American Journal of Pathology, Vol. 166, No. 6, June 2005

Tumorigenesis and Neoplastic Progression

Microtubule-Associated Protein 2, a Marker of Neuronal Differentiation, Induces Mitotic Defects, Inhibits Growth of Melanoma Cells, and Predicts Metastatic Potential of Cutaneous Melanoma

Mohammad H. Soltani,* Rita Pichardo,† Ziqui Song,‡ Namrata Sangha,‡ Fabian Camacho,§ Kapaettu Satyamoorthy,¶ Omar P. Sangueza,† and Vijayasaradhi Setaluri*‡

From the Departments of Cancer Biology, Pathology,† Dermatology,‡ and Public Health Sciences,§ Wake Forest University School of Medicine, Winston-Salem, North Carolina; and the Wistar Institute,¶ Philadelphia, Pennsylvania*

Dynamic instability of microtubules is critical for mitotic spindle assembly and disassembly during cell division, especially in rapidly dividing tumor cells. Microtubule-associated proteins (MAPs) are a family of proteins that influence this property. We showed previously that MAP2, a neuron-specific protein that stabilizes microtubules in the dendrites of postmitotic neurons, is induced in primary cutaneous melanoma but is absent in metastatic melanomas. We proposed that induction of a microtubule-stabilizing protein in primary melanoma could disrupt the dynamic instability of microtubules, inhibit cell division and prevent or delay tumor progression. Here we show, by Kaplan-Meier survival and multivariate Cox regression analysis, that patients diagnosed with MAP2$^+$ primary melanomas have significantly better metastatic disease-free survival than those with MAP2$^-$ disease. Investigation of the mechanisms that underlie the effect of MAP2 on melanoma progression showed that MAP2 expression in metastatic melanoma cell lines leads to microtubule stabilization, cell cycle arrest in G2-M phase and growth inhibition. Disruption of microtubule dynamics by MAP2 resulted in multipolar mitotic spindles, defects in cytokinesis and accumulation of cells with large nuclei, similar to those seen *in vivo* in MAP2$^+$ primary melanomas cells. These data suggest that ectopic activation of a neuronal differentiation gene in melanoma during early tumor progression inhibits cell division and correlates with inhibition or delay of metastasis. ***(Am J Pathol 2005, 166:1841–1850)***

Although a number of clinical and pathological factors that influence melanoma progression have been identified, to date there is no single histological, immunohistochemical, serological, or molecular marker that accurately predicts aggressive behavior of melanoma.[1] Tumor thickness, which is considered the best predictor of melanoma aggressiveness, is not always a reliable parameter and is not relevant for more advanced primary tumors and metastatic disease.[1,2] Therefore, there is a need for identification of molecular markers that predict biological behavior of melanoma cells independent of tumor thickness.

Melanoma exhibits plasticity of differentiation and is known to differentiate along multiple, including endothelial and neuronal, cellular pathways.[3] However, the effects of transdifferentiation of melanoma cells on tumor progression are not well understood. Earlier, we showed that MAP2 (microtubule-associated protein 2), a neuron-specific protein, is expressed abundantly in early invasive primary melanoma lesions (by immunohistochemistry) and primary melanoma cell lines (by Northern and western blot analyses) but is absent in metastatic melanomas lesions and cell lines.[4] In addition to primary melanomas[5] expression of MAP2 has been reported in other cutaneous tumors with neuroendocrine differentia-

Supported in part by the Comprehensive Cancer Center of Wake Forest University (PUSH grant to V.S.) and the National Institutes of Health (cancer biology training grant T32 CA79448).

Accepted for publication March 3, 2005.

Address reprint requests to Vijayasaradhi Setaluri, University of Wisconsin Medical School, Department of Dermatology, 1300 University Ave., B-25, Madison, WI 53706. E-mail: vsetaluri@dermatology.wisc.edu.

tion such as Merkel cell carcinomas but not in basal or squamous cell carcinomas.[6]

MAPs are a family of proteins that bind to and stabilize microtubules (MTs). Whereas MAP4 is expressed ubiquitously, isoforms of MAP1, MAP2 and tau are expressed primarily in neurons.[7] MAP2 exists in 3 alternatively spliced isoforms that are developmentally regulated. These are the juvenile MAP2c (70 kd) and mature MAP2a and b (280 kd). Whereas the juvenile MAP2c form is found in only in immature neurons, the mature MAP2ab forms are found throughout the life of the neurons. In postmitotic terminally differentiated neurons MAP2 is localized to the dendrites.[8] Expression of MAP2 *in vitro* in heterologous cells induces rapid formation of stable MT bundles and cellular processes.[9,10] Effects of ectopic MAP2 expression *in vivo* have not been investigated. Recently, other novel embryonic isoforms of MAP2 have also been described.[11,12]

MTs play a critical role during mitosis. Segregation of sister chromatids depends on spatially and temporally coordinated polymerization/depolymerization of MTs, and activities of MT motors and non-motor MT associated proteins (MAPs).[13] Disruption of dynamic instability of MTs can lead to mitotic block, cell cycle arrest and cell death. Agents (such as vinca alkaloids and taxols) that target MTs are, therefore, ideal for treatment of cancer.[14,15] Since the only known function of MAP2 is to stabilize MTs, we hypothesized that ectopic induction of MAP2 in early primary melanoma cells disrupts MT dynamics, and delays/arrests mitosis resulting in restricted tumor growth, and that absence or loss of MAP2 correlates with melanoma tumor progression. Thus, MAP2 expression can be an indicator of metastatic potential of melanoma. In this paper, we show that adenoviral mediated expression of the mature form of MAP2 leads to mitotic spindle defects, cell cycle arrest and apoptosis of metastatic melanoma cells *in vitro*, and that MAP2 expression *in vivo* in primary tumors correlates with metastatic disease-free survival of patients diagnosed with cutaneous melanoma.

Materials and Methods

Materials

All tissue culture reagents were purchased from GIBCO BRL, Grand Island, NY. Mouse pSVMAP2a was a generous gift of Dr. N. Cowan, New York University, NY[16] and pRC/CMV MAP2C plasmid[17] was provided by Dr. B. Shafit-Zagado, Albert Einstein College of Medicine, Bronx, NY. MAP4 adenovirus and MAP4 antibody were generous gifts from Dr. George IV Cooper, Medical University of South Carolina, Charleston, SC. Anti-MAP2 mAb (HM-2), anti-α-tubulin clone DM1A mAb, anti-α-tubulin mAb-FITC conjugate, and propidium iodide (PI) were purchased from Sigma, St. Louis, MO. AIM-1 (Aurora and Ipl1-like midbody associated protein) mIgG1, Mad2 (mitotic arrest-deficient) mIgG2a, and anti-cytochrome *c* mAB were purchased from BD Biosciences, San Jose, CA. Caspase 8 mAb (1C12) and poly (ADP-ribose) polymerase (PARP) antibody were purchased from Cell Signaling Technology, Beverly, MA. Paclitaxel (unlabeled, 98% purity) was purchased from Sigma, St. Louis, MO. Fluorescent paclitaxel conjugate (BODIPY FL) was purchased from Molecular Probes, Eugene, OR.

