Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases

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Abstract

Although >66% of melanomas harbor activating mutations in BRAF and exhibit constitutive activity in the mitogen-activated protein kinase/extracellular signal–regulated kinase kinase (MEK)/extracellular signal–regulated kinase signaling pathway, it is unclear how effective MEK inhibition will be as a sole therapeutic strategy for melanoma. We investigated the anticancer activity of MEK inhibition in a panel of cell lines derived from radial growth phase (WM35) and vertical growth phase (WM793) of primary melanomas and metastatic melanomas (1205Lu, 451Lu, WM164, and C8161) in a three-dimensional spheroid model and found that the metastatic lines were completely resistant to MEK inhibition (U0126 and PD98059) but the earlier stage cell lines were not. Similarly, these same metastatic melanoma lines were also resistant to inhibitors of the phosphatidylinositol 3-kinase/Akt pathway (LY294002 and wortmannin). Under adherent culture conditions, the MEK inhibitors blocked growth through the induction of cell cycle arrest and up-regulation of p27, but this was readily reversible following inhibitor washout. However, when the phosphatidylinositol 3-kinase and MEK inhibitors were combined, the growth and invasion of the metastatic melanoma three-dimensional spheroids were blocked. Taken together, these results suggest that the most aggressive melanomas are resistant to strategies targeting one signaling pathway and that multiple signaling pathways may need to be targeted for maximal therapeutic efficacy. It is further suggested that BRAF mutational status is not predictive of response to MEK inhibition under three-dimensional culture conditions. [Mol Cancer Ther 2006;5(5):1136–44]

Introduction

The finding that >66% of melanomas harbor mutations in BRAF leading to constitutive activity in the mitogen-activated protein kinase (MAPK) pathway has raised expectations for targeted therapy in melanoma (1–3). Most of the reported mutations in BRAF are the activating V600E mutation, which works through the destabilization of the inactive form of the kinase, shifting the equilibrium towards the active form (4). *In vitro* studies have shown that BRAF is an oncogene in immortalized mouse melanocytes (5) and that selective down-regulation of BRAF V600E using RNA interference causes apoptosis and the reversal of the melanoma phenotype (6). Although BRAF would seem to be the obvious target in melanoma, it is likely that most of the oncogenic effects of BRAF are mediated through MAPK/extracellular signal–regulated kinase (ERK) kinase (MEK). Inhibitors of MEK are readily available for preclinical and clinical evaluation. A recent study showed that BRAF mutation status is associated with selectivity to treatment with MEK inhibition compared with those cells with wild-type BRAF, and it was suggested that MEK inhibition would be a viable strategy for melanoma therapy (3).

Although much of recent melanoma therapy research has focused on the BRAF/MEK/ERK pathway, a number of other pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor κB, Janus-activated kinase/signal transducers and activators of transcription, and β-catenin are also known to be active in melanoma (7). Of these, the PI3K/Akt pathway plays a critical role in the oncogenic behavior of melanoma through its ability to suppress apoptosis (8) and control cell cycle entry via the regulation of both cyclin D1 and myc (9, 10). In the present study, we have investigated whether targeting either the MEK/ERK pathway or the PI3K/Akt pathway is a viable approach to melanoma therapy. As preclinical studies *in vitro* are often poorly predictive of the outcome of clinical studies, we have developed a novel cell culture model wherein human tumor cells are grown as three-dimensional spheroids and then implanted into collagen gels to mimic the tumor architecture and microenvironment. Most other preclinical cell culture models fail to account for the fact that tumor cells exist embedded within a three-dimensional stromal matrix that includes other cell types. The demonstration that anti-β1-integrin antibodies reverse the malignant phenotype of breast cancer cells in

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three-dimensional, but not two-dimensional, culture is a pertinent example of marked differences seen under the different experimental conditions (11). In the current study, we have developed a novel model of melanoma whereby melanoma cells are grown under nonadherent conditions, which permits the formation of three-dimensional aggregations or spheroids. Once formed, the spheroids were harvested and implanted into a matrix of collagen I, which mimics the microenvironment of human skin. Using this three-dimensional spheroid model, it was found that cell lines derived from melanoma metastases were highly resistant to both PI3K and MEK inhibitors. This was in marked contrast to the responses seen to the same cell lines grown under standard two-dimensional cell culture conditions. Further studies revealed that only the combination of PI3K and MEK inhibitors had any antitumor activity in three-dimensional culture and led us to conclude that targeting only one signaling pathway, such as MEK, may not be a viable strategy for treating the most aggressive of melanomas.

Materials and Methods

Cell Culture

Human melanoma cells were isolated and cultured as described in ref. 12. The dominant-negative p85 PI3K adenovirus was provided by W. Ogawa (Kobe University, Kobe, Japan) and used as previously described (13).

Adherent Cell Proliferation Analysis

Cells were plated into a 96-well plate at a density of 2.5×10^4/mL and left to grow overnight. Cells were treated with increasing concentrations of LY294002 (0.01–30 μmol/L; Calbiochem, Darmstadt, Germany) or U0126 (0.01–30 μmol/L; Calbiochem) in triplicate. In each instance, cells were left to grow for 72 hours before being treated with 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 hours (Sigma, St. Louis, MO). After this time, the medium was rapidly removed and the MTT crystals were solubilized using DMSO. The resulting absorbance was read in a plate reader at 560 nm. Absorbance readings were subtracted from the value of blank wells; the reduction in cell growth was calculated as a percentage of control absorbance in the absence of any drug. Data show the mean of at least three independent experiments ±SE.

