HER3 Is a Determinant for Poor Prognosis in Melanoma

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Abstract

Purpose: The epidermal growth factor receptor family member HER3 is overexpressed in diverse human cancers and has been associated with poor prognosis in breast, lung, and ovarian cancer. However, the relevance of HER3 with regard to its prognostic significance and function in primary melanoma and metastases remains largely elusive.

Experimental Design: HER3 protein expression was analyzed immunohistochemically using tissue microarrays of 130 primary melanoma and 87 metastases relative to established clinical variables. The possibility of an influence of HER3 on melanoma cell proliferation, migration, invasion, and chemotherapy-induced apoptosis was studied in human melanoma cell lines.

Results: We show that HER3 is frequently expressed in malignant melanoma and metastases at elevated levels. High HER3 expression may serve as a prognostic marker because it correlates with cell proliferation, tumor progression, and reduced patient survival. Suppression of *HER3* expression by RNA interference reduces melanoma cell proliferation, migration, and invasion *in vitro*. In addition, down-regulation of *HER3* synergistically enhances dacarbazine-induced apoptosis. Moreover, monoclonal antibodies specific for the extracellular portion of HER3 efficiently block heregulin-induced proliferation, migration, and invasion of melanoma cell lines.

Conclusion: Our results provide novel insights into the role of HER3 in melanoma and point out new possibilities for therapeutic intervention.

Melanoma is a common type of skin cancer, which develops from the malignant transformation of melanocytes, and accounts for 80% of deaths arising from skin cancer (1). The underlying molecular mechanisms of melanocyte transformation have been studied extensively in the past (2, 3). However, to date no drugs are available that significantly prolong patient survival once melanoma progresses to the metastatic state (4). Thus, there is an urgent need for novel therapeutic agents and prognostic markers in the treatment of melanoma patients.

The human epidermal growth factor receptor (EGFR; HER) family of receptor tyrosine kinases regulates a large variety of biological processes including cell proliferation, migration, invasion, and survival (5). The family consists of four members: EGFR (HER-1), HER-2 (neu or ErbB2), HER3 (ErbB3), and HER-4 (ErbB4). To date, 11 ligands have been reported including EGF, heparin-binding EGF-like growth factor, transforming growth factor-α, amphiregulin, epiregulin, betacellulin, and the heregulins. These ligands bind directly to their cognate receptors, which leads to the formation of receptor homodimers or heterodimers that trigger the activation of multiple signaling pathways (6). Dysregulation of members of the HER family by either activating mutations, receptor overexpression, or aberrant ligand release leads to the development of a variety of human tumors (7). HER3 is overexpressed in breast, ovarian, and lung cancer and this genetic feature has been correlated with poor prognosis (8–10). On activation by heregulins, HER3 dimerizes with HER-2 and EGFR to form potent oncogenic receptor heterodimers (11–13). Within this complex, HER3 preferentially recruits phosphatidylinositol 3-kinase (PI3K) to its cytoplasmic docking sites thereby regulating cell proliferation and survival (14, 15). Thus far, it was assumed that HER3 is kinase inactive due to apparently aberrant sequence characteristics in its kinase domain and that it requires heterodimerization with a kinase-intact member of the HER family to initiate signaling events (16). Consistent with this, it was shown that HER-2 requires HER3 to drive breast tumor cell proliferation (17). However, recent findings of van der Horst et al. showed that HER3 is able to phosphorylate Pyk2, which results in the activation of the mitogen-activated protein kinase pathway in human glioma cells (18). Furthermore, monoclonal antibodies specific for HER3 can inhibit the proliferation and migration of cancer cell lines (19). Interestingly, it was shown recently that cancer cells escape HER family inhibitor therapy by up-regulation of HER3 signaling (20) and that HER3 inhibition abrogates HER-2-driven tamoxifen resistance in breast cancer cells (21). Moreover,

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resistance to gefitinib (Iressa) therapy, an EGFR small-molecule inhibitor, was shown to be connected to HER3 signal activation (22).

In this study, we analyzed the expression and prognostic significance of HER3 in malignant melanoma. We show that HER3 is up-regulated in melanoma and that high HER3 levels correlate with proliferation, tumor progression, and reduced overall patient survival. In addition, we show that HER3 regulates proliferation, migration, invasion, and cell survival of human melanoma cell lines. Moreover, blocking HER3 activation with specific monoclonal antibodies substantially reduced the proliferation, migration, and invasion of these cell lines *in vitro*. In summary, our findings support a prognostic relevance of HER3 in melanoma and validate this HER family member as a potential target for the development of novel cancer therapies.

Materials and Methods

Melanoma patients. Formalin-fixed, paraffin-embedded tissue of 130 primary cutaneous melanoma and 87 metastases was immunohistochemically analyzed for HER3. The patient age ranged from 19 to 90 years. Clinical follow-up was available in all of the patients (mean clinical follow-up was 56 ± 25 months). There were 60 nodular, 42 superficial spreading, 3 lentiginous, 9 acral lentiginous, and 16 not otherwise specified melanoma. All melanoma had a Breslow tumor thickness between 0.4 and 17 mm. Fifty-three of 130 (41%) patients had metastases during follow-up and 24 of 130 (18%) patients died. Matched tumor samples of primary melanoma and metastases were available for 20 patients. Fifty-four of the 130 patients with primary cutaneous melanomas were reported previously in a sentinel lymph node study (23). Approval was obtained from a local institutional ethical committee and written informed consent signed by all study participants.

Tissue microarray construction and immunohistochemistry. A morphologically representative region of a paraffin "donor" block was chosen to prepare the melanoma tissue microarrays. The representative region was taken with a core tissue biopsy (diameter, 0.6 mm; height, 3-4 mm) and precisely arrayed into a new "recipient" paraffin block using a customer-built instrument (24). After the block construction was completed, 4.0 μm sections of the resulting tumor tissue microarray block were cut with a microtome and used for further analysis.

HER3 and Ki-67 immunohistochemistry was done using a Ventana Benchmark automated staining system (Ventana Medical Systems). For antigen retrieval, slides were heated with cell conditioner 1 (standard procedure). Endogenous biotin was blocked with the appropriate kit. Primary antibodies against HER3 (clone C-17, 1:50 dilution; Santa Cruz) and Ki-67 proliferation antigen (clone MIB-1, 1:20 dilution) were applied and revealed with the iVIEW DAB detection kit, yielding a brown reaction product. The signal was enhanced with the Ventana amplification kit. Slides were counterstained with hematoxylin before glass cover-slipping. The specificity of the staining was controlled by using isotype antibody controls, secondary antibody controls, or blocking peptides. The analysis of the tissue microarrays was done using a Zeiss Axiovert 300 microscope.

