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Over-expression of cofilin-1 suppressed growth and invasion of cancer cells is associated with up-regulation of let-7 microRNA

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ABSTRACT

Cofilin-1, a non-muscle isoform of actin regulatory protein that belongs to the actin-depolymerizing factor (ADF)/cofilin family is known to affect cancer development. Previously, we found that over-expression of cofilin-1 suppressed the growth and invasion of human non-small cell lung cancer (NSCLC) cells in vitro. In this study, we further investigated whether over-expression of cofilin-1 can suppress tumor growth in vivo, and performed a microRNA array analysis to better understand whether specific microRNA would be involved in this event. The results showed that over-expression of cofilin-1 suppressed NSCLC tumor growth using the xenograft tumor model with the non-invasive reporter gene imaging modalities. Additionally, cell motility and invasion were significantly suppressed by over-expressed cofilin-1, and down-regulation of matrix metalloproteinase (MMPs) -1 and -3 was concomitantly detected. According to the microRNA array analysis, the let-7 family, particularly let-7b and let-7e, were apparently up-regulated among 248 microRNAs that were affected after over-expression of cofilin-1 up to 7 days. Knockdown of let-7b or let-7e using chemical locked nucleic acid (LNA) could recover the growth rate and the invasion of cofilin-1 over-expressing cells. Next, the expression of c-myc, LIN28 and Twist-1 proteins known to regulate let-7 were analyzed in cofilin-1 over-expressing cells, and Twist-1 was significantly suppressed under this condition. Up-regulation of let-7 microRNA by over-expressed cofilin-1 could be eliminated by co-transfected Twist-1 cDNA. Taken together, current data suggest that let-7 microRNA would be involved in over-expression of cofilin-1 mediated tumor suppression in vitro and in vivo.

1. Introduction

Cancer development involves uncontrolled cell growth followed by increased motility and invasion for distant metastasis [1]. Targeting on both cancer growth and metastasis is one of the strategies for cancer treatment, such as the adjuvant radiotherapy and chemotherapy. Because adjuvant radio-chemotherapy usually leads to resistance, it is of interest to investigate whether concomitant suppression of cancer growth and metastasis can be raised by certain biomolecules for cancer control.

Actin cytoskeleton has long been regarded a therapeutic target because it plays a role in cellular transformation through accelerated cell proliferation and migration [2,3]. However, the anti-actin agents isolated from fungi or sea sponges possess universal toxic problems so that the clinical application is limited [4]. Because actin cytoskeletal organization is dependent on various actin regulatory proteins (ARPs), targeting on ARPs may be applicable for influence actin organization and cancer development. Although ARP is believed to regulate actin dynamics for cell division and migration, little is known whether ARP affects specific intracellular signaling pathways for malignancy.

Cofilin-1 is a ~19 kD, nonmuscle isoform of actin regulatory protein that belongs to the actin-depolymerizing factor (ADF)/cofilin family [5].

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The primary function of cofilin-1 is to accelerate the actin turnover via severing the actin filaments or depolymerizing the ADP bound actin at the pointed end of the actin filaments. Cofilin-1 is essential for cell cycle progression, migration, intravasation and invasion of cancer cells [6,7]. Interestingly, Yap et al. found a biphasic effect between the cofilin level and locomotory rate because over-expression of cofilin in transfected clones up to 4.5 times that of wild-type cells will increase the locomotory rate of glioblastoma tumor cells, but clones producing over this amount displayed reduced motile speed [8]. Consistent with this finding, we have found that the cell invasion was inhibited by cofilin-1 over-expression of up to 15 folds in H1299 cells [9]. The cell growth and cell cycle progression were also suppressed by over-expressed cofilin-1 within a dose- and time-dependent manner.[6]. However, the underlying mechanisms of cofilin-1 over-expression caused suppression of growth rate and invasion remain to be addressed.

MicroRNA is a small non-coding RNA with approximately 22 nucleotides and participates in diverse biological functions, including differentiation, development, cell cycle, metabolism and apoptosis [10]. Additionally, accumulated literatures have indicated that microRNA is important for cancer progression [11–13]. Although different types of microRNA can suppress downstream oncogenes or tumor suppressor genes, the total amount of microRNA is decreased in cancers compared to normal tissues [14]. This phenomenon suggests that most of microRNAs play roles in suppressing tumor progression. For instance, hsa-let-7 (let-7) microRNA family and hsa-miR-29 (miR-29) family are down-regulated in lung cancer and breast cancer, and are regarded tumor suppressors by inducing apoptosis [15–17]. Other microRNA families such as hsa-miR-34, hsa-miR-200, hsa-miR-185, hsa-miR-107 and hsa-miR-335 are reported to exhibit tumor suppression capacity by cooperating with p53, inhibition of epithelial–mesenchymal transition and angiogenesis, cell cycle arrest, or inhibition of tumor re-initiation [18–23]. Therefore, restoration or maintenance of endogenous microRNA with tumor suppressor ability would be an important strategy for the design of cancer treatment strategy.

Destabilization of actin cytoskeleton by cofilin-1 or actin inhibitors has been reported to suppress the growth and invasion of a cancer cell [6,24,25]. MicroRNA has also been reported to regulate the actin cytoskeleton to modulate tumor growth, mobility, invasion and metastasis [26–28]. Whether cofilin expression caused tumor response is associated with microRNA regulation is of interest to investigate. In this study, we first examined the effects of over-expressed cofilin-1 on tumor growth using a xenograft tumor model. The cell motility was also compared before and after over-expression of cofilin-1. Subsequently, a survey of microRNA profiles after over-expression of cofilin-1 was performed. It revealed that let-7 microRNA was apparently up-regulated by over-expressed cofilin-1. Interestingly, over-expression of cofilin-1 caused suppression of growth rate and invasion could be compromised by silencing let-7. Additionally, Twist-1 transcription factor that can regulate let-7 expression was found to mediate the signaling between cofilin-1 and let-7. The potent crosstalk among these molecules was discussed.

2. Materials and methods

2.1. Cell culture

Human non-small cell lung adenocarcinoma H1299 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamate, 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA). H1299/*tet-on*-cofilin cell line was maintained in the medium containing 100 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA), and H1299/*tet-on*-cofilin /EGFP-tk cell line was maintained in medium containing 100 μg/ml hygromycin B and 1 μg/ml puromycin (Sigma-Aldrich Co., St. Louis, MO, USA). The pH of DMEM was adjusted to 7.0–7.2 using hydrogen chloride because the antibiotics could change the pH of medium. Cells were maintained in a 37 °C, humidified incubator (5% CO_2 and 95% air), and routinely passaged every two days.

2.2. Soft agar colony formation assay

This assay is performed according to previous reports with modifications [29]. Cells (1×10^4) with or without doxycycline treatment were suspended in 2× DMEM with 20% FBS and mixed with 0.66% soft agar (Sigma-Aldrich Co., St. Louis, MO, USA) at equal volumes. The mixture was then seeded into 6-well plates and incubated under 4 °C for 15–20 min, and then maintained in a humidified incubator (5% CO_2 and 95% air) at 37 °C. During incubation, 0.5 ml of 2× medium was added to wells every 7 days. After 14 days of incubation, the 6-well plates were collected, stained with crystal violet for 10 min, rinsed and subjected to microscopic examination for counting colony number (each colony should be ≥50 cells).