Western Blot Analysis

Proteins were extracted and blotted as described in ref. 12. After analysis, Western blots were stripped once and reprobed for β-actin to show even protein loading. Antibodies to phospho-glycogen synthase kinase 3β (GSK-3β), cyclin D, and phospho-ERK were from Cell Signaling Technology (Beverly, MA). The antibody for $p27^{Kip-1}$ was from BD PharMingen (San Jose, CA) and the monoclonal antibody to β-actin was from Sigma.

Three-Dimensional Spheroid Growth

Melanoma spheroids were prepared using the liquid overlay method. Briefly, 200 μL of melanoma cells (25,000/mL) were added to a 96-well plate coated with 1.5% agar (Difco, Sparks, MD). Plates were left to incubate for 72 hours, by which time cells had organized into three-dimensional spheroids. Spheroids were then harvested using a P1000 pipette. The medium was removed and the spheroids were implanted into a gel of bovine collagen I containing EMEM, L-glutamine, and 2% fetal bovine serum. Normal 2% melanoma medium was overlaid on top of the solidified collagen. Spheroids were treated with either LY294002 (1–50 μmol/L), U0126 (1–50 μmol/L), or LY294002 and U0126 in combination before being left to grow for 72 hours. Spheroids were then washed twice in PBS before being treated with calcein-AM and ethidium bromide (Molecular Probes, Eugene, OR) for 1 hour at 37°C according to the instruction of the manufacturer. After this time, pictures of the invading spheroids were taken using a Nikon-300 inverted fluorescence microscope.

Immunofluorescence Microscopy

Melanoma cells were seeded onto glass coverslips in six-well plates and incubated overnight. Cells were then fixed in 4% formaldehyde solution (Electron Microscopy Systems, Hatfield, PA) and permeabilized with Triton X-100 (0.2% v/v) before being blocked in PBS containing 1% bovine serum albumin. Primary antibody incubations (1:200) were done at 37°C in a humidified atmosphere for 1 hour. Coverslips were then washed thrice in PBS before being incubated with secondary antibodies for 1 hour under similar conditions to the primary antibody (dilution factor, 1:250). The monoclonal antibody to the p85α PI3K subunit was from BD PharMingen and the antimouse Texas red–conjugated secondary antibody was from Vector Laboratories (Burlingame, CA). Coverslips were then further washed in PBS and sterile water before being treated with VectorShield (Vector Laboratories) antifade and analyzed using either immunofluorescence or confocal microscopy.

Cell Cycle Analysis

Cell cycle analysis was done after treatment with kinase inhibitors for 24 hours (U0126, 10 μmol/L; LY294002, 20 μmol/L) and after incubation with kinase inhibitors for 24 hours followed by a further 24, 48, or 72 hours without inhibitors. Cells (1×10^6–2×10^6) grown adherently on a culture dish were harvested, washed in cold PBS, and resuspended in 200 μL cold PBS. Cells were fixed by adding 200 μL of above-mentioned cell solution to 4 mL of 70% ethanol and incubated on ice for at least 1 hour (typically overnight). Intracellular DNA was labeled with 200 μL propidium iodide solution containing 40 μg/mL propidium iodide and 100 μg/mL RNase in PBS and incubated at 37°C for 30 minutes in the darkness. Samples were analyzed using EPICS XL (Beckman-Coulter, Inc., Miami, FL). The cell cycle profile was obtained by analyzing 15,000 cells.

Analysis

Unless otherwise stated, all data show the mean of at least three independent experiments and, where appropriate, ±SE.

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Results

Characterization of the Melanoma Spheroids

Initial studies revealed that a high proportion (18 of 26) of melanoma cell lines formed organized spheroids when grown on top of hard (1.5%) agar for 72 hours (Supplementary Fig. S1A).[3] After this time, the spheroids were harvested and implanted into a gel of bovine collagen, which was then overlaid by medium. It was noted that the spheroids invaded into the collagen and the extent of invasion was correlated with the melanoma tumor stage. Radial growth phase WM35 primary melanoma cells were poorly invasive, the vertical growth phase WM793 primary melanoma cells invaded slowly, whereas the metastatic 1205Lu line invaded rapidly (Supplementary Fig. S1B).[3] Viability testing using a mixture of calcein-AM/ethidium bromide revealed that the spheroids were viable and metabolically active both when on top of the agar and once implanted into the collagen gels (Supplementary Fig. S1C).[3]

Inhibition of MEK Blocks the Growth of Aggressive Melanoma Cells in Two-Dimensional but not Three-Dimensional Culture

One of the best characterized signaling pathways in melanoma is the MAPK pathway (1), which is activated by BRAF V600E mutations as well as autocrine growth factor loops (1). With the exception of the C8161 cell line (14), all of the melanoma cell lines used in this study harbored the V600E BRAF mutation. Increasing concentrations of the MAPK inhibitor U0126 reduced the growth of the four melanoma cell lines in monolayer culture (Fig. 1A). When grown adherently, the radial growth phase melanoma WM35 was especially susceptible to the growth inhibitory effects of U0126 whereas WM793, 1205Lu, and C8161 were less sensitive but were still inhibited with a similar potency (Fig. 1A). However, phospho-ERK levels were reduced in 1205Lu cells at concentrations much lower than those needed to block growth in adherent culture (Fig. 1B). U0126 reduced cell growth and viability only in the radial growth phase and vertical growth phase primary melanoma cell lines in three-dimensional spheroid culture (Fig. 1C). In this instance, the effects were very striking with virtually no viable cells remaining after 72 hours of treatment (Fig. 1C). In contrast, U0126 had no effect on the growth and invasion of three of four (1205Lu, C8161, and 451Lu) melanoma lines derived from metastases (Fig. 1C). Treatment of the WM164 cell line, which was also derived from a metastasis, resulted in reduced growth and survival (data not shown). In all cases, similar results were seen with another MEK inhibitor, PD 98059 (data not shown).

