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Epha2 is a Critical Oncogene in Melanoma

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Abstract

EphA2 is a member of the Eph family of receptor tyrosine kinases and is highly expressed in many aggressive cancer types, including melanoma. We recently showed that EphA2 is also upregulated by ultraviolet radiation and is able to induce apoptosis. These findings suggest that EphA2 may have different, even paradoxical, effects on viability depending on the cellular context and that EphA2 mediates a delicate balance between life and death of the cell. To functionally clarify EphA2's role in melanoma, we analyzed a panel of melanoma cell lines and found that EphA2 levels are elevated in a significant fraction of the samples. Specific depletion of EphA2 in high-expressing melanoma cells using shRNA led to profound reductions in cellular viability, colony formation and migration in vitro and a dramatic loss of tumorigenic potential in vivo. Stable introduction of EphA2 into low-expressing lines enhanced proliferation, colony formation and migration further supporting its pro-malignant phenotype. Interestingly, transient expression of EphA2 and/or $Braf^{V600E}$ in non-transformed melanocytes led to significant and additive apoptosis. These results verify that EphA2 is an important oncogene and potentially a common source of "addiction" for many melanoma cells. Moreover, acute induction of EphA2 may purge genetically-susceptible cells thereby uncovering a more aggressive population that is in fact dependent on the oncogene.

Keywords

Melanoma; EphA2; oncogene; survival

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Introduction

EphA2 belongs to the large Eph family of receptor tyrosine kinases (RTKs), which are known to participate in various developmental processes including mammary epithelial growth and branching morphogenesis and patterning of the visual system (Andres et al 1994, Connor et al 1998, Ganju et al 1994, Moore-Scott et al 2007, Ruiz and Robertson 1994, Vaught et al 2009). Like other developmental genes, EphA2 has also been implicated in tumor formation. The *EPHA2* locus maps to chromosomal region 1p36, which is a region frequently altered in tumors of neuroectodermal origin such as neuroblastoma and melanoma (Sulman et al 1997). Earlier studies have reported that EphA2 is overexpressed in many cancer types including cutaneous melanoma (Cui et al 2010, Easty et al 1999, Hendrix et al 2003, Kamat et al 2009, Kataoka et al 2004, Liu et al 2010, Lu et al 2008, Miyazaki et al 2003, Nemoto et al 1997, Seftor et al 2002, Walker-Daniels et al 1999, Zelinski et al 2001). There is some evidence that EphA2 might be involved in tumor cell differentiation (Bogan et al 2009) and plasticity (Parri et al 2009). Mechanistically, both kinase-dependent and independent roles for EphA2 have been implicated in cancer progression (Taddei et al 2009). Also, EphA2 appears to participate in cross-talk between the Ras-PI3k-Akt and Ras-MAPK pathways (Menges and McCance 2008). In melanomas, EphA2 levels correlate with aggressiveness (Hess et al 2006) though lucid details of its downstream effects are still lacking. Of note, Margaryan et al. recently demonstrated that downregulation of EphA2 in C8161 melanoma cells by anti-sense oligonucleotides led to suppression of growth (Margaryan et al 2009).

In a separate but related line of investigation, we recently found that EphA2 is an essential mediator of apoptosis in response to ultraviolet radiation (UVR) (Zhang et al 2008) - the leading environmental carcinogen involved in melanoma formation. We reported that the UVR-mediated upregulation of EphA2 occurs in human melanocytes, keratinocytes, and murine fibroblasts in a p53-independent but MAPK-dependent mechanism (Yang et al 2006, Zhang et al 2008). We also demonstrated that ectopic expression of EphA2 without UVR led to a brisk induction of apoptosis in primary melanocytes. Given the seemingly paradoxical nature of EphA2's acute apoptotic effects in melanocytes and its correlation to aggressiveness in melanoma cells, we set out to investigate the impact of EphA2 on melanoma cell survival and tumorigenicity. Our results suggest that melanoma cells, which harbor high EphA2 levels, are in fact "addicted" to this pro-survival factor thereby positioning EphA2 as a bona fide melanoma oncogene.

Results

Melanoma cells often exhibit high levels of EphA2

In prior studies (Yang et al 2006), we demonstrated a trend towards higher EphA2 transcript levels in melanoma cells compared to non-transformed melanocytes. To expand upon this characterization at the protein level, we tested a panel of 17 melanoma cell lines (Figure 1A) and found that most melanoma lines (11/17) exhibited much higher levels of EphA2 expression compared to melanocytes. Analysis of the *EPHA2* locus in these cell lines did not show any evidence of amplification by SNP-CGH arrays (data not shown).