MAP2a Adenovirus

A 5.8 kb coding region of the MAP2a cDNA was isolated by digesting pSVMAP2 plasmid with Ase I followed by Klenow fill-in reaction and digestion with *Sal*I. Gel purified insert fragment was cloned into *Sal*I-EcoR V digested pShuttle-CMV (BD Biosciences, San Jose, CA). Construction, preparation and titering of the adenovirus were done as described.[18]

Cell Culture

Metastatic human melanoma cell lines, 451Lu and SK-MEL-23, were grown and passaged as described earlier.[4] MAP2 stable transfectants were selected with 1 mg/ml G418, and clones were isolated and maintained in G418-containing medium.

Flow Cytometry

451Lu cells were plated (5×10^4/well) in six-well dishes (Fisher Scientific, Pittsburgh, PA), and incubated for 24 hours before infection with Ad-GFP and Ad-MAP2 (MOI: 200–300). At 24, 48, and 72 hours postinfection, the cells were collected, washed, fixed and stained with of propidium iodide (PI) and analyzed using the FACS Star Plus flow cytometry (Becton Dickinson, Mansfield, MA). Cell cycle kinetics and DNA content were determined using the Modfit Analysis (Verity Software, Inc).[19]

Cytosolic and Mitochondrial Extract Preparation

Melanoma cells were infected with Ad-GFP and Ad-MAP2 at an MOI 200–300, and after a 48 hours of incubation, cytosolic and mitochondrial fractions were isolated,[20] and analyzed by western blot using anti-cytochrome *c* mAb.

Caspase 8 and PARP Cleavage Analysis

451Lu cells grown to 50 to 60% confluence in 100 mm culture dishes were infected with Ad-GFP and Ad-MAP2 (MOI 200). After 48 hours of incubation, the cells were scraped in the presence of protease inhibitors and lysed and in detergent lysis buffer. The supernatants were collected and protein concentration was determined using BCA protein assay kit, analyzed by SDS-PAGE and western blot using anti-caspase 8 and anti-PARP antibodies.

Monomeric (Soluble) and Polymeric (Insoluble) Tubulin

The soluble (S) and insoluble (P) fractions of cellular tubulin were prepared and analyzed as described.[21] Pro-

tein concentration was determined and the α-tubulin in soluble and insoluble fractions was detected by western blotting. Tubulin P/S ratio was estimated using densitometric measurements of the band intensities (Scion Corp., Frederick, MA).

Western Blot Analysis

Cells were lysed in lysis buffer, and protein concentration was estimated using BCA protein Assay kit (Pierce, Rockfod, IL). Cell extracts were resolved on 9 to 12% sodium dodecyl sulfate-polyacrylamide gels, and blotted onto PVDF membranes (NEN Life Science Inc., Boston, MA). The blots were blocked in 5% nonfat dry milk or BSA for 1 hour and incubated overnight at 4°C with primary antibodies at dilutions recommended by the supplier, followed by appropriate horseradish peroxidase-conjugated IgG at 1:5000 dilution (Amersham Biosciences, Piscataway, NJ). Bound antibodies were detected using an ECL chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Immunostaining and Microscopy

Melanoma cells grown on glass coverslips were infected with adenoviruses. After 48 hours of incubation at 37°C, the cells were fixed with cold methanol for 5 minutes and nonspecific sites were blocked with 2% BSA in PBS at 37°C for 15 minutes. Cells were incubated with mouse mAb against α-tubulin (1:500) followed by rabbit anti-mouse IgG–FITC (1:100), and then with mouse monoclonal anti-MAP2 mAb and by Alexa 647-conjugated goat anti-mouse IgG (1:500) (Molecular Probes, Eugene, OR), and finally with PI. Coverslips were mounted with mounting medium containing anti-bleaching agent and examined with confocal laser-scanning microscope (Carl Zeiss Laser Scanning System LSM).

Immunohistochemistry (IHC)

Paraffin-embedded tumor specimens from patients diagnosed with cutaneous primary melanoma at the Dermatology clinics of Wake Forest University School of Medicine were obtained from the archives of the Dermatopathology section and processed for MAP2 IHC as described.[4] MAP2 staining pattern and intensity were evaluated independently by two pathologists and scored as negative (0), weak heterogeneously positive (1+) and positive (2+ to 3+).

Melanoma Patients and Statistical Analysis

We evaluated all patients diagnosed with cutaneous primary melanoma at the Wake Forest University Baptist Medical Center between 1981 and 1999. Criteria for inclusion for analysis were: a) availability of the original paraffin blocks of the primary tumor specimen and b) clinical follow up for at least 5 years with or without diagnosis of metastatic melanoma or death due to metastatic disease. The frequency of follow up visits for recurrence ranged from once every 3–6 months to once a year depending on the thickness of the primary lesion. Patients diagnosed with melanoma *in situ*, those without a 5-year follow up and those patients who died of causes other than melanoma were not included in this study. All human subject research protocols were approved by the Institutional Review Board of WFUSM. We conducted multivariate Cox proportional hazards analysis using STATA data analysis program (STATA Corp. LP, College Station, TX). Variables included age, sex, Breslow thickness and Clark level. Kaplan-Meier survival curves and adjusted survival curves describing the survival function for MAP2 were plotted using GraphPad Prism software v. 3.00 for Windows (San Diego, CA).

Results

MAP2 Is a Prognostic Indicator for Melanoma Tumor Progression

Based on immunohistochemical analysis that showed variable expression of MAP2 in primary melanomas and its absence in metastatic lesions, earlier we proposed that MAP2 expression in primary tumor may have implications for progression of melanoma.[4] Here, we investigated the relationship between MAP2 expression and metastatic disease free survival (DFS) of patients diagnosed with primary melanoma. We evaluated MAP2 expression in primary melanomas from 37 patients (Supplementary data Table 1) by immunohistochemistry using a mouse mAb M13 (Zymed) that recognizes only the mature MAP2ab (Figure 1A). Based on staining pattern found in at least 3 sections per tumor, we scored tumors as negative (0), weakly positive (1+), positive (2+) and strongly positive ($\geq$3+) for MAP2 expression. Tumors that were negative or only weakly positive for MAP2 expression were uniformly negative or homogeneously weakly positive, respectively. MAP2-positive tumors (2+ and $\geq$3+), on the other hand, often displayed heterogeneity in staining intensity. Semiquantitative analyses showed that >70% cells in these tumors showed staining intensity of 2+ or 3+ (data not shown). Kaplan-Meier survival analysis and log-rank test showed that patients with $MAP2^+$ primary tumors had significantly better ($P = 0.01$) DFS than those patients with $MAP2^-$ primary melanomas (Figure 1B). Multivariate Cox proportional hazard analysis showed that MAP2 is a significant predictor ($P = 0.003$) of time to metastatic recurrence with a hazard ratio of 0.18. Median DFS, adjusted to age, sex, Breslow thickness and Clark level, was 32.5 months for $MAP2^-$ group compared to >60 months for $MAP2^+$ group (Figure 1C; and Supplementary data, Table 2). Also, there was a statistically significant correlation (two-sided Fisher's exact test, $P = 0.02$) between MAP2 expression and met-