2.3. Plasmids and transfection

H1299/*tet-on*-cofilin stable cell line harbors a *tet-on* inducible gene expressive construct that can over-express human cofilin-1 by adding doxycycline as described previously [30]. A bidirectional *tet-on* inducible gene expressive plasmid named pBI-EGFP-tk-puro was constructed from pBI-EGFP plasmid (BD Biosciences Clontech Inc., Worcester, MA, USA) digested using PvuII and NheI restriction enzymes. The herpes simplex virus type 1-thymidine kinase (HSV1-tk) cDNA was digested from pORF-HSV1-tk plasmid (a kind gift from Dr. Jeng-Jong Hwang, National Yang-Ming University) using the same enzymes and cloned into pBI-EGFP plasmid to obtain the pBI-EGFP-tk plasmid. The puromycin resistant cassette was obtained from RNAi-Ready pSIREN-RetroQ plasmid (BD Biosciences Clontech Inc., Worcester, MA, USA) and subcloned into pBI-EGFP-tk plasmid through Klenow enzyme filled-in blunt ligation. The resultant pBI-EGFP-tk-puro plasmid was then transfected into H1299/*tet-on*-cofilin cells to produce H1299/*tet-on*-cofilin /EGFP-tk stable cells using the jetPEI transfection reagent (Polyplus-transfection, SA, Illkirch, France). Cells were trypsinized and split after 24 h of transfection and then selected using puromycin (2 μg/ml). The survival clones were pooled after one week of selection, and were validated by functional analysis of EGFP and HSV1-tk expression (see results).

2.4. Transfection of miRNA inhibitors and luciferase reporter gene assay

For miRNA inhibition, 30 nM chemically modified locked nucleic acid (LNA™) (Exiqon, Los Angeles, CA, USA) specifically targeting on let-7b (5′-ACCACACAACCTACTACCTC-3′) or let-7e (5′-ACTATACAACCTCCTACCTC-3′) was transfected into cells using JetPEI transfection reagent as described above. To determine the inhibitory effect of let-7 miRNA, a 260 bp 3′-untranslated region (3′-UTR) of human transformation growth factor beta receptor 1 (TGFBR1) mRNA encompassing a second seed sequence of let-7 binding site [31] was amplified using polymerase chain reaction (PCR) and cloned into pMIR-REPORT™-Luciferase plasmid to generate pMIR-TGFBR1-reporter construct through SpeI and HindIII restriction enzymes. The primer set for amplifying this 3′UTR fragment was 5′-ACTAGTCTGAATATCATGAACCATG-3′ and 5′-ATACAGTACCATTTGAGTAACTGAGC-3′. For luciferase reporter gene assay, cells from different treatments were harvested with passive lysis buffer and the luciferase activity was analyzed using reporter assay buffer (50 mM glycylglycin, 1 M magnesium sulfate, 10 mg/ml bovine serum albumin, and 0.5 M EDTA) mixed with 100 mM adenosine 5′-triphosphate disodium salt (Sigma-Aldrich Co., St. Louis, MO, USA), 1 M dithiothreitol, 50 mM D-luciferin (Promega Co., Madison, WI, USA). The mixture was immediately measured using a Wallac-1420 VICTOR^2 multilabel reader (PerkinElmer Co., Waltham, MA, USA).

2.5. In vitro invasion assay

H1299/*tet-on*-cofilin cells were treated with or without doxycycline treatment for 48 h and then trypsinized for counting the total cell number. Subsequently, 1×10^4 cells were resuspended in serum-free DMEM mixed with Matrigel (BD biosciences, San Jose, CA, USA) at 3:1 ratio and were seeded into transwells. For determination of migration ability, each transwell was put on a well of a 24-well dish which contained DMEM with 10% FBS. After 2 days of incubation, the Matrigel inside a transwell was removed with a cotton stub and each transwell was fixed by 4% paraformaldehyde for 10 min followed by 30 min staining in hematoxylin, rinsed, visualized and counted under a bright-field microscope. The cell number in each well in serum-free DMEM was used to normalize transversed cell counts after two days incubation.

2.6. Time lapse microscopy and motility analysis

H1299/*tet-on*-cofilin cells (2×10^4) were seeded in a 35 mm dish and treated with doxycycline or left untreated for 48 h. Cell motility was then detected by acquiring continuous images at 5-min intervals using a humidified, CO_2-equilibrated chamber with a Leica DM-IRBE combining a cooled CCD camera and the AQUA COSMOS imaging analysis software (ORCA-ER, C4742-95, Hamamatsu Photonics, Hamamarsu, Japan). The moving pathways of individual cells were tracked by positioning cell nuclei using MetaMorph® software (Molecular Devices, Inc., CA, USA). A new stack file was established to make a movie by acquiring an image every 5 min over a period of 16 h. The travel distance of each cell was then calculated by taking the positions of cell centroid during the tracking period. Fifteen cells of each group were randomly selected for analysis of movement distance.

2.7. Western blot analysis and antibodies

Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl; 120 mM NaCl and 0.5% NP-40) containing 20 μg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich Co., St. Louis, MO, USA). The crude protein lysates were collected by centrifugation (12,000 ×g) and the protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of crude protein lysates from different treatments were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The fractionated proteins were electro-transferred to polyvinylidine fluoride (PVDF) membrane in transfer buffer (25 mM Trizma-base;192 mM glycine and 20% v/v methanol) for 120 min. The membrane was blocked using TBST buffer (0.8% w/v NaCl; 0.02% w/v KCl; 25 mM Tris-HCl and 0.1% v/v Tween20) containing 4% skim milk and then incubated with a primary antibody at 4 °C overnight. The membrane was then rinsed with TBST buffer followed by horseradish peroxidase (HRP)-conjugated secondary antibody. Protein signals were detected using ECL™ detection reagents (PerkinElmer Inc., Waltham, MA, USA). The primary antibodies used in this study included anti-cofilin-1 (Genetex Inc., Irvine, CA, USA), anti-ser3-phospho-cofilin-1 (Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-Twist-1 (Genetex Inc., Irvine, CA, USA), anti-MMP1 (Genetex Inc., Irvine, CA, USA), anti-MMP3 (Genetex Inc., Irvine, CA, USA), anti-MMP9 (Genetex Inc., Irvine, CA, USA), anti-GAPDH (Sigma-Aldrich Co., St. Louis, MO, USA), anti-c-myc (Genetex Inc., Irvine, CA, USA), anti-LIN28 (Genetex Inc., Irvine, CA, USA), anti-flag (Sigma-Aldrich Co., St. Louis, MO, USA), and anti-HSV1-tk (Santa Cruz Biotechnology Inc., Dallas, TX, USA) antibodies.

2.8. Cell uptake assay

H1299/*tet-on*-cofilin-EGFP/tk cells (1.5×10^6) were seeded in two 150 mm^2 dishes with or without doxycycline. The serum-free medium containing radioactive probe ^{131}I-FIAU (0.5 μCi/ml) was then used to replace culture medium after 24 h of incubation. Cells were then collected at different time points using centrifugation (3500 rpm) for 2 min. Supernatant and pellets were separately frozen with dry ice, and the radioactivities of these two components were counted using a γ-scintillation counter (Wallac Wizard 1470, Perkin Elmer, Waltham, MA, USA). The specific radioactivity was normalized with the weight of each component. The final accumulation of ^{131}I-FIAU in cells was calculated using the formula:

$$\frac{\text{Radioactivity of cell pellet} \,/\, \text{Net weight of cell pellet}}{\text{Radioactivity of medium} \,/\, \text{Net weight of medium}}.$$

2.9. Xenograft tumor model

Six weeks old male nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were anesthetized by intra-peritoneal injection of a mixture of ketamine chloride (50 mg/kg) and xylazine (15 mg/kg). Subsequently, 5×10^6 cells re-suspended in 100 μl medium were subcutaneously injected in both thighs of those NOD/SCID mice using 27-gauge insulin syringes. Tumor volume was caliperly measured and calculated using this formula: length × $(\text{width})^2$ / 2 [32].