Three of 17 lines showed relatively low expression (C32, Mel-Juso, MEWO) while another 3 lines lacked any appreciable amounts of EphA2 (WM164, SK-Mel-119 and K19) altogether. Since there was no evidence of *EPHA2* deletion by SNP-CGH in these non-expressors (data not shown), we speculated that at least some melanoma cell lines may have epigenetically silenced *EPHA2*. As shown in Figure 1B, addition of 5′-Aza 2′-deoxycytidine increased EphA2 in the low expression melanoma cells but not in the immortalized normal human melanocytes (NHMs). However, when proliferation was scored, there was a general trend towards growth suppression although these results are hard to interpret given the non-selective nature of 5′-Aza 2′-deoxycytidine treatment (Figure S1). Overall, these observations suggest that some melanomas do not require EphA2 for survival while others may possibly depend upon EphA2 for growth. In order to directly test the latter hypothesis, we set out to selectively deplete EphA2 in several high-expressing lines.

EphA2 is essential for melanoma survival and tumorigenicity

To assess for survival, we selectively suppressed EphA2 in 3 independent cell lines using shRNA against the molecule (Figure 2A). Within 96 hours after infection, there was a dramatic increase in apoptosis in all 3 lines as measured by the percentage of cells in sub-G1 fraction (Figure 2B; whole tracings Figure S2) and the percentage of FITC-Annexin positive cells (Figure 2C). Consonant with these apoptotic effects, cellular viability was also significantly impaired (Figure 2D) along with colony formation (Figure 2E).

We then speculated that p53 might be involved in the early apoptotic signal upon EphA2 deprivation. As shown in Figure 3A, EphA2 downregulation in UACC903 cells (*TP53* wildtype) led to a concomitant increase in p53, a finding replicated in two additional melanoma lines (Figure 3B, western analysis, Figure S3). However, does the apoptotic response due to EphA2 withdrawal require p53? In p53-depleted A375 melanoma cells ($A375^{shp53}$; Figure 3C, lanes 2 and 4), there was still a comparable increase in apoptosis when EphA2 was suppressed (Figure 3D) though the induction of p53 was appropriately blunted (Figure 3C, lanes 3 vs. 4). It is worth noting that the constitutive levels of EphA2 appear to be lower in the $A375^{shp53}$ cells consistent with prior evidence that EphA2 is also regulated by p53 (Dohn et al 2001). These results speak to a complex interaction between EphA2 and p53 and that the observed cellular effects of EphA2 withdrawal are not solely due to p53.

As shown in Figure 4A, transient EphA2 silencing in MM455 and WM115 cells significantly reduced transwell migration compared to control cells ($p=0.0005$ and $p=0.009$, respectively). Moreover, in 3-D cultures, reduction in EphA2 levels correlated with a loss of anchorage-independent growth (Figure 4B). In order to further validate EphA2 addiction in vivo, we next determined the effect of EphA2 suppression on tumorigenicity (Figure 4C). Compared to $UACC903^{shCON}$ cells, when $UACC903^{shEphA2}$ cells were injected into female Balb/C nude mice, there was a highly significant decrease in tumor formation (average tumor volume 394.4 mm^3 vs. 21.32 mm^3, $p<0.001$). Taken together, these in vitro and in vivo assays support the contention that EphA2 is a potent determinant of invasiveness and survival in melanoma and that a percentage of these tumors can be considered dependent on this oncogene.

Ectopic expression of EphA2 in melanoma cells promotes a more malignant phenotype

We next sought to study the effects of overexpressing EphA2 in non-expressing cells. Immediately after introduction of EphA2 into non-transformed immortalized melanocytes, there was a notable increase in the subG1 fraction, which was additive with ectopic $Braf^{V600E}$ expression (Figure 5A). This acute apoptotic effect was noted in our prior studies (Zhang et al 2008).

To investigate EphA2's longer-term effects, we selected two melanoma lines with low endogenous EphA2 (ie. WM164 and SK-Mel-119, Figure 1) and stably introduced EphA2. As shown in Figure 5B, both $WM164^{EphA2}$ and SK-Mel-119^{EphA2} cells exhibited significantly higher proliferation rates (1.33-fold and 1.45-fold, respectively) compared to their vector control counterparts.

EphA2 expression also led to significant increases in colony formation for both cell lines (WM164, p=0.015 and SK-Mel-119, p=0.012; Figure 5C). Lastly, we also found increased chemotactic migration in response to NIH3T3-conditioned media, a rich source of matrix proteins including fibronectin and vitronectin (Albini et al 1987) (Figure 5D) along with enhanced random migration in the scratch assay (Figure S5). The increased migration is also preserved even in the presence of mitomycin-C (Figure S4).