Inhibition of PI3K Blocks the Growth of Aggressive Melanomas in Two-Dimensional but not Three-Dimensional Culture

Treatment of melanoma cells with the PI3K inhibitor LY294002 led to concentration-dependent decreases in cell growth in the WM35, WM793, 1205Lu, and C8161 cell lines when grown in adherent culture (Fig. 2A). The decreases in cell growth were paralleled by decreases in the activity of the downstream mediator of PI3K, phospho-GSK-3β (Fig. 2B). The inhibition of melanoma cell growth following LY294002 treatment was not associated with induction of apoptosis as shown by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining (data not shown). When these experiments were repeated on the spheroids, LY294002 blocked growth and reduced viability of the biologically earlier WM35 and WM793 cells, as shown by increased red ethidium bromide staining and smaller spheroid size (Fig. 2C). In contrast, increasing concentrations of LY294002 had no effect on 1205Lu, 451Lu, or C8161 metastatic cells when grown as collagen-implanted spheroids, indicating that, under these conditions, the cells were resistant (Fig. 2C).

Inhibition of PI3K or MEK Induces a Reversible Cell Cycle Arrest in Metastatic Cells

Treatment of adherent 1205Lu or C8161 cells with 10 μmol/L U0126 or 10 μmol/L LY294002 for 72 hours led to a growth inhibition of ~40% as shown by MTT assays. Simultaneous treatment with both inhibitors reduced growth by 60%. This growth inhibition was readily reversible, as cells incubated without inhibitors for a further 72 hours showed the same extent of growth as untreated cells (Fig. 3A). Under adherent culture conditions, the MEK inhibitor U0126 blocked growth through the up-regulation of the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip-1}$ (Fig. 3B) whereas the PI3K inhibitor LY294002 did not induce an up-regulation of p27. Cell cycle analysis revealed that this reduction in growth was associated with a G_1-phase cell cycle arrest but not apoptosis (Fig. 3C and D). The MEK inhibition–induced cell cycle arrest was readily reversible with the melanoma cells reentering the cell cycle 24 hours after removal of the drug (Fig. 3E and F).

Expression of the Dominant-Negative p85 Subunit of PI3K Does Not Reduce Viability of Metastatic Melanoma Cells in Three-Dimensional Culture

It was unclear whether growing the metastatic melanoma cells as spheroids increases active drug transport and therefore reduces the intracellular concentrations of PI3K/MEK or there was less reliance on these pathways in three-dimensional culture. To answer this question, we infected 1205Lu cells with an adenoviral vector encoding for dominant-negative p85 subunit of PI3K. Infection with the adenovirus increased dominant-negative p85 protein expression and reduced the activity of phospho-GSK-3β (Fig. 4A and B). It also reduced the growth of adherent 1205Lu cells by 40%, which was similar to the growth reduction seen following LY294002 (10 μmol/L) treatment. Spheroids were also made from 1205Lu cells infected with dominant-negative p85 PI3K and implanted into collagen. After 72 hours, the dominant-negative p85 PI3K spheroids were less invasive but still viable (Fig. 4C and D). Further studies using higher concentrations of LY294002 (50 μmol/L) also showed a reduction in melanoma cell invasion. These data show that dominant-negative p85 PI3K, like LY294002, reduced 1205Lu cell growth in two-dimensional but not three-dimensional culture.