2.10. In vivo fluorescent imaging

Tumor-bearing mice were anesthetized using 2% isoflurane and the IVIS-50 system (Caliper Co., Hopkinton, MA, USA) was used for image acquisition. The level and quantification of fluorescence was determined by circling the region of interests (ROIs) of animal image with photon signals at tumor inoculating sites. The intensity of fluorescent signals was expressed as photons per second per centimeter squared per steradian (p/s/cm^2/sr). Imaging was performed weekly to monitor the development of tumor growth in mice.

2.11. Positron emission tomography (PET) imaging

The radioactive tracers ^{18}F-2′-fluoro-2′deoxy-1β-D-arabinofuranosyl-5-ethyl-uracil (^{18}F-FEAU) and 2-deoxy-2-fluoro-D-glucose (^{18}F-FDG) were exploited for detecting HSV1-tk expression and tumor viability using PET imaging, respectively. Tumor-bearing mice were i.v. injection of ^{18}F-FEAU or ^{18}F-FDG (100 μCi per mouse) for 60 min, and whole body PET imaging was performed with a microPET R4 system (Concorde Microsystems, Knoxville, TN, USA). Mice were anesthetized using 2% isoflurane and images were acquired for 15 min. The level of radioactivity in the tumors (% dose/g) was determined by the analysis software (ASIPro VM 6.3.3.0) bundled with PET. The above-mentioned animal studies and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) in National Yang-Ming University. The IACUC approval number is 951201.

2.12. Microarrays analysis for microRNA expression

Total RNA was extracted using the Trizol reagent (Life-Technologies Co., Carlsbad, CA, USA) and quantified using the NanoDrop ND-1000 (ThermoFisher Scientific Inc., Waltham, MA, USA). RNA quality was determined using agarose gel electrophoresis. Agilent Human miRNA V3 array was used to determine the global expression of microRNA. The data obtained from microarray analysis have been deposited to the Gene Expression Omnibus of National Center for Biotechnology Information (NCBI-GEO) database (accession no.: GSE63699).

2.13. Quantification of microRNA and mRNA

To measure microRNA levels before and after cofilin-1 overexpression, quantitative PCR (qPCR) of targeted miRNA was performed.

In brief, complementary DNA (cDNA) was generated from 5 μg total RNA using SuperScript II reverse transcriptase (Life-Technologies Co., Carlsbad, CA, USA). The cDNA products were then mixed in the Fast SYBR Green Master Mix (Life-Technologies Co., Carlsbad, CA, USA) and subjected to the StepOnePlus Real-Time PCR System (Life-Technologies Co., Carlsbad, CA, USA) according to the manufacturer's instructions. The stem loop primers used for let-7b, let-7e and let-7i were 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACcac-3′, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTAT-3′, and 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACAGC-3′, respectively. The forward primers of qPCR used for let-7b, let-7e and let-7i were 5′-GCCGCTTGAGGTAGTAGGTTGT-3′, 5′-GCCGCTTGAGGtAGGAGGTTGT-3′, and 5′-GCCGCTTGAGGTAGTAGTTTGT-3′, respectively. The universal reverse primer was used for all three let-7: 5′-CCAGTGCAGGGTCCGAGGT-3′. The vertebrate U6 small nuclear RNA (snRNA) gene was used as an internal control, which was amplified using the primer set: 5′-CGCTTCGGCAGCACATATAC-3′ and 5′-TTCACGAATTTGCGTGTCAT-3′. The forward and reverse qPCR primers of Twist-1 were 5′-AGCTACGCCTTCTCGGTCT-3′ and 5′-CCTTCTCTGGAAACAATgACATC-3′, respectively. The forward and reverse qPCR primers of actin were 5′-GGAAATCGTGCGTGACATTAAG-3′ and 5′-GGCCATCTCTTGCTCGAAGT-3′, respectively. The forward and reverse qPCR primers of cofilin-1 were 5′-CTCCTCTGGCGTTGAAGACT-3′ and 5′-GTGCCCTCTCCTTTTCGTTT-3′, respectively.

2.14. Immunohistochemistry

The paraformaldehyde-fixed, paraffin-embedded xenograft tumor samples were used for immunohistochemical staining. Samples were deparaffinized using xylene and then sequentially re-hydrated by 90%, 80% and 70% ethanol. Tissue sections (5 μm) were treated with an antigen retrieval solution [10 mM sodium citrate buffer (pH 6.0) containing 0.05% Tween 20] under boiling water for 25 min. These samples were then incubated with anti-cofilin-1 antibody (Santa Cruz Biotechnology Inc., Dallas, Texas, USA) at 37 °C for 1.5 h. Tissue sections were washed by cold PBS 3 times and then incubated with secondary anti-goat HRP antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Finally, tissue sections were incubated with 3′,3′-diaminobenzidine (Dako Denmark A/S, Denmark) until a brown color developed and the tissue sections were further counterstained with hematoxylin. In a negative control, all steps were followed except the addition of primary antibody.

2.15. Statistical analysis

Three independent experiments were conducted and each data set represented the mean ± S.D. Statistical analysis between controls and experimental groups were determined using the *t* test, and $p < 0.05$ was considered a significant difference.

3. Results

3.1. Over-expression of cofilin-1 suppresses the tumor formation in the xenograft tumor model

To investigate whether over-expression of cofilin-1 would suppress tumor formation in vivo, we used H1299 cells harboring the tetracycline-inducible (*tet-on*) gene expression system to conditionally express exogenous cofilin-1 using doxycycline [9]. Exogenous cofilin-1 could be induced over 7 folds in this stable H1299/*tet-on*-cofilin cell line compared to untreated cells (Fig. 1A). Consistent with a previous study, [6] the cell growth rate of cofilin-1 over-expressing cells (+Doxy) was slower than that of normal cells (-Doxy) as determined by the hemocytometry of up to five days of cell culture (Supplementary fig. 1). Prior to implantation of the tumor cells to the NOD/SCID mice, we used the soft-agar colony formation assay to compare the in vitro tumorigenic ability with or without cofilin-1 over-expression in H1299 cells. The results showed that cofilin-1 over-expressing cells failed to form colonies in soft agar (Fig. 1B). Thereafter, H1299/*tet-on*-cofilin cells and H1299/*tet-on*-vector cells were subcutaneously implanted in right and left thighs of NOD/SCID mice, respectively. These mice were separated into two groups (N = 3 each) fed with or without doxycycline (2 mg/ml v/v in 5% sucrose solution) right after cells were implanted. The results showed that H1299/*tet-on*-vector cells could form tumors in both conditions, while H1299/*tet-on*-cofilin cells could not form tumors in mice treated with doxycycline after five weeks of implantation (Fig. 1C). The tumor growth rate was also measured using the caliper to compare the tumor formation by H1299/tet-on-cofilin cells and H1299/tet-on-vector cells with or without doxycycline (Fig. 1D). Notably, the tumor growth rate of H1299/*tet-on*-vector cells was somehow accelerated in doxycycline fed group (Fig. 1E). Because high concentration of doxycycline (10 mg/pellet) has been reported to induce cytotoxicity of tumor cells, [33] the amount of doxycycline used here should not suppress tumor growth. Whether accelerated tumor growth is caused by the sucrose solution or low dose doxycycline remains to be examined. Additionally, it is observed that the tumor growth rate of H1299/*tet-on*-cofilin cells was slower than that of vector transfected H1299 cells in the absence of doxycycline. Because the basal levels of cofilin-1 in these two cell lines are similar, [30] the different tumor growth rates may be due to the effects of clonal variance after antibiotic selections [34]. We had compared the cell growth of different single stable clones and the result conformed to this assumption (Supplementary fig. 2). Although the growth of H1299/*tet-on*-cofilin cells formed xenograft tumors were suppressed by doxycycline, the tumor was re-grown when doxycycline was removed from drinking water for another four weeks (Supplementary fig. 3). These results suggest that over-expression of cofilin-1 can suppress tumor growth in vivo, but the tumor formation will recover when this stress is removed.

