RNA-interference-mediated downregulation of Pin1 suppresses tumorigenicity of malignant melanoma A375 cells

J. JIN1, Y. ZHANG1, Y. LI1, H. ZHANG1, H. LI1, X. YUAN1, X. LI1, W. ZHOU1, B. XU2, C. ZHANG2, Z. ZHANG2, L. ZHU2, X. CHEN1,*

1Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, PR China; 2Department of Medicinal Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, PR China

*Correspondence: chenxg@imm.ac.cn, rebeccagold@imm.ac.cn

Received June 15, 2012 / Accepted August 5, 2012

The peptidyl-prolyl isomerase Pin1 is overexpressed in many human cancers, including melanoma. To investigate its possible role in oncogenesis of melanoma and as a therapeutic target, we suppressed Pin1 expression in the human melanoma cell line A375 by microRNA (miRNA) interference technology. Two stable clones with suppressed Pin1 were established by stable transfection of miRNA plasmid targeting Pin1 into A375 cells. Both clones showed reduced proliferation and invasion in vitro and suppressed tumorigenic potential in athymic mice. Furthermore, Pin1 inhibition also resulted in decreased phosphorylation of Akt and repressed expression of C-Jun N-terminal kinase and pro-matrix metalloproteinase 2, which were associated closely with the development of melanoma. These findings indicate that Pin1 plays an important role in the tumorigenesis of melanoma and might serve as a promising therapeutic target.

Key words: malignant melanoma, A375, Pin1, RNA interference, cancer therapy

Malignant melanoma is a cancer of melanocytes, and is the most serious form of skin cancer. Its incidence has been rising steadily throughout the past few decades.[1, 2] It is considered curable when detected at an early stage, but once it has entered the advanced stage, it disseminates widely and soon becomes an incurable malignancy with extremely poor prognosis.[3] The median survival time for patients with malignant melanoma is <1 year and the 5-year survival rate after initial presentation is <5%.[4] As a result of the special characteristics and usual resistance to standard chemotherapy, there is no systemic and effective therapy that has a clear effect on overall survival of patients with malignant melanoma. Although our understanding of the molecular biology of malignant melanoma has increased in recent years, detailed knowledge of the molecular mechanisms involved in its formation and progression remains elusive. Furthermore, although after many decades of progress in treating malignant melanoma, many promising new targets are entering the clinic, only a small percentage of patients get benefit from these targeted drugs.[5, 6] Therefore, a penetrating understanding of the pathogenesis of malignant melanoma and search for potent molecular targets based on its etiology might lead to effective strategies for the treatment of this cancer.

Oncogenesis is a complex multistep and multifactorial process that ultimately results in uncontrolled cell proliferation and transformation. One of the most important signaling mechanisms involved in this event is phosphorylation of proteins on serine or threonine residues that precede proline (Ser/Thr-Pro).[7] The peptidyl-prolyl isomerase Pin1, which specifically recognizes phosphorylated Ser/Thr-Pro motifs, is an enzyme that promotes the cis–trans isomerization of the peptide bond of its target proteins. These conformational changes can have profound effects on Pin1 substrates, such as modulating their activity and stability.[8, 9] Therefore, Pin1 has been demonstrated to be involved in the regulation of diverse cellular events, especially proliferation and transformation. A number of studies have suggested that Pin1 plays an important role in tumorigenesis and is overexpressed in most types of human cancer.[10-15] Furthermore, increased levels of Pin1 have been shown to correlate with poor clinical outcome, which indicates that the expression level of Pin1 could have become a prognostic marker for diseases such as prostate cancer.[16-18]

Bao et al.[10] have shown that Pin1 is overexpressed in melanoma. However, it is not known whether Pin1 is involved
in the oncogenesis of melanoma, and it is unclear whether Pin1 inhibition affects cell growth or blocks development of melanoma. In the present study, we suppressed the expression of Pin1 in human malignant melanoma A375 cells by microRNA interference (miRNAi) to explore the role and mechanism of Pin1 in the progression of malignant melanoma.

Materials and methods

Cell culture. Human malignant melanoma cell line A375, preserved by our laboratory, was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL Life Technologies, Grand Island, NY, USA), and incubated at 37°C in a humidified atmosphere that contained 5% CO2.