[3] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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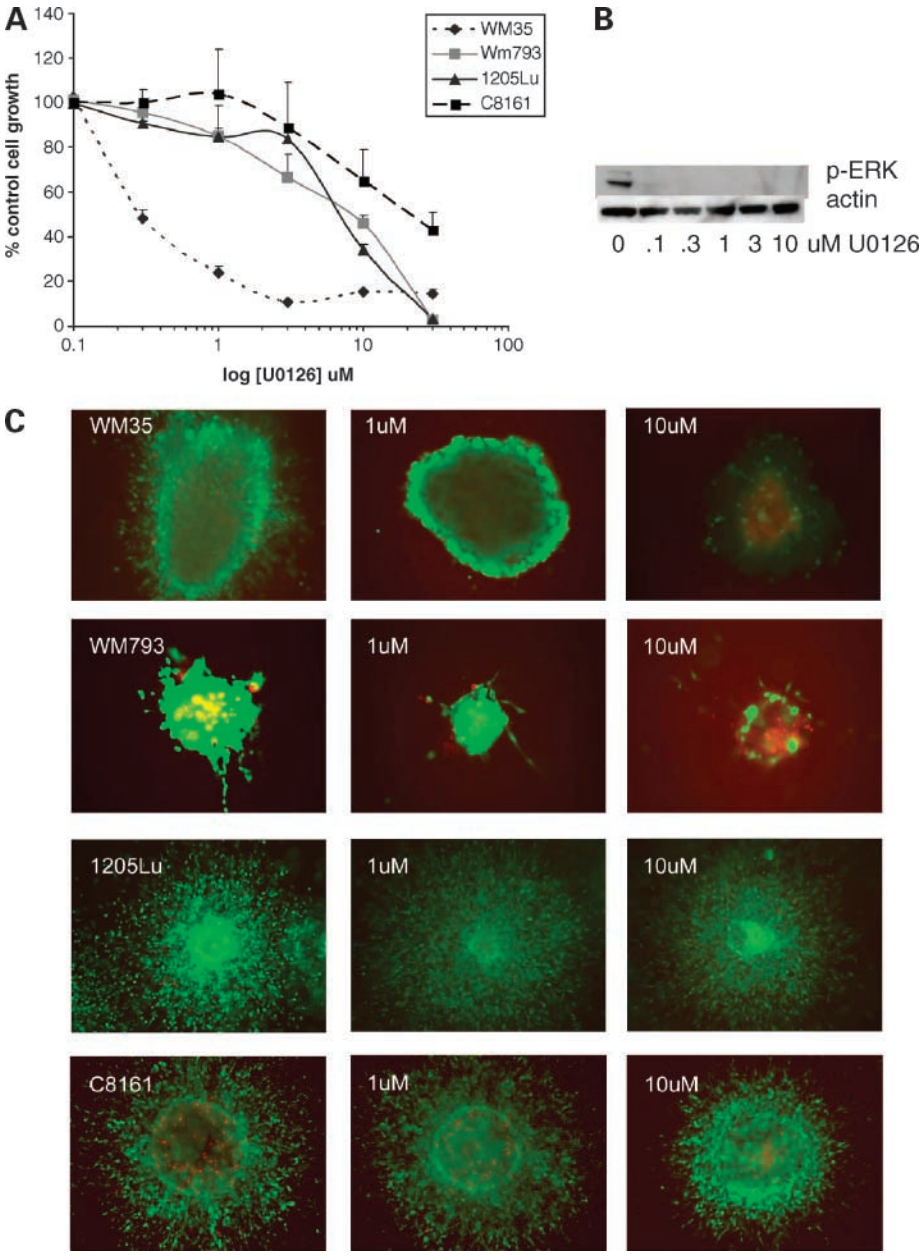

Figure 1. Inhibiting the MAPK pathway blocks the growth of radial growth phase and vertical growth phase, but not metastatic, melanoma in three-dimensional culture. **A,** cells were treated with increasing concentrations of UO126 (0.1 – 30 μmol/L) for 72 h before being treated with MTT. The resulting changes in absorbance were read in a plate reader at 480 nm and expressed as a percentage of control absorbance. *Points,* mean of three independent experiments; *bars,* SE. **B,** reduction of phospho-ERK activity following U0126 treatment (0.1 – 10 μmol/L) in 1205Lu cells. Blots were stripped and reprobed with an anti-actin antibody to confirm equal protein loading. **C,** melanoma cells were grown under nonadherent conditions for 72 h until spheroids had formed. Spheroids were then harvested and implanted into a collagen gel before being treated with UO126 (1 and 10 μmol/L). After 72 h, cells were treated with the cell viability kit wherein living cells stain green and dead cells stain red. Representative of three independent experiments. Magnifications, ×10 (WM35 and WM793); ×4 (1205Lu and C8161).

Evidence for Synergy between MEK and PI3K Inhibitors in Aggressive Melanoma Lines Grown in Three-Dimensional Culture

As multiple signaling pathways are known to be active in metastatic melanoma, the cells were treated with inhibitors to PI3K and MAPK simultaneously (Fig. 5A). In 1205Lu cells grown under adherent conditions, addition of either LY294002 or U0126 reduced cell growth by either 24.4 ± 7.0% or 25.0 ± 7.4%, respectively. Addition of both inhibitors at the same time was additive, reducing cell growth by 54.0 ± 3.2%. In three-dimensional culture, addition of the two inhibitors showed synergy, inhibiting both the growth and invasion of 1205Lu cells (Fig. 5A). However, the cells remained viable and removal of the inhibitors was followed by regrowth of the spheroids. The resistance of the metastatic melanoma lines in this model was also shown in 451Lu and C8161 cells (data not shown).

The Tumor Environment Modulates Drug Resistance

The influence of the tumor microenvironment on drug resistance was examined in 1205Lu cells. Survival signals are often received from the serum in cell culture medium. To investigate this, the spheroids were grown in serum-free collagen and were supplemented with serum-free medium. The control 1205Lu spheroids were still viable and invaded into the collagen (Fig. 5B). However, they

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were no longer resistant to U0126 and became nonviable, indicating that serum-derived signals can overcome inhibition of the MAPK pathway. Interestingly, 1205Lu cells were still resistant to LY294002, suggesting that this pathway was less critical than the MAPK pathway.

The role of the collagen gel in modulating drug resistance was investigated by treating the spheroids on top of agar with the PI3K and MAPK inhibitors. In this instance, both LY294002 and U0126 reduced cell viability. The combination of the two drugs was synergistic and completely blocked cell survival (Fig. 5C). In the absence of the collagen matrix, the spheroids were no longer resistant to the signal transduction inhibitors, suggesting that critical survival signals involved in drug resistance are received from the stromal microenvironment (15).