3.2. Use of reporter gene imaging to monitor the growth of xenograft tumor in response to induced expression of cofilin-1

To simultaneously monitor the cofilin-1 induction and tumor suppression in vivo, we stably transfected a *tet-on* pBI-EGFP-tk-puro dual reporter plasmid into H1299/*tet-on*-cofilin cell to obtain a stable cell line named H1299/*tet-on*-cofilin-EGFP/tk cells (see Materials and methods). Doxycycline induced EGFP in this novel cell model was visualized using the fluorescent microscopy (Fig. 2A). These cells (5×10^6) were then subcutaneously implanted to the left thigh of each of the NOD/SCID mice. After six weeks of implantation, the tumor-bearing mice (~100 mm^3 size) were fed with doxycycline supplemented water, and compared to those fed with normal water. Using in vivo fluorescent imaging, the EGFP signal at tumor lesion was detected two weeks after doxycycline, but it gradually decreased and completely diminished after six weeks of treatment (Fig. 2B). The photon flux was quantified at each time point and compared between mice with or without doxycycline (Fig. 2C). Concomitantly, the radionucleotide based reporter gene imaging using HSV1-tk was performed. Induction of co-expressed HSV1-tk reporter gene in H1299/*tet-on*-cofilin-EGFP/tk cells was confirmed using Western blot analysis (Supplementary fig. 4A). The activity of co-expressed HSV1-tk was also detected using cell uptake assay that ^{131}I-FIAU was used as a radioactive substrate for HSV1-tk (Supplementary fig. 4B). For PET imaging, tumors formed by H1299/*tet-on*-cofilin-EGFP/tk cells were detected in tumor-bearing mice after they were intravenously injected with ^{18}F-FEAU (Supplementary fig. 4C and D). Moreover, tumor viability was reduced because tumor uptake of ^{18}F-FDG was reduced in doxycycline treated tumor-bearing mice (Supplementary Fig. 4E–F). Tumor growth of H1299/*tet-on*-cofilin-EGFP/tk cells was also suppressed in tumor-bearing mice after they were treated with doxycycline compared to untreated control (Fig. 2D). Furthermore, the immunohistochemical staining of the tumors biopsies obtained from tumor-bearing mice showed that cofilin-1 was induced in tumor treated with doxycycline compared to untreated control (Fig. 2E). Taken together, current data suggest that over-

expression of cofilin-1 would suppress the growth of xenograft tumors in vivo.

3.3. Over-expression of cofilin-1 reduces cell invasion and motility

We have previously shown that over-expression of cofilin-1 causes morphological change accompanied by destabilization of actin cytoskeleton [30]. Here the effects of over-expressed cofilin-1 on cell mobility was examined. H1299/*tet-on*-cofilin cells were induced to over-express cofilin-1 for 48 h and then subjected to the time-lapse microscopy to record the cellular traveled path and distance for 16 h (Supplementary video 1 and video 2). The results showed that cofilin-1 over-expressing cells traveled a shorter distance and fewer paths than normal cells (Figs. 3A and B). Consistent with previous finding, [9,30] the in vitro invasion assay showed that over-expression of cofilin-1 prohibited cell invasive ability using the Matrigel coated transwells (Figs. 3C and D). Subsequently, we analyzed whether the expressions of matrix metalloproteinase (MMP), including MMP-1, MMP3 and MMP-9 were affected by over-expressed cofilin-1. The Western blot data showed that these MMPs were differently suppressed after over-expression of cofilin-1 (Fig. 3E). Among them, MMP-1 and MMP-3 were significantly suppressed by over-expressed cofilin-1 (Fig. 3F). Therefore, over-expression of cofilin-1 not only suppresses tumor formation in vivo but also inhibits cell motility and invasion in vitro.

3.4. Over-expression of cofilin-1 influences the expressive profile of microRNA

The molecular mechanisms of growth and migration inhibition caused by cofilin-1 were largely unknown. Accumulated literatures

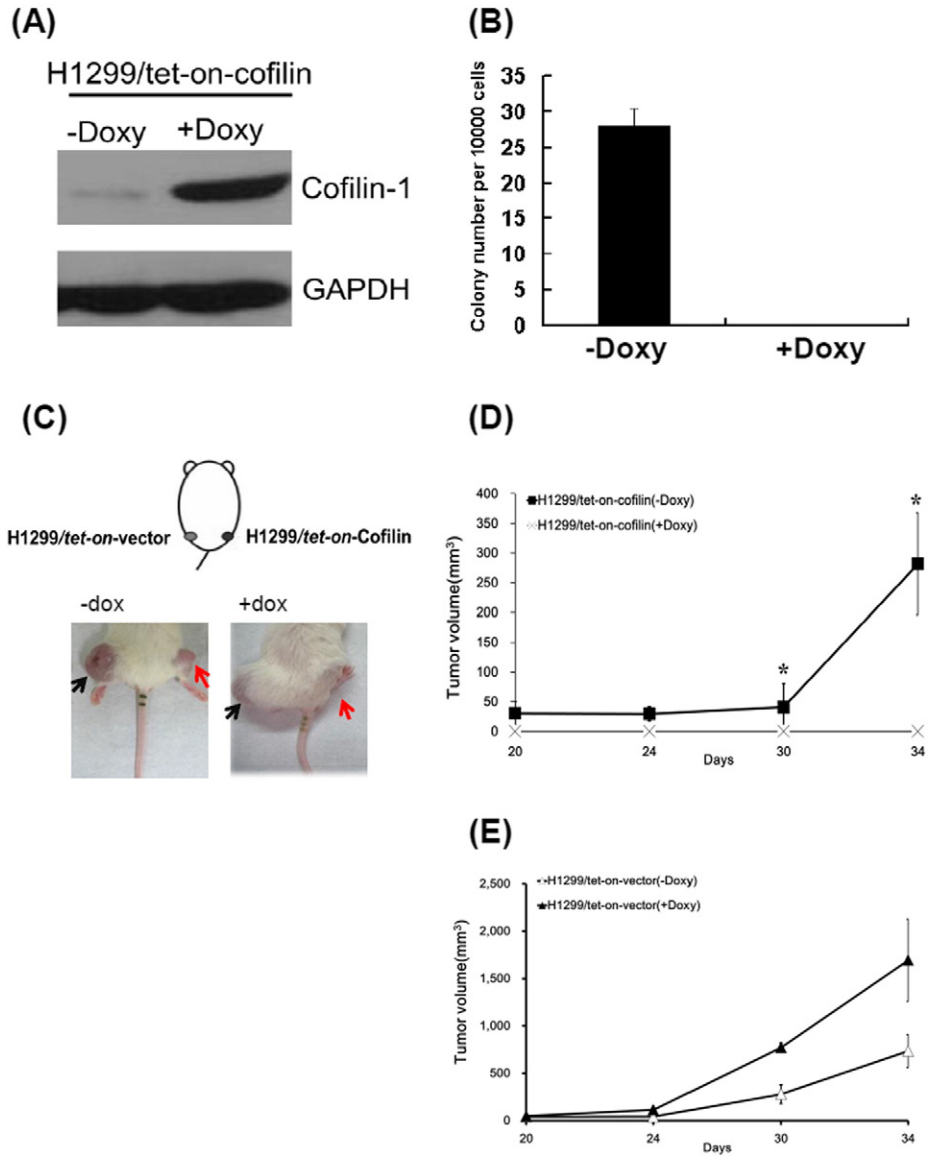


Fig. 1. Effects of cofilin-1 over-expression on xenograft tumor. (A) Induction of exogenous cofilin-1 in H1299/*tet-on*-cofilin cells using doxycycline (1 μg/ml). (B) Sphere formation of H1299/*tet-on*-cofilin cells in three-dimensional soft agar before and after induction of cofilin-1 over-expression. (C) The xenograft tumor formed by s.c. implanting H1299/*tet-on*-cofilin cells and vector transfected H1299 cells to right thighs and left thighs of NOD/SCID mice (N = 6), respectively. Three of these mice were fed with doxycycline supplemented drinking water (2 mg/ml), and others were fed with normal water. For each photo, arrows at left and right represent the tumor formed by H1299/tet-on-vector and H1299/tet-on-cofilin with or without doxycycline, respectively. (D) Comparison of growth curves of H1299/*tet-on*-vector tumor in NOD/SCID mice treated with or without doxycycline up to 34 days after implantation of tumor cells. (E) Comparison of growth curves of H1299/*tet-on*-cofilin tumor in NOD/SCID mice treated with or without doxycycline up to 34 days after implantation of tumor cells.