Pin1 miRNA plasmids and cell transfection. Four pcDNA6.2-GW/EmGFP-miR-Pin1 plasmids and the pcDNA6.2-GW/EmGFP-miR-Negative control plasmid were constructed and provided by Invitrogen (Grand Island, NY, USA). The sequences of the four synthesized miRNA oligonucleotides that targeted Pin1 (NM_006221) cloned into the vector, pcDNA6.2-GW/EmGFP-miR, were miRNA1 (sense sequence: 5'-TGCTGCTGCCGGTCTGGCTCTTCCTCGTTTTGGCACCAGTCTGAGAGAGAGACGAGGAGGCCGCAGC-3', antisense: 5'-CCTGTCGCGGCTCAGTCAG-3'), miRNA2 (sense sequence: 5'-TGCTGAGACCGAGAAGGCGTAGCTGACGATGGCAAGCTGCAGCTATGC-3', antisense: 5'-TGGCTGAGACCGAGAAGGCGTAGCTGACGATGGCAAGCTGCAGCTATGC-3'), miRNA3 (sense sequence: 5'-TGCTGCATAGCTGCAGCTTGGCCAAAACCAGGACTACATCGACTTCCTCTAC-3', antisense: 5'-CCTGCACTGACGATGGCAAGGCTACTACATCTCGCTCT-3'), miRNA4 (sense sequence: 5'-TGCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACCAGGACTACATCGACTTCCTCTAC-3', antisense: 5'-CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACCAGGACTACATCGACTTCCTCTAC-3'). The sequence of the negative control oligonucleotides was (sense sequence: 5'-TGCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACCAGGACTACATCGACTTCCTCTAC-3', antisense: 5'-TGCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACCAGGACTACATCGACTTCCTCTAC-3').

A375 cells were transiently transfected with pcDNA6.2-GW/EmGFP-miR-Pin1 plasmids and the pcDNA6.2-GW/EmGFP-miR-Negative control plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 4 hr and then recording the absorbance at 490nm with complete medium. According to the manufacturer's protocol, assays were performed by adding 10μL MTS reagent (Promega, Madison, USA) directly into culture wells, incubating for 4 hr and then recording the absorbance at 490nm with complete medium containing Blastidin (Invitrogen) at 7ug/ml for clonal selection. Selection was maintained in a medium containing Blastidin at 3.5ug/ml to ensure stable transfection.

MTS assay. Cells (1×105) were seeded into 96-well plates with complete medium. According to the manufacturer’s protocol, assays were performed by adding 10μL MTS reagent (Promega, Madison, USA) directly into culture wells, incubating for 4 hr and then recording the absorbance at 490nm with a 96-well plate reader every 24 hr for 5 days. All experiments were performed in triplicate.

Colony formation assay. A total of 200 cells were plated in six-well plates, which yielded 150–200 colonies per well after approximately 14 days of culture. The growth of the colonies was examined 10 days later after crystal violet staining. Colonies that consisted of more than 50 cells were scored. The number of colonies was examined as the mean and standard deviation (SD) of triplicate experiments.

Flow cytometry analysis. Cells (1 × 105) were collected from the flasks with 10% serum were trypsinized, washed three times with phosphate-buffered saline (PBS), and fixed with cold 70% ethanol for 24 hr at –20°C. Before testing, the fixed cells were washed with PBS and re-suspended in PBS that contained 50 mg/ml propidium iodide and 0.5 mg/ml RNase.
A for 30 min at 37°C. Cell cycle distribution was measured subsequently.

**Western blot analysis.** Total cellular protein was extracted using lysis buffer. Protein concentration was measured by the Bio-Rad protein assay. An equal amount of protein was separated using 10% and 12% SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Bucks, UK). The membranes were blocked with 5% skimmed milk in PBS and incubated overnight with primary antibodies, followed by horseradish-peroxidase-conjugated antibodies at room temperature. β-Actin was used as internal positive control. Primary antibodies included Pin1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pAKT (Cell Signaling Technology, Danvers, MA, USA), AKT (Cell Signaling Technology), C-Jun N-terminal kinase (JNK; Cell Signaling Technology), matrix metalloproteinase (MMP)-2 (Santa Cruz Biotechnology), β-catenin (Santa Cruz Biotechnology) and β-actin (Santa Cruz Biotechnology). Signals were visualized using an enhanced chemiluminescence system (Amersham).