Evaluation of HER3 expression. To determine the expression frequencies of HER3, a semiquantitative scoring system was applied following the German immunohistochemical scoring (GIS) system in which the final immunoreactive score equaled the product of the percentage of positive cells times the average staining intensity. Percentage of positive cells was graded as follows: 0, negative; 1, up to 10% positive cells; 2, 11% to 50%; 3, 51% to 80%; and 4, >80%. Staining intensity was graded as follows: 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive (25–27). The extent of KI-67 staining was recorded as the Ki-67 labeling index (number of marked nuclei per 100 melanoma cells). All stainings were evaluated by two different investigators (M.R. and D.M-P.).

Statistical analysis. The protein expression frequency for HER3 was analyzed by dividing the GIS score in three groups: GIS 1-4, no/low; GIS 5-8, moderate; and GIS 9-12, high expression. For statistical analysis, two groups were considered which were divided through GIS dichotomization at the median (low: GIS ≤ 6 and high: GIS > 6). Correlations between HER3 and Ki-67 were analyzed using Spearman's rank correlation. The overall survival of melanoma patients was estimated by the Kaplan-Meier method and differences between groups were assessed by the log-rank test. The overall survival was defined as the time of primary tumor diagnosis to the last follow-up visit or patient death. All P values were calculated using the two-sided Fisher's exact test or the paired Student's t test and P values < 0.05 were considered statistically significant. A multivariable Cox regression model was adjusted, testing the independent prognostic relevance of HER3 in melanoma patients. The following clinical variables were considered: age (≤60 versus >60 years), sex (male versus female), tumor thickness (≤2 versus >2 mm), and metastases during follow-up. The proportionality assumption for all variables was assessed with log-negative-log survival distribution functions. For the analysis of matched tumor-metastases pairs, the GIS values of HER3 in primary melanoma and melanoma metastases were directly compared and analyzed. The statistical analysis was done with the SPSS 12.0 software (SPSS).

Cell culture and compounds. Human Colo 829 melanoma cells were maintained in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, 1% sodium bicarbonate, 10 mmol/L HEPES, and 4.5 g/L glucose. Human MM-358, Mel Gerlach, and Mel Juso melanoma cells were maintained in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin. Dacarbazine and propidium iodide were purchased from Sigma. Heregulin β1 was purchased from R&D Systems and diluted in PBS before use.

RNA interference. *HER3* small interfering RNA (siRNA) were obtained from Ambion. Two independent siRNA were used in all experiments. Sequences for *HER3* siRNA were sense1 5′-GGCUAUGUCCUCGUGGCCAtt-3′, antisense1 5′-UGGCCACGAGGACAUAGCCtg-3′, sense2 5′-GGCAGUGUGUCCUGGGACUtt-3′, and antisense2 5′-AGUCCCAGGACACACUGCCtg-3′. The *GL-2* siRNA (Dharmacon) was used as a negative control in all experiments: *GL-2*_sense 5′-CGUACGCGGAAUACUUCGAtt-3′ and *GL-2*_antisense 5′-UCGAAGUAUUCCGCGUACGtt-3′. Transfection of siRNA was done using Oligofectamine (Invitrogen) according to the manufacturer's recommendation.

Antibodies, reverse transcription-PCR, and Western blot analysis. Antibodies against phospho-HER3 (Tyr^{1289}), p85, phospho-AKT (Ser^{473}), cyclin B1, phospho-extracellular signal-regulated kinase 1/2, phospho-mammalian target of rapamycin (mTOR; Ser^{2448}), and phospho-Rb (Ser^{780}) were all purchased from Cell Signaling. Horseradish peroxidase–conjugated rabbit secondary antibodies were from Bio-Rad. Anti-tubulin, anti-β-actin, and horseradish peroxidase–conjugated mouse secondary antibodies were from Sigma. The anti-HER3 (clone 2F12) antibody was from Upstate and anti-HER3 (C-17) for immunohistochemistry as well as AKT1/2 (H-136) were from Santa Cruz. Anti-p27 was purchased from Abcam. Western blot analysis and immunoprecipitations were done as described previously (28). Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and c-DNA was synthesized using the AMV Reverse Transcriptase (Roche) according to the manufacturer's recommendations. Reverse transcription-PCR primers for HER3: HER3_forward 5′-CTCCGCCCTCAGCCTACCAGTT-3′ and HER3_reverse 5′-TGCTCCGGCTTCTACACATTGACA-3′ (T_m = 64°C). Reverse transcription-PCR primers for tubulin: tubulin_forward 5′-AAGTGACAAGACCATTGGGGGAGG-3′ and tubulin_reverse 5′-GGGCATAGTTATTGGCAGCATC-3′ (T_m = 55°C). All PCR were done in an Eppendorf thermocycler (Eppendorf).

Proliferation assay. Cells (75,000 or 250,000) were seeded in 24-well or 6-cm plates and transfected with *HER3* or *GL-2* siRNA using Oligofectamine (Invitrogen). The cells were either grown in the presence of medium containing 10% FCS or serum starved in medium containing 1% FCS and stimulated with 100 ng/mL heregulin β1. The cell number was counted (Coulter counter, Becton Dickinson) at the indicated time points. The data are shown as mean ± SD.

Migration and invasion assay. Cells (200,000) were seeded in six-well plates and transfected with *HER3* or *GL-2* siRNA using Oligofectamine (Invitrogen). The cells were serum starved in medium containing 0.1% FCS for 24 h and cells (25,000) were seeded either onto a membrane or onto a growth factor–reduced Matrigel-coated membrane with 8 μm pores of a modified Boyden chamber (Schubert and Weiss) containing 500 μL serum-free medium; 10% FCS served as chemoattractant. The cells were allowed to migrate or invade for 20 or 24 h, respectively. Migrated or invaded cells were stained with crystal violet and washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope. For quantification, cells in at least 10 random fields were counted. The values for control cells were set to 100% and results are shown relative to controls. The data are shown as mean ± SD.