Discussion

The Eph system has been implicated in various cellular processes ranging from control of normal development, angiogenesis and vascularization (Walker-Daniels et al 2003) to oncogenesis of various tumor types (Han et al 2005, Herath et al 2006, Kataoka et al 2004, Miyazaki et al 2003, Nemoto et al 1997) including melanoma (Easty et al 1999, Hendrix et al 2003, Walker-Daniels et al 2003). In this study, we performed a comprehensive phenotypic analysis of EphA2 functionality and provide substantiating evidence that EphA2 is an essential survival factor in a subset of melanomas.

The most striking finding is the profound loss of viability and tumorigenicity in melanoma cells upon depletion of EphA2 levels; a subset of melanomas is thus clearly "addicted" to this oncogene. We also found an effect on migration and invasion in melanoma cells. This echoes some recent findings where EphA2 was reported to also promote migration of glioma and prostate cancer cells in a ligand-independent manner (Miao et al 2009). Miao et al. further implicated *PTEN* loss and Akt-mediated phosphorylation of EphA2 in their mechanism. Interestingly, in our melanomas, the 3 cell lines that showed profound growth suppression by EphA2 depletion all harbor *PTEN* lesions - one mutation (UACC903-p.Y76X) and two deletions (WM115-del^{Exon6}; MM455- del^{Exon6}). Although, differences in EphA2 function may exist between tumor cell types (glioma vs. melanoma) and or physiological effects (migration vs. survival), some element of mechanistic overlap is also likely.

Introduction of EphA2 into low-expressing melanoma cells enhanced proliferation, invasiveness and colony formation. With the absence of frequent structural changes at the *EPHA2* locus (i.e. amplification or recurrent mutations), *EPHA2* belongs to a class of

growth-promoting oncogenes distinct from *BRAF*^{V600E} or *NRAS*^{Q61}. EphA2's survival influence is likely mediated through quantitative, rather than qualitative, changes.

As demonstrated in our previous study (Zhang et al 2008) and replicated in the current one, acute overexpression of EphA2 in non-transformed melanocytes elicited an apoptotic response; this effect is similar to and additive with those observed for acute Braf^{V600E} expression (Figure 5A). It thus appears that, in the proper cellular context, EphA2 can behave as both a pro-apoptotic factor and a pro-survival factor. One potential unifying model is that of "oncogene overdose-addiction." In other words, cells that develop compensatory mechanisms to survive the oncogenic stress conferred by EphA2 induction subsequently become reliant upon the survival-promoting effects of EphA2. This life/death duality is not unique to EphA2. Both apoptosis (Wajapeyee et al 2008) and addiction (Hingorani et al 2003, Wellbrock et al 2004) have also been associated with the common melanoma oncogene, Braf^{V600E}. Also, in a recent study of the prototypic death receptor, CD95 (i.e. Fas or APO-1), abrogation of CD95 expression resulted in substantial growth retardation of ovarian, colon, liver, renal and breast cancer lines (Chen et al); these various tumors have apparently come to rely on some element of the "pro-apoptotic" CD95 system for survival. Thus, susceptible cells which succumb to an initial oncogenic stress or pro-apoptotic signal (i.e. "overdose") may be less adapted for survival and purged from the tumor population; the stress-resistant cells exist in a genetic context which favors oncogenic signaling and then become reliant on the oncogene for growth (i.e. "addiction").

While many melanoma lines exhibit greater expression of EphA2 compared to non-transformed melanocytes, some cells appear to epigenetically silence *EPHA2*. The control of intracellular EphA2 levels is clearly complex and may be heterogeneous even within a single tumor. There are studies that showed that EphA2 levels may be higher among ulcerated and mitogenic melanomas (Straume and Akslen 2002) and along invasive fronts of the tumor (Margaryan et al 2009); however, overall prognosis does not appear to be influenced by EphA2 expression (Straume and Akslen 2002). Given the large number of Eph RTKs, it is possible that other members of the Eph family may be the source of oncogenic sustenance in cells where EphA2 is not expressed.

In summary, we provide compelling data that EphA2 behaves as an essential survival factor in melanoma cells. In cells that overexpress EphA2, genetic suppression of the molecule leads to rapid loss of viability. However, in non-transformed cells, acute induction of EphA2 triggers a profound apoptotic response that may purge a less adaptive population of cells thereby establishing one possible mechanism of melanocytic tumor promotion after UV exposure. This overdose-addiction model could potentially be leveraged for therapeutic gain in the clinical setting.

Materials and Methods

Antibodies and inhibitors

Antibodies used in this study were as follows: polyclonal anti-EphA2 (C-20), mouse monoclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse

conjugated horseradish peroxidase and goat anti-rabbit IgG-HRP (Cell Signaling Technologies, Beverly, MA).