have supported that several small non-coding microRNAs can regulate actin cytoskeleton and suppress the tumorigenesis [35]. Thus, we used the microarray analysis for microRNA to examine the expression of approximately 1000 microRNAs. Among them, 248 microRNAs were differentially affected in H1299/*tet-on*-cofilin cells after they were treated with doxycycline for 2 days and 7 days. We focused on several microRNAs, including hsa-miR29, [16] hsa-miR34a, [36] hsa-miR335, [37] hsa-miR200 family, [38] hsa-miR185, [39] hsa-miR107 [19] and hsa-let7 family [40] that have been reported to negatively regulate tumorigenesis, and found that let-7b and let-7e microRNA were apparently up-regulated (>2 folds) after over-expression of cofilin-1 for 7 days (Fig. 4A). Actually, expression of all let-7 family members were up-regulated by over-expressed cofilin-1 (Fig. 4B). Over-expression of cofilin-1 affected expressions of let-7b, let-7e, and let-7i were further confirmed using qPCR analysis, and the results were consistent with microarray data (Fig. 4C). Therefore, current data suggest that long-term over-expression of cofilin-1 would influence microRNA let-7b and let-7e of H1299/*tet-on*-cofilin cells.

3.5. Inhibition of let-7b and let-7e expression compromises the cofilin-1 over-expression suppressed cell growth and invasion

To investigate whether let-7b and let-7e are involved in mediating cofilin-1 caused tumor suppression, commercially available locked nucleic acids (LNA) targeting on let-7b and let-7e were transfected to cofilin over-expressing cells to examine if the cell growth rate and invasion ability would be changed. Because the TGFBR1 mRNA is a direct target of let-7, [31,41] we constructed a pMIR-TGFBR1-luc reporter plasmid to estimate the inhibitory efficiency of LNAs. After transfection of the reporter plasmid into H1299/*tet-on*-cofilin cells, the luciferase activity was suppressed after doxycycline, but it was recovered by co-transfected LNAs targeting on let-7b or let-7e (Fig. 5A). These data suggest that over-expression of cofilin-1 up-regulated let-7 can be suppressed by LNA. We next examined the cell growth rate under these conditions, and the results were consistent with the data of LNA mediated let-7 inhibition that either LNA could compromise the effects of over-expressed cofilin-1 on suppression of cell growth (Fig. 5B). Moreover, inhibition of both let-7 types by LNAs could recover the invasion ability that had been inhibited by over-expressed cofilin-1 (Fig. 5C). It appeared that the invasive ability of cofilin-1 over-expressing cells was even higher than that of normal cells after LNA treatment, suggesting that let-7 may influence cell invasion and growth at different pathways. Together, these data demonstrate that let-7 tends to mediate over-expressed cofilin-1 suppressed cell growth and invasion.

3.6. Involvement of Twist-1 transcription factor in cofilin-1 mediated up-regulation of let-7

The expression of the let-7 family has been reported to be regulated by Twist-1, c-myc and LIN28 via different mechanisms [42,43]. We then analyzed the expression of these proteins after over-expression of cofilin-1. The Western blot results showed that Twist-1 was significantly

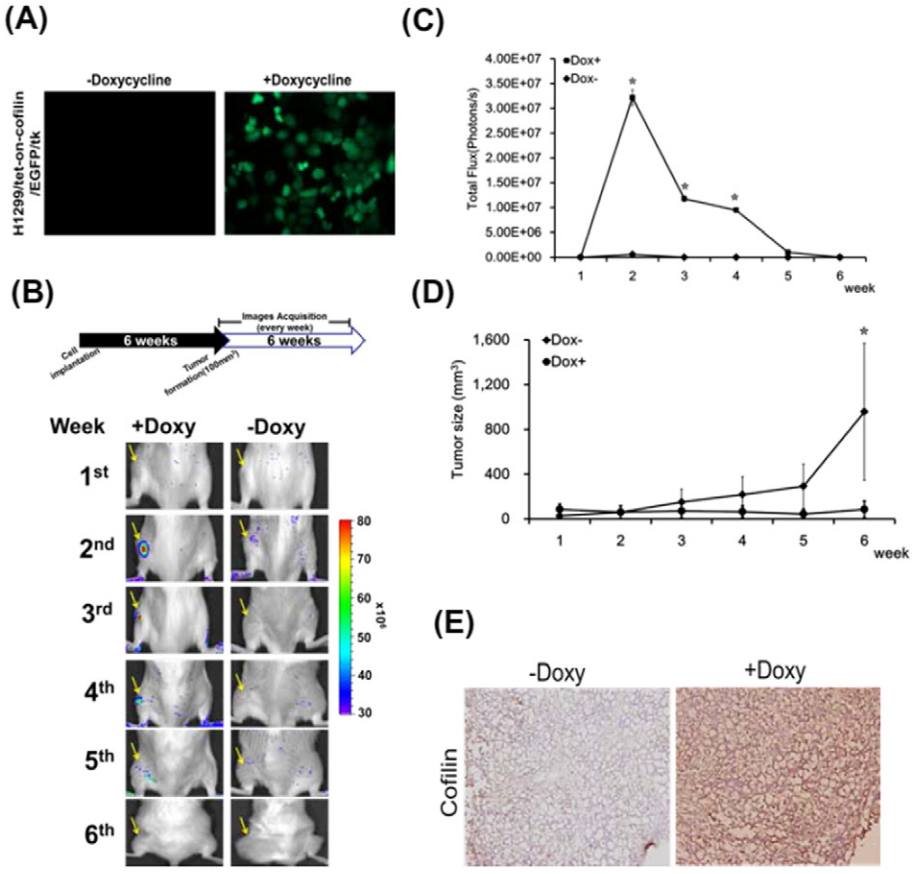


Fig. 2. Use of optical imaging to monitoring the tumor suppressive kinetics by over-expressed cofilin-1. (A) Visualization of induced EGFP in H1299/*tet-on*-cofilin-EGFP/tk cells using doxycycline (1 μg/ml). (B) Design of in vivo experiments for optical imaging is shown. Doxycycline treatment induced maximum fluorescent signals at formed tumors (~100 mm^3, arrow head) in tumor-bearing mice (N = 6) after 2 weeks of doxycycline treatment. (C) Quantification of fluorescent signals by selected region of interest (ROI) in tumor-bearing mice with or without doxycycline up to 6 weeks. *: $p < 0.05$. (D) Comparison of tumor growth rates of H1299/*tet-on*-cofilin-EGFP/tk cells in tumor-bearing mice with or without doxycycline treatment. (E) Immunohistochemical staining of co-expressed cofilin-1 in tumor sections obtained from tumor-bearing mice with or without doxycycline treatment for 2 weeks.