HER3 blocking antibody—proliferation, migration, and invasion experiments. The HER3 blocking antibody (clone 105.5) was purchased from Upstate. The second HER3 blocking antibody (clone 2D1D12) was generated in the Department of Molecular Biology at the Max-Planck Institute of Biochemistry. The HER-2 blocking antibody (clone 4D5) used was the nonhumanized form of trastuzumab.

The proliferation assay was done using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolimbromide assay. Briefly, 7,500 cells were seeded in 48-well plates. The cells were serum starved in medium containing 1% FCS for 24 h, preincubated with either 10 μg/mL HER3 blocking antibody (clone 105.5) or an isotype control antibody for 1 h, and stimulated with 100 ng/mL heregulin β1. The cells were allowed to grow for 48 h, and at that time point, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolimbromide (thizolyl blue, Sigma) was added to each well at a final concentration of 1 mg/mL. The plates were incubated for 2 h. The yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolimbromide dye is reduced by mitochondrial dehydrogenase activity to a purple formazan, which was then solubilized (SDS, 2-butanol, and HCl) and absorbance was measured at 570 nm on a microplate reader.

MDA-MB-468 breast cancer cells were already described to be inhibited by the anti-HER3 antibody (clone 105.5; ref. 19) and served as a positive control. The data are shown as percent of inhibition relative to control cells.

The migration and invasion assays were done as described previously (19, 29). Briefly, 300,000 cells were seeded in 6-cm plates and serum starved in medium containing 0.1% FCS for 24 h. Cells (200,000/mL) were incubated with 10 μg/mL HER3 blocking antibody or an isotype control antibody for 1 h and 50,000 cells were then seeded either onto a membrane or onto a growth factor–reduced Matrigel coated membrane with 8 μm pores of a modified Boyden chamber (Schubert and Weiss) containing 500 μL serum-free medium. Conditioned NIH 3T3 medium containing 0.01% ascorbic acid and heregulin β1 (100 ng/mL) were used as a chemoattractant. The cells were allowed to migrate or invade for 20 or 24 h, respectively. Migrated or invaded cells were stained by crystal violet, washed in PBS, and analyzed using a Zeiss Axiovert 300 microscope. For quantification, at least 10 random fields were counted. The values for control cells were set to 100% and results are shown relative to controls. The data are presented as mean ± SD.

To asses the HER3 phosphorylation state, cells were serum starved for 24 h, incubated with 10 μg/mL blocking antibody for 1 h, stimulated with 50 ng/mL heregulin β1 for 2 h, lysed, and subjected to Immunoprecipitations using a specific HER3 antibody.

Apoptosis assay (propidium iodide staining). Cells (200,000) were seeded into six-well plates (Nunc) and transfected with *HER3* or *GL-2* siRNA. Apoptosis was induced by adding either 10 or 20 μmol/L dacarbazine in DMSO to the medium. After 48 h, the supernatant of each reaction was collected and the cells were trypsinized. After centrifugation, the cells were incubated for 2 h in a propidium iodide buffer (0.1% sodium citrate, 0.1% Triton X-100, and 20 μmol/L propidium iodide) and thereafter subjected to flow cytometric analysis (Becton Dickinson Biosciences). Apoptotic cells were identified as the sub-G_0-G_1 peak and quantified using the CellQuest Pro software (Becton Dickinson).

Results

HER3 is frequently expressed in primary melanoma and metastases. We investigated HER3 protein expression in 130 primary malignant melanoma and 87 metastases using tissue microarrays. HER3 immunoreactivity was accentuated at the cell membrane. In primary melanoma, no or low HER3 expression was found in 45 of 130 (35%) cases (Fig. 1A). Strikingly, moderate to high HER3 expression levels were found in 85 of 130 (65%) cases (Fig. 1B and C). Furthermore, HER3 was highly expressed in 35 of 87 (40%) melanoma metastases (Fig. 1D). Taken together, HER3 is frequently expressed in primary melanoma and metastases, indicating that HER3 may contribute to melanoma development and progression (Fig. 1E). Importantly, HER3 expression was undetectable in primary melanocytes (Fig. 1F). Interestingly, moderate to high HER3 expression significantly correlated with increased tumor cell proliferation (Ki-67 labeling index) in primary malignant melanoma ($P = 0.008$; data not shown).

HER3 expression in melanoma progression. To investigate the relevance of HER3 expression in primary melanoma, we analyzed the relationship between HER3 and clinical variables. In addition, matched tumor samples of primary melanoma and metastases were studied in 20 patients. Remarkably, 10 of 20 (50%) patients showed an increase of HER3 expression in the metastases compared with the primary tumor. Six of 20 (30%) matched tumor samples showed a similar expression of HER3 and only 4 of 20 (20%) patients showed less HER3 expression in the metastases when compared with the primary tumor (Fig. 2A). These results show that in a majority of cases HER3 expression remains either stable or even increases during disease progression.

Importantly, Kaplan-Meier analysis showed that HER3 expression was significantly associated with tumor specific survival ($P = 0.014$; Fig. 2B). In a multivariate analysis, an adjusted Cox regression model was developed for the assessment of the overall survival rate. The characteristics of the variables are shown in Table 1. The clinical variables used in the analysis were age, sex, metastases during progression, tumor thickness, and HER3 expression (HER3 immunohistochemistry score). In this model, metastases ($P = 0.000$) and HER3 expression ($P = 0.041$) were correlated with poor prognosis. The hazard ratio for death from melanoma concerning HER3 status was 2.6 (95% confidence interval, 1.042-6.671); accordingly, in cases with high HER3 expression, the probability of tumor-related death was almost three times higher than in cases with low HER3 staining. Because of the assumption of proportional hazards, the probability of melanoma-related death was consistently valid during the entire observation period. Notably, HER3 expression did not significantly correlate with any other clinical variables (e.g., tumor thickness and