Cell culture

Both normal human melanocytes (NHM) (Invitrogen, Gibco Cell Culture, Portland, OR) and immortalized NHMs (gift from Hans Widlund, Harvard Medical School, Boston, MA) were maintained in Medium-254 (Invitrogen, Gibco Cell Culture, Portland, OR) supplemented with human melanocyte growth supplement and 1% penicillin/streptomycin. Melanoma cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) containing 1% penicillin/streptomycin. Cells were treated with methylation inhibitor 5′-Aza-2′-deoxycytidine (Sigma Aldrich, St. Louis, MO) at the indicated concentrations for 48 hours.

Immunoblot analysis

SDS-PAGE was done according to standard protocols. Total cell lysates (20–25 μg) were diluted in 6x Laemmle buffer (Boston Bioproducts, Inc, Ashland, MA) and loaded onto 4–20%, or 10% precast gels (Bio-Rad Laboratories, Hercules, CA), transferred onto Nitrocellose membrane (Bio-Rad Laboratories, Hercules, CA), and blocked with 5% nonfat milk (Bio-Rad Laboratories, Hercules, CA d) in Tween 0.1%/TBS or 5%BSA (Fisher Scientific, Pittsburgh, PA) in TBS/Tween20 0.1%. Primary antibody dilutions used in this study were as follows: polyclonal anti-EphA2 (1:300) and polyclonal anti-p53 (1:500). Loading equivalence was monitored with monoclonal anti–α-tubulin (1:150) or monoclonal anti-GAPDH (1:3,500) and gels were visualized by enhanced chemiluminescence (GE Healthcare formerly, Amersham Bioscience, Piscataway, NJ) after application of 1:2,000 goat anti-rabbit-HRP, or goat anti-mouse HRP.

Plasmid construction and transfection

The eukaryotic expression plasmid of the human *EPHA2* gene used in this study was generated previously in our laboratory (Zhang et al 2008). The cell lines were transfected with 4 μg of the EphA2 plasmid or the control vector using either Lipofectamine (Invitrogen, Carlsbad, CA) or the Nucleofector (Lonza, Walkersville, MD) according to manufacturer's protocols. The cells were lysed with RIPA buffer and the extracts were either collected at 48 hours for analysis or the cells were trypsinized and re-plated at 1:10 dilution and selected with G418 (1mg/ml) for stable incorporation. The stable clones were isolated at 14 days and the protein expression levels were confirmed with immunoblotting as described above.

Cell viability assay

The melanoma cells were plated in 96 well plates at a density of 1×10^4 cells per well in corresponding growth media in triplicates. After 24 hours, AlamarBlue® reagent (Invitrogen, Carlsbad, CA) was added at 1:10 (reagent: volume of media in the well) dilution and incubated at 37 °C for 48 hours. The fluorescence intensity measured at excitation wavelength of 550 nm and emission at 590 nm.

Gene silencing

Hairpin shRNAs targeting EphA2 (5 clones) mRNA were purchased from Open Biosystems, Huntsville, AL. The shRNA plasmids were mixed with the lentivirus packaging plasmids (Invitrogen, Carlsbad, CA) in the ratio according to the manufacturer's protocol. They were then mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and transfected into 293T cells for 48 hours in order to generate virus. After 48 hours, the viral supernatants were filtered and used for target cell infection in the presence of 8 μg/ml Polybrene (American Bioanalytical, Natick, MA). Infection was maintained for 24 hours and the cells were selected with Puromycin and experiments were performed after selection. SiRNAs (Ambion/Applied Biosystems, Austin, TX) were nucleofected using the Lonza protocol; these experiments were carried out 24–48 hours post nucleofection.

Cell proliferation and colony formation assay

For assaying proliferation, cells were plated on a 24-well cell culture plates (Corning Inc, Corning, NY), 4×10^4 cells were plated per well in DMEM supplemented with 10% FBS. Cells were then counted on the days indicated in the respective Figures. For colony formation assays, the cells were plated in 6-well plates at a density of 400 cells/well. The media was changed every other day and colonies were counted at day 14 after staining with 0.05% crystal violet. The experiments were carried out in triplicates and the results were validated with two different cell lines.

