Relocalized p27Kip1 Tumor Suppressor Functions as a Cytoplasmic Metastatic Oncogene in Melanoma

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Abstract
The p27 tumor suppressor negatively regulates G1 cell cycle progression. However, human malignancies rarely select for deletion/inactivation of p27, a hallmark of tumor suppressor genes. Instead, p27 is degraded or relocalized to the cytoplasm in aggressive malignancies, supporting the notion that p27 sequestration from its nuclear cyclin/cyclin-dependent kinase (cdk) targets is critical. However, emerging cell biology data suggest a novel cdk-independent cytoplasmic function of p27 in cell migration. Here, we find cytoplasmic p27 in 70% of invasive and metastatic melanomas. In contrast, no cytoplasmic p27 was detected in noninvasive, basement membrane–confined melanoma in situ, suggesting a late oncogenic role for cytoplasmic p27 in metastasis. Targeted cytoplasmic expression of wild-type or non–cdk-binding p27 at subphysiologic levels induced melanoma motility and resulted in numerous metastases to lymph node, lung, and peritoneum. These observations point to a prominent role of cytoplasmic p27 in metastatic disease that is independent of cyclin/cdk regulation or mere nuclear loss. [Cancer Res 2007;67(19):9238–43]

Introduction
The p27 cyclin-dependent kinase (cdk) inhibitor was identified as a negative regulator of G1 phase cell cycle progression (1). Based on tumor predisposition of p27-null mice, p27 is regarded as a tumor suppressor protein (2–4). However, human malignancies rarely select for deletion/inactivation of the p27 gene, a hallmark of tumor suppressor genes (5–7). Instead, p27 is often found either absent from the nucleus or relocalized to the cytoplasm in multiple aggressive malignancies. For example, in primary human breast cancer, activated AKT induces nuclear export of p27 into the cytoplasm, effectively partitioning p27 away from its nuclear target cyclin/cdk complexes and thereby providing the tumor cell with a growth advantage. Indeed, nonnuclear cytoplasmic p27 is correlated with poor patient prognosis in multiple malignancies (8–11). Taken together, these observations suggest that, in the absence of p27 inactivating mutations or genetic deletions, tumor cells de facto inactivate p27 by shuttling it into the cytoplasm to sequester it from its nuclear cyclin/cdk targets.

In contrast to the well-characterized nuclear cyclin/cdk inhibitory function of p27, emerging data suggest that cytoplasmic p27 has a cell cycle–independent function. Activation of the Met receptor by hepatocyte growth factor (HGF) leads to alteration of cell proliferation and motility and is associated with tumor metastasis (12). HGF signaling in human hepatocellular carcinoma cells results in actin cytoskeletal rearrangements, increased cell migration, and translocation of p27 to the cytoplasm (13, 14). In the cytoplasm, p27 colocalizes with actin at the plasma membrane, suggesting a role for p27 in regulation of cell migration (14). Moreover, reintroduction of p27 into p27-deficient mouse embryonic fibroblasts (MEF), which are defective for cell migration, rescues the phenotype and results in wild-type levels of cell migration. Furthermore, p27 has also been linked to cytoskeletal reorganization and neuronal migration during corticogenesis (15). Thus, tumor cell relocalization of p27 into the cytoplasm may have resulted in not only the loss of nuclear tumor suppressor function but also a cytoplasmic gain of function in promoting cell motility, invasion, and metastasis.

To directly test for a p27 role in induction of metastasis, we expressed a non–cdk-binding cytoplasmic form of p27 at subphysiologic levels in a low metastatic melanoma cell line. Surprisingly, these cytoplasmic p27 cell lines showed dramatic increases in both cell motility in vitro and metastasis in vivo. Moreover, 71% of invasive and metastatic human melanomas presented with cytoplasmic p27, whereas basement membrane–confined melanoma in situ showed no cytoplasmic p27. Our results show a gain of function for p27 when in the cytoplasm in promoting metastasis.