**Transwell migration assay.** Transwell migration assay was performed by the Boyden chamber system (Neuro Probe, Gaithersburg, MD, USA) with a fibronectin-precoated (0.5 mg/ml) polycarbonate membrane (8μm pore size). The lighter side of the polycarbonate membrane was precoated with 250 μg/ml Matrigel (BD Labware, Franklin Lakes, NJ, USA). The bottom chambers were filled with 30 μl RPMI 1640 serum-free medium that contained 2% bovine serum albumin (BSA), and the top chambers were filled with 50 μl RPMI 1640 serum-free medium that contained 0.2% BSA. Cells (1×10^5/well) were added to the top chamber, followed by 16 hr incubation at 37°C in a 5% CO₂ incubator. Three independent experiments were performed in triplicate. The cells were fixed in methanol and stained with hematoxylin. The top surface of the membrane was gently scrubbed with a cotton bud. The cells that had migrated to the lower side of the membrane were counted under a microscope (OLYMPUS IX70; Tokyo, Japan), and the percentage of migration were calculated.

**Tumor formation in nude mice.** Six to eight-week-old male nude mice (BALB/c-nu) were obtained from the Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All animals in our study were housed under pathogen-free conditions and maintained according to the guidelines of the Committee on Animals of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College. Three groups of cells (NC, Clone1 and Clone2) were washed and resuspended with normal saline. The cell suspensions (1×10^7 cells) were injected subcutaneously into the BALB/c-nu mice. One week later, tumor formation was monitored and recorded every 3 or 4 days. Tumor volume was calculated by the formula: \(a^2b/2\), where \(a\) and \(b\) are the two maximum diameters measured by a sliding caliper. The mice were sacrificed after 21 days and

![Figure 2. Pin1 inhibition decreased proliferation and colony formation in A375 cells.](image-url)
the tumors were weighed. A total of four nude mice were used for each group.

**Statistical analysis.** All experiments were repeated at least three times. One way ANOVA was used to evaluate the differences between experimental and control groups with SPSS version 13.0 (SPSS, Chicago, IL, USA).

**Results**

**Inhibition of Pin1 expression by RNAi in A375 cells.** To examine the role of Pin1 in carcinogenesis of malignant melanoma, we depleted Pin1 using the RNAi method in human malignant melanoma A375 cells, which have the advantage of high-proliferative and invasive activity that makes experimental protocols possible in vitro and in vivo. Four pcDNA6.2-GW/EmGFP-miR-Pin1 plasmids against Pin1 with four different synthesized miRNA oligonucleotides were constructed. Western blot analysis of transient transfection revealed that the pcDNA6.2-GW/EmGFP-miR-Pin1 plasmid with miRNA4 oligonucleotides showed the strongest inhibitory activity against Pin1 expression, whereas the pcDNA6.2-GW/EmGFP-miR-Negative plasmid showed no effect (Fig. 1). Subsequently, stable transfection using the pcDNA6.2-GW/EmGFP-miR-Pin1 plasmid with miRNA4 oligonucleotides was performed and two single clones designated Clone1 and Clone2 were established. The A375 cells stably transfected with pcDNA6.2-GW/EmGFP-miR-Negative control plasmid were designated NC. As shown in Figure 1B, expression of Pin1 was significantly suppressed in Clone1 and Clone2 (84.9% and 83.9% inhibition respectively, analyzed by Image Pro, P < 0.001), but unchanged in NC compared with the parental cells.

**Pin1 suppression retards proliferation of A375 cells.** To investigate whether inhibition of Pin1 expression can inhibit cell proliferation, we recorded cell growth curves by MTS assay. As shown in (Fig.2A), Pin1 inhibition resulted in a significant decrease in cell proliferation, whereas the NC grew normally as parental A375 cells. Compared with NC, the inhibitory rate of Clone1 and Clone2 were 55.5% and 59.8% for 72 hour (P < 0.01), 51.8% and 61.0% for 96 hour (P < 0.01), 41.2% and 55.5% for 120 hour (P < 0.01), respectively.

**Pin1 suppression abrogates anchorage-dependent colony formation in A375 cells.** To examine the effect of Pin1 inhibition on tumorigenic properties in A375 cells in vitro, we tested the anchorage-dependent colony formation ability of different cells. As shown in (Fig.2B), compared with the NC...
group, colony formation of Clone1 and Clone2 was reduced by 59.0% (P < 0.05) and 90.3% (P < 0.01), respectively. In contrast, there was no significant difference between NC and parental A375 cells (P > 0.05).