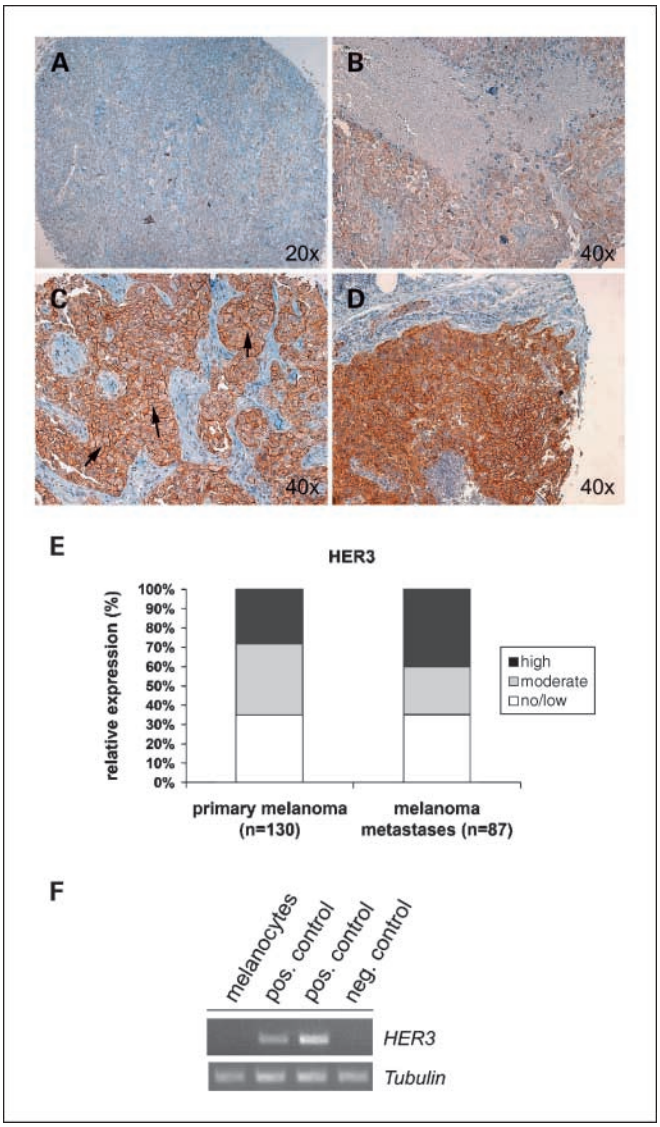

Fig. 1. HER3 expression in primary melanoma and metastases. Immunohistochemical staining of HER3 in primary melanoma and metastases. Low HER3 expression (×20; *A*), moderate HER3 expression (×40; *B*), and high HER3 expression (×40; *C*) in primary melanoma. HER3 immunoreactivity is accentuated at the cell membrane (*black arrows*). *D,* high HER3 expression in melanoma metastases (×40). *E,* HER3 expression frequencies in primary melanoma and metastases. *F,* absence of HER3 expression in primary melanocytes. HER3-expressing and nonexpressing melanoma cell lines served as positive and negative controls, respectively. Tubulin served as a loading control.

ulceration) tested. Taken together, these results indicate that HER3 is a critical variable for melanoma prognosis and progression.

HER3 knockdown inhibits melanoma cell proliferation. To further characterize the role of HER3 in melanoma, we down-regulated *HER3* by specific siRNA in Colo 829, MM-358, Mel Gerlach, and Mel Juso human melanoma cell lines (Fig. 3A; Supplementary Figs. S1A and S2A). Strikingly, depletion of *HER3* strongly inhibited proliferation in these cell lines (Fig. 3B; Supplementary Figs. S1A and S2B). To further characterize the mechanism of the growth inhibition, we analyzed proteins implicated in cell cycle control. As shown in Fig. 3B, *HER3* knockdown led to increased p27 levels, reduced cyclin B1 levels, and decreased Rb phosphorylation, suggesting that HER3 controls melanoma cell proliferation by interfering with key cell cycle regulators. In addition, *HER3* knockdown strongly inhibited heregulin-induced cell proliferation (Fig. 3B; Supplementary Fig. S1A). Importantly, HER3 predominantly signals via the PI3K-AKT pathway in the regulation of cell proliferation and survival. Indeed, AKT activation was impaired in heregulin β1 stimulated knockdown cell lines, indicating that HER3 may signal via the PI3K-AKT pathway in melanoma cells (Fig. 3C; Supplementary Fig. S1B). In contrast, phospho-extracellular signal-regulated kinase and phospho-mTOR levels remained unchanged (Fig. 3C; Supplementary Fig. S1B). Notably, HER-2 protein levels were not altered in *HER3* down-regulated cells, suggesting that the effects are specific for HER3 (Supplementary Fig. S3). Our data show that HER3 appears to promote melanoma cell proliferation, which most likely involves the modulation of key cell cycle regulators.

HER3 knockdown inhibits melanoma cell migration and invasion and sensitizes melanoma cells to dacarbazine-induced apoptosis. Melanoma metastases frequently express high levels of HER3 (Fig. 1D and E). Given the association between HER3 expression and poor survival, one might hypothesize that HER3 plays a role in melanoma progression. Increased tumor cell migration and invasion are important prerequisites for

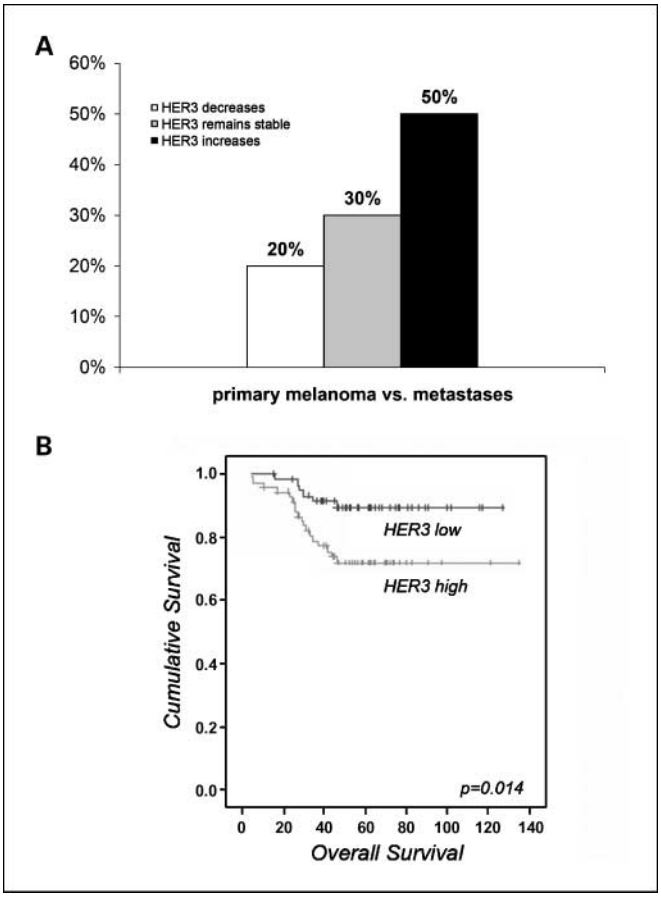

Fig. 2. HER3 protein expression confers poor prognosis in melanoma patients. *A,* HER3 expression increases during melanoma progression. Twenty primary tumors with matching melanoma metastases were evaluated based on their GIS score for HER3. *B,* Kaplan-Meier analysis of tumor-specific survival according to HER3 expression levels ($P = 0.014$).

