Monoclonal Antibody 4C5 Immunostains Human Melanomas and Inhibits Melanoma Cell Invasion and Metastasis

Dimitris Stellas,1,2 Andreas Karameris,3 and Evangelia Patsavoudi1,2

Abstract

Purpose: Tumor cell metastasis constitutes a major problem in the treatment of cancer. Because the cure rate of metastatic tumors is very low, new therapeutic approaches are needed. Heat shock protein 90 (HSP90) is a molecular chaperone that is recognized as a new target for the treatment of cancer. Here, we examine the value of a monoclonal antibody (mAb) against HSP90, mAb 4C5, as a potential marker in malignant melanomas. Moreover, we investigate the possibility to use mAb 4C5 as an inhibitor of melanoma cell invasion and metastasis.

Experimental Design: Paraffin blocks of formalin-fixed human melanoma tumor tissues were used to prepare tissue microarrays. The B16 F10 melanoma cell line was used in all the in vitro experiments. To assess melanoma cell invasion, the wound-healing assay and the Matrigel invasion assay were applied. To evaluate the effect of mAb 4C5 on tumor metastasis, we used an experimental model of metastatic melanoma.

Results: Immunohistochemical studies done on a panel of malignant melanomas showed positive immunostaining with mAb 4C5 in all cases. mAb 4C5 inhibits B16 F10 cell invasion by binding to surface HSP90 because it is not internalized. mAb 4C5 significantly inhibits melanoma metastasis in C57BL/6 mice inoculated with B16 F10 cells.

Conclusions: mAb 4C5 could be potentially used as a novel specific marker for malignant melanomas. mAb 4C5 inhibits melanoma cell invasion in vitro by binding to cell surface HSP90 expressed on B16 F10 melanoma cells. Finally, this antibody significantly inhibits melanoma metastasis, thus rendering it a potential therapeutic agent for the treatment of cancer metastasis.

Metastasis is one of the major causes of mortality in cancer patients. Inhibition of invasion and metastasis of cancer cells is of great importance in cancer treatment. However, the development of new drugs with antimetastatic activity is still not satisfactory. Thus, it is critical to discover potent agents with low cytotoxicity and high efficiency to treat tumor metastasis.

Heat shock protein 90 (HSP90) is a chaperone protein that is essential for the cell to stabilize the form and the function of a variety of substrates, including molecules involved in signal transduction pathways (1–3), in the cell cycle (reviewed in ref. 4) and in the function of steroid hormone receptors (5, 6). Moreover, HSP90 interacts with a great number of molecules that are involved in the development and/or survival of cancer cells (reviewed in refs. 7, 8), allowing mutant proteins to retain or gain function while permitting cancer cells to tolerate the imbalanced signaling that such oncoproteins create. HSP90 is now considered as a new target for the development of molecular cancer therapeutics (9–12). Indeed, highly specific inhibitors of this molecule have been identified, which modify its chaperoning activity and decrease the cellular levels of the cancer-related client proteins that depend on HSP90 for their function. Among these, the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin has recently entered clinical trials, giving encouraging results (13–16).

We have previously produced a monoclonal antibody (mAb), mAb 4C5, after immunization of mice with a brain membrane fraction of 15-day-old rat embryos (17). Mass spectrometry analysis and immunoprecipitation experiments showed that mAb 4C5 recognizes both the α isoform and, to a lesser extent, the β isoform of the HSP90 protein (18). Using mAb 4C5, we have shown that HSP90 is localized not only in the cytoplasm but also on the cell surface and that it contributes to cell migration processes during nervous tissue development (18–21). Finally, data have been presented suggesting the involvement of this protein in actin cytoskeletal dynamics of migrating cells (18, 20).

Taking all the above into consideration in addition with recent data showing that HSP90 is localized on the surface of melanoma cells derived from melanocytic lesions (22), we examined immunohistochemically a group of human melanocytic tumors using mAb 4C5 and compared the results with those obtained using other commercially available antibodies against...
HSP90. Moreover, and taking into account that the migration mechanisms occurring in normal nonneoplastic cells during embryonic development are similar if not identical to the invasion processes of tumor cells during metastasis, in this work, we sought to investigate the possible involvement of cell surface HSP90 in the invasion process of B16 F10 melanoma cells using the function blocking mAb 4C5. Finally, we examined the capacity of mAb 4C5 to inhibit melanoma metastasis using a lung metastasis experimental model.