Materials and Methods

Cell culture. Low metastatic potential murine B16F0 melanoma cells [American Type Culture Collection (ATCC)] and human HEK 293T cells (ATCC) were maintained in DMEM high glucose (Invitrogen), 10% fetal bovine serum (FBS; Sigma), and penicillin-streptomycin (Invitrogen). All cells were grown at 37°C in 5% CO2. Cell growth curves were done by plating 5 × 104 cells per well in six-well tissue culture plates and counting trypsin blue–negative cells with a hemocytometer every 24 h. Immunolocalization studies were done by growing cells on glass coverslips followed by 4% paraformaldehyde fixation and 0.1% Triton X-100 permeabilization. Cell were then incubated with anti-Myc antibodies (9E10, Santa Cruz Biotechnology) and fluorescently labeled Alexa Fluor 488 secondary goat anti-mouse antibodies (Molecular Probes) and imaged on a Zeiss Axiovert 200M microscope.

Cell migration assays. Cell migration assays were done using Transwell inserts (Costar) of 8.0-µm pore size coated (underside of filter only) with 10 µg/mL fibronectin in PBS for 24 h and blocked with 2.5% bovine serum albumin in PBS. Cells were serum starved overnight (0.5% FBS), trypsinized, and washed once in serum-free medium. Cells were resuspended in serum-free medium and 1 × 105 cells were added to the upper chamber of the Transwell. The lower chamber of the Transwell was filled with complete medium (10% FBS). Cells on the upper surface of the filter were removed after 16 h, and the cells that migrated to the lower surface of the filter were fixed in 10% formalin and stained with 1% crystal violet. The number of cells that migrated was counted under a microscope in four different fields.

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p27 constructs. p27<sup>Kip1</sup>-expressing constructs were made by inserting the human p27 cDNA into pCMV-Myc vector (Clontech). All p27<sup>Kip1</sup> mutant constructs were generated using the QuikChange II Mutagenesis kit (Stratagene). Nuclearexportsequence (NES)-p27 was constructed by fusing two NES of protein kinase inhibitor (residues 35–49) in tandem to the NH<sub>2</sub> terminus. Stable transfectants were generated by pBABE-puro cotransfection and placing the cells under puromycin selection.

Immunoprecipitation, kinase assays, and immunoblotting. For coimmunoprecipitation experiments, B16F0 cells were transfected with the different Myc-tagged p27<sup>Kip1</sup> constructs using LipofectAMINE 2000 (Invitrogen). Twenty-four hours after transfection, cells were lysed in embryo lysis buffer [50 mmol/L HEPES (pH 7.2), 250 mmol/L NaCl, 2 mmol/L EDTA, 0.5% NP40] and Myc-tagged p27 proteins were immunoprecipitated using 2 μg of anti-Myc (9E10) antibody and protein G-Sepharose beads (Zymed). Immunoblots were done using anti-cyclin A (H432), cdk2 (M20), p27 (C19), and Myc (9E10) antibodies (Santa Cruz Biotechnology) and kinase assays using the antibodies anti-cdk2 (M2) and Ccd2 p34 (Santa Cruz Biotechnology) were done as described (16).

Experimental in vivo metastasis assay. S.c. tumors were generated by injection of 2 × 10<sup>6</sup> or 5 × 10<sup>6</sup> B16F0 melanoma cells in 100 μL HBSS into 4- to 8-week-old syngeneic C57BL/6J mice (Charles River). Tumor volume was estimated by V = (a<sup>2</sup> × b) / 2, where a is the short axis and b is the long axis of the tumor. Mice were euthanized on day 13 or day 21, and the tumors were dissected and weighed. For lung metastasis model, 2 × 10<sup>5</sup> murine B16F0 melanoma cells were resuspended in 0.2 mL HBSS and injected i.v. into the lateral tail vein of 3- to 6-month-old syngeneic C57BL/6J mice. Mice were euthanized at day 15 after inoculation, and the lungs were removed and macroscopically inspected for metastasis on the surface. For histologic examination, murine lungs were fixed in Bouin's solution for 24 h, sectioned (University of California at San Diego (UCSD) Histology Core), H&E stained, and examined microscopically. All animal studies were approved by the UCSD Institutional Animal Care and Use Committee.

Human melanoma immunohistochemistry. Case files from the UCSD Department of Pathology were retrospectively reviewed, and we randomly selected 29 cases of melanoma in situ and 24 cases of malignant melanocytic lesions (13 cases of primary invasive tumors and 11 cases of metastatic melanoma). Samples were sectioned, counterstained with hematoxylin, and probed with anti-p27 antibodies per the manufacturer's instruction and developed with 3-amino-9-ethylcarbazole peroxidase substrate. Tumor samples were independently evaluated by two investigators and scored as cytoplasmic p27 positive if >25% of tumor cells were positive. Interobserver variation (<10%) was addressed by averaging individual values. All tumor samples were obtained under an approved UCSD Institutional Review Board protocol with no patient identifiers. Statistical significance was determined by pairwise Fisher exact tests, adjusting the significance for the three comparisons via Bonferroni’s correction.