Table 1. Multivariate analysis of factors possibly influencing overall survival (forward LR method)

Variable	Characteristics	Hazard ratio (95% confidence interval)	*P*
Age	0 = ≤60 y 1 = >60 y	—	NS
Sex	0 = male 1 = female	—	NS
Tumor thickness	0 = ≤2 mm 1 = >2 mm	—	NS
HER3	0 = low 1 = high	2.6 (1.04-6.6)	0.041*
Metastases during follow-up	0 = no metastases 1 = metastases	22.2 (5.2-95.5)	0.000*

*$P < 0.05$.

metastasis. To address this question, we analyzed the migration and invasion of Colo 829, MM-358, and Mel Gerlach melanoma cells on interference with *HER3* expression. To exclude the possibility of measuring inhibition of proliferation, we used serum-starved melanoma cells and monitored migration and invasion after 20 and 24 h, respectively. As shown in Fig. 4A, *HER3* knockdown efficiently blocked melanoma cell migration in all three cell lines. In invasion experiments, Colo 829 and Mel Gerlach cells were markedly inhibited on *HER3* suppression (Fig. 4B), whereas MM-358 cells did not invade the matrix even in untransfected controls (data not shown). These results establish HER3 as a potent mediator of melanoma cell migration and invasion.

We next asked the question whether *HER3* down-regulation can induce melanoma cell death. However, in contrast to previously published data in lung cancer cells, which undergo apoptosis in the absence of *HER3* (15), suppression of *HER3* did not induce major apoptosis in melanoma cells (Supplementary Fig. S4). Nevertheless, we reasoned that inhibition of *HER3* might synergize with chemotherapy in the induction of apoptosis in melanoma cells. Indeed, we found that dacarbazine-induced apoptosis was significantly increased in *HER3* knockdown melanoma cells (Fig. 4C; Supplementary Fig. S1C). These results suggest that a combination therapy with HER3 and dacarbazine-like drugs might be useful for the treatment of malignant melanoma.

Anti-HER3 monoclonal antibodies block heregulin-induced HER3 activation and melanoma cell proliferation, migration, and invasion. We have shown that HER3 is frequently overexpressed in primary melanoma and melanoma metastases and that high HER3 levels confer poor prognosis for melanoma patients. In addition, the RNA interference experiments suggest that HER3 may be a potential target for melanoma therapy. To test this hypothesis *in vitro*, we treated Colo 829, MM-358, Mel Gerlach, and Mel Juso melanoma cells with anti-HER3 monoclonal antibodies. Remarkably, heregulin-induced activation of HER3 and its association with the PI3K subunit p85 was completely abrogated in antibody-treated cells when compared with controls (Fig. 5A). In addition, anti-HER3 monoclonal antibodies seem to cause receptor degradation or internalization similar to previously obtained results in breast cancer cells (19). Importantly, anti-HER3 monoclonal antibodies are able to block heregulin-induced proliferation, migration, and invasion of human melanoma cell lines (Fig. 5B-D; Supplementary Fig. S2C), indicating that such antibodies may be effective anti-melanoma therapeutics. Importantly, in Mel Juso melanoma cells, HER-2 inhibition by a specific monoclonal antibody had no effect on tumor cell invasion, showing that inhibition of HER3 is sufficient to block melanoma invasivity (Supplementary Fig. S2C).

Taken together, these results suggest that targeting HER3 may be a promising new opportunity for melanoma therapy.

Discussion

The discovery of animal oncogenes that are derived from genes encoding receptor tyrosine kinases has led over the past 20 years to the development of several targeted therapeutics with the HER-2 monoclonal antibody trastuzumab being the first in clinical application for the treatment of metastatic breast carcinoma with HER-2 gene amplification. However, in spite of further advances in the development of side effect–poor therapies for major malignancies such as breast cancer, there is still a great unmet need for better, more effective therapies for other cancer types. Melanoma is a highly aggressive skin cancer and current therapies only show limited efficacy in patients with late-stage disease (4, 30). Thus far, it is known that the Ras-Raf-mitogen-activated protein kinase and the PI3K-AKT pathways are frequently activated in malignant melanoma and that this contributes to tumor progression (30, 31). However, the role of receptor tyrosine kinases in melanoma development remains poorly characterized.

HER3 is highly expressed in many human tumor types and has been associated in some cases with poor prognosis (8–10). Moreover, recent studies suggest that breast tumors exposed to EGFR or HER-2 targeted therapies escape this inhibition by persistent activation of HER3 and the PI3K-AKT pathway (20–22), suggesting that agents targeting HER3 could provide a novel and promising approach toward the treatment of some cancers. We have shown recently that *HER3* is frequently expressed in human melanoma cell lines (32) and macroarray analysis revealed that *HER3* is the most prominently expressed HER family receptor in these cell lines.[7] Interestingly, HER3 and HER-2 have been described to be expressed in human

[7] P. Knyazev, unpublished data.

melanoma cell lines and both receptors were implicated in the control of melanoma cell growth (33). In the present study, we show HER3 expression in primary melanoma and its significant association with tumor cell proliferation. Furthermore, we show frequent and high HER3 expression in melanoma metastases, suggesting that HER3 may be involved in disease progression. In addition, high levels of HER3 significantly correlated with decreased life expectancy of patients establishing HER3 as a novel prognostic marker for melanoma. Importantly, HER3 expression is undetectable in primary melanocytes,

