

Angiopoietin-2 Levels Are Associated with Disease Progression in Metastatic Malignant Melanoma

Iris Helfrich,¹ Lutz Edler,² Antje Sucker,³ MarkusThomas,¹ Sven Christian,¹ Dirk Schadendorf,³ and Hellmut G. Augustin¹

Abstract Purpose: The blood vessel-destabilizing Tie2 ligand angiopoietin-2 (Ang-2) acts in concert with the vascular endothelial growth factor/vascular endothelial growth factor receptor system to control vessel assembly during tumor progression. We hypothesized that circulating soluble Ang-2 (sAng-2) may be involved in melanoma progression.

Experimental Design: Serum samples ($n = 98$) from melanoma patients (American Joint Committee on Cancer stages I-IV), biopsies of corresponding patients, and human melanoma cell lines were analyzed for expression of Ang-2 and S100 β . Multiple sera of a subcohort of 33 patients were tested during progression from stage III to IV. Small interfering RNA-based loss-of-function experiments were done to assess effects of Ang-2 on melanoma cells.

Results: Circulating levels of sAng-2 correlate with tumor progression in melanoma patients ($P < 0.0001$) and patient survival ($P = 0.007$). Analysis of serum samples during the transition from stage III to IV identified an increase of sAng-2 up to 400%. Comparative analyses revealed a 56% superiority of sAng-2 as predictive marker over the established marker S100 β . Immunohistochemistry and reverse transcription-PCR confirmed the prominent expression of Ang-2 by tumor-associated endothelial cells but identified Ang-2 also as a secreted product of melanoma cells themselves. Corresponding cellular experiments revealed that human melanoma-isolated tumor cells were Tie2 positive and that Ang-2 acted as an autocrine regulator of melanoma cell migration and invasion.

Conclusions: The experiments establish sAng-2 as a biomarker of melanoma progression and metastasis correlating with tumor load and overall survival. The identification of an autocrine angiopoietin/Tie loop controlling melanoma migration and invasion warrants further functional experiments and validate the angiopoietin/Tie system as a promising therapeutic target for human melanomas.

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Tumor progression and metastasis are critically dependent on the tumor's sustained ability to induce a host neovascular response that leads to the growth of new blood vessels to nourish the growing tumor and to remove metabolic waste products. The Notch/Delta and the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) systems are key regulators of the angiogenic cascade. Consequently, most tumors, including melanomas, express high levels of VEGF (1). Although VEGF has recently been described as an autocrine endothelial cell regulator of angiogenesis and vascular homeostasis (2), VEGF is produced by most tumor cells and acts paracrine on tumor-associated endothelial cells (3). Tumor cell VEGF production results as a consequence of oncogenic activation and the increasing hypoxia of a growing tumor nodule with an avascular or hypovascular center (4, 5).

VEGF is a master regulator of the angiogenic cascade and is involved in the progression of most solid tumors. Yet, it is not capable on its own to induce the formation of an anastomosing network of functional capillaries that is capable of sustaining perfusion (6). Instead, it acts in concert with later regulators of the angiogenic cascade. Vascular guidance

Translational Relevance

The present study established circulating soluble angiopoietin-2 as a potential biomarker of melanoma progression and metastasis correlating with tumor load and overall survival. Soluble angiopoietin-2 measurements may be useful to improve the clinically established biomarker S100 β for the identification of a higher fraction of stage III patients and for the monitoring of the progression from stage III to IV. Moreover, the experiments identified an autocrine angiopoietin/Tie loop controlling melanoma migration and invasion. These expression profiling, biochemical, and cellular experiments contribute to validating the angiopoietin/Tie system as a promising dual antiangiogenic and anti-tumor therapeutic target for malignant melanoma.

molecules such as the ephrins, semaphorins, netrins, and slits control vascular assembly, network formation, and arteriovenous differentiation (7, 8). Subsequently, the immature vascular network matures, recruits mural cells (pericytes and smooth muscle cells), and differentiates in an organ- and caliber-specific manner. These later steps of the angiogenic cascade are controlled by the angiopoietins and platelet-derived growth factors (9, 10).

The angiopoietins angiopoietin-1 (Ang-1) and Ang-2 have been identified as agonistic and antagonistic ligands of the vascular receptor tyrosine kinase Tie2, respectively (11, 12). Ang-1 acts as an endothelial cell survival factor and promotes vascular maturation. Constitutive Ang-1/Tie2 signaling is required to maintain the quiescent phenotype of the vascular endothelium. Ang-2 acts as a context-specific antagonist of Ang-1/Tie2 signaling. As such, it destabilizes the quiescent endothelial cell phenotype and primes it for exogenous cytokines including angiogenic and inflammatory stimuli (13). Yet, Ang-2 has also been reported to be capable of acting as an agonist of Tie2 (14).