Materials and Methods

Reagents. mAb 4C5 was produced in our laboratory as described previously (17). In the present study, mAb 4C5 was used as concentrated serum-free supernatant in all experiments. Polyclonal antibodies against the α and the β isoforms of mouse HSP90 were obtained from Chemicon International (Temecula, CA). Polyclonal antibodies against the α and the β isoforms of human HSP90, polyclonal antibodies against human HMB45, mAb against cyclin D1, and polyclonal biotinylated anti-rabbit immunoglobulins were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary FITC-conjugated anti-mouse and anti-rabbit immunoglobulins, poly-l-lysine, and 3,3′-diaminobenzidine were purchased from Sigma Chemical Co. (Munich, Germany). DMEM and fetal bovine serum were obtained from Life Technologies, Inc. (Rockville, MD).

Tissue microarray construction. Ten melanoma cases were retrieved from the tissue bank of 417 Veterans Administration Hospital (Athens, Greece). The sites of origin of the biopsies were as follows: (a) one case of an in situ melanoma from skin of tibia; (b) three cases of primary melanomas from skin of back, buttock, and pubic area; and (c) six cases of metastatic melanomas from chest (three cases), maxilla, scalp (primary site in brain), and inner thigh. Areas of interest were identified on H&E-stained slides by a conventional microscope (Olympus BX 50, Tokyo, Japan). The corresponding paraffin blocks from the previously mentioned melanomas were used for the construction of one tissue microarray block. Using a tissue microarrayer ATA-100 (Chemicon International), all of the source blocks were cored twice to increase the capacity of mAb 4C5 to inhibit melanoma metastasis using a lung metastasis experimental model.

Materials and Methods

Antibody internalization assay. B16 F10 cells were incubated while in culture with 120 μg/mL of mAb 4C5, anti-HSP90α, and anti-HSP90β antibodies for 1, 4, and 6 h. The cells were then washed in DMEM and fixed in cold methanol for 3 min. For detection of possible internalization of the antibodies, the cells were permeabilized with 0.1% Triton X-100 in PBS followed by incubation with fluorescein-conjugated anti-mouse and anti-rabbit immunoglobulins, respectively, diluted 1:200 in PBS containing 10% fetal bovine serum and 0.1 mol/L lysine. Finally, the cells were mounted on glass slides in Citifluor to prevent fading of the fluorescence. For all experiments, controls were done by omitting the primary antibodies. All preparations were viewed by a Leica confocal microscope.

Wound-healing assay. For the wound-healing assay, 2 × 10^5 B16 F10 cells per well, which had been serum starved for 12 h, were plated in serum-free DME medium and in 48-well plates (Costar, Schiphol-Rijk, the Netherlands). A plastic pipette tip was drawn across the center of the well to produce a clean 1-mm-wide wound area after the cells had reached confluence followed by addition of 120 μg/mL of either mAb 4C5 or anti-HSP90α or anti-HSP90β antibodies in the culture medium. The migration and cell movement throughout the wound area was examined after 24 h. The migration distances were calculated and compared with the use of Image J.

Matriplag invasion assay. Subconfluent B16 F10 cells were detached by scraping. Cells (8 × 10^4 per well) were cultured in serum-free DME medium in the presence of 120 μg/mL of either mAb 4C5 or anti-HSP90α or anti-HSP90β antibodies, which were added into the upper compartment of BioCoat Matriplag invasion chambers (Becton Dickinson Bioscience, Bedford, MA). The 8-μm filter pores were precoated with Matriplag basement membrane matrix. As a chemoattractant, 10% fetal bovine serum was used in the lower chamber. After 24 h of culture, the noninvasive cells of the upper surface of the filter were removed and the cells on the lower surface of the filter were fixed with cold methanol, stained with Giemsa staining, and visualized with a Zeiss microscope. Cells from 18 random fields were counted for each filter.