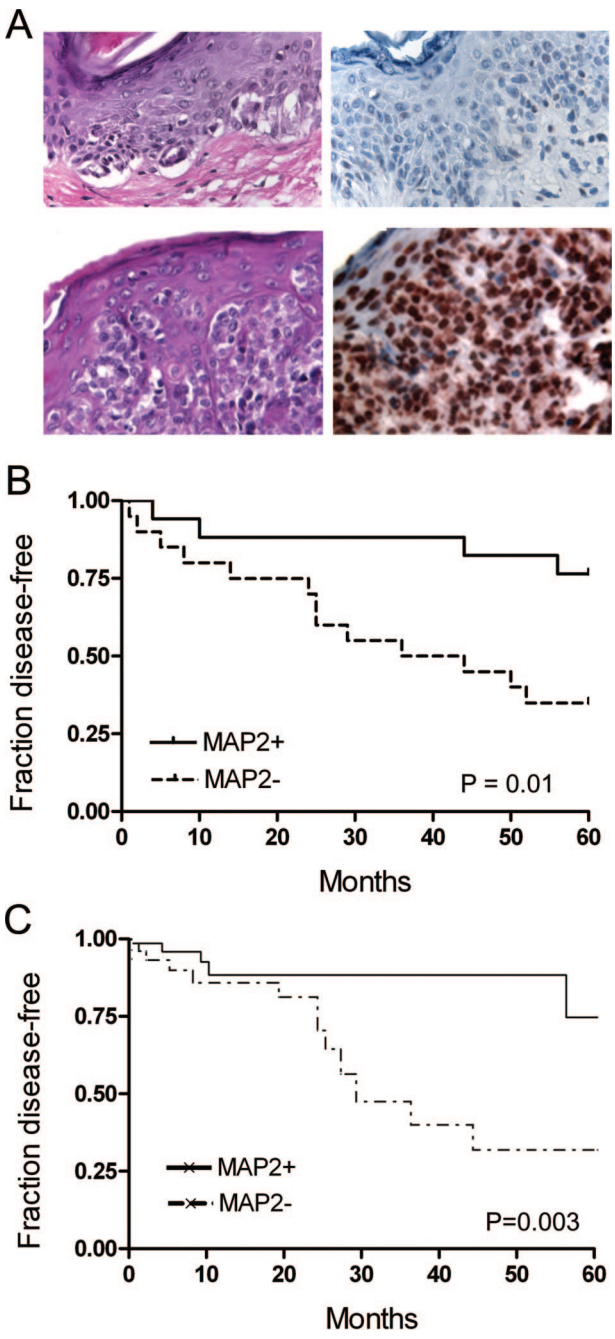

Figure 1. MAP2 as melanoma prognostic indicator. **A:** MAP2 expression in paraffin-embedded primary melanoma specimens was evaluated by IHC using anti-MAP2 mAb HM-2. MAP2 expression in two representative specimens is shown. **Top**: $MAP2^-$ primary melanoma (right back of 53-year-old male; Breslow thickness, 0.55 mm). **Bottom**: $MAP2^+$ melanoma (right cheek of 77-year-old male; Breslow thickness, 1.5 mm). **Left**: H&E staining; **right**: MAP2 staining. **B:** Melanoma specimens with variable Breslow thickness (0.2 to 11 mm) from 37 individual patients (see Supplementary Information, Table 1) with clinical follow-up of at least 5 years for recurrence were studied.[7] Kaplan-Meier analysis of 5-year DFS in patients diagnosed with $MAP2^-$ ($n = 20$) and $MAP2^+$ ($n = 17$) primary melanoma. Data on censored patients (ie, with follow-up beyond 5 years) are not shown. **C:** Estimated survivor functions of patients diagnosed with $MAP2^+$ and $MAP2^-$ primary cutaneous melanoma adjusted to age, sex, Breslow thickness, and Clark level.

astatic recurrence within five years. These data suggest that MAP2 is a predictor of aggressiveness of cutaneous primary melanomas.

MAP2 Inhibits Growth of Melanoma Cells in Vitro

To understand the role MAP2 plays in melanoma progression, we first tested the effect of expression of MAP2 on the growth of two representative MAP2-negative metastatic melanoma cell lines SK-MEL-23 and 451Lu. Infection of cells growing in monolayer culture with the recombinant adenovirus Ad-MAP2a resulted in abundant expression of MAP2 protein (as seen by immunofluorescence staining and western blot analyses with anti-MAP2 monoclonal antibody) similar to the expression observed, by immunohistochemistry, in many primary melanomas lesions.[4] Cells transduced with Ad-MAP2a grew significantly slowly, while cells infected with a control virus Ad-GFP (green fluorescent protein) grew at a rate similar to that of uninfected cells (Figure 2A). In agreement with the role of MAP2 in formation of dendrites,[9] Ad-MAP2a-transduced cells exhibited distinct morphology with long branched, dendritic extensions (Figure 2C).

Attempts to establish stable 451Lu melanoma cell lines expressing MAP2 isoforms yielded, in 3 independent transfections, relatively fewer MAP2a-expressing clones compared to MAP2c, the juvenile form of MAP2. Stable MAP2a clones exhibited variable growth inhibition (20 to 80%) compared to untransfected cells. On continuous culture beyond 15 passages, MAP2a stable clones progressively lost MAP2 expression and showed normal growth (data not shown). Since primary melanoma cells and some metastatic melanoma cell lines in culture also express the alternatively spliced immature isoform MAP2c, we tested the effect of expression of MAP2c on MAP2-negative 451Lu cells. In contrast to MAP2a, transfection with MAP2c cDNA yielded several stable clones that expressed abundant 70 kd MAP2c protein. However, the growth rate of MAP2c-expressing clones was similar to that of control 451Lu cells (Supplementary data, Figure 1; A to C), suggesting that MAP2a, the mature and predominant from in postmitotic neurons, but not MAP2c, the immature form found in immature neurons inhibits melanoma cell growth. Interestingly, overexpression of MAP4, which is ubiquitously expressed but not up-regulated in melanoma, also resulted in growth inhibition at higher MOI (Supplementary data, Figure 1D).

MAP2 Induces Cell-Cycle Arrest in Melanoma Cells

Flow cytometric analyses of control uninfected cells, Ad-GFP or Ad-MAP2-transduced cells showed that Ad-MAP2a cells accumulated in G2-M phase of cell cycle accounting for >50% of total cell population by 48 hours postinfection, compared to ~15% in control and Ad-GFP infected cells. MAP2a expression also resulted in accumulation, up to 15% by 72 hours, of cells with >4N DNA (Figure 2B; and Supplementary data, Figure 1E). In Ad-MAP2-transduced cells, progressive accumulation, up to 25% by 72 hours, of apoptotic subG1 population could also be seen. These data show that MAP2a expression