Angiogenesis as evidenced by the determination of intratumoral microvessel densities and detection of VEGF expression has in recent years been solidly established as hallmark of melanoma progression (15–17). In contrast, much less is known about the role of the angiopoietin/Tie system during melanoma progression. Ang-2 is almost exclusively produced by activated endothelial cells, making it an autocrine regulator of endothelial cell function (18–20). Consequently, given the luminal location of endothelial cells within blood vessels, the concentrations of circulating Ang-2 may be a good biomarker of endothelial cell activation. Significantly elevated circulating levels of soluble Ang-2 (sAng-2) have in recent years been associated with tumor angiogenesis, for example, in hepatocellular carcinoma (21), lung tumors (22), and colorectal cancers (23), but also during severe sepsis (24). Based on these findings, the aim of this study was (a) to analyze sAng-2 serum levels in melanoma patients in various tumor stages as well as in sequential sera from progressing patients over time, (b) to test whether sAng-2 levels correlate with overall survival, (c) to evaluate the prognostic value of sAng-2 compared with the established marker S100 β , (d) to analyze the expression and localization of Ang-2 in biopsies of melanoma samples and corresponding human melanoma cell lines, and (e) to assess

functional effects of Ang-2 on human melanoma cells by loss-of-function experiments. These experiments identified sAng-2 as a potential biomarker of melanoma progression with better predictive power in advanced disease as the established melanoma progression marker S100 β . Surprisingly, Ang-2 was also identified as a gene product of Tie2-positive melanoma cells in which Ang-2 acted as a positive regulator of melanoma migration and invasion.

Patients, Materials, and Methods

Patients and sera. All 65 patients included in this study had histologically confirmed cutaneous malignant melanoma. Informed patient consent and the appropriate institutional review board approval were obtained for all patients. All clinical information including age, gender, stage of disease, tumor load, and survival time was documented and retrieved from an electronic database (Achiever Anyware Medical, Achiever Software). Tumor load was measured by physical examination, X-ray or computed tomography of the chest, ultrasound, or computed tomography of the abdomen and lymph nodes as well as the brain. For serum collection, blood was drawn from each patient and healthy volunteer into uncoated serum tubes (8 mL) at the Skin Cancer Unit of the Medical Faculty Mannheim of the University of Heidelberg. Following a resting period of at least 30 min and a maximum of 60 min, the tubes were centrifuged at $2,500 \times g$ for 10 min. Serum was harvested, aliquoted, and stored at -20°C until usage (25). All serum samples were collected between January 2000 and July 2003 and selected randomly from the database retrospectively. Group 1 ($n = 18$) contained patients with primary melanoma [American Joint Committee on Cancer (AJCC) stage I and II according to current AJCC criteria; ref. 26]. Group 2 ($n = 37$) included cases with histologically verified regional lymph node, satellite, and/or in-transit metastases (clinical stage III). Group 3 included 47 serum samples collected from patients characterized by visceral metastases (AJCC stage IV). A group of 82 healthy blood donors (group 4) was included in the study as control. To analyze disease progression over time, additional sera were analyzed from a cohort of 33 patients with one serum taken in stage III and one in stage IV over a period of 2 years. All sera were taken from patients without treatment. The follow-up time of patients was calculated from date of first diagnosis up to date of last contact or rather death of patient.

Cell culture. Human umbilical vein endothelial cells were purchased from PromoCell and cultured in endothelial cell medium (EGM-2; Cambrex). Blood endothelial cells (BEC) were isolated from human dermal microvascular endothelial cells of human foreskin and cultured as described (6). A375 melanoma cells (CRL-1619) were purchased from the American Type Culture Collection and cultured in DMEM. Early-passage human melanoma cell lines (MA-Mel-142, MA-Mel-48a, and MA-Mel-141b) established from stage IV melanoma at the Skin Cancer Unit (German Cancer Research Center) were cultured in RPMI 1640. All cells were maintained at 37°C and 5% CO_2 . DMEM and RPMI 1640 were supplemented with 10% FCS, L-glutamine (2 mmol/L), and penicillin/streptomycin solution (5 units/mL; all from PAA Laboratories).

Immunoassays. Quantikine immunoassays were used for the quantitation of sAng-2 in serum samples and cell culture supernatants (R&D Systems). Serum samples were thawed and diluted 5-fold. All serum samples were measured in triplicates according to the manufacturer's instructions (R&D Systems). A microplate reader was used for measuring absorbance at ~ 450 nm within 30 min, with correction wavelength at 540 nm. A standard curve of Ang-2 was established in each experiment and used to calculate human Ang-2 concentrations in individual samples. The minimal detectable concentration of Ang-2 detection was 8.3 pg/mL according to the manufacturer's information. The average value intra-assay coefficient of variation for Ang-2 and the average value interassay coefficient was $<5\%$. The assay sensitivity

threshold was 0.2 ng/mL. No cross-reactivity or interference for angiogenic factors and additional angiopoietins were observed according to the manufacturer's information. Immunoanalysis for protein S100β in serum samples of patients and healthy blood donors was done by using Elecsys 2100 (Roche Diagnostics). Serum samples were thawed and measured without dilution following the manufacturer's instructions. The lowest measurable S100β concentration was determined as 0.02 μg/L; intra-assay and interassay variations were <10%.