It is important to note that, for the two in vitro assays mentioned above, control cultures were grown either in culture medium alone or in culture medium containing 120 μg/mL of rabbit immunoglobulins or an irrelevant antibody, mAb LD33, which was a kind gift of Dr. K. Soteriadou (Hellenic Pasteur Institute, Athens, Greece; ref. 24). All of these controls had similar results.

Bromodeoxyuridine incorporation assay. Serum-starved B16 F10 cells were exposed to bromodeoxyuridine at a concentration of 10⁻³ μM for 4 h. Cells were subsequently rinsed in PBS and fixed in 4% paraformaldehyde for 15 min. After washing, the cells were incubated in 2 mol/L HCl containing 0.1% Triton X-100 for 10 min followed by 20-min incubation in 0.1 mol/L sodium borate and then processed for indirect immunofluorescence using mouse anti-bromodeoxyuridine antibody (DAKO) followed by Alexa Fluor 488–conjugated goat secondary antibody.

Trypan blue staining. At the end point of the wound-healing assay, B16 F10 cells were incubated for 5 min with 0.4% trypan blue in PBS.
before washing of cells with PBS. Excess staining was removed and the cells were visualized in a Leica microscope.

Assay of melanoma metastasis. C57BL/6 mice were originally purchased from Harlan Laboratories (Harlan Italy, S. Pietro al Natisone, Italy) and bred and maintained under specific pathogen-free conditions at the Experimental Animal Unit of the Hellenic Pasteur Institute (Athens, Greece). All of the experiments with animals were done in accordance with the guidelines approved by the Ethical Committee of the Hellenic Pasteur Institute.

For the lung metastasis experimental model, the following procedure was done: cultured B16 F10 melanoma cells were detached from their culture flasks with a cell scrapper, washed twice with PBS, and made up to the cell density of 7.5 × 10^5/mL in PBS. Thirty 8- to 10-week-old female C57BL/6 mice were injected through the tail vein with 0.1 mL of the above cell preparation. The next day, the animals were divided into three equal groups: the control untreated group injected with PBS or the above cell preparation. The next day, the animals were divided into three equal groups: the control untreated group injected with PBS or 100 µg/mouse of an irrelevant antibody named mAb LD33 (24) and the two mAb 4C5–treated groups (100 and 200 µg/mouse, respectively). I.p. injections were done daily for a period of 2 weeks. The same experiment was done twice with similar results.

Evaluation of pulmonary metastases. For estimation of pulmonary metastases, a second set of 30 C57BL/6 mice was divided into three equal groups and treated as described above. To compare the melanocytic nodules between the control and the mAb 4C5–treated groups, mice were sacrificed on the 26th day after inoculation with the B16 F10 melanoma cells, which was the day that the first two deaths occurred in the control group. The lungs were retrieved, formalin fixed, and paraffin embedded before sectioning at a thickness of 8 µm and staining with H&E. Melanocytic nodules were counted with a Zeiss microscope for each mouse in five randomly chosen sections covering the whole of the lung tissue.

Statistical analysis. For the experimental groups that were statistically analyzed for differences, Student’s t test was used, in which P < 0.05 was defined as statistically significant. For the in vivo survival experiments, the Kaplan-Meier statistical analysis of survival was done using the MedCalc software in which P was calculated by the log-rank test.

Results

mAb 4C5 immunostains human melanomas. We examined immunohistochemically mAb 4C5 on a panel of human melanomas. All lesions were also stained with commercially available polyclonal antibodies against the α and β isoforms of human HSP90. Our results showed that mAb 4C5, in contrast to what was observed with the commercial antibodies, positively immunostained all primary melanomas as well as metastatic lesions (Table 1; Fig. 1). It is noteworthy that one case of in situ melanoma tested gave negative results for all three antibodies used (Table 1; Fig. 1). It should be noted that immunostaining of control tissues with mAb 4C5 gave negative results (Fig. 1). In all cases studied, negative controls were constantly used. Moreover, positive controls using HMB45, a well-documented melanoma marker (23), always resulted in significant immunostaining of the melanoma cells (Table 1).