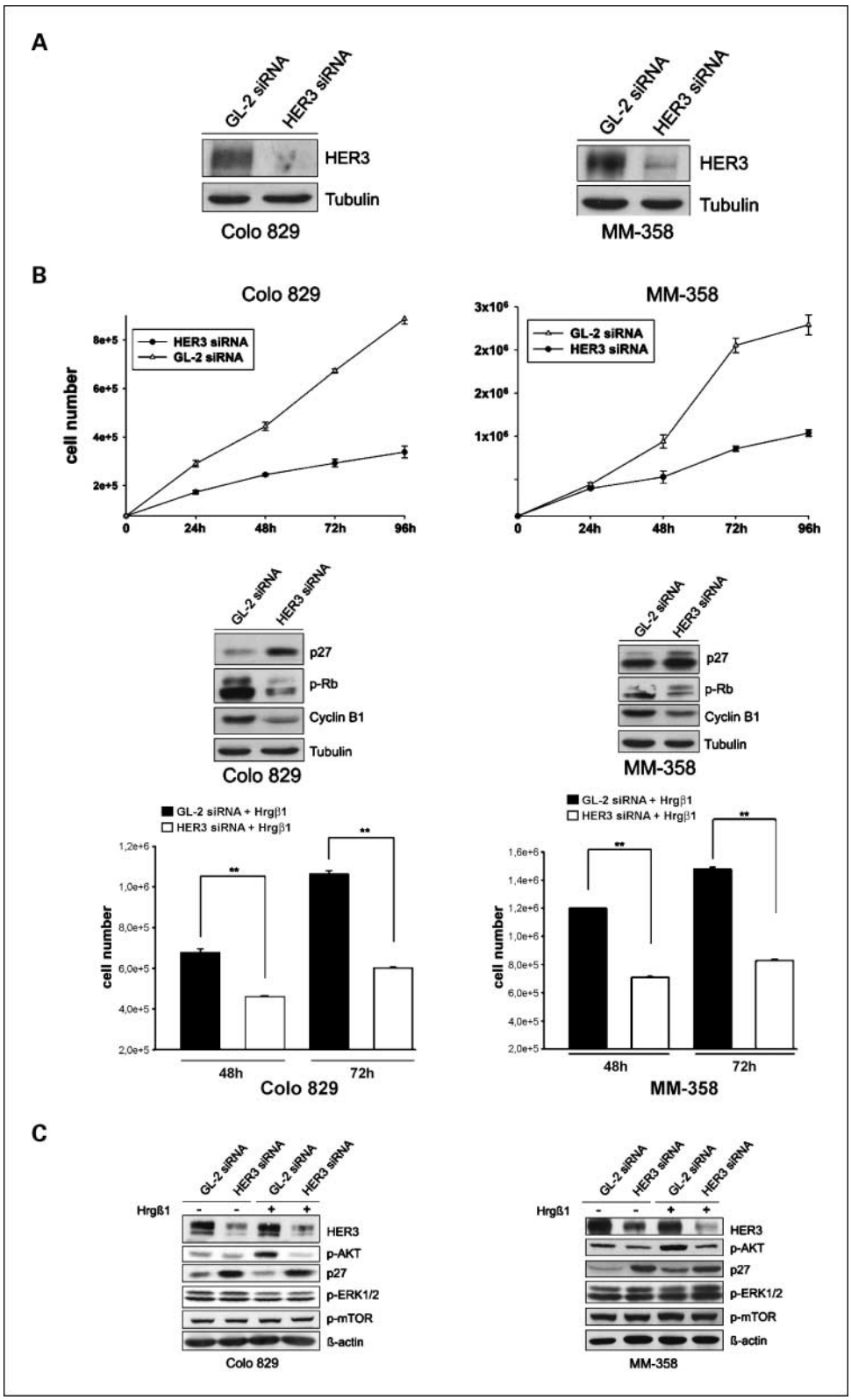

Fig. 3. *HER3* knockdown inhibits melanoma cell proliferation. *A, HER3* knockdown in Colo 829 and MM-358 melanoma cells. Western blots are shown for HER3 and tubulin. *B, HER3* knockdown inhibits the proliferation of Colo 829 and MM-358 melanoma cells. The growth curves were done as described in Materials and Methods. Mean ± SD. Western blots for p27, phospho-Rb, and cyclin B1 are shown. Tubulin served as a loading control. *C, HER3* knockdown impairs AKT activity on heregulin β1 stimulation in Colo 829 and MM-358 melanoma cells. Western blots are shown for HER3, phospho-AKT, p27, phospho-extracellular signal-regulated kinase 1/2, and phospho-mTOR. β-Actin served as a loading control.