For the analysis of Ang-2 secretion from the cultured human melanoma lines MA-Mel-48a, MA-Mel-141b, MA-Mel-142, and A375, cells were serum starved for 2 days and medium was changed to serum-free RPMI (10 mL/10 cm dish). Cells were unstimulated or stimulated with either VEGF (25 μg/L) or basic fibroblast growth factor (25 μg/L). Supernatants were harvested and concentrated using Vivaspin 20 following the manufacturer's protocol (VWR). Conditioned media were diluted 1:10 and analyses were done in triplicates according to the protocol described for the detection of Ang-2 in serum samples. Protein concentrations of cell lysates were determined using bovine serum albumin as a standard. Ang-2 background levels of culture medium were subtracted from samples of conditioned medium.

Immunocytochemistry and immunohistochemistry. Cells were acetone/methanol fixed (1:1) and blocked for 30 min with 1% bovine serum albumin followed by a 1 h incubation with goat anti-human Ang-2 (R&D Systems) or ChromPure goat IgG (Dianova) as negative control. Following extensive washings with PBS, bound antibody was detected after 45 min incubation with the corresponding fluorescently labeled secondary antibody. Nuclei were counterstained with propidium iodide. Samples were analyzed by fluorescence and confocal microscopy (TCS SP2; Leica Microsystems).

Cryosections from human stage III or IV melanoma metastases were processed for Ang-2 immunostaining. Sections were fixed with 4% formaldehyde, and endogenous peroxidase was blocked with 3% H₂O₂

followed by avidin/biotin blocking. Nonspecific binding was blocked with 5% rabbit serum/1% bovine serum albumin in PBS + 0.02% Tween for 30 min. Goat anti-human Ang-2 (R&D Systems) or ChromPure goat IgG (Dianova), used as negative control, were incubated overnight and visualized using DAB Substrate Chromogen System (DAKO). Nuclei were counterstained with Meyer's Hemalaun solution (Merck). Anti-human Ang-2/anti-human CD31 double stainings were done using sheep anti-human CD31 (DAKO) for cellular localization of Ang-2 expression. Primary antibodies were detected by rabbit anti-goat Alexa Fluor 546, donkey anti-sheep Alexa Fluor 488, rabbit anti-goat Alexa Fluor 488, or rabbit anti-sheep Alexa Fluor 546 (all from Invitrogen). Slides were examined by fluorescence and confocal laser scanning microscopy (TCS SP2; Leica Microsystems).

Small interfering RNA. Validated control small interfering RNA (siRNA) or human Ang-2 siRNA (Ambion) were transfected using Oligofectamine reagent (Invitrogen) following the manufacturer's instructions. Efficacy of gene silencing was controlled by reverse transcription-PCR (RT-PCR) for all indicated time points and transfections.

RT-PCR. Expression of human Ang-2 and additional angiogenic factors in human cell lines and melanoma sections was analyzed by RT-PCR using 1 μg total cellular RNA for reverse transcription. The following primers were used: *hAng-2* forward 5'-tggcagcgttgatttcagag-3' and reverse 5'-cgttgctccatcttctgtgc-3', *hTie2* forward 5'-tgtctctctccag-gatgg-3' and reverse 5'-tgttcacactgcagacccaaa-3', *hTie1* forward 5'-accatctccactgtccg-3' and reverse 5'-tggcgtggagggtgtcg-3', *hVEGF* forward 5'-cttgctctgctctactct-3' and reverse 5'-ggcacacaggattga-3', *hVEGFR-1* forward 5'-gcttggcccaataatcaga-3' and reverse 5'-acacgactccatgttgta-3', *hVEGFR-2* forward 5'-catgtggtctctctgtgtg-3' and reverse 5'-ctggtcactg-gaaggagat-3', *hGAPDH* forward 5'-accacagtcctcatcac-3' and reverse 5'-tcaccacctgtgatga-3', and *hActin* forward 5'-acctgaagtacccat-3' and reverse 5'-tagaagcatttgcggtg-3'.