HSP90 is expressed on the cell surface of B16 F10 melanoma cells. To show the cell surface localization of HSP90, unfixed B16 F10 melanoma cells were immunostained with mAb 4C5 and commercially available polyclonal antibodies against the α and β isoforms of mouse HSP90. The cells were then washed, fixed, and labeled with fluorescent secondary antibody. Thus, the primary antibody had access to the external surface of the cell. The observed typical punctuate immunostaining confirmed the cell surface localization of HSP90 in all three cases studied (Fig. 2). Control experiments using an antibody to the nuclear protein cyclin D1 gave negative results (data not shown).

mAb 4C5 inhibits B16 F10 melanoma cell invasion. Increased cell motility is one of the characteristics associated with malignancy and is involved in the genesis of metastatic disease. We therefore examined the effect of mAb 4C5, anti-HSP90α, and anti-HSP90β on cell motility using the wound-healing assay. After 24 h of culture, a significantly slower rate of wound closure was observed for cells cultured in the presence of all three antibodies (Fig. 3A and B) when compared with controls. Interestingly, when the cells in all cases studied were visualized at a higher magnification, although no cell death was observed in the cases of the controls and the cells cultured in the presence of mAb 4C5, a significant number of cells with a round shape containing pyknotic bodies that are characteristics of cell death were detected in the cultures containing anti-HSP90α and anti-HSP90β (Fig. 3C). Trypan blue staining carried out in all four cases at the end point of the wound-healing assay showed that, indeed, a much greater number of cells were stained in the cultures done in the presence of anti-HSP90α and anti-HSP90β when compared with the controls and the cultures done in the presence of mAb 4C5 (Fig. 3C, arrowheads).

It is important to note that, in this assay, the effect of mAb 4C5 is directed toward cell invasion and not toward cell proliferation because very low (9.3%) bromodeoxyuridine incorporation was observed in B16 F10 cultures treated as above with no apparent differences between the different experimental conditions (data not shown).

As an additional measure of the inhibition of B16 F10 cell motility and invasiveness due to mAb 4C5, we assessed the ability of B16 F10 cells to invade through a Matrigel matrix, which serves as a reconstituted basement membrane in vitro. The layer occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells are able to detach themselves from and invade through the Matrigel matrix. Cells were plated on one side of the membrane in the presence and absence of mAb 4C5, anti-HSP90α, and anti-HSP90β, and after 24 h, cells on the opposite side of the membrane were stained and counted. Results of such an experiment confirmed the ability of mAb 4C5 to inhibit B16 F10 cell invasion (Fig. 4). Addition of anti-HSP90α and anti-HSP90β in the culture medium also resulted in a similar reduction of cell invasion (Fig. 4).

mAb 4C5 is not internalized in B16 F10 melanoma cells. The binding of mAb 4C5 to B16 F10 cells was examined at various

### Table 1. Immunohistochemical analysis of human primary and metastatic melanomas

<table>
<thead>
<tr>
<th>Type of tumors</th>
<th>Antibodies</th>
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<tbody>
<tr>
<td></td>
<td>mAb 4C5</td>
</tr>
<tr>
<td>Primary melanoma</td>
<td>+</td>
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<tr>
<td>Primary melanoma</td>
<td>+</td>
</tr>
<tr>
<td>Primary melanoma</td>
<td>+</td>
</tr>
<tr>
<td>In situ melanoma</td>
<td>+</td>
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<tr>
<td>Metastatic melanoma</td>
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<td>Metastatic melanoma</td>
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The observed typical punctuate immunostaining confirmed the cell surface localization of HSP90 in all three cases studied (Fig. 2). Control experiments using an antibody to the nuclear protein cyclin D1 gave negative results (data not shown).
intervals. Binding of the antibody was analyzed after fixation and permeabilization of the cells by confocal microscopy. Our results showed that, at all time intervals studied and even after 6 h of incubation, mAb 4C5 was not internalized and remained localized on the surface of the cells (Fig. 5). Interestingly, when a similar procedure was followed using anti-HSP90α and anti-HSP90β polyclonal antibodies and in contrast to what was observed with mAb 4C5, high intracellular levels of both antibodies were detected already after 4 h of incubation of the cells with the antibodies (Fig. 5). It should be noted that similar results were obtained when internalization of all three antibodies was monitored for 24 h (data not shown).