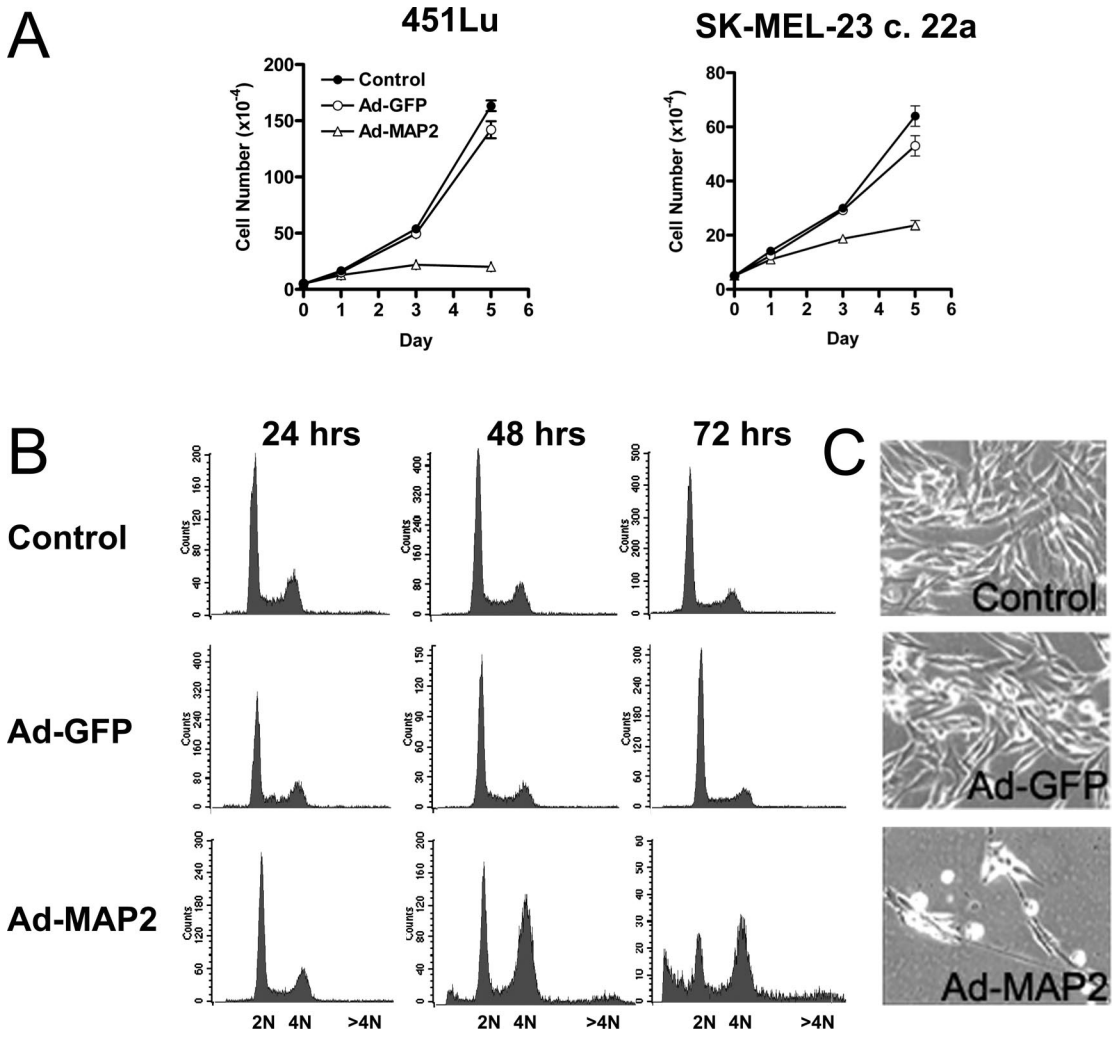

Figure 2. The effect of MAP2 expression on proliferation (**A**), cell cycle distribution (**B**), and morphology (**C**) of human metastatic melanoma cells. **A:** 451Lu (5×10^4/well in triplicate in six-well plates) and SK-MEL-23.cl 22a (1×10^4/well in triplicate in 24-well plates) cells were plated and grown under routine growth conditions (uninfected) or after transduction with control virus Ad-GFP or MAP2 virus Ad-MAP2a (200 to 300 MOI). Cells were counted by trypan blue exclusion method. Representative data of three independent experiments are shown. Numbers shown are mean ± SD. **B:** 451Lu cells in parallel wells as in **A** were trypsinized 24, 48, and 72 hours after transduction, fixed in ethanol, stained with PI, and analyzed by flow cytometry for DNA content. Note the predominant peak representing cells with 4 N DNA at 48 hours and appearance of population of sub-G_1 DNA and >4 N DNA at 72 hours in Ad-MAP2-transduced cells. **C:** Rounded-up cells and fewer attached cells remained in Ad-MAP2-transduced dishes 72 hours after transduction compared to uninfected or Ad-GFP-infected wells. Long dendritic processes in Ad-MAP2 cells can be seen.

arrests metastatic melanoma cells in G2-M phase of the cell cycle, and suggest that a proportion of G2-M arrested cells complete mitosis without cytokinesis to produce cells with >4N DNA, which may ultimately undergo apoptosis.

Intracellular Distribution of MAP2 in Melanoma Cells

In neurons, MAP2 is primarily localized in the dendrites. This localization is achieved by targeting MAP2 mRNA into the dendrites by a signal in the 3′ untranslated region of MAP2 mRNA and its translation in the dendrite. To test whether MAP2 is also restricted to the dendrites in melanoma cells, we examined intracellular distribution of MAP2. Immunofluorescence staining of Ad-MAP2 infected 451Lu melanoma cells showed co-localization of MAP2 with dense arrays of cytoplasmic MTs (stained by tubulin) adjacent nuclei (Figure 3a) and in the cytoplasm in postmitotic cells (Figure 3d). Biochemically, altered stability of MTs can be measured as ratio of polymerized (P)/soluble (S) tubulin by western blotting followed by densitometry of tubulin bands in P and S fractions (data not shown). The P/S ratio of tubulin in Ad-MAP2 was 1.40 ± 0.21 compared to 0.93 ± 0.12 ($P = 0.005$) in uninfected and Ad-GFP infected 451Lu cells showing that MAP2a expression significantly increased the amount of tubulin in the polymerized fraction. However, MAP2 expression did not alter the total amount of cellular tubulin (data not shown).

Nuclear Abnormalities in MAP2-Expressing Melanoma Cells

MAP2-expressing cells were readily distinguishable by the presence of large nuclei, both *in vivo* in primary mel-

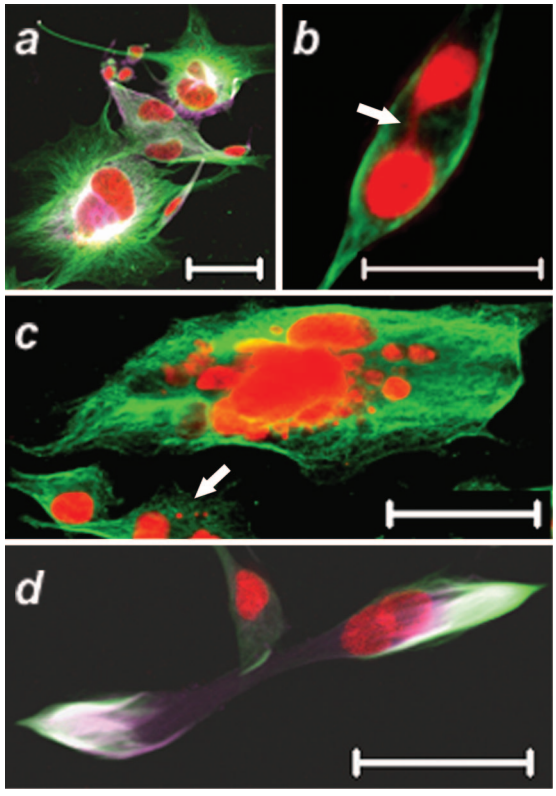

Figure 3. Localization of MAP2 and nuclear abnormalities in Ad-MAP2 451Lu cells. Cells on glass coverslips were fixed and stained with (in **a** and **d**) anti-MAP2 mAb HM-2, goat anti-mouse IgG-Alexa 647, followed by α-tubulin mAb DM1A-FITC and finally with PI (in **b** and **c**) or with anti-α-tubulin mAb followed by PI and examined with Zeiss 310 LSM310 confocal microscope. **Arrows** in **b** and **c** show chromosomal bridges and lagging chromosomes, respectively. Scale bars: 50 μm (**a**, **c**, **d**); 20 μm (**b**).