**Pin1 suppression induces G0/G1 arrest.** We determined whether loss of Pin1 expression affected cell cycle progression. As shown in (Fig.3), the proportion of cells residing in the G0/G1 phase of parental A375 and NC cells was 46.6% and 49.5%, respectively. However, this proportion was significantly increased to 66.1% and 70.2% in Clone1 and Clone2, respectively, which indicated that depletion of Pin1 resulted in arrest in G0/G1 phase.

**Pin1 suppression inhibits proliferation of A375 in vivo.** The results presented above suggest that Pin1 plays a dominant role in the proliferation of A375 cells in vitro. To investigate whether Pin1 suppression could decrease tumorigenesis in vivo, xenograft studies were carried out using NC, Clone1 and Clone2. As shown in (Fig.4), Clone1 and Clone2 with silenced Pin1 expression showed an obvious decrease in tumor size (52.2% and 30.8% decrease respectively) and weight (59.2% and 45.5% decrease respectively) at day 21 after subcutaneous inoculation, especially Clone1, compared with NC cells (P < 0.01).

**Pin1 suppression inhibits the invasive ability of A375 cells.** To evaluate further the role of Pin1 in cell invasion, we performed an invasion assay to investigate any difference between these cells. As shown in (Fig.5), Clone1 and Clone2 showed a dramatic decrease in migration, in which the percentage of invaded cells was 42.3% (P < 0.05) and 27.4% (P < 0.01), respectively, compared with A375 cells. These results suggested that Pin1 depletion could significantly suppress invasion of A375 cells.

**Effects of Pin1 suppression on related protein expression.** It has been reported that Pin1 regulates a series of target proteins, many of which are often deregulated during the course of oncogenesis.[19, 20] To identify which proteins are inhibited by Pin1 suppression in malignant melanoma, phosphorylation of Akt, and expression of JNK, MMP2 and β-catenin were measured by western blotting. Compared with NC, Clone1 and Clone2 showed a significant decrease in activated Akt (shown as pAkt-Ser473/Akt) (42.3% and 46.2% down-regulation, P<0.05), JNK2/3 (59.6% and 76.6% down-regulation, P<0.01), JNK1 (60.8% and 55.4% down-regulation, P<0.01) and pro-MMP2 (59.6% and 76.6% down-regulation, P<0.01), whereas there was no conspicuous change in the expression of β-catenin (Fig. 6).
Discussion

Although much research has indicated that Pin1 is markedly overexpressed in many different human cancers, including melanoma, it is still unclear whether Pin1 is involved in the carcinogenesis of malignant melanoma, and whether Pin1 suppression blocks tumorigenesis of malignant melanoma.

In the present study, we inhibited Pin1 expression in A375 cells with miRNA plasmid and investigate whether Pin1 suppression could reverse the malignant phenotype. The cells of Clone1 and Clone2 with silenced Pin1 showed retarded proliferation and decreased ability of colony formation. The xenografted tumors from the cells of Clone1 and Clone2 were observed decreased in size and mass. These observations demonstrated the crucial role of Pin1 in the regulation of the proliferation of melanoma. Further, we investigated whether this growth inhibition caused by Pin1 depletion was related to changes in cell cycle distribution. Flow cytometry analysis showed that both clones with silenced Pin1 expression had an increased proportion of cells residing in the G0/G1 phase, which could partially elucidate the growth inhibition phenomenon, and indicated that Pin1 was mainly involved in the transition between G0/G1 and S phase in malignant melanoma A375 cells.

Malignant melanoma, one of the most malignant cutaneous tumors, is characterized by its high potential for invasiveness and metastasis. It is these characteristics that lead to extremely poor prognosis and high mortality.[4, 21, 22] As shown in the present study, Pin1 suppression greatly inhibited the invasive ability of A375 cells.

AKT/protein kinase B is a cardinal node in many signal cascades of receptor tyrosine kinase downstream pathways, and plays a pivotal role in diverse cellular functions such as proliferation, differentiation and migration[23-25]. Numerous studies have testified that AKT is frequently aberrantly regulated in a wide variety of human cancers including malignant melanoma, and increased phosphorylation of AKT in malignant melanoma is associated with tumor progression and shorter survival. Importantly, AKT has been recognized as a potential target in therapy of malignant melanoma[5, 26-29]. In a study of breast cancer, Liao et al. [30] have found that the expression level of AKT phosphorylation at S473 is correlated with the level of Pin1, and their combined expression levels predict poorer prognosis than does either one alone.