Results

Human invasive and metastatic melanomas have cytoplasmic p27. Loss of p27 from the nucleus in cancer patient biopsies is correlated with poor clinical outcomes for multiple malignancies (17, 18). Primarily based on the theory that separation of p27 from its nuclear cyclin-cdk targets results in deregulated cell cycle progression and oncogenesis, p27 immunohistologic analyses combine loss of p27 with cytoplasmic p27. However, emerging
evidence suggests a cytoplasmic role of p27 in cell motility (14, 19). Therefore, we examined melanoma patients staged from noninvasive melanoma in situ to metastatic melanoma for the presence of cytoplasmic p27. Melanoma in situ and benign nevi are noninvasive, premalignant precursor lesions of melanoma that have not crossed the basement membrane but remain confined in the melanocyte niche (20). Surprisingly, none of the noninvasive benign nevi or melanoma in situ patients (0 of 29) had cytoplasmic p27 staining (Fig. 1). In contrast, we found that 69% (9 of 13; P < 10^{-5}) of invasive malignant melanomas and 73% (8 of 11; P < 10^{-5}) of metastatic melanomas were strongly positive for cytoplasmic p27 (Fig. 1). In addition, we did not detect any invasive primary or metastatic melanoma patients with exclusively nuclear p27. Consistent with the human cytoplasmic p27 localization correlation with the metastatic phenotype, p27 was predominately present in the nucleus of low metastatic murine B16F0 melanoma cells, whereas we detected both cytoplasmic and nuclear p27 in high metastatic B16F10 melanoma cells (Fig. 1C). Taken together, these results show a selection for p27 relocalization to the cytoplasm at the transition from noninvasive, premalignant in situ lesions to invasive malignant and metastatic melanoma.

**Cytoplasmic p27 induces cell migration.** To evaluate a cytoplasmic role for p27 in metastasis, we generated a p27 construct that was constitutively localized in the cytoplasm of cells. We placed a NES and Myc epitope tag at the NH2 terminus of a previously characterized p27CK- mutation that fails to bind cyclin/cdk complexes or induce a cell cycle arrest (Fig. 2A; ref. 21). Consistent with endogenous p27, both p27WT and p27CK- proteins were detected in the nucleus of B16F0 melanoma cells (Fig. 2B). In contrast, both NESp27WT and NESp27CK- proteins were predominantly present in the cytoplasm of cells. Both NESp27CK- and p27CK- failed to form complexes with cyclin Acdk2 complexes, whereas control p27WT and NES-p27WT bound to cyclin Acdk2 complexes when ectopically expressed in cells (Fig. 2C). We have previously shown that ectopic introduction of p27 into p27-deficient MEFs results in rescue of the cell motility defect and also cytoskeletal remodeling (14). To test for induction of cell migration, we expressed the various p27 constructs in murine B16F0 melanoma cells and assayed for migration in a chemotactic Transwell assay. Expression of either NESp27WT or NESp27CK- resulted in a significant increase in cell migration (Fig. 2D). In contrast, expression of nuclear p27WT or p27CK- did not result in an increase in cell migration above control. These observations support a role for cytoplasmic p27 in cell motility that is independent of cell cycle (cyclin/cdk binding) regulation.

We generated stable cell lines expressing the NESp27CK- construct in low metastatic B16F0 melanoma cells. To ensure physiologic relevance, stable NESp27CK- clones were isolated based on subphysiologic expression relative to endogenous p27. Three representative clones, L9 (low, ~5% of endogenous p27), M12 (medium, ~15% of endogenous p27), and H8 (high, ~60% of endogenous p27), were selected for further analysis (Fig. 3A). All three clones expressed NESp27CK- below the level of endogenous