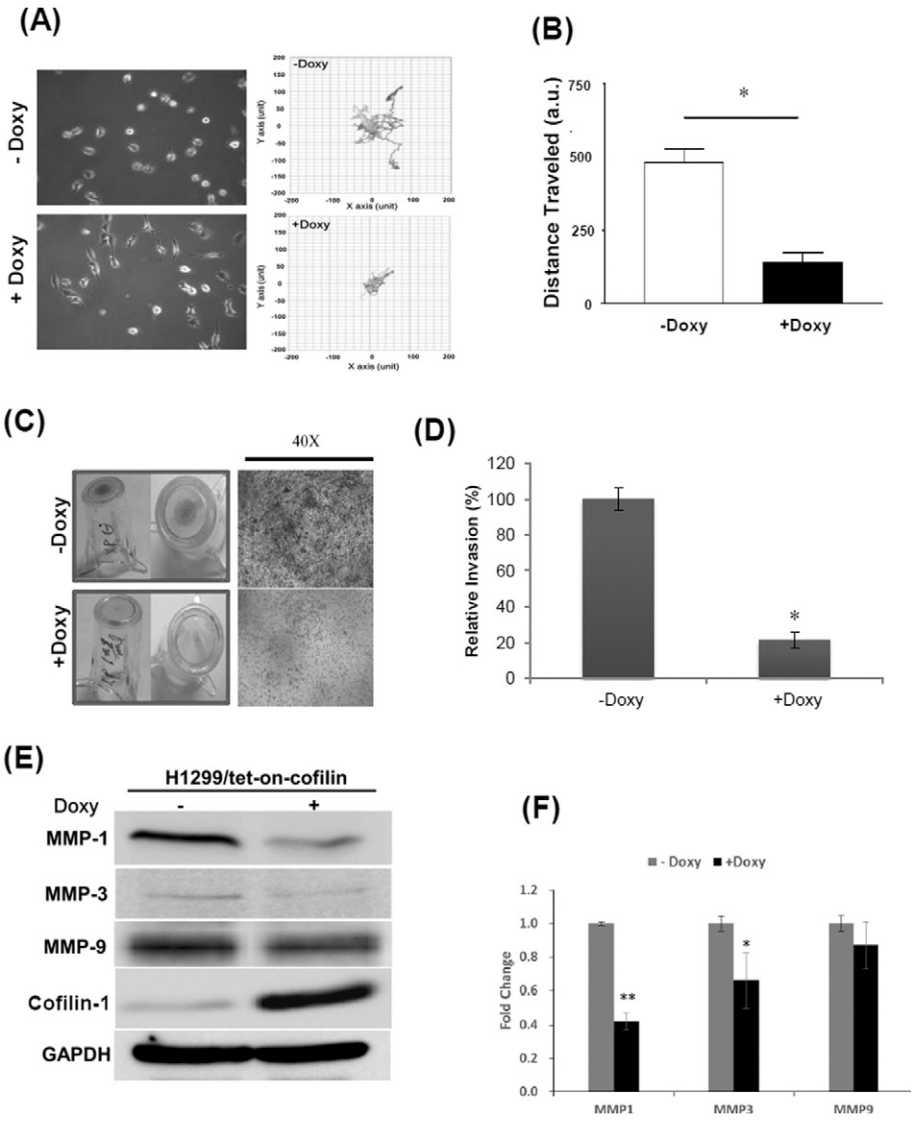


Fig. 3. Effects of cofilin-1 over-expression on mobility and invasion of H1299/*tet-on*-cofilin cells. (A) Visualization and recording of cells movement with or without doxycycline using the time lapse microscopy. (B) Quantification and comparison of distance traveled of cells. a.u.: arbitrary unit. *: $p < 0.05$. (C) In vitro invasion assay using the Matrigel coated transwells. (D) Comparison of the ratio of transversed cells between doxycycline treated and untreated groups. *: $p < 0.05$. (E) Western blot analysis of MMP-1, MMP-3 and MMP-9 levels before and after induction of cofilin-1 over-expression. (F) Densitometric quantification of MMPs using Image J software. The result of each band was normalized to GAPDH, and each datum represented the mean ± S.D. of three independent experiments. *: $p < 0.05$;**: $p < 0.001$.

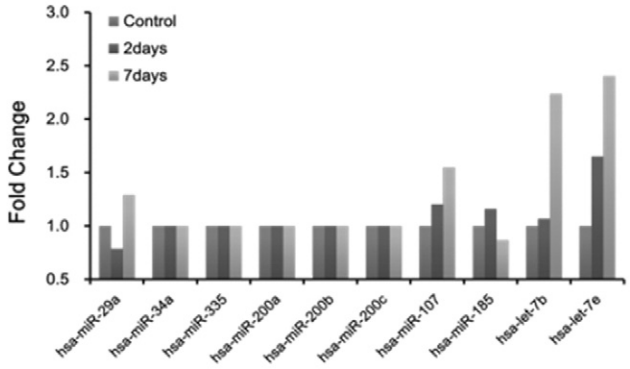
down-regulated by over-expressed cofilin-1, but c-myc and LIN28 were not affected (Fig. 6A). Quantitative PCR showed that over-expression of cofilin-1 could down-regulate basal level of Twist-1 mRNA, whereas over-expression of Twist-1 did not significantly influence the basal level of cofilin-1 mRNA (Fig. 6B). Over-expression of Twist-1 was achieved by transfection of pFLAG-Twist-1 plasmid and the expression of flag tagged Twist-1 was confirmed using anti-flag and anti-Twist-1 antibodies (Fig. 6C). Furthermore, over-expression of cofilin-1 mediated up-regulation of let-7 could be eliminated by concomitant over-expression of Twist-1 (Fig. 6D). We also showed that over-expression of Twist-1 or inhibition of let-7 using LNA increased cellular invasion (Fig. 6E). These data suggest that cofilin-1 induced let-7 expression is associated with regulation of Twist-1 transcription factor, and this signaling pathway may explain the effects of over-expressed cofilin-1 on inhibition of cell invasion.

4. Discussion

Accumulated evidence have supported that cofilin-1 should play a role in cancer progression [44,45]. Whether cofilin-1 over-expression can affect cancer progression in a small animal is rarely studied. Here we used NOD/SCID mice that were subcutaneously implanted with H1299 cells harboring a *tet-on* system to demonstrate that doxycycline induced exogenous cofilin-1 could suppress tumor growth compared to parental H1299 cells. Interestingly, tumors were re-grown after doxycycline was removed from the water (Supplementary fig. 3). Additionally, in vitro studies showed that over-expressed cofilin-1 reduced cell motility and invasion, although we did not detect any distant metastasis in the xenograft tumor model (data not shown). Therefore, cofilin-1 over-expression would suppress the growth of tumor cells in vivo and in vitro, at least in our experimental model.