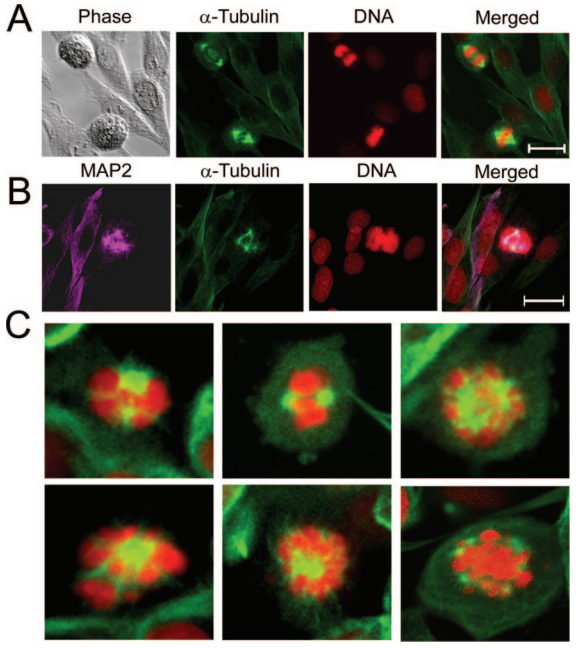

Figure 4. Mitotic spindle defects in Ad-MAP2 451Lu cells. Control 451Lu melanoma cells and Ad-MAP2 451Lu cells on glass coverslips were ethanol-fixed and stained with FITC-conjugated anti-α-tubulin mAb DM1A followed by PI (**A** and **C**), or anti-MAP2 mAb HM-2, goat anti-mouse IgG-Alexa 647 followed by anti-α-tubulin DM1A-FITC, and finally with PI (**B**), and examined with confocal microscope. Cells undergoing mitosis with normal appearing metaphase and anaphase in control (**A**), a cell with multipolar mitotic spindle in MAP2-expressing cells (**B**), and several abnormal mitotic spindles intermingled with chromosomes (**C**) in Ad-MAP2 451 cells can be seen. Scale bar, 20 μm.

anomas [mean diameter of 10.92 ± 2.9 μm (n = 100) for MAP2+ melanoma cells compared to 7.39 ± 1.51 μm (n = 100) for MAP2- cells; two-tailed t-test P = <0.0001] and *in vitro* in AdMAP2-transduced melanoma cells [mean nuclear diameter of 14.01 ± 3.54 μm (n = 100) in Ad-MAP2 cells compared to 11.54 ± 2.37 μm (n = 100) in Ad-GFP cells, P = <0.0001; Supplementary data, Figure 2, A to C]. Ad-MAP2a cells exhibited a variety of nuclear abnormalities including multilobed (Figure 3a) or fragmented nuclei (Figure 3c), chromosomal bridges (Figure 3b, arrow), lagging chromosomes (Figure 3c, arrow) and abnormal cytokinesis (Figure 3d). The nuclear abnormalities are consistent with the flow cytometric data that showed accumulation of cells with >4N DNA.

MAP2 Induces Mitotic Spindle Defects

A close examination Ad-MAP2-transduced 451Lu melanoma cells showed that compared to uninfected or Ad-GFP infected cells, MAP2 infected cells had fewer mitoses (average of 6 mitoses per 1000 cells in Ad-MAP2 cells compared to 20 mitoses per 1000 cells in controls). Analysis of number of mitoses in primary melanoma sections showed that compared to $MAP2^-$ melanomas $MAP2^+$ primary melanomas had fewer fields with ≥1 mitoses/field (two-sided Fisher's exact test, P = 0.03). Further, the mitoses in control uninfected cells exhibited typical bipolar spindle morphology (Figure 4A), whereas in Ad-MAP2a cells the majority of mitoses appeared abnormal, ie, misaligned, unipolar or multipolar spindles (Figure 4, B and C). Presence of aberrant mitoses and bi- and multinucleated cells suggests that, in MAP2a-expressing cells, spindle checkpoint fails to sustain mitotic arrest and cells exit from mitosis without cytokinesis. To understand the status of spindle checkpoint in MAP2a cells, we investigated cellular localization of checkpoint proteins Mad2 and Aurora kinase B (AIM-1) during mitosis (Figure 5). Mad2 protein is associated with the kinetochores of chromosomes at prometaphase and its localization correlates with tension. Mad2 is important for the M phase arrest in mammalian cells in response to microtubule inhibitors.[22] In Ad-MAP2a 451Lu cells, Mad2 showed patchy staining near poorly formed spindle poles, and was localized along randomly arrayed spindle MTs (Figure 5A).

AIM-1 is involved in chromosomal segregation and cytokinesis. It follows a typical "passenger protein" pattern of distribution during mitosis, ie, it is localized to centromere region of metaphase chromosomes, and transferred to central spindle MTs during anaphase and finally to the midbody.[23] AIM-1 showed similar distribution on metaphase chromosomes in both control and MAP2a melanoma cells (Figure 5B, top and bottom), although assembly of MTs into spindle is delayed or inhibited in MAP2a cells (compare green panels in 451Lu and 451-MAP2). During anaphase, AIM-1 is localized to