Discussion

Standard chemotherapy drugs have failed in large-scale clinical trials for melanoma. To date, the only Food and Drug Administration–approved drug for melanoma is the alkylating agent dacarbazine (DTIC), which when given as a single agent has a response rate of 5% to 10% (16). Novel approaches to melanoma therapy are therefore urgently needed. Recent work has focused on targeting signaling pathways which are known to be active in melanoma (7). Although many signaling cascades are known to be active in melanoma, most interest has centered on the BRAF/MEK/ERK pathway (2). Questions still remain over whether this pathway would represent a suitable therapeutic target in melanoma and there is still controversy on whether BRAF is a strong enough oncogene to drive

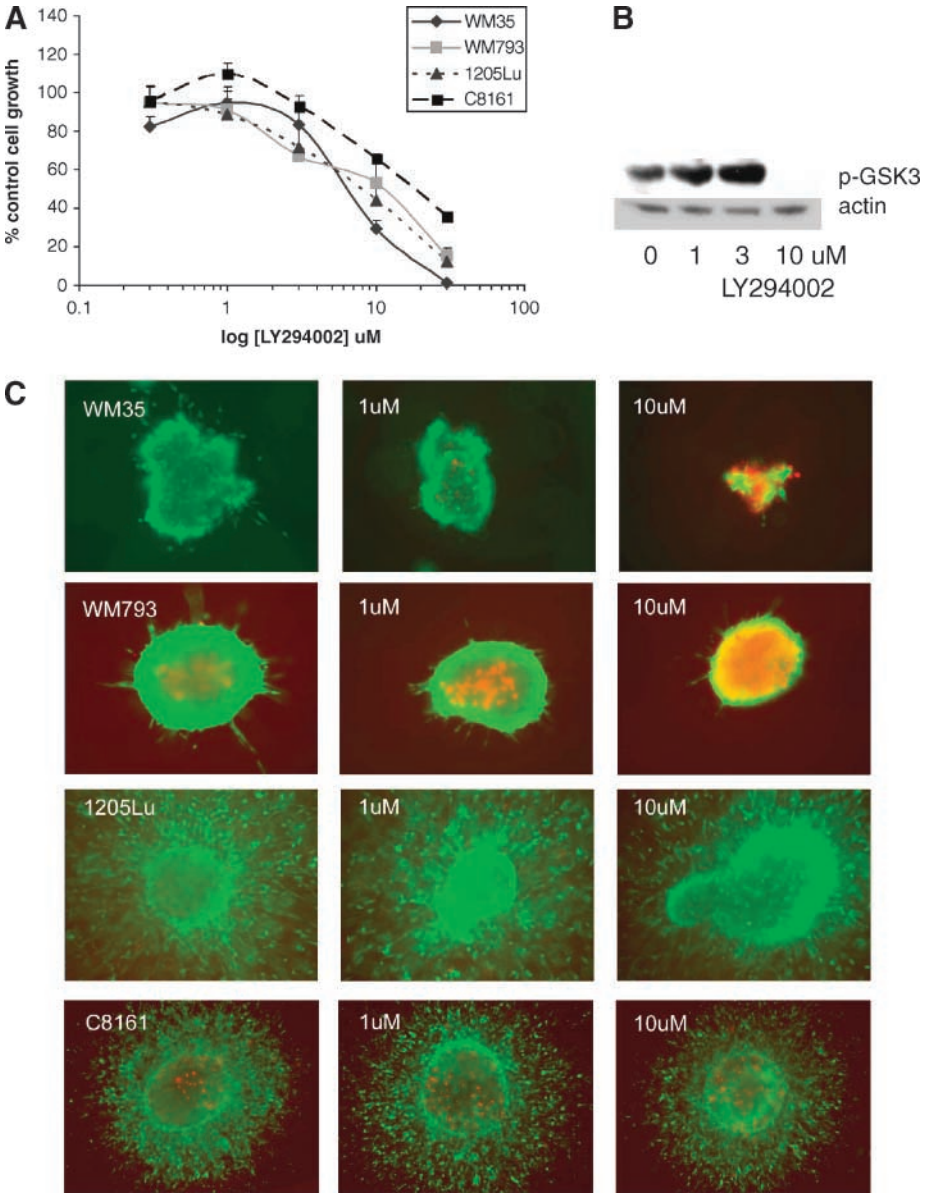

Figure 2. Inhibiting the PI3K pathway blocks the growth of radial growth phase and vertical growth phase, but not metastatic, melanoma in three-dimensional culture. **A,** cells were treated with increasing concentrations of LY294002 (0.3–30 μmol/L) for 72 h before being treated with MTT. The resulting changes in absorbance were read in a plate reader at 480 nm and expressed as a percentage of control absorbance. *Points,* mean of three independent experiments; *bars,* SE. **B,** reduction of phospho-GSK-3β activity following LY294002 treatment (1–10 μmol/L) in 1205Lu cells. Blots were stripped and reprobed with an antiactin antibody to confirm equal protein loading. **C,** melanoma cells were grown under nonadherent conditions for 72 h until spheroids had formed. Spheroids were then harvested and implanted into a collagen gel before being treated with LY294002 (1 and 10 μmol/L). After 72 h, cells were treated with the cell viability kit wherein living cells stain green and dead cells stain red. Representative of three independent experiments. Magnifications, ×10 (WM35 and WM793); ×4 (1205Lu and C8161).