**mAb 4C5 inhibits B16 F10 melanoma metastasis in C57BL/6 mice.** Based on the in vitro studies, we next examined the effect of mAb 4C5 on metastatic tumor growth in an experimental metastasis model. To this purpose, we injected B16 F10 melanoma cells i.v. in C57BL/6 mice. Treatment of mice with mAb 4C5 (100 and 200 μg/animal) resulted in a significant improvement of survival ($P < 0.0001$) when compared with untreated control mice (Fig. 6A). Interestingly, the survival rate
of the mAb 4C5–treated mice was dose dependent (P = 0.0075). Median survival for the control untreated mice and the mAb 4C5–treated mice was 27 days, 39 days (100 μg/mouse), and 47.5 days (200 μg/mouse), respectively. Lung autopsy of a mouse from the untreated control group, which died on the 27th day after challenging with B16 F10 melanoma cells, showed extensive tumor growth both at the macroscopic and microscopic level (Fig. 6B). In contrast, no lung metastases were observed in a mouse derived from the group treated with 100 μg of mAb 4C5/animal and sacrificed on the same day. This animal presented a similar profile with a mouse that had not been challenged with B16 F10 melanoma cells (Fig. 6B).
Pulmonary metastasis analysis at the microscopic level of the control and mAb 4C5–treated mice confirmed the above (Table 2). It is important to note that lung autopsy of a mAb 4C5–treated mouse (200 μg) at the time of its death, 47 days after inoculation with B16 F10 cells, revealed similar tumor growth as described above in a control untreated mouse (data not shown).

Discussion

In the present study, we present data showing that mAb 4C5, a mAb against the chaperone protein HSP90, immunostains specifically a panel of human melanoma tumors. Moreover, we show that mAb 4C5 inhibits B16 F10 melanoma cell invasion in vitro and melanoma metastasis in a lung metastasis experimental model.

Previously reported experiments using mAb 4C5 have shown that the corresponding antigen (i.e. HSP90) participates in cell migration processes during development of the nervous system (18–21) and that it may be associated with actin cytoskeletal dynamics during cell migration (18, 20).

To evaluate the potential diagnostic value of mAb 4C5 in malignant melanoma, a panel of melanomas was immunostained with mAb 4C5 and the results were compared with those obtained with commercially available anti-HSP90α and anti-HSP90β polyclonal antibodies against human HSP90. Interestingly, mAb 4C5 reacted positively in all the primary and metastatic melanomas examined, whereas anti-HSP90α and anti-HSP90β failed to give positive results in all of the cases tested. It is also noteworthy that one case of in situ, nonmetastatic melanoma gave negative results with all three antibodies. Our results suggest that mAb 4C5 may be a new specific marker for melanomas both for primary as well as for metastatic. However, more cases should be tested to confirm the above.

Similar results to ours have recently been reported (22) showing, with the use of a polyclonal anti-HSP90 antibody, overexpression of HSP90 in most of the skin melanomas (10 of 14) and in all of the metastatic cases examined, whereas most of the melanocytic nevi gave negative results (7 of 9).

Cell surface localization of HSP90 was shown immunohistochemically on live B16 F10 melanoma cells using mAb 4C5, anti-HSP90α, and anti-HSP90β polyclonal antibodies. Comparable results have recently been reported by Becker et al. (22) who observed, using a polyclonal anti-HSP90 antibody, surface expression of HSP90 on cells derived from melanoma metastases.

Inhibition of B16 F10 melanoma cell invasion by mAb 4C5 was shown in vitro with two different bioassays (i.e., the
mAb 4C5 Inhibits Melanoma Cell Invasion and Metastasis

In contrast, a mAb 4C5 treated mouse (100 μg/mouse) had not been inoculated with B16 F10 melanoma cells. Bar, 1,000 μm. Day showed no melanoma metastasis and had the same profile as a mouse that was sacrificed on the same day. These results suggest that arrest of B16 F10 cell motility with the use of anti-HSP90α and anti-HSP90β polyclonal antibodies may be at least in part due to cell death.