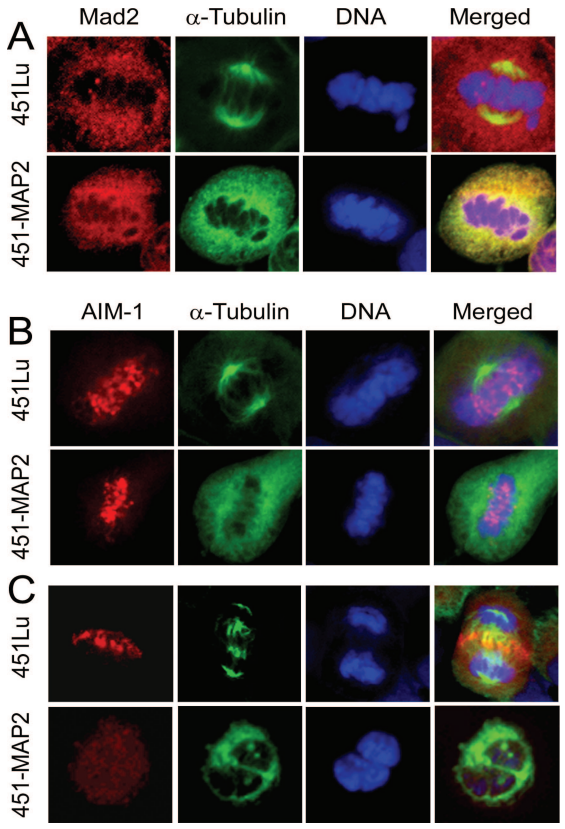

Figure 5. Abnormal localization of spindle checkpoint protein AIM-1in MAP2-expressing melanoma cells. Control and MAP2-expressing cells on coverslips were fixed and stained with abnormal localization of spindle checkpoint protein Mad2 in MAP2-expressing melanoma cells. Control and MAP2-expressing cells on coverslips were fixed and stained with anti-Mad2 mAb followed by anti-α-tubulin mAb DM1A-FITC and finally with PI (**A**) and anti-AIM-1 mAb followed by anti-α-tubulin mAb DM1A-FITC and finally with PI (**B**) and examined with confocal microscope. In **A**, the colors of the images were altered, pink to red for Mad2, and red to blue for DNA in LSM Image Browser for optimal contrast. In MAP2-expressing cells, diffuse cytoplasmic localization of Mad2 at metaphase can be seen (**bottom left**). Bipolar spindles are absent at metaphase in MAP2 cells. Compare **top** (control 451 cells) and **bottom** (451-MAP2 cells) panels marked α-tubulin. In **B**, the colors of the images were altered, pink to red for AIM-1, and red to blue for DNA. In MAP2-expressing cells, diffuse cytoplasmic localization of AIM-1 at anaphase can be seen (**bottom left** in **B**). Bipolar spindles are absent at metaphase in MAP2 cells. Compare **top** (control 451 cells) and **bottom** (451-MAP2 cells) panels marked α-tubulin. At anaphase, whereas in control cells localization of AIM-1 at the cleavage furrow is clearly visible, in 451-MAP2 cells absence of cleavage furrow and diffuse cytoplasmic distribution of AIM-1 can be seen (compare **top** and **bottom**, **left**, in **B**).

the central spindle and cleavage furrow in control 451Lu cells (Figure 5C, top), whereas in MAP2a cells the protein displays a diffuse staining pattern and the cell appears to fail to undergo cytokinesis (Figure 5C, bottom).

Induction of Apoptosis by MAP2

What is the consequence of G2-M arrest caused by MAP2? Time-lapse video microscopy showed that by 72 hours post infection Ad-MAP2 infected cells round up and detach from the dish. FACS analysis showed accumulation of cells with sub G1 DNA content suggesting induction of cell death. To determine whether these cells are undergoing apoptosis we examined PARP cleavage in control Ad-GFP or Ad-MAP2 infected cells. An increase

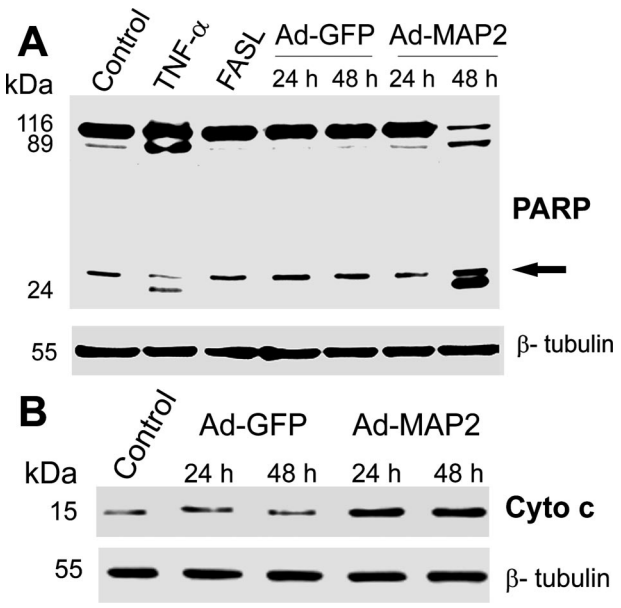

Figure 6. Induction of apoptosis by MAP2 expression. Cleavage of PARP (**A**) and release of cytochrome *c* into cytosol (**B**) in control uninfected, Ad-GFP-transduced or Ad-MAP2-transduced (24 hours and 48 hours) cells was monitored by Western blotting. Whole cell detergent lysates (for PARP) and cytosolic fraction (for cytochrome *c*) were separated by SDS-PAGE, blotted onto PVDF membrane, incubated with anti-PARP or anti-cytochrome *c* antibody, and bands were detected by chemiluminescence. Immunoblotting with anti-β-tubulin shows protein-loading variability. A significant increase in the release of cytochrome *c* into the cytosol in Ad-MAP2-transduced cells could be seen as early as 24 hours, and PARP cleavage products, both 89- and 24-kd bands, were detectable at 48 hours. PARP cleavage induced by TNF-α treatment is shown for comparison. Numbers on the **left** show molecular weights (kd) and **arrow** on the **right** indicates a nonspecific band.

in the 85 kd PARP cleavage product could be seen 48 hours after infection in Ad-MAP2, but not in Ad-GFP, cells (Figure 6A). Western blot analysis using anti-cytochrome *c* antibody showed presence of significantly larger amounts of this mitochondrial protein in cytosolic fraction of MAP2a cells (Figure 6B). No significant cleavage of caspase 8, which is activated by death receptors, could be seen in Ad-MAP2 cells (Supplementary data, Figure 3). These data show that expression of MAP2a in melanoma cells causes spindle assembly defects, mitotic exit without cytokinesis, endoreduplication and apoptosis by mitochondrial pathway.

Discussion

Progression of cutaneous melanoma from the locally invasive, but metastasis-incompetent radial growth phase to rapidly proliferating, metastasis-competent vertical growth phase is accompanied by invasion of tumor cells into the dermis and subcutaneous tissue and a dynamic interaction between tumor cells and stroma.[24] This reciprocal interaction results in differentiation of melanoma cells along different pathways including neuronal pathways.[3,25,26] Our data suggest that neuronal differentiation of primary melanoma cells in the dermis, as indicated by MAP2 expression, can profoundly affect cell cycle progression (Figure 2B), induce apoptosis (Figure 6), and influence clinical outcome (Figure 1). Interestingly,

$MAP2^+$ melanoma cells in vivo and in vitro share certain morphological features, specifically large nuclear size (Supplementary data, Figure 2). However, earlier investigations of nuclear size as a prognostic factor for cutaneous melanoma have resulted in contradictory conclusions.[27,28] Additional features such as relative difficulty of establishing early primary melanoma cells *in vitro* and their slow growth even in growth factor-enriched media,[29,30] and their dendritic appearance (data not shown) suggest a role for MAP2 in the biology of primary melanoma cells.