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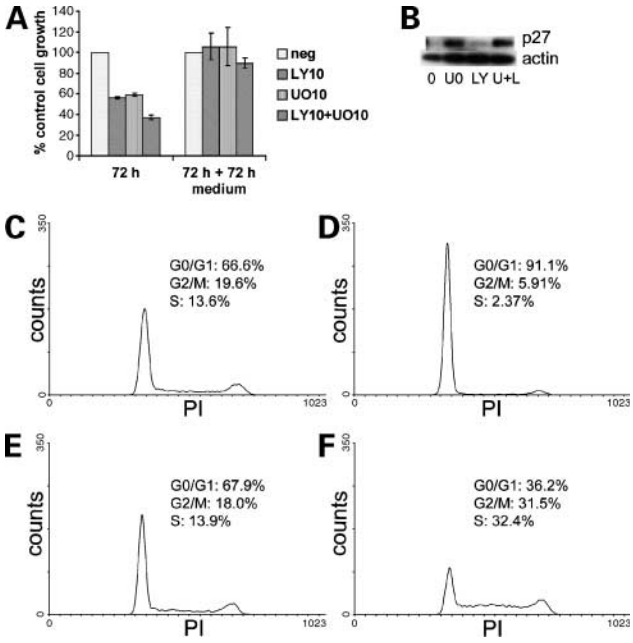

Figure 3. Inhibition of cell growth by U0126 is caused by reversible G_1 cell cycle arrest and up-regulation of the CDK inhibitor $p27^{Kip-1}$. **A,** adherent 1205Lu (or C8161 cells) were treated with DMSO (*neg*), 10 μmol/L U0126 (*U010*), 10 μmol/L LY294002 (*LY10*), or both simultaneously (*LY10 + U010*) for 72 h or for 72 h and a further 72 h after removal of the drugs and analyzed by MTT assays. Data were normalized to the negative controls. Note the reversibility of the growth inhibition. **B,** adherent 1205Lu cells were treated with DMSO (*0*), 10 μmol/L U0126 (*U0*), 10 μmol/L LY294002 (*LY*), or both simultaneously (*U + L*) for 24 h before total protein extractions. Total protein was analyzed via Western blotting with an antibody against p27. Blots were stripped and reprobed with an antiactin antibody to confirm equal protein loading. **C** to **F,** adherent cells were treated with DMSO (**C**) and 10 μmol/L U0126 (**D**) for 24 h. Cells treated with U0126 were found to enter into G_1-phase cell cycle arrest. In other experiments, cells were treated with DMSO (**E**) for 48 h or 10 μmol/L U0126 (**F**) for 24 h followed by washout and no drug for a further 24 h. It was noted that after the removal of U0126 (**F**), the cells reentered S phase. Samples were analyzed using EPICS XL (Beckman-Coulter). The cell cycle profile was obtained by analyzing 15,000 cells.

melanocytic transformation (17). The finding that there are V600E BRAF mutations in nontumorigenic melanocytic nevi (18) then lacking in early-stage radial growth phase melanomas, only to appear at the later vertical growth phase melanomas (19), is difficult to interpret and requires further study.

The role of constitutive MEK activity in melanoma is more well defined and includes increased cell proliferation, enhanced matrix metalloprotease secretion, and invasion (reviewed in ref. 1). A recent study has claimed that the presence of BRAF mutations in melanoma confers selectivity to MEK inhibitors and that MEK inhibition offers a rational therapeutic approach to melanomas with BRAF mutations (3). However, there is little evidence to suggest that MEK inhibition induces apoptosis in tumor cells and it seems more likely that these drugs would instead only induce cell cycle arrest. The current study has investigated whether targeting MEK is a viable strategy for treating melanoma cell lines derived from radial growth phase, vertical growth phase, and metastatic lesions. With the exception of the C8161 cell line, all of the melanoma cell lines tested harbor the V600E mutation in BRAF.

In agreement with other work, it was noted that increasing concentrations of the MEK inhibitor U0126 reduced the growth of all of the melanoma cell lines tested under normal adherent culture conditions (3). Cell cycle analysis revealed that this reduction in growth was associated with loss of cells in S phase and up-regulation of the CDK inhibitor $p27^{Kip-1}$ and G_1-phase cell cycle arrest. Cell proliferation is regulated during the first pause in the cell cycle at the G_1 checkpoint. Under normal conditions, cell growth is tightly controlled by the growth-promoting cyclins and CDKs of which the activity is opposed by the CDK inhibitors. The progression through the G_1-phase checkpoint is driven by CDK4, CDK6, and their interaction with the cyclin D family of proteins (20). Activation of the MAPK pathway via ERK-induced regulation of cyclin D1 expression is a key regulator of the G_1 transition (21). The ability of U0126 to arrest melanoma cells in the G_1 phase of the cell cycle is consistent with a requirement for constitutive MAPK activity in the progression of melanoma cells through this checkpoint. The effects of U0126 on G_1 cell cycle arrest were readily reversible with the melanoma cells reentering the cell

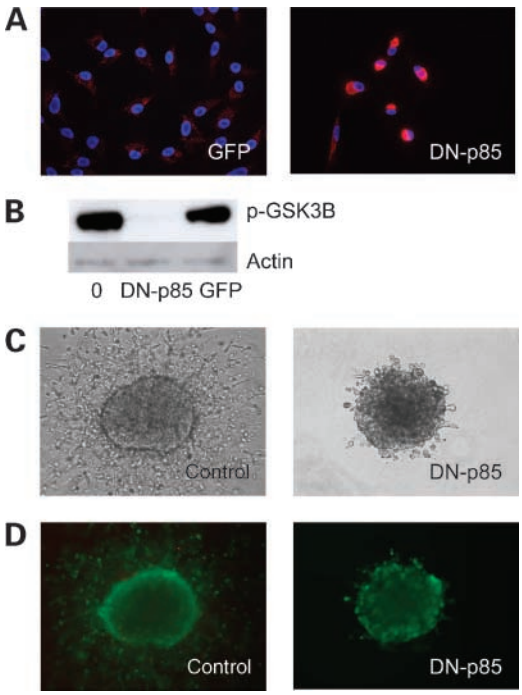

Figure 4. Dominant-negative p85 PI3K reduces the activity of GSK-3β and spheroid invasion but not viability. **A,** infection with adenovirus for dominant-negative p85 (*DN-p85*) PI3K increases the expression of the p85 subunit in 1205Lu cells (*red*). **B,** infection of dominant-negative p85 PI3K reduces downstream phospho-GSK-3β activity. **C,** dominant-negative p85 PI3K reduces the ability of 1205Lu spheroid cells to invade. **D,** dominant-negative p85 PI3K does not affect cell viability. Magnifications, ×40 (**A**); ×10 (**C** and **D**).