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**Figure 2.** Expression of cytoplasmic NESp27CK- results in increased migration. A, schematic representation of p27 constructs used in this study. Cyclin/cdk asterisk, location of two amino acid changes that prevent cyclin/cdk binding. Note all constructs contain a NH2-terminal Myc epitope tag. B, B16F0 melanoma cells transfected with p27 constructs in A were analyzed for p27 localization with anti-Myc antibodies by immunohistochemical staining. C, p27CK- fails to bind cyclin Acdk2 complexes. Top, B16F0 cells transfected with p27 constructs were immunoprecipitated (IP) with anti-Myc antibodies and immunoblotted with anti-cyclin A, anti-cdk2, and anti-Myc p27 antibodies; bottom, corresponding whole-cell lysates (WCL) were immunoblotted with anti-cyclin A antibodies. D, in vitro cell migration analysis of B16F0 cells in Transwell chambers transiently transfected with p27 constructs from A. Migrated cells were quantified by counting four independent microscopic fields at 16 h by counting crystal violet-stained cells on opposite side of filter with a microscope. Cell number represents the mean number of cells per field (P < 0.001).
p27 and the protein was present predominately in the cytoplasm (Fig. 3D). All three clones showed similar proliferation capacity and cell cycle profiles (Fig. 3C). Consistent with the observations above and the inability of the CK expression to bind to and inactivate cyclin:cdk complexes, we did not detect any measurable differences in cdk2 or cdk1 kinase activity between the NESp27CK-expressing clones and the parental cells (data not shown). We next assayed for altered in vitro cell migration properties of NESp27CK-melanoma clones in Transwell migration assays (Fig. 3D). Cells were seeded onto fibronectin-coated Transwell filters and assayed for migration. Surprisingly, although the cytoplasmic NESp27CK expression levels represent only a fraction of the endogenous nuclear p27 level, all of the NESp27CK-expressing clones showed a significantly enhanced ability to migrate on fibronectin-coated filters in a concentration-dependent manner (Fig. 3D). Importantly, cells expressing a cyclin:cdk binding NES-p27WT also dramatically increased cell migration (data not shown). Although all three NESp27CK clones have similar growth characteristics to the parental cells, expression of even a small fraction of cytoplasmic p27 resulted in marked increases of cell migration.

Cytoplasmic p27 induces melanoma metastasis. To test for any differences in tumorigenic potential, we s.c. inoculated 2 × 10^6 cells of the three NESp27CK clones and control B16F0 cells into syngeneic C57BL/6 mice and assayed for tumor growth on day 13. All three NESp27CK clones generated s.c. tumors of size and latency that was comparable with the parental B16F0 melanoma cells (Fig. 4A). These observations suggest that cytoplasmic p27 does not alter the tumorigenic potential of melanoma cells. Unlike many tumors inoculated s.c. that remain confined in the s.c. space, metastatic melanoma can invade across the abdominal wall and metastasize throughout the peritoneal cavity (22). To test for increased invasion and metastasis, we s.c. inoculated a reduced number (5 × 10^5) of NESp27CK-H8 and parental B16F0 cells and then analyzed the peritoneal cavity for metastasis on day 21. Mice inoculated with NESp27CK-H8 cells resulted in a high number of mice (9 of 12) that had invaded across the abdominal muscle wall and metastasized into the peritoneum forming macrocolonies on the mesenteric membrane (8 of 12; Fig. 4B and C). In contrast, parental B16F0 cells showed a low number of invasive tumors (3 of 12) and failed to metastasize into the peritoneum (0 of 12). These observations show that cytoplasmic p27 confers a more aggressive invasive and metastatic melanoma phenotype in a cell cycle-independent manner.

We next tested the metastatic potential in a separate experimental metastasis model. Syngeneic C57BL/6 mice were inoculated with 2 × 10^5 cells from NESp27CK clones and parental B16F0 lines into the lateral tail vein and assayed for lung and lymph node metastasis. As expected, parental B16F0 melanoma cells showed less than three lung metastases per mouse (Fig. 5A). In contrast, i.v. inoculation of each NESp27CK melanoma clone resulted in significant (P < 0.05) increases in both the number and size of lung metastases (Fig. 5). The number of metastases strongly correlated with the amount of cytoplasmic NESp27CK expressed in each clone (r = 0.88) with >20 lung metastases per mouse in clone H8 that expressed ~60% of endogenous cytoplasmic p27. Furthermore, we detected NESp27CK metastases in axillary, brachial, renal, and mesenteric lymph nodes, whereas animals inoculated with parental B16F0 melanoma cells showed no lymph node