The notion that MAPs might be involved in development, survival and progression of tumors is supported by the following observations. 1) pVHL protein, the product of the tumor suppressor gene *Von Hippel Lindau* is a microtubule-associated protein that can protect MT from depolymerization *in vivo*. Inactivating mutations in *VHL* that disrupt MT stabilizing function are associated with the dominantly inherited cancer syndrome suggesting a link between MT stabilizing function of pVHL and pathogenesis of VHL disease.[31] 2) A somatic mutation in oncoprotein Op18/Stathmin, a MT destabilizing protein, identified in esophageal adenocarcinoma has been shown to confer transforming properties to this protein[32] and deletion of this region is commonly observed in cancers including breast and colon carcinomas,[33,34] and in breast carcinomas overexpression of stathmin is associated with highly proliferative tumors.[35] 3) Expression of survivin, an inhibitor of apoptosis that also influences MT dynamics and spindle check point,[36] in tumors including melanoma is associated with increased aggressiveness and decreased patient survival.[37–39]

Induction of a MT stabilizing protein in the primary tumor and its absence or silencing in metastatic disease has implications for not only tumor progression but also the sensitivity of tumors to MT agents. While drugs (such as vinca alkaloids and taxols) that target MTs are in active use for treatment of several cancers, toxicity and development of drug resistance are major drawbacks associated with their use. Resistance to killing by these drugs reflects, in part, the ability of cancer cells to activate mechanisms that counteract the action of these drugs.[40] For example, resistance of cancer cells to MT stabilizing drug taxol has been shown to correlate with covalent modification of tubulins or overexpression of MT disrupting proteins.[41,42] Several changes in tubulin/MAPs, including increase in MT-destabilizing form of stathmin and decrease in MT stabilizing form of MAP4, seem to be related to the Taxol-resistant phenotype of human lung carcinoma cell lines.[42] In a murine pancreatic ductal adenocarcinoma model, sensitivity of the tumors to MT targeting agent, docetaxel, appears to be determined by proteolysis of MAP2.[43] Therefore, it is possible that the notorious unresponsiveness of metastatic melanoma to MT targeting drugs reflects a selection process during which cellular mechanisms that disrupt MT dynamics, such as MAP2 expression, are silenced in primary melanoma cells. Intrinsic resistance of melanoma cells to apoptosis is also thought to contribute to the poor response of this tumor to chemotherapy.[44] In this context, it is interesting to note that re-expression of MAP2 overcomes such resistance and induces significant apoptosis as early as 24 hours postinfection as indicted by cytochrome *c* release.

MAP2 is expressed mainly in neuronal dendritic extensions where the protein is known to stabilize MTs and influence the density of MTs and the length of dendrites.[8] MAP2 also acts as scaffold for signaling proteins and has a binding domain for the regulatory subunit II of cAMP-dependent protein kinase PKA.[8,45] A bulk of MAP2 expressed in melanoma remains associated with the cellular MTs and co-localizes to spindle MTs during mitosis (Figure 2). Our observation that disruption of MT dynamics by MAP2 has a profound effect on cytokinesis is consistent with recent findings that MTs are the only structural constituents of the spindle apparatus required for induction of cell cleavage.[46]

How is MAP2 gene activated in primary melanoma? Although early tumor development is driven for most part by discrete genetic alteration in the transformed cells, it is now accepted that later steps in tumor progression are dependent on reciprocal interactions between the tumor cells and host microenvironment.[47] We find that in primary melanomas that consist of both *in situ* and invasive components, MAP2 expression is mostly restricted to the invasive cells in the dermis, suggesting a role for dermis in MAP2 induction. The possibility that stromal response to invading tumor cells could exert an initial inhibitory constraint on tumor growth has been raised earlier,[48,49] but the nature and mechanisms of inhibitory effects have not been investigated. In this context, it is worth noting that a majority of benign dermal melanocytic nevi also express abundant MAP2 and only a small proportion of melanomas are associated with such pre-existing nevi.[4,50] That tumor-stromal interactions need not always favor tumor progression is also indicated by the observation that tumors prefer to grow and metastasize from orthotopic sites rather than ectopic sites.[51]

Since, MAP2 appears to act *in vivo* mainly on primary melanoma cells during early tumor progression in the context of dermal environment, we surmised that evaluating the correlation between MAP2 expression in primary melanoma and metastasis directly using human tumors is relevant for understanding its role in melanoma progression. Accordingly, our immunohistochemical evaluation and survival analysis data suggest that MAP2 is a good prognostic indicator of aggressiveness of melanoma. However, long term follow-up of a larger population of melanoma patients is needed to further confirm the utility of MAP2 in predicting clinical outcome. Additionally, understanding the mechanisms of MAP2 gene regulation might allow design and development of therapeutic strategies (for eg, treatment with agents such as hexamethylene bisacetamide[4]) to induce/up-regulate MAP2 expression in advanced melanomas.

Acknowledgments

We thank Dr. A. Thorburn for help with time-lapse video microscopy and the generous gift of antibodies, Dr. K.

Bhat for help with figures, Dr. G.L. Prasad for discussions, and Inez Evans of Wake Forest University Cancer Registry for her help.

