease in Matrigel invasiveness. $^{*}p < 0.01$. (C) The morphology change of A549 cells treated with jasplakinolide. Consistent with treatment with U0126, jasplakinolide induced flattened morphology. Jas: jasplakinolide. (D) Jasplakinolide inhibits Matrigel invasiveness. $^{*}p < 0.01$.

reagent according to the manufacturer's protocol. Following transfection, the cells were harvested for experiments as indicated.

2.7. Immunofluorescence analysis

A549 cells were plated on glass coverslips, treated with DMSO or U0126 for 48 h, washed twice with PBS, fixed with 60% acetone/3.7% formaldehyde in PBS, and blocked with 3% non-fat dry milk in PBS for 30 min at room temperature. Actin filaments were stained with rhodamine-labeled phalloidin (1:500; Sigma), and nuclei were stained with DAPI (1:1000; Invitrogen). Fluorescent images were obtained using an Olympus inverted microscope.

2.8. Statistics

The statistical significance between groups was determined with a two-tailed Student's *t*-test.

3. Results and discussion

3.1. U0126 modifies cell morphology and induces the formation of actin stress fibers in A549 cells

A549 cells normally have a human alveolar basal epithelial appearance. However, they acquired a different phenotype following U0126 treatment and appeared to be flat and spread (Fig. 1A). Because actin microfilaments and associated proteins determine the cell shape [11], the actin skeleton was stained in A549 cells. Control cells showed a diffuse distribution of actin; however, U0126 increased the sizes and promoted the formation of actin stress fibers in A549 cells (Fig. 1A). Indeed, treatment of A549 cells with U0126 resulted in cell flattening marked by the reestablishment of actin stress fibers. We addressed whether the U0126-induced morphology change was due to inhibition of MEK/ERK signaling activation. Thus, A549 cells were serum starved in the

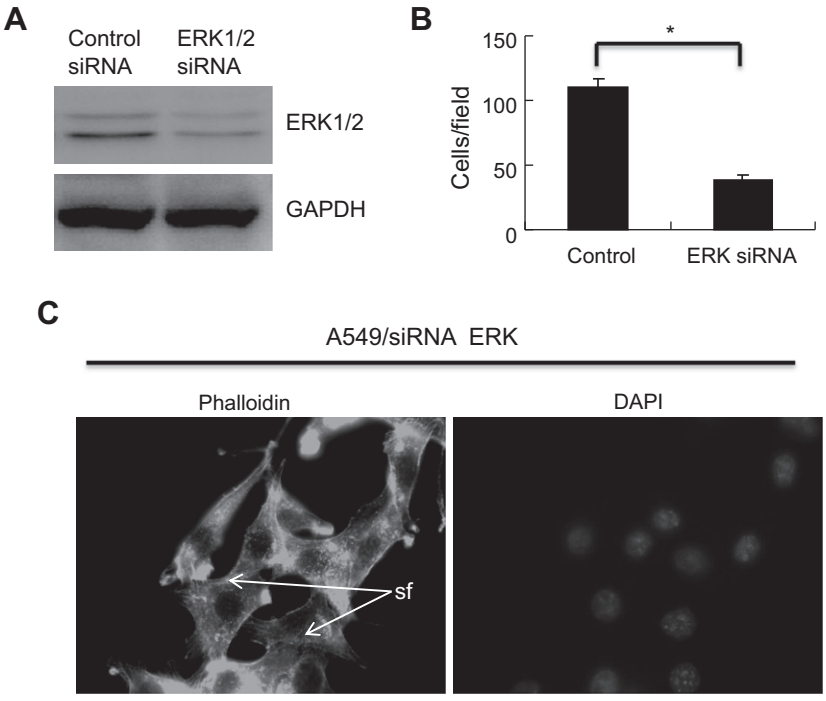

Fig. 3. siRNA ERK inhibits invasion and induces stress fiber formation in A549 cells. (A) Immunoblot analysis showing decreased ERK1/2 protein levels in cells transfected with siRNA ERK. (B) siRNA ERK inhibits Matrigel invasiveness in A549 cells. $^{*}p < 0.01$. (C) siRNA ERK induces actin stress fiber formation. sf: stress fibers.

presence or absence of U0126, stimulated with 10% FBS for 10 min, and the cell lysates were probed for phospho-MEK or -ERK relative to total MEK or ERK protein levels as an indicator of activated MEK/ERK signaling. As shown in Fig 1B, MEK and ERK phosphorylation was decreased after U0126 treatment after serum stimulation.

3.2. U0126 inhibits migration and Matrigel invasion

Cell adhesion and migration are controlled by complex changes in the cytoskeleton, particularly the actin cytoskeleton, which plays a pivotal role in controlling cell motility, including cell morphology, adhesion and migration [12–14].