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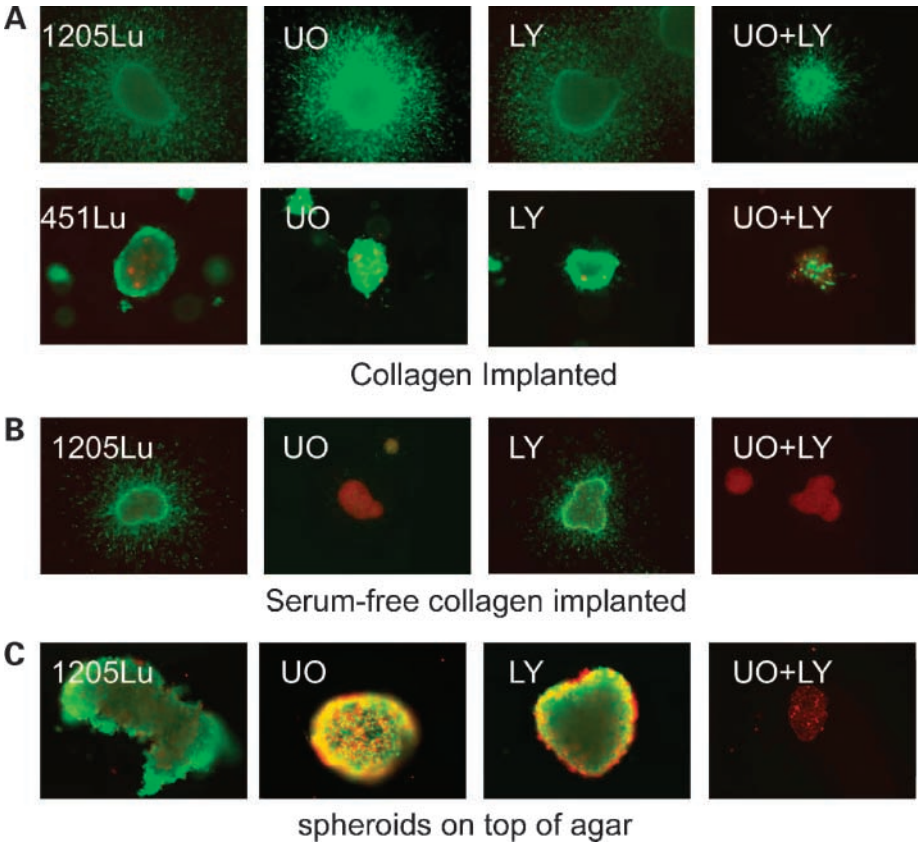

Figure 5. Synergy between LY294002 and UO126 in metastatic melanoma lines in three-dimensional culture. **A,** melanoma cells were grown under nonadherent conditions for 72 h until spheroids had formed. Spheroids were then harvested and implanted into a collagen gel before being treated with either UO126 (10 μmol/L), LY294002 (10 μmol/L), or the two drugs in combination. After 72 h, cells were treated with the cell viability kit wherein living cells stain green and dead cells stain red. Magnification, ×10. **B,** removal of serum reduces drug resistance in 1205Lu cells. Spheroids were prepared as above and implanted into serum-free collagen. Serum-free medium was overlaid before being treated with either UO126 (10 μmol/L), LY294002 (10 μmol/L), or the two drugs in combination. Cell viability was measured as above. **C,** lack of collagen implantation reduces drug resistance. Spheroids were prepared as above and left on top of the agar layer before being treated with either UO126 (10 μmol/L), LY294002 (10 μmol/L), or the two drugs in combination. Cell viability was measured as above. Representative of three independent experiments.

cycle 24 hours after removal of the drug. Consistent with these results, it was also noted that U0126 did not induce apoptosis in any of the melanoma cells grown in two-dimensional culture, as assessed by propidium iodide staining and the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay (data not shown).

Interestingly, there was no correlation between the concentrations of U0126 required to block phospho-ERK activity and to inhibit cell growth. Indeed, for the 1205Lu cell line, the concentrations of U0126 required to inhibit cell growth were nearly 100-fold higher than those required to block kinase activity. In other studies, it was shown that sensitivity to MEK inhibition was correlated with BRAF mutational status (3) and that cell lines in which MEK inhibition and growth arrest were disconnected were wild-type for both BRAF and N-Ras (3). However, in our study, there was only a close correlation between MEK inhibition and attenuation of cell growth in the early-stage WM35 cells, which harbored the V600E BRAF mutation. Therefore, in our cell lines, it seemed unlikely that the correlation between the U0126 concentrations required for MEK inhibition and those for growth inhibition was due to BRAF mutational status.