To assess the participation of cell surface and not intracellular HSP90 in the invasion process of B16 F10 cells, internalization of mAb 4C5, anti-HSP90α, and anti-HSP90β was examined at various time intervals of B16 F10 cultures. Interestingly, mAb 4C5 remained bound to the cell surface even after 6 h of culture, whereas both anti-HSP90α and anti-HSP90β were internalized after 4 h of incubation. These results strongly suggest that the previously mentioned inhibitory effect of mAb 4C5 on B16 F10 cell invasion is due to binding of the antibody to the cell surface pool of HSP90. On the other hand, internalization of the anti-HSP90α and anti-HSP90β polyclonal antibodies renders it difficult to distinguish whether the effect of these antibodies on cell motility is due to their transient binding to cell surface HSP90 or to their binding to the intracellular pool of this molecule. Moreover, and taking into consideration that HSP90 exerts its chaperone activity on a variety of intracellular molecules necessary for cell survival, it is tempting to speculate that binding of these antibodies to the cytoplasmic pool of HSP90 causes the cell death observed in the wound-healing assay. Participation of extracellular HSP90α in cancer cell invasiveness has recently been reported by Eustace et al. (25). In particular, they show that inhibition of extracellular HSP90α using a mAb decreases the invasiveness of HT-1080 fibrosarcoma cells and the activity of matrix metalloproteinase 2, a molecule activated by HSP90α outside the cell.

Metastasis is one of the most important factors related to cancer therapeutic efficacy and survival. In line with the in vitro results in this work, we show that mAb 4C5 can significantly inhibit experimental melanoma metastasis in C57BL/6 mice inoculated with B16 F10 melanoma cells. More precisely, the survival rate of mice treated with mAb 4C5 was significantly greater when compared with control untreated mice. The dose-dependent effect of mAb 4C5 on the survival rate of treated mice confirms the specificity of the antibody. Furthermore, melanocytic nodule formation was greatly reduced in the mAb 4C5–treated mice compared with the control mice. To our knowledge, the above-mentioned result shows for the first time that a mAb targeted against HSP90 has antimetastatic capacity. Interestingly Price et al. (26) have recently reported that the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AM) is highly active in vivo against experimental metastatic melanoma.

Table 2. Effect of mAb 4C5 on pulmonary metastases of B16 F10 melanoma cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. tumor nodules</th>
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<tr>
<td>Control</td>
<td>359.5 ± 12.91</td>
</tr>
<tr>
<td>mAb 4C5 (100 μg/mouse)</td>
<td>6 ± 2.36*</td>
</tr>
<tr>
<td>mAb 4C5 (200 μg/mouse)</td>
<td>2 ± 1.35*</td>
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Note: Ten mice from each group were analyzed for pulmonary metastases 26 d after inoculation with B16 F10 melanoma cells. Values represent mean number of tumor nodules counted ± SD.

*Different from control mice at P < 0.0001.

†Different from mice treated with 100 μg/mouse at P < 0.001.
enhanced bone metastasis and osteolytic lesions in nude mice, which had been inoculated with a human breast cancer cell line. However, it is important to note that 17-allylamino-17-demethoxygeldanamycin, in contrast to what is observed with mAb 4C5, is targeted against the entire pool of HSP90, including the cytoplasmic, which as previously mentioned with mAb 4C5, is targeted against the entire pool of HSP90, including the cytoplasmic, which as previously mentioned interacts with a large number of intracellular molecules. Recently, studies have been reported using HSP90 inhibitors in in vivo experimental models and showing their inhibitory effect on tumor cell proliferation (27) and factors required for tumor angiogenesis (28).

In conclusion, we have presented data suggesting that mAb 4C5 may be a novel specific marker for malignant melanomas. Moreover, we have shown that mAb 4C5 inhibits B16 F10 melanoma cell invasion by binding to cell surface HSP90. Finally, we have shown that mAb 4C5 can significantly inhibit melanoma metastasis in an experimental metastatic model. Our combined data indicate that mAb 4C5 has therapeutic potential for the treatment of cancer metastasis. The precise mode of action of mAb 4C5 in the multistep process of melanoma progression remains unclear and merits further investigation. Moreover, future studies include humanization of mAb 4C5.

Acknowledgments

We thank G. Vilas for technical assistance, Dr. C. Baxevanis for kindly providing the B16 F10 melanoma cell line, and Drs. G. Panayotou and C. Baxevanis for critically reading the manuscript.

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