Figure 5. Depletion of Pin1 suppressed cell invasion. (N=3) (A) Invasion of melanoma cells (A375, NC, Clone1 and Clone2) through reconstituted basement membrane (original magnification, 40×). (B) The percentage of invaded cells in the transwell migration assay.

![Image](image.png)

Figure 6. Pin1 inhibition induced downregulation of the phosphorylation of Akt (P < 0.05), the expression of JNK (P < 0.01) and pro-MMP2 (P < 0.01), whose expression was associated with development of melanoma. Western blot analysis with antibodies specific for p-AKT, AKT, JNK, MMP2 and β-catenin is shown. β-actin levels are shown as an internal control. (N=3)
They have also shown that Pin1 can regulate AKT stability and phosphorylation at S473 through the phosphorylated Thr-Pro motifs of AKT.[30] Similarly, our study showed that Pin1 suppression inhibited phosphorylation of AKT at S473 in A375 cells. Thus, specific Pin1 depression could be an appealing strategy to inhibit deregulation of AKT in malignant melanoma.

JNKs, also referred to as stress-activated kinases, are an evolutionarily conserved sub-group of mitogen-activated protein kinases and are implicated in oncogenic transformation.[31-34] Although its role in tumor development remains controversial, several studies have suggested that JNK is essential to the survival of melanoma, and JNK inhibition can suppress melanoma cell proliferation in vitro and in vivo.[35, 36] In a study of Alzheimer’s disease, Rudrabhatla et al.[37] have reported that Pin1 can regulate the activity of JNK3, a JNK isoform that is restricted to brain, heart and testes.[38, 39] However, we do not know whether Pin1 is involved in regulation of JNKs in cancer. In the present study, we found for the first time that Pin1 inhibition suppressed JNK expression dramatically. Although further detailed analysis, such as the exact mechanism of direct JNK inhibition, is necessary, our results indicated that Pin1 can affect JNK expression in malignant melanoma, and the growth inhibition of malignant melanoma that is mediated by Pin1 suppression might be related to JNK inhibition.

It is well established that upregulation of MMPs in melanoma is one of the most crucial factors that lead to tumor invasion and metastasis.[40-43] In particular, patients with high expression of MMP2 often have worse prognosis.[40, 44] In fact, Ryo et al. have found that inhibition of Pin1 suppresses expression of MMP2 in prostate cancer cells.[45] Our study showed that, in melanoma A375 cells, Pin1 inhibition decreased expression of MMP2 in prostate cancer cells. Although the cleaved MMP2 did not change significantly, there was a tendency to decline. Further research is under way in our laboratory to investigate this phenomenon.

β-catenin is a key component of the Wnt signaling pathway, and is closely associated with tumor cell proliferation and invasion.[46] However, its exact role in melanoma remains controversial.[47, 48] β-catenin is also one of the most important substrates regulated by Pin1.[12, 13, 49, 50] However, in our study, we did not find any changes in the expression of β-catenin followed by Pin1 interference. The fact that Pin1 did not affect expression of β-catenin in A375 cells indicated that the relationship between Pin1 and β-catenin could have tumor origin specificity.

In summary, Pin1 suppression by miRNAi prevented human melanoma A375 cell proliferation and invasion in vitro and tumor growth in vivo, that was accompanied with the inhibition of a variety of tumorigenesis-related oncoproteins. These findings indicate that Pin1 might be a potential molecular target for malignant melanoma therapy. Our further studies are going to be focused on the mechanism of Pin1 regulating proliferation, apoptosis and angiogenesis.

Acknowledgments: This work is supported by the National Natural Science Foundation of China (Grant No. 30500634) and the National Science and Technology Major Project of the Ministry of Science and Technology of China (Grant No. 2009ZX09301-003-9-1).

References


YEH ES, MEANS AR. PIN1, the cell cycle and cancer. Nat Rev Cancer 2003; 3: 918–928. http://dx.doi.org/10.1038/nrc1091


LIAO Y, WEI Y, ZHOU X, YANG JY, DAI C, et al. Ptd-2-prolyl cis/trans isomerase Pin1 is critical for the regulation of PKB/Akt stability and activation phosphor-