References

1. Li N, Mangini J, Bhawan J: New prognostic factors of cutaneous melanoma: a review of the literature. J Cutan Pathol 2002, 29:324–340
2. Kalady MF, White RR, Johnson JL, Tyler DS, Seigler HF: Thin melanomas: predictive lethal characteristics from a 30-year clinical experience. Ann Surg 2003, 238:528–535
3. Hendrix MJ, Seftor EA, Hess AR, Seftor RE: Molecular plasticity of human melanoma cells. Oncogene 2003, 22:3070–3075
4. Fang D, Hallman J, Sangha N, Kute TE, Hammarback JA, White WL, Setaluri V: Expression of microtubule-associated protein 2 in benign and malignant melanocytes: implications for differentiation and progression of cutaneous melanoma. Am J Pathol 2001, 158:2107–2115
5. Blumcke I, Becker A, Normann S, Hans V, Riederer B, Krajewski S, Wiestler O, Reifenberger G: Distinct expression pattern of microtubule-associated protein-2 in human oligodendrogliomas and glial precursor cells. J Neuropathol Exp Neurol 2001, 60:984–993
6. Liu Y, Mangini J, Saad R, Silverman AR, Abell E, Tung MY, Graner SR, Silverman JF: Diagnostic value of microtubule-associated protein-2 in Merkel cell carcinoma. Appl Immunochem Mol Morphol 2003, 11:326–329
7. Cassimeris L, Spittle C: Regulation of microtubule-associated proteins. Int Rev Cytol 2001, 210:163–226
8. Harada A, Teng J, Takei Y, Oguchi K, Hirokawa N: MAP2 is required for dendrite elongation, PKA anchoring in dendrites, and proper PKA signal transduction. J Cell Biol 2002, 158:541–549
9. Kalcheva N, Rockwood JM, Kress Y, Steiner A, Shafit-Zagardo B: Molecular and functional characteristics of MAP-2a: ability of MAP-2a versus MAP-2b to induce stable microtubules in COS cells. Cell Motil Cytoskeleton 1998, 40:272–285
10. Nguyen H, Chari S, Gruber D, Lue C, Chapin S, Bulinski J: Overexpression of full- or partial-length MAP4 stabilizes microtubules and alters cell growth. J Cell Sci 1997, 110:281–294
11. Shafit-Zagardo B, Davies P, Rockwood J, Kress Y, Lee S: Novel microtubule-associated protein-2 isoform is expressed early in human oligodendrocyte maturation. Glia 2000, 29:233–245
12. Suzuki S, Kitai R, Llena J, Lee S, Goldman J, Shafit-Zagardo B: MAP-2e, a novel MAP-2 isoform, is expressed in gliomas and delineates tumor architecture and patterns of infiltration. J Neuropathol Exp Neurol 2002, 61:403–412
13. Wittmann T, Hyman A, Desai A: The spindle: a dynamic assembly of microtubules and motors. Nat Cell Biol 2001, 3:E28–E34
14. Checchi PM, Nettles JH, Zhou J, Snyder JP, Joshi HC: Microtubule-interacting drugs for cancer treatment. Trends Pharmacol Sci 2003, 24:361–365
15. Wassmann K, Benezra R: Mitotic checkpoints: from yeast to cancer. Curr Opin Genet Dev 2001, 11:83–90
16. Lewis S, Gu W, Cowan N: Free intermingling of mammalian beta-tubulin isotypes among functionally distinct microtubules. Cell 1987, 49:539–548
17. Albala JS, Kalcheva N, Shafit-Zagardo B: Characterization of the transcripts encoding two isoforms of human microtubule-associated protein-2 (MAP-2). Gene 1993, 136:377–378
18. Satyamoorthy K, Li G, Vaidya B, Patel D, Herlyn M: Insulin-like growth factor-1 induces survival and growth of biologically early melanoma cells through both the mitogen-activated protein kinase and {beta}-catenin pathways. Cancer Res 2001, 61:7318–7324
19. Bagwell C: Flow cytometry data analysis in clinical cytometry. Clinical Flow Cytometry: Theoretical Aspects of Flow Cytometry Data Analysis. Edited by Bauer K, Shankey V. New York, Valez-Liss, 1992, pp 41–61
20. Bossy-Wetzel E, Green DR: Assays for cytochrome c release from mitochondria during apoptosis. Methods in Enzymology. Edited by Reed JC. New York, Academic Press, 2000, pp 235–238
21. Joshi H, Cleveland D: Differential utilization of beta-tubulin isotypes in differentiating neurites. J Cell Biol 1989, 109:663–673
22. Gorbsky GJ, Chen R-H, Murray AW: Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. J Cell Biol 1998, 141:1193–1205
23. Bischoff JR, Plowman GD: The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. Trends Cell Biol 1999, 9:454–459
24. Ruiter D, Bogenrieder T, Elder D, Herlyn M: Melanoma-stroma interactions: structural and functional aspects. Lancet Oncol 2002, 3:35–43
25. Sangueza OP, Requena L: Neoplasms with neural differentiation: a review. Part II: malignant neoplasms. Am J Dermatopathol 1998, 20:89–102
26. Slutsky SG, Kamaraju AK, Levy AM, Chebath J, Revel M: Activation of myelin genes during transdifferentiation from melanoma to glial cell phenotype. J Biol Chem 2003, 278:8960–8968
27. Heenan PJ, Jarvis LR, de Klerk NH, Armstrong BK: Nuclear indices and survival in cutaneous melanoma. Am J Dermatopathol 1989, 11:308–312
28. Pierard-Franchimont C, Pierard GE: Karyometry of primary and metastatic malignant melanoma. Am J Dermatopathol 1994, 16:247–252
29. Rodeck U, Herlyn M, Menssen H, Furlanetto R, Koprowski H: Metastatic but not primary melanoma cell lines grow in vitro independently of exogenous growth factors. Int J Cancer 1987, 15:687–690
30. Satyamoorthy K, DeJesus E, Linnenbach A, Kraj B, Kornreich D, Rendle S, Elder D, Herlyn M: Melanoma cell lines from different stages of progression and their biological and molecular analyses. Melanoma Res 1997, 7:S35–S42
31. Hergovich A, Lisztwan J, Barry R, Ballschmieter P, Krek W: Regulation of microtubule stability by the von Hippel-Lindau tumor suppressor. Nat Cell Biol 2003, 5:64–70
32. Misek DE, Chang CL, Kuick R, Hinderer R, Giordano TJ, Beer DG, Hanash SM: Transforming properties of a Q18–>E mutation of the microtubule regulator Op18. Cancer Cell 2002, 2:217–228
33. Farabegoli F, Ceccarelli C, Santini D, Trere D, Baldini N, Taffurelli M, Derenzini M: Chromosome 1 aneusomy with 1p36 under-representation is related to histologic grade, DNA aneuploidy, high c-erb B-2 and loss of bcl-2 expression in ductal breast carcinoma. Int J Cancer 1996, 69:381–385
34. Tanaka K, Tanoshita R, Konishi M, Oshimura M, Maeda Y, Mori T, Miyaki M: Suppression of tumourigenicity in human colon carcinoma cells by introduction of normal chromosome 1p36 region. Oncogene 1993, 8:2253–2258
35. Curmi PA, Nogues C, Lachkar S, Carelle N, Gonthier MP, Sobel A, Lidereau R, Bieche I: Overexpression of stathmin in breast carcinomas points out to highly proliferative tumours. Br J Cancer 2000, 82:142–150
36. Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, Villa A, Marchisio PC, Altieri DC: Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat Cell Biol 1999, 1:461–466
37. Swana HS, Grossman D, Anthony JN, Weiss RM, Altieri DC: Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. N Engl J Med 1999, 341:452–453
38. Kawasaki H, Altieri D, Lu C, Toyoda M, Tenjo T, Tanigawa N: Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 1998, 58:5071–5074
39. Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC: Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet 1998, 351:882–883
40. Dumontet C, Sikic BI: Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. J Clin Oncol 1999, 17:1061–1070
41. Alli E, Bash-Babula J, Yang J-M, Hait WN: Effect of Stathmin on the sensitivity to antimicrotubule drugs in human breast cancer. Cancer Res 2002, 62:6864–6869
42. Martello LA, Verdier-Pinard P, Shen H-J, He L, Torres K, Orr GA, Horwitz SB: Elevated levels of microtubule destabilizing factors in a taxol-resistant/dependent A549 cell line with an {alpha}-tubulin mutation. Cancer Res 2003, 63:1207–1213
43. Veitia R, David S, Barbier P, Vantared M, Gounon P, Bissery M, Fellous A: Proteolysis of microtubule associated protein 2 and sensi-tivity of pancreatic tumours to docetaxel. Br J Cancer 2000, 83:544–549
44. Soengas MS, Lowe SW: Apoptosis and melanoma chemoresistance.

Oncogene 2003, 22:3138–3151
45. Lim RWL, Halpain S: Regulated association of microtubule-associated protein 2 (MAP2) with Src and Grb2: evidence for MAP2 as a scaffolding protein. J Biol Chem 2000, 275:20578–20587
46. Alsop GB, Zhang D: Microtubules are the only structural constituent of the spindle apparatus required for induction of cell cleavage. J Cell Biol 2003, 162:383–390
47. Barsky S, Gopalakrishna R: Increased invasion and spontaneous metastasis of BL6 melanoma with inhibition of the desmoplastic response in C57BL/6 mice. Cancer Res 1987, 47:1663–1667
48. Ronnov-Jessen L, Petersen OW, Bissell MJ: Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev 1996, 76:69–125
49. Kenny PA, Bissell MJ: Tumor reversion: correction of malignant behavior by microenvironmental cues. Int J Cancer 2003, 107:688–695
50. Harley S, Walsh N: A new look at nevus-associated melanomas. Am J Dermatopathol 1996, 18:137–141
51. Stephenson RA, Dinney CPN, Gohji K, Ordonez NG, Killion JJ, Fidler IJ: Metastatic model for human prostate cancer using orthotopic implantation in nude mice. J Natl Cancer Inst 1992, 84:951–957