Figure 3. Characterization of stable cytoplasmic NESp27CK cells. A, immunoblot analysis of NESp27CK-expressing stable clones with anti-Myc (top) and anti-p27 (bottom) antibodies. NESp27CK levels relative to endogenous p27 levels are quantified below each lane. B, clone expressing ectopic cytoplasmic p27 (green) retains nuclear localization of endogenous p27 (red). B16F0 melanoma and clone H8 were analyzed for exogenous p27 localization with anti-Myc antibodies and for endogenous p27 with anti-p27 antibody. C, cell cycle DNA profiles of parental B16F0 melanoma cells and NESp27CK-expressing clones (asynchronous culture). Cells were analyzed for DNA content by propidium iodide and flow cytometry analysis. D, in vitro cell migration analysis in Transwell chambers of NESp27CK-expressing clones. Migrated cells were quantified by counting four independent microscopic fields at 16 h by counting crystal violet–stained cells on opposite side of filter with a microscope. Cell number represents the mean number of cells per field (P < 0.0001).
metastases (Fig. 5C). Taken together, these observations show that subphysiologic amounts of cytoplasmic p27 are sufficient to induce significant and marked increases in the metastatic potential of melanoma cells in vivo independent of cyclin-cdk regulation.

Discussion

In the world of tumor suppressor genes, p27<sup>Kip1</sup> remains an enigma. Unlike classic tumor suppressor genes that acquire genetic mutations during oncogenesis resulting in functional inactivation, p27 is rarely, if ever, deleted or inactivated in cancer (5–7). In contrast, p27 is often relocalized to the cytoplasm, suggesting that cytoplasmic p27 may confer a selective advantage to tumor cells. Indeed, cytoplasmic p27 serves a prognostic indicator of poor outcomes in multiple cancer types, including breast and ovarian cancer, and melanomas (11, 18, 23). Relocalization of p27 out of the nucleus was interpreted as a mechanism to alleviate its tumor-suppressive properties by separating it from its nuclear cyclin-cdk targets. Here, we present in vivo data showing that even low...
cytoplasmic level of p27 promotes dramatic increases in the metastatic potential of melanomas. Moreover, we found that ~70% of invasive and metastatic melanomas from patients were positive for cytoplasmic p27 compared with noninvasive forms of melanoma, suggesting that patients with a cytoplasmic p27 biomarker may be at an increased risk for tumor invasiveness and metastasis. These observations show that p27 does at least two functions dependent on its cellular location: a nuclear cyclin/cdk binding dependent function and a cytoplasmic motility function that is independent of cyclin/cdk binding. Consistent with a cytoplasmic gain-of-function role for p27, we have previously identified a COOH-terminal domain outside of the cyclin/cdk binding domain that promotes cell migration in vitro and actin cytoskeletal rearrangements (13, 14). In addition, Besson et al. reported that cytoplasmic p27 down-regulates Rho activity leading to an increase in cellular motility. Moreover, p27 has been linked to neuronal cell migration (15).

The cytoplasmic relocalization and involvement of p27 in actin cytoskeletal rearrangement are evolutionarily reminiscent of α factor pheromone signaling in yeast. Far1p, the yeast p27 cyclin/cdk inhibitor, mediates both a G1 cell cycle arrest when present in the nucleus and induction of schmoo formation when present in the cytoplasm that orients the actin cytoskeleton toward the opposite mating partner (24). In response to α factor, Far1p translocates from the nucleus to the cytoplasm and interacts with Cdc24p (25), a guanine nucleotide exchange factor for Cdc42p. Consistent with this functional analogy, mutation of a Far1p-like sequence motif present in the p27 COOH-terminal domain inactivates both cell migration and actin rearrangement but preserves the cell cycle arrest functions of nuclear p27 (14). However, it remains unclear if aggressive tumors are selecting for cytoplasmic p27 localization to harness a preexisting p27-regulated motility pathway or are using cytoplasmic p27 to pirate an unrelated pathway. Thus, relocalization of p27 to the cytoplasm in tumor cells results both in a nuclear loss of p27-mediated cyclin/cdk inhibition and in a cytoplasmic motility induction resulting in increased metastatic potential.

In summary, our observations offer a molecular basis for the selective advantage that aggressive tumors, such as melanoma, relocalize p27 to the cytoplasm rather than selecting for its genetic inactivation. Taken together, our observations reveal a previously unforeseen molecular paradox in cancer where a nuclear tumor suppressor protein functions as a cytoplasmic metastatic oncogene.

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