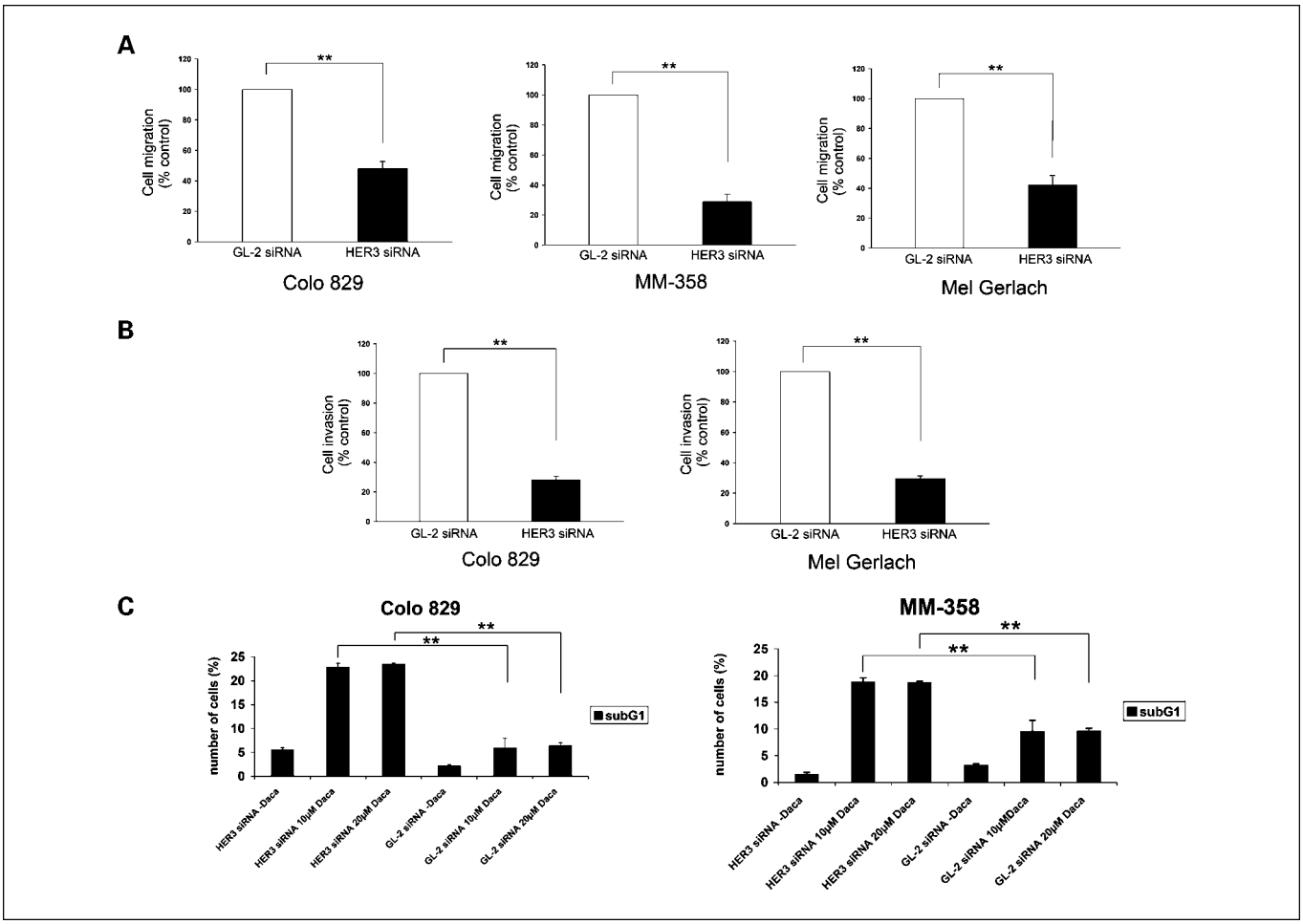

Fig. 4. *HER3* knockdown inhibits melanoma cell migration and invasion and induces apoptosis in response to chemotherapeutic drugs. *A, HER3* knockdown inhibits the migration of Colo 829, MM-358, and Mel Gerlach melanoma cells. For quantification, pictures of migrated cells were taken on a Zeiss Axiovert 300 microscope and cells were counted in at least 10 random fields. The values for control cells were set to 100% and results are shown relative to controls. Mean ± SD. *B, HER3* knockdown inhibits the invasion of Colo 829 and Mel Gerlach melanoma cells. Quantification of invaded cells was done as described in *A. C,* induction of apoptosis in Colo 829 and MM-358 *HER3* knockdown cells on treatment with increasing amounts (10 and 20 μmol/L) of dacarbazine. Mean ± SD.

suggesting that HER3 overexpression specifically occurs during melanoma development. The mechanism of HER3 overexpression appears to be caused by increased gene transcription because Southern blot analysis of 54 human melanoma cell lines did not reveal HER3 gene amplification (data not shown). Thus far, HER3 gene amplifications could only be detected in non-small cell lung and breast cancer as well as in synovial sarcomas (34–36), whereas no amplification was reported for pancreatic, stomach, head and neck, and brain tumors (37–40). To specifically address the role of HER3 in melanoma development and progression, we analyzed human melanoma cell lines on siRNA interference with *HER3* expression. Reduction of *HER3* expression resulted in reduced cell proliferation, migration, and invasion. On the molecular level, suppression of *HER3* led to increased p27 protein levels and reduced Rb phosphorylation, which seems to be the cause for the observed growth inhibition. Interestingly, HER3 can inhibit heregulin-induced cell proliferation, which may be due to an impaired AKT activity. In this context, it is important to note that AKT is known to modulate the cellular localization of p27, thereby promoting cell cycle progression (41–44); however, in melanoma cells, p27 seems to be regulated by an AKT-independent pathway because p27 up-regulation could also be seen in unstimulated *HER3* knockdown cells. Interestingly, p27 proteolysis can be regulated by oncogenic signaling through the EGFR and HER-2, which can be reversed by targeted inhibition of both receptors (45). It will be the topic of further studies to elucidate the mechanism of HER3-mediated p27 regulation in the control of human melanoma cell proliferation, which may be similar to EGFR and HER-2-mediated p27 regulation. Notably, *HER3* ablation did not affect the extracellular signal-regulated kinase 1/2 and mTOR kinases, suggesting that inhibition of other downstream pathways seems to be sufficient to block melanoma cell proliferation, migration, and invasion. Interestingly, mTOR phosphorylation at Ser^{2448} is known to be mediated by the PI3K-AKT pathway; however, inhibition of AKT in heregulin-stimulated cells did not result in down-regulation of phospho-mTOR. In fact, mTOR seems to be constitutively activated as shown in unstimulated melanoma cells (Fig. 3C). A possible explanation for this observation is that mTOR might be either activated through yet unknown mutations or directly