Migration assays: scratch migration and Boyden chamber transwell migration assay

Scratch migration assay, an *in vitro* wound model, was performed as described (Liang et al 2007). Briefly, cells were grown to confluence in 12-well culture plates (Corning Inc, Corning, NY). A wound was then created by scratching across the well with a thin pipette tip. Cells were then washed once with PBS, re-nourished with fresh media and monitored for 72 hours. The migration of cells was recorded by phase-contrast microscopy (Carl Zeiss Inc., Dublin) at time 0 (immediate after the scratch) and every 24 hours for 3 days. The area of open gap left was measured using the software ImageJ. Chemotactic migration assay was performed using 24-well transwell inserts with 8 μm membrane. 1×10^5 cells were placed on the top of the chamber in media supplemented with 0.1% FBS and NIH3T3 conditioned media was applied on the bottom chamber. The cells were allowed to migrate at 37°C for 4 hours; subsequently, the bottom side of the membrane was stained with crystal violet and the migrated cells were counted under an inverted microscope. The assays were carried out in duplicate.

Three dimensional organotypic cultures and the anchorage-independent growth assay

The 3-D cultures were carried out as described (Debnath et al 2003). The cells were plated on a mixture of collagen: matrigel (Becton Dickinson Biosciences, San Jose, CA) in the ratio (1:1) and monitored for 10–14 days. The cells were fixed with 100% methanol at −20°C for 20 min. followed by staining by immunofluorescence. The cells were blocked with 5% goat serum/PBS/Triton X blocking solution and the primary antibody incubated overnight. FITC-anti-mouse secondary antibody and DAPI were used to label the cells, which were then visualized with a confocal imaging system (Olympus, Center Valley, PA).

Anchorage-independent growth was assessed by growing the cells in ultra-low attachment tissue culture plates for 4–6 days followed by visualization with an inverted microscope (Olympus, Center Valley, PA).

Cell viability analysis: annexin-FITC staining and propidium iodide (PI) staining

For cell cycle analysis and propidium iodide (PI) staining, 1×10^6 cells were harvested and then fixed with 70% ice-cold ethanol overnight. After fixation, cells were washed with PBS and then stained with 100 μg/mL propidium iodide (PI) in PBS containing 100 μg/mL RNase A and 0.1% NP40 (all from Sigma). PI–stained cells were analyzed by FACSCalibur (Becton Dickinson Biosciences, San Jose, CA). For quantitation of apoptotic cell dealth, the Annexin IV-FITC Apoptotic Kit was used (Becton Dickinson Biosciences, San Jose, CA). Cells were trypsinized, collected, washed twice with 1× Binding buffer provided with the kit, incubated with 5 μl each of FITC- Annexin and PI (reference for dead cells) for 15 min at RT, diluted with binding buffer and analyzed by FACSCalibur within 1 hour of staining.

In vivo mice tumor studies

Experiments involving mice were approved by the Institutional Animal Care and Use Committee, at the Massachusetts General Hospital. 1×10^6 UACC903 melanoma cells (with or without EphA2 overexpression) were injected into the subcutaneous region of the Balb/c female nu/nu mice, and the tumors were allowed to grow. Four mice were used for each category. Palpable tumors were measured between 4–8 weeks. At 8 weeks, the mice were sacrificed, and the tumor size was measured with microcalipers, and recorded. The final tumor volume was calculated by multiplying the height, length and width of each tumor, expressed as mm^3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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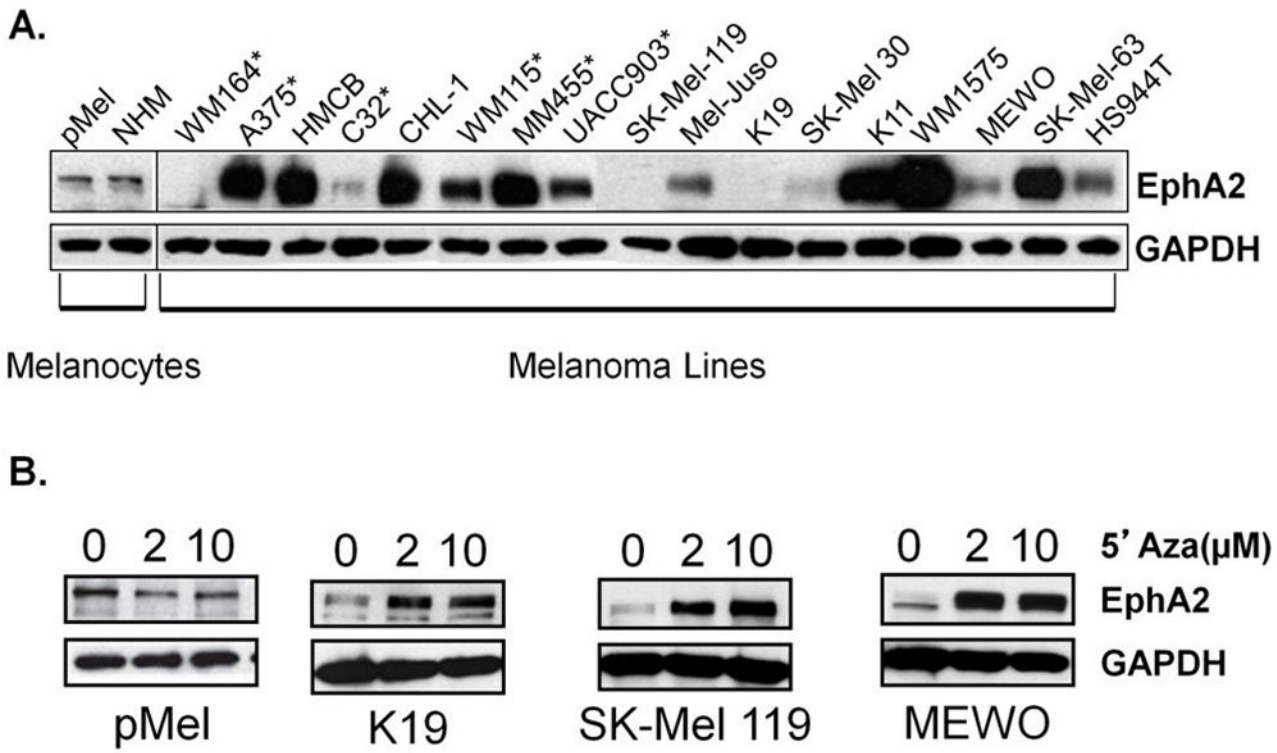