When the same panel of melanoma cell lines were grown under three-dimensional collagen-implanted spheroid culture conditions, the results were strikingly different. It was found that U0126 treatment completely inhibited the growth and survival of the cells derived from earlier melanoma lesions (radial growth phase and vertical growth phase) but had little effect on any of the metastatic lesions. It seems that under three-dimensional culture conditions, the metastatic melanoma lines were very resistant to MEK inhibition. Further experiments using U0126 concentrations up to 50 μmol/L also showed a lack of inhibitory effects on cell growth or invasion. These results were in marked contrast to those from the recent study of Solit et al. (3), which showed that melanoma cells with BRAF mutations were susceptible to MEK inhibition. One possible explanation for this discrepancy is the choice of melanoma lines. In our study, three of four of the most aggressive melanoma lines (1205Lu, 451Lu, and C8161) were resistant to MEK inhibition whereas one (WM164) was not, suggesting that not all metastatic melanoma lines are equally resistant. Of these lines, two of the three resistant lines were BRAF mutant (1205Lu and 451Lu) and one was BRAF wild-type (C8161). As even the nonresistant line (WM164) harbored the V600E BRAF mutation, this seemed unlikely to provide the explanation for the difference in sensitivity observed. It is also pertinent to note that the resistance was only seen under three-dimensional culture conditions and was not predicted by any of the assays done using two-dimensional cell culture techniques.

To ascertain whether this acquired resistance seen under three-dimensional culture conditions was specific to the

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MEK/ERK pathway, we did similar experiments targeting the PI3K/Akt pathway using LY294002. In these studies, LY294002 blocked the growth of all four of the melanoma cell lines with a similar potency in two-dimensional culture. Again, like U0126, the metastatic melanoma lines (three of four tested) were completely resistant to the inhibitor when grown in three-dimensional spheroid culture. To investigate the mechanism of this acquired resistance in the three-dimensional culture, the 1205Lu melanoma cell lines were infected with a dominant-negative form of the p85 subunit of PI3K and grown as spheroids. In this instance, we found that the melanoma cells were less invasive but still viable after 72 hours of growth, showing that the melanoma cells were able to survive in three-dimensional culture in the absence of PI3K activity. Similar results were also seen when the 1205Lu spheroids were treated with higher concentrations of LY294002 (50 μmol/L; data not shown). This suggested that resistance to LY294002 in three-dimensional culture was not through altered drug transport mechanisms. The underlying mechanisms of drug resistance of the metastatic cell lines are likely due to high activity in multiple prosurvival signaling pathways. The data presented in this study have shown that blocking one signaling pathway may not be a viable therapeutic strategy for the most aggressive melanomas and that multiple pathways may have to be targeted simultaneously. To further investigate this idea, we treated the aggressive melanoma lines with both LY294002 and U0126 simultaneously and found an additive effect in two-dimensional adherent culture. When the same drug treatment was applied to the aggressive melanoma lines, there was some synergism; the spheroids were smaller in size and were less invasive. However, some viable cells still remained, suggesting that this approach may not be curative.

It is possible that the observed resistance seen with the aggressive melanoma lines in three-dimensional culture is a result of the collagen matrix sequestering the drug or being somehow less permeable. We would argue that this is unlikely to be the case. The fact that spheroids derived from the radial growth phase and vertical growth phase lesions, which are the same size as those from the metastatic lines, are exquisitely sensitive to these inhibitors suggests that there are no issues with either permeability or drug absorbance onto the matrix. In addition, previous studies using fluorescent drug conjugates, such as BODIPY-taxol, have shown the rapid (<360 minutes) transport of drugs to the core of the spheroid (22).

In the final part of the study, we looked at the effects of modulating the microenvironment on melanoma sensitivity to MEK and PI3K inhibition and whether this played a role in the observed resistance. Removing the serum from the medium and the surrounding collagen completely reversed the resistance of 1205Lu spheroids to U0126 but not to LY294002. It therefore seemed that other extrinsic serum-derived survival signals were responsible for mediating resistance to MEK but not to PI3K/Akt inhibition and suggested that activity in the MEK/ERK pathway is more critical than that in PI3K/Akt for melanoma survival. The fact that serum removal reversed the resistance of 1205Lu cells to U0126 further adds to the argument that the drug is able to reach the spheroid.

We further showed a role for the collagen mediating resistance to both U0126 and LY294002. When spheroids on top of agar were treated with either of the inhibitors, the cells underwent substantial cell death, which was not seen when the spheroids were implanted into the collagen. It is likely that integrin-collagen engagement activates outside-in signaling, which is critical for cell survival. Recent work has shown that engagement of α_v integrin with a three-dimensional collagen matrix increases melanoma survival via suppression of p53 activity (23). The observation that modulating the microenvironment alters drug sensitivity suggests that a better understanding of the tumor-stromal interaction will allow the development of strategies to overcome drug resistance in melanoma.

Although the preliminary *in vitro* data look promising (5, 24), it is unclear whether BRAF or MAPK inhibition will translate into the clinical treatment of melanoma. This contention is supported by a limited number of clinical studies on the MEK inhibitor CI-1040, which has shown pharmacologic activity at the level of phospho-ERK inhibition but little clinical benefit above stable disease (25, 26). It seems that the responses to MEK inhibition *in vivo* are more complex than those seen under normal tissue culture conditions and that the microenvironment may modulate drug resistance in unforeseen ways. Clearly, these results have important implications for the future therapeutic use of MEK inhibitors and suggest that a greater knowledge of signaling within the tumor microenvironment is required to overcome therapeutic resistance.

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Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases

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