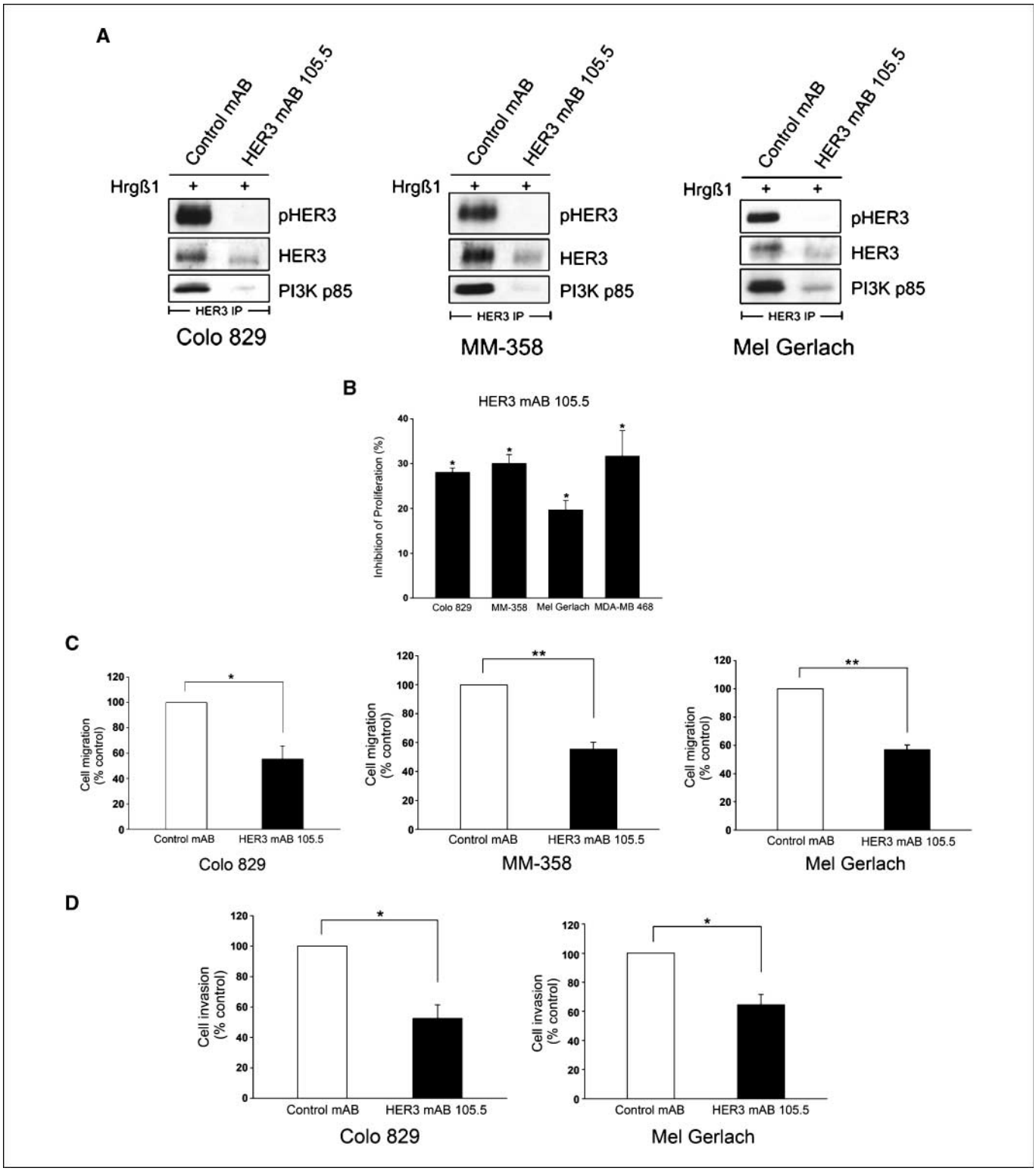

Fig. 5. An anti-HER3 monoclonal antibody (clone 105.5) inhibits HER3 activation and blocks melanoma cell proliferation, migration, and invasion. *A,* anti-HER3 antibody treatment blocks heregulin-induced HER3 activation and its association with p85 and leads to receptor internalization or degradation. Colo 829, MM-358, and Mel Gerlach melanoma cells were serum starved, incubated with 10 μg/mL HER3 blocking antibody (clone 105.5) or an isotype control antibody, stimulated with heregulin β1, and lysed and equal amounts of protein were subjected to immunoprecipitations using a specific HER3 antibody. Western blots for phospho-HER3 (Tyr^{1289}), HER3, and p85 are shown. *B,* an anti-HER3 antibody (clone 105.5) inhibits heregulin-induced melanoma cell proliferation *in vitro*. Cell proliferation was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolimbromide assay as described in Materials and Methods. MDA-MB-468 breast cancer cells served as a positive control as described previously (19). *C,* an anti-HER3 antibody (clone 105.5) blocks melanoma cell migration *in vitro*. The cells were either incubated with 10 μg/mL anti-HER3 antibody or an isotype control antibody. The migration assay was done in a modified Boyden chamber. Conditioned NIH3T3 medium containing 100 ng/mL heregulin β1 was used as a chemoattractant. The quantification was done as described in Fig. 4. Mean ± SD. *D,* an anti-HER3 antibody (clone 105.5) blocks melanoma cell invasion *in vitro*. The assay was done as in *B* using growth factor – reduced Matrigel in a modified Boyden chamber.

modulated by the Ras-related GTPase Rheb as described recently (46–48).

Furthermore, *HER3* suppression led to a marked decrease in melanoma cell migration and invasion. It will be of importance to elucidate the exact role of HER3 in these processes and to identify factors which mediate HER3 responses during melanoma metastasis (e.g., matrix metalloproteinases).

Interestingly, *HER3* knockdown in lung cancer cells did lead to a significant increase in apoptosis (15). However, interference with HER3 function was not sufficient to induce major apoptosis of melanoma cells. Based on these results, we tested whether a combination of *HER3* down-regulation with chemotherapeutic drug treatment would increase cell death. To date, dacarbazine is the only Food and Drug Administration–approved drug for the treatment of advanced melanoma with a tumor response rate of ~15% to 20% (4). Indeed, *HER3* knockdown cells were highly sensitive to dacarbazine-induced apoptosis, suggesting that a combination with agents interfering with HER3 might prove effective in the treatment of malignant melanoma.

To further analyze whether HER3 may qualify as a novel target in melanoma therapy, we treated human melanoma cells lines with anti-HER3 monoclonal antibodies. We found that such antibodies can inhibit heregulin-induced HER3 phosphorylation leading to receptor internalization or degradation. Furthermore, we show that binding of p85, the regulatory subunit of PI3K, to HER3 is abrogated on antibody-incubation, showing that signaling via the PI3K/AKT signaling pathway is inhibited in these cells. Importantly, melanoma cell proliferation, migration, and invasion are significantly reduced in antibody-treated cells when compared with controls. These data show that anti-HER3 antibodies can inhibit HER3 signaling most likely through the PI3K-AKT pathway in melanoma cell lines and thereby seem to block melanoma cell functions. Importantly, we could show in Mel Juso melanoma cells that a monoclonal antibody specific for HER-2 did not affect melanoma cell invasion, suggesting that inhibition of HER3 alone is sufficient to block melanoma invasiveness (Supplementary Fig. S2C). It will be essential in the future to test the efficacy of anti-HER3 blocking antibodies on melanoma development and metastasis in preclinical animal models to further validate HER3 as a possible target for melanoma therapy.

Taken together, our results establish HER3 as a potential target for melanoma therapy development and interference with its function may offer a new and promising approach to improve clinical patient outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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