Figure 1. EphA2 is overexpressed in a majority of melanoma lines

(A) Western analysis demonstrating the relative protein levels of EphA2 in normal human melanocytes (NHM), immortalized NHMs (pMel) and a collection of 17 melanoma lines as indicated; melanoma lines with a BRAF mutation are designated by an asterisk. The vertical lines separate the melanocytes from the melanomas; both were analyzed on the same gel but in discontinuous lanes. **(B).** Treatment of immortalized NHMs (pMel) and 3 melanoma lines (K19, SK-Mel 119, and MEWO) with 5′Aza-2′-deoxycytidine for 48 hrs leads to increased EphA2 in the 3 melanoma lines but not in the melanocytes.

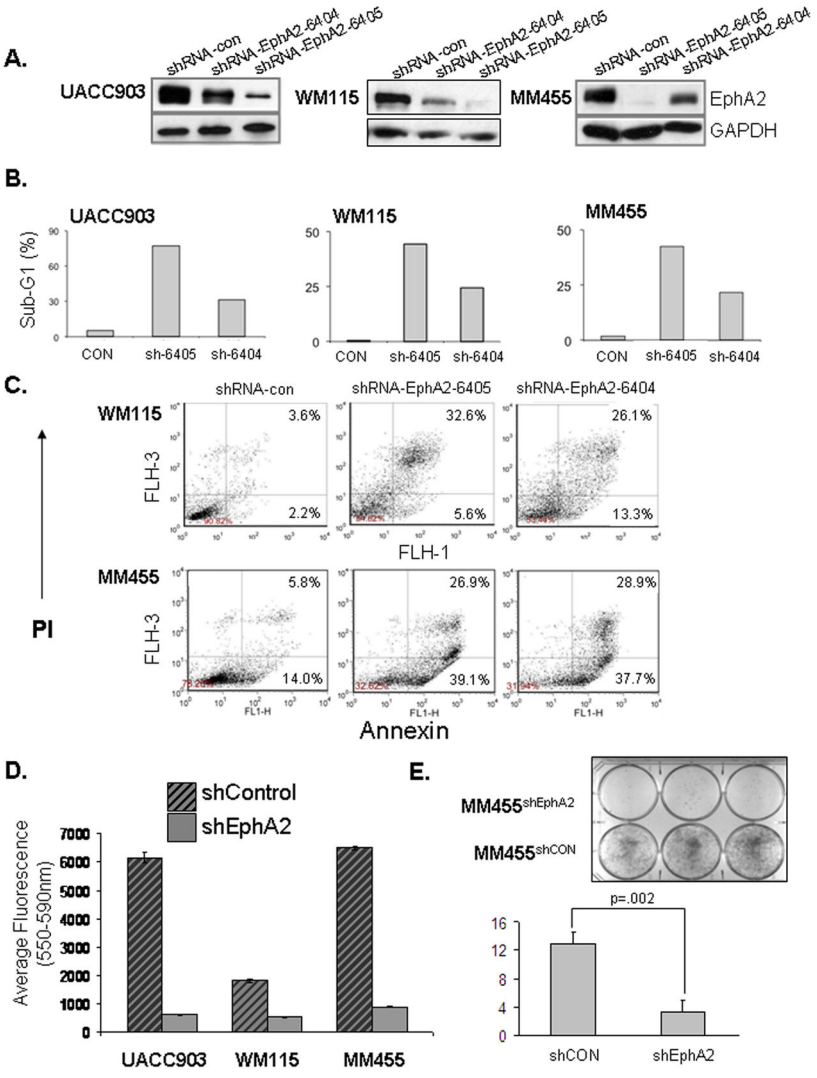

Figure 2. EphA2 depletion leads to loss of viability