On the other hand, siRNA mediated knockdown of cofilin-1 in H1299/tet-on-cofilin cells led to cell death but not accelerated cell growth (Supplementary fig. 5). This is consistent with previous reports that cofilin-1 is essential for cell division and viability [46,47]. Actually, our previous study has revealed that over-expression of cofilin-1 can arrest cell cycle but not promote apoptosis [6]. Therefore, over-expression and knockdown of cofilin-1 would cause cell growth suppression and death, respectively. These observations further emphasize that strict regulation of cofilin-1 level is important for cell growth under

(A)



(B)

Fold changes

	-Doxy	+Doxy 2 days/-Doxy	+Doxy 7 days/-Doxy
hsa-let-7a	1	1.04	1.29
hsa-let-7b	1	1.07	2.24
hsa-let-7c	1	1.12	1.95
hsa-let-7d	1	1.12	1.34
hsa-let-7e	1	1.65	2.40
hsa-let-7f	1	1.07	1.31
hsa-let-7g	1	1.20	1.47
hsa-let-7i	1	1.08	1.24

(C)

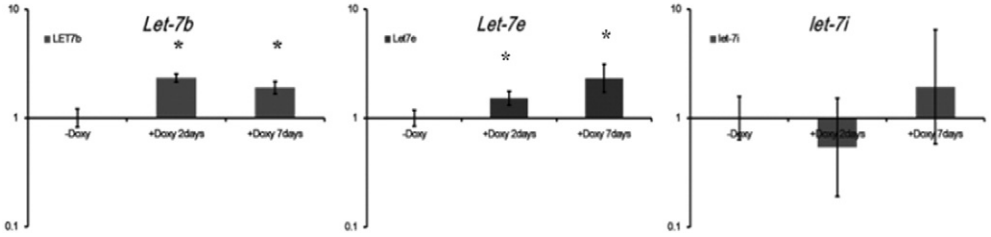


Fig. 4. Effects of cofilin-1 over-expression on the expressive profile of microRNA. (A) Comparing the change of selected microRNAs in H1299/*tet-on*-cofilin cells with doxycycline treatment for 2 days and 7 days. The levels of these microRNAs were obtained from the microarray analysis. (B) The fold change of let-7 microRNA family in H1299/*tet-on*-cofilin cells after doxycycline treatment. Let-7b and let-7e increased over 2 folds at 7 days of doxycycline treatment compared to untreated control. (C) Use of qPCR to confirm the results of microarray analysis for let-7b, let-7e, and let-7i.

physiological conditions. Whether knockdown of cofilin-1 will influence the cell motility as well as the let-7 expression would be of interest to study using a conditional gene knockdown system.

To track the tumor suppression by cofilin-1 over-expression in vivo, we introduced the reporter gene imaging because this novel technique has been widely used for noninvasively monitoring tumor growth and metastasis in small animals [48–50]. We constructed a bidirectional *tet-on* dual reporter plasmid that was transfected to H1299/*tet-on*-cofilin cells to concomitantly track the expression of cofilin-1 and tumor suppression in tumor-bearing mice by optical imaging and radionucleotide based imaging. This reporter gene system was actually designed to report both gene-of-interest and its effects on tumor progression. Indeed, we could detect the tumor progression using the optical imaging. The co-induced cofilin-1 was further confirmed by IHC analysis of tumor sections. Using this imaging approach, we could reduce animal amount to track the effect of cofilin-1 on tumor suppression because the sacrifice of animals at each time point is not required.

Whether over-expression of cofilin-1 can suppress the motility is controversial. Yap. et al. have reported that over-expression of cofilin-1 mediated motility of glioblastoma cells is concentration dependent [8]. However, another report shows that over-expression of serine-3 phosphorylated mutant cofilin-1 (S3D) results in an almost complete inhibition of cell motility in human colon adenocarcinoma cells [51]. Although wild-type cofilin-1 was induced to express in our cell model, it can be normally phosphorylated on serine-3 [6]. Therefore, modulation of cell motility by over-expressed cofilin-1 may not only depend on the protein concentration but also the phosphorylation status of cofilin-1. Although it has not been proven, different types of cancer cells may also exhibit different motilities in response to over-expressed cofilin-1. The invasive ability of cancer cells is usually associated with the secretion of MMPs. Here we found that MMP-1 and MMP-3 were significantly down-regulated by over-expressed cofilin-1. Because over 20 types of MMP are known, whether other MMPs can also be influenced by over-expressed cofilin-1 remains to be examined.

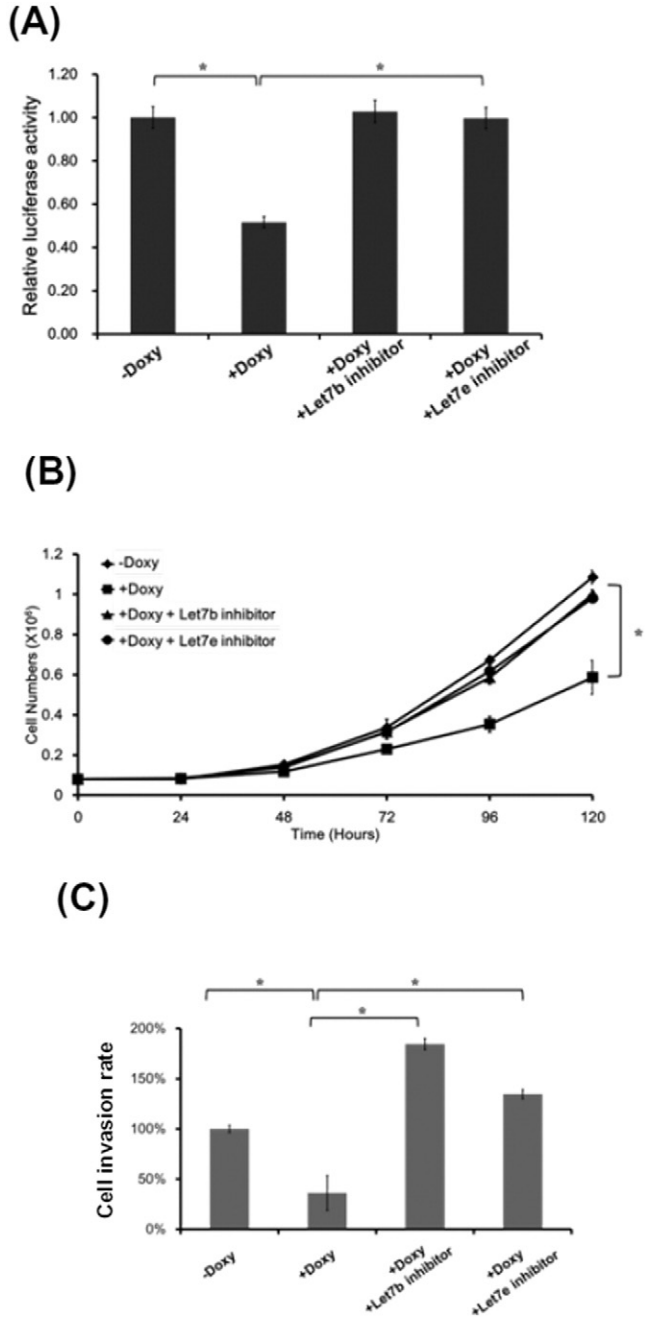


Fig. 5. Inhibition of let-7b and let-7e compromises over-expressed cofilin-1 mediated suppression of cell growth and invasion. (A) Use of pMIR-TGFBR1-luc reporter plasmid confirms that let-7b or let-7e can be inhibited by LNA in cofilin-1 over-expressing cells. (B) Effects of let-7b and let-7e specific inhibitors on the growth rate of cofilin-1 over-expressing cells. (C) Effects of let-7b and let-7e specific inhibitors on the invasive ability of cofilin-1 over-expressing cells *: $p < 0.05$.