MAPK is involved in cell motility and invasion [15,16]. We determined whether U0126-induced remodeling of the cytoskeleton affected cell motility. Chemotaxis was evaluated in A549 cells. The cells were incubated in serum-free media with or without U0126 in the upper chamber of 8 μm pore membranes of Boyden chambers with 10% FBS as a chemoattractant in the lower wells. Fig. 1A shows that U0126 treatment decreased chemotaxis approximately 3-fold ($p < 0.01$; Fig. 2A). To further explore invasive activity, similar Boyden chamber assays were performed, except the cells were placed in the upper chamber of inserts with Matrigel-coated membranes and then assayed for movement toward the bottom chamber containing 10% FBS. Inhibition of MEK/ERK activity also decreased invasiveness 6-fold ($p < 0.01$; Fig. 2B). Indeed, the inhibition of MEK/ERK signaling in A549 cells resulted in cell flattening marked by the reestablishment of actin stress fibers. We determined whether the ability of U0126 to suppress invasion was associated with the actin cytoskeleton. The cells were treated with jasplakinolide, which stabilizes actin stress fibers primarily by decreasing the dissociation rate of actin subunits at a low concentration [17], and jasplakinolide showed similar results as U0126, inducing cell flattening and the formation of stress fibers (Fig. 2C) and decreasing cell invasion (Fig. 2D). Taken together, these data indicate that inhibiting MAPK can inhibit specialized *in vitro* parameters of motility associated with remodeling of the cytoskeleton.

3.3. Knockdown of ERK decreased invasion and established the actin cytoskeleton

To further determine whether ERK plays a role in A549 cell migration and invasion, siRNA-ERK1/2 was used to knockdown ERK1/2 expression in A549 cells, as confirmed with immunoblot analysis (Fig. 3A). siRNA-ERK decreased A549 cell invasion upon serum chemical attraction and induced actin stress fiber formation (Fig. 3B and C), suggesting that the inhibition of ERK-suppressed A549 cell invasion may be due to changes in the cytoskeleton.

It has become clear that MEK/ERK signaling is an important regulator of cell motility, including migration and invasion. In this context, U0126 inhibited MEK/ERK activity and promoted the formation of actin stress fibers, leading to reduced cell migration and invasion. Because these mechanisms are fundamental for cell structure and force generation, they are associated with numerous cellular processes, such as intracellular trafficking, cell morphology maintenance and changes, cytokinesis, secretion, cell adhesion and migration.

Thus, inhibition of ERK may provide a mechanism of blocking tumor cell motility and invasion and metastasis in lung cancer.

Acknowledgment

We are grateful to Hongwei Wang for his skillful and outstanding help.

References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, Global cancer statistics, CA Cancer J. Clin. 61 (2011) 69–90.
[2] J. Sangodkar, S. Katz, H. Melville, G. Narla, Lung adenocarcinoma: lessons in translation from bench to bedside. Mt. Sinai, J. Med. 77 (2010) 597–605.
[3] J.S. Sebolt-Leopold, R. Herrera, Targeting the mitogen-activated protein kinase cascade to treat cancer, Nat. Rev. Cancer 4 (2004) 937–947.
[4] S. Schubbert, K. Shannon, G. Bollag, Hyperactive Ras in developmental disorders and cancer, Nat. Rev. Cancer 7 (2007) 295–308.
[5] G. Pages, P. Lenormand, G. L'Allemain, J.C. Chambard, S. Meloche, J. Pouyssegur, Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation, Proc. Natl. Acad. Sci. USA 90 (1993) 8319–8323.

[6] C.P. Webb, A.L. Van, M.H. Wigler, G.F. Woude, Signaling pathways in Ras-mediated tumorigenicity and metastasis, Proc. Natl. Acad. Sci. USA 95 (1998) 8773–8778.
[7] K. Lehmann, E. Janda, C.E. Pierreux, M. Rytomaa, A. Schulze, M. McMahon, C.S. Hill, H. Beug, J. Downward, Raf induces TGFbeta production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells, Genes Dev. 14 (2000) 2610–2622.
[8] E. Janda, K. Lehmann, I. Killisch, M. Jechlinger, M. Herzig, J. Downward, H. Beug, S. Grunert, Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways, J. Cell Biol. 156 (2002) 299–313.
[9] O. Takahashi, R. Komaki, P.D. Smith, J.M. Jurgensmeier, A. Ryan, B.N. Bekele, I.I. Wistuba, J.J. Jacoby, M.V. Korshunova, A. Biernacka, B. Erez, K. Hosho, R.S. Herbst, M.S. O'Reilly, Combined MEK and VEGFR inhibition in orthotopic human lung cancer models results in enhanced inhibition of tumor angiogenesis, growth, and metastasis, Clin. Cancer Res. 18 (2012) 1641–1654.
[10] S. Ueno, M. Mojic, Y. Ohashi, N. Higashi, Y. Hayakawa, T. Irimura, Asialoglycoprotein receptor promotes cancer metastasis by activating the EGFR–ERK pathway, Cancer Res. 71 (2011) 6419–6427.
[11] E. Knust, Control of epithelial cell shape and polarity, Curr. Opin. Genet. Dev. 10 (2000) 471–475.
[12] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, A.R. Horwitz, Cell migration: integrating signals from front to back, Science 302 (2003) 1704–1709.
[13] D. Pantaloni, C.C. Le, M.F. Carlier, Mechanism of actin-based motility, Science 292 (2001) 1502–1506.
[14] E.S. Chhabra, H.N. Higgs, The many faces of actin: matching assembly factors with cellular structures, Nat. Cell Biol. 9 (2007) 1110–1121.
[15] J.S. Krueger, V.G. Keshamouni, N. Atanaskova, K.B. Reddy, Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion, Oncogene 20 (2001) 4209–4218.
[16] R.L. Klemke, S. Cai, A.L. Giannini, P.J. Gallagher, L.P. de, D.A. Cheresh, Regulation of cell motility by mitogen-activated protein kinase, J. Cell Biol. 137 (1997) 481–492.
[17] M.R. Bubb, I. Spector, B.B. Beyer, K.M. Fosen, Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain in vivo observations, J. Biol. Chem. 275 (2000) 5163–5170.