(A) Western analysis to confirmed significant EphA2 suppression (40–80%) in 3 melanoma lines. Depletion of EphA2 was associated with **(B)** increased sub-G1 accumulation as determined by PI staining. **(C)** EphA2 downregulation induces apoptosis in human melanoma cells. FITC-Annexin and PI staining of melanoma cells, as indicated, after suppression by EphA2 shRNAs. In the 2 cell lines shown, apoptosis was increased more than 25% at 72 hours after EphA2 downregulation. The sh-6405 was selected for further analysis and indicated as **shEphA2. (D).** EphA2 depletion also led to diminished cell viability as measured by Alamar Blue metabolic labeling (in triplicate) and **(E)** reduced colony formation (p=0.002; 2-tail T-test).

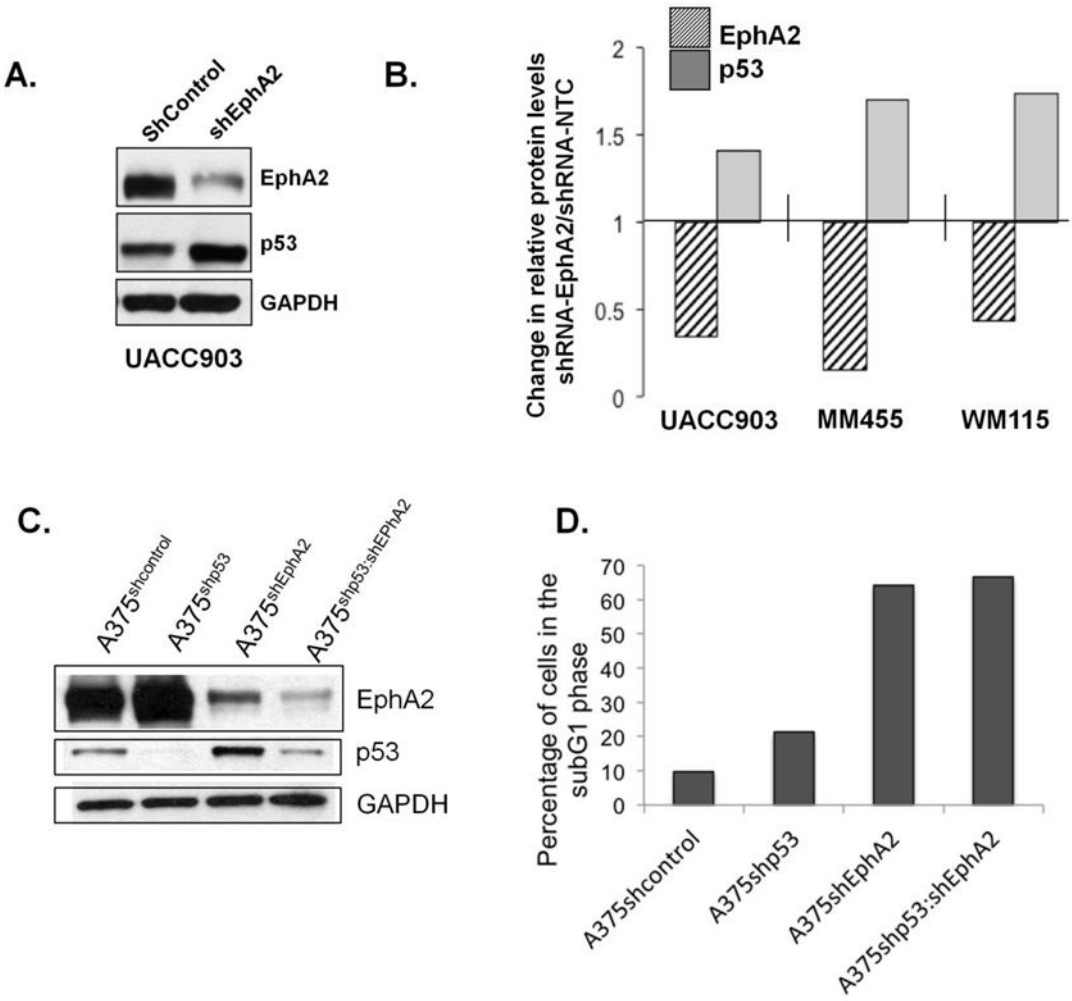

Figure 3. EphA2 withdrawal increases p53

Suppression of EphA2 by sh-RNA is also correlated with an increase in p53 levels **(A and B). (C).** A375 cells devoid of p53 still undergo subG1 accumulation upon EphA2 silencing.