Because the motility and the invasion of tumor cells were suppressed by cofilin-1 over-expression, it is of interest to further investigate the putative molecular mechanisms for this observation. Here we investigated the microRNA expressive profile after over-expression of cofilin-1 up to 7 days, and showed that the let-7 microRNA family, particularly let-7b and let-7e were apparently up-regulated compared to other microRNA. The let-7 microRNA family has been reported to be down-regulated in various cancer types, and re-introduction of let-7 into cancer cells leads to suppression of the tumorigenesis [52,53]. Here we also showed that inhibition of let-7 using LNAs in cofilin-1 over-expressing cells could recover their growth rate and invasive ability (Fig. 5B and C). One of the interesting observations is that use of either let-7b or let-7e targeted LNA could recover nearly 90% of cell growth rates in cofilin over-expressing cells. It is unclear whether the effects of let-7b and let-7e on cell growth are independent or additive. We investigated whether transfection of either LNA would influence the expression of both let-7b and let-7e. The results showed that the expression of both let-7b and let-7e were inhibited by transfection of either LNA, suggesting that a potent interaction between let-7 members may exist (Supplementary fig. 6). Because only three nucleotides are different between these two LNAs and they have the same seed sequence (5′-TACCTC-3′), it is also not excluded that the specificity of LNA is insufficient to distinguish the functions of different let-7 members on cell growth. Nevertheless, it is believed that let-7b and let-7e should play a role in mediating the functions of cofilin-1 on cell growth.

It has been reported that enforced expression of let-7b can affect actin dynamics related genes and inhibit cell motility [28]. Therefore, although cofilin-1 can directly bind to actin filaments and affect actin dynamics, it seems that over-expressed cofilin-1 can also regulate actin dynamics via up-regulate let-7 microRNA. Because over-expression of cofilin-1 can destabilize actin cytoskeleton, it remains unclear that let-7 is up-regulated before or after destabilization of actin cytoskeleton. Investigation of let-7 expression using actin inhibitors may answer this question.

Little is known about the molecular pathway for let-7 regulation, although c-myc and LIN28 has been reported to be the regulators of the let-7 family [54,55]. A recent report also showed that the epithelial mesenchymal transition (EMT) regulator Twist-1 is involved in regulation of let-7i, a member of the let-7 family [42]. We examined the expression of these three molecules after over-expression of cofilin-1. Surprisingly, Twist-1 is the only molecule to be affected by over-expressed cofilin-1. Over-expression of Twist-1 could suppress the up-regulated let-7 induced by over-expressed cofilin-1, suggesting that Twist-1 down-regulation is required for cofilin-1 mediated up-regulation of let-7. Although the levels of c-myc and LIN28 are not affected by over-expressed cofilin-1, their activity or subcellular distribution may need to be further investigated to conclude their importance on cofilin-1 mediated let-7 expression.

In summary, we used a xenograft tumor model to demonstrate that forced expression of cofilin-1 could suppress the formation of subcutaneous tumor formed by human NSCLC cells. Over-expression of cofilin-1 also reduced the invasion and motility of cancer cells in vitro. We focused on the change of microRNA profiles, and found that let-7b and let-7e microRNAs were apparently up-regulated by long-term over-expression of cofilin-1. Concomitantly, the potent let-7 regulator Twist-1 transcription factor was down-regulated by over-expressed cofilin-1. Transduction of Twist-1 into cofilin-1 over-expressing cells could inhibit let-7 level that was up-regulated by over-expressed cofilin-1. Therefore, current data suggest that over-expression of cofilin-1 would affect tumor progression through elevation of let-7 microRNA. This may provide a novel signaling pathway between cofilin and let-7 to influence tumor development.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.01.007.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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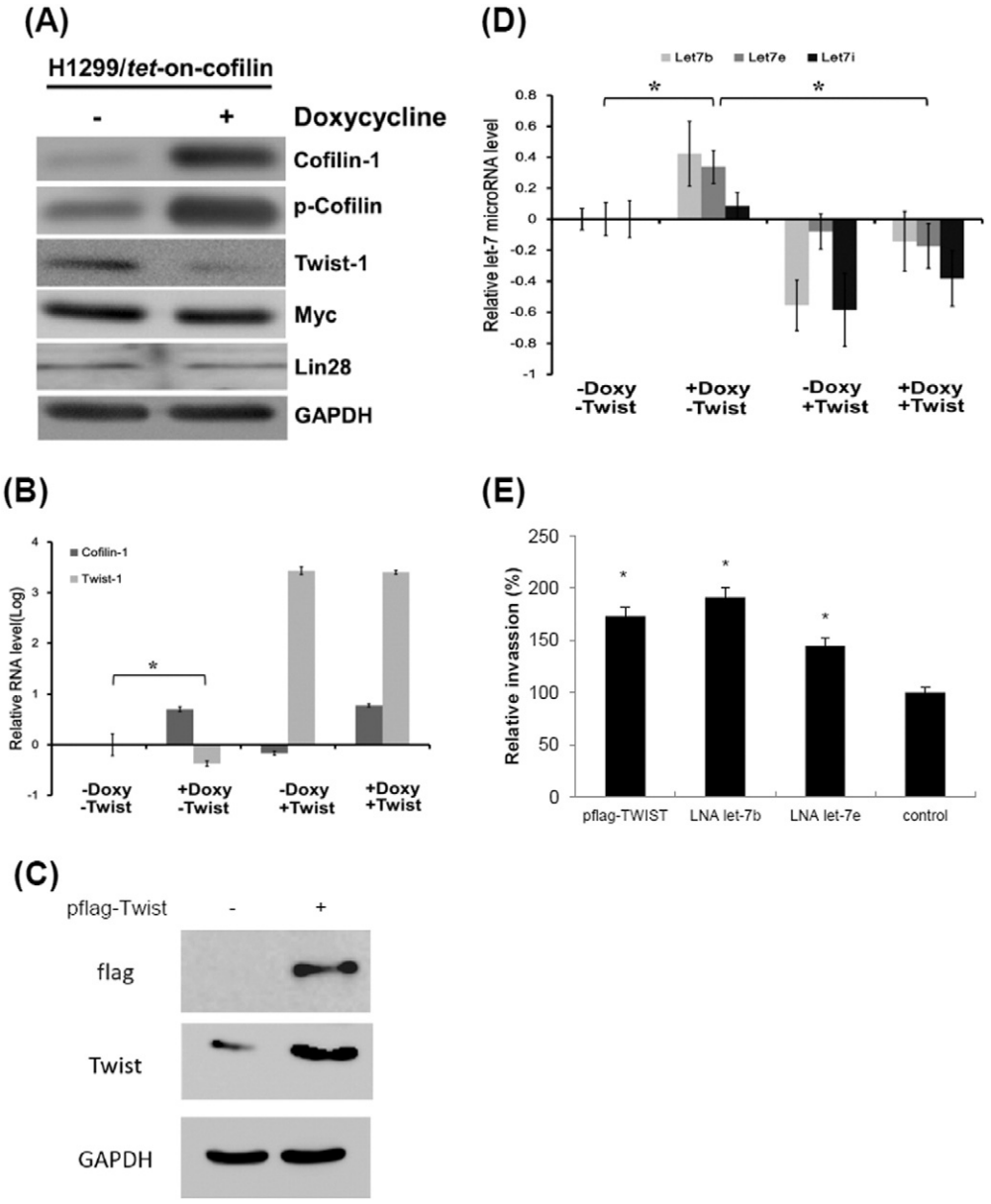

Fig. 6. Effects of cofilin-1 over-expression on molecules that regulate let-7 expression. (A) Western blot analysis of Twist-1, c-myc and LIN28 expression after H1299-*tet-on*-cofilin cells were treated with doxycycline (1 μg/ml) for 48 h. (B) Over-expression of cofilin-1 suppressed the expression of Twist-1 mRNA as demonstrated by qPCR. (C) Western blot analysis for detecting ectopic expression of flag tagged Twist-1 using anti-Flag and anti-Twist-1 antibodies. (D) Over-expression of cofilin-1 mediated up-regulation of let-7 could be suppressed by ectopic expression of Twist-1. (E) In vitro invasion assay showed that over-expression of Twist-1 or transfection of LNA to inhibit let-7b and let-7e could increase the invasion rate. *: $p < 0.05$.

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