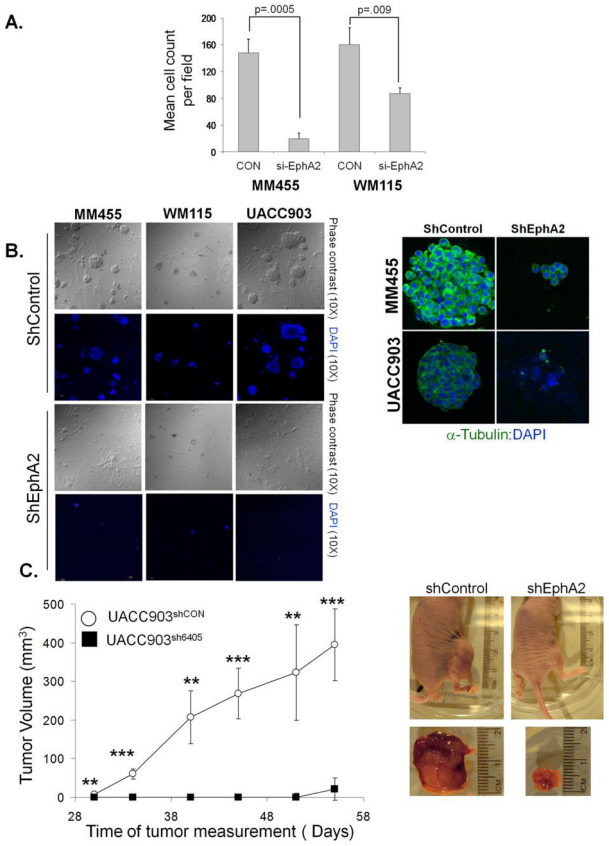

Figure 4. EphA2 loss reduces cell migration and suppresses melanoma growth

(A) EphA2 silencing significantly decreased transwell migration of MM455 and WM115 melanoma cells (MM455: p=0.0005 and WM115: p=0.009, both by T-test). **(B).** Melanoma growth on a 3-D matrix of collagen:matrigel (1:1) is dramatically reduced with EphA2 suppression. The left panel shows the 10X magnification of the control and EphA2-silenced MM455, UACC903 and WM115 cells while the right panel shows a representative magnified image of the individual tumor nodule of UACC903 and MM455 (Day 14) stained with alpha-tubulin and DAPI. **(C).** Depletion of EphA2 inhibits tumor growth in vivo (**=p<.01; ***=p<.001, T-test).

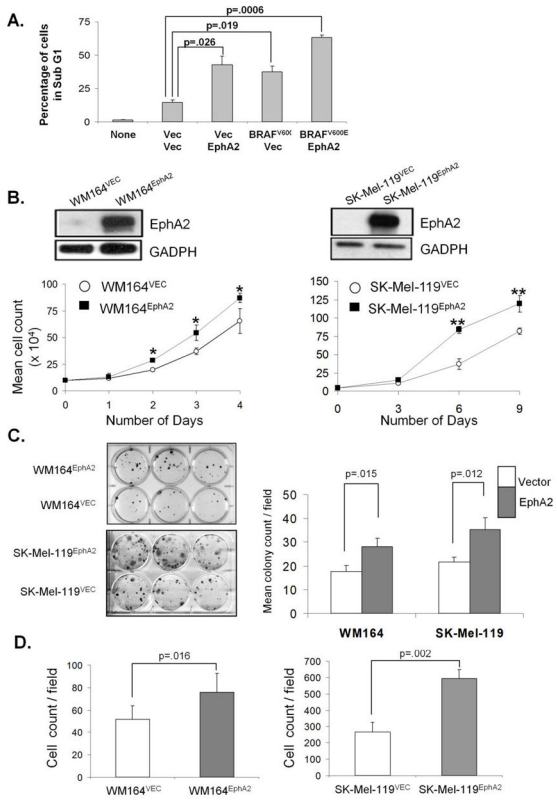

Figure 5. EphA2 overexpression in melanocytes and melanomas

(A) Immortalized normal human melanocytes were transfected with combinations of EphA2, BRAFV600E or vector (Vec). At 48 hours, there were increases in sub G1 accumulation with EphA2 and BRAFV600E; the effect was additive when both molecules were expressed. P values are shown. Ectopic introduction of EphA2 into WM164 and SK-Mel-119 melanoma lines led to **(B)** increased proliferation (*=p<.05; **=p<.01), **(C)** greater colony formation and **(D)** and heightened transwell migration.