Table 1. Patients characteristics

	No. cases	Age (y)	Ang-2 (ng/mL), median (25%/75%)	S100β (μg/L), median (25%/75%)	Follow-up time (mo)
Patients	65	60 ± 13	2.03 (1.71/3.28)*	0.05 (0.03/0.09)*	72 ± 52
Sex					
Male	36	60 ± 12	1.96 (1.71/3.15)	0.04 (0.02/0.08)	66 ± 45
Female	29	59 ± 15	2.16 (1.89/3.28)	0.06 (0.04/0.01)	79 ± 59
Serum samples †					
No.	98				
AJCC I	9	57 ± 18	1.11 (1.03/1.81) ‡	0.04 (0.03/0.05) §	97 ± 25
AJCC II	9	60 ± 12	1.68 (1.19/1.93) ‡	0.04 (0.00/0.07) §	101 ± 82
AJCC III	37	62 ± 11	2.11 (1.93/2.79) ‡	0.06 (0.04/0.10) §	67 ± 47
AJCC IV	43	61 ± 13	3.26 (2.76/4.29) ‡	0.16 (0.05/0.91) §	64 ± 51
Tumor thickness (mm)					
pT ₁ (≤1.00)	11	—	—	—	—
pT ₂ (1.01-2.00)	16				
pT ₃ (2.01-4.00)	17				
pT ₄ (>4.00)	10				
Unknown	11				
Disease progression during follow-up	42/65	—	—	—	—
Mortality					
Alive	17	—	—	—	—
Dead	36				
Last contact > 1 y	12				
Controls	82	43 ± 25	1.24 (0.93/1.57)	0.03 (0.02/0.06)	—

NOTE: Statistical analyses were done using the Wilcoxon rank-sum test (patients versus controls for Ang-2: *P* < 0.0001 and S100β: *P* < 0.0001, males versus females) and the Kruskal-Wallis test to analyze the specificity of Ang-2 or S100β for different stages (Ang-2: *P* = 0.0016 and S100β: *P* = 0.015).

**P* ≤ 0.001.

† Multiple sera were tested in 33 patients during disease progression from AJCC stage III to IV.

‡ *P* ≤ 0.01.

§ *P* ≤ 0.05.

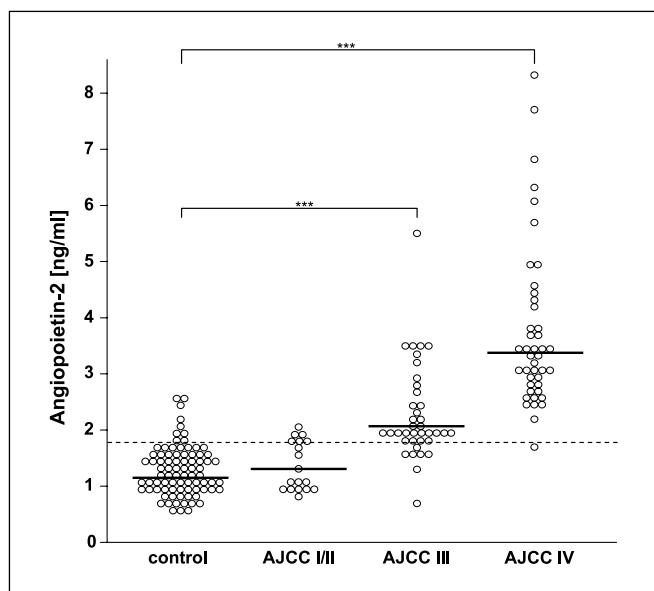


Fig. 1. Detection of circulating Ang-2 levels (sAng-2) in different stages of melanoma patients (AJCC stage I/II-IV) compared with healthy control patients (*ctrl*). sAng-2 levels in patients with malignant melanoma stage III ($n = 37$) and IV ($n = 43$) were significantly elevated compared with the control population ($n = 82$; Wilcoxon rank-sum test control versus stage III, $P < 0.0001$; control versus stage IV, $P < 0.0001$). sAng-2 was not elevated in serum of stage I/II melanoma patients ($P = 0.08$). Dotted line, cutoff for Ang-2 (1.8 ng/mL) determined as 90% quantile of the control population; horizontal lines, median values of the experimental groups.

Migration and invasion assays. To study lateral migration, human melanoma cell lines (MA-Mel-141b, MA-Mel-48a, and MA-Mel-142) were seeded in triplicate into 24-well tissue culture plates and grown to confluence. A scratch wound through the central axis of the plate was gently made using a pipette tip 4 h after the cells were transfected with validated control siRNA or human Ang-2 siRNA. Migration of melanoma cells into the denuded area was determined by repetitive measurements of the distance/scratch at four fixed marks at indicated time points (24 and 36 h). The efficacy of siRNA-mediated gene silencing was monitored by RT-PCR.

For invasion assays, Ang-2-silenced or control siRNA-transfected human melanoma cells (MA-Mel-142, MA-Mel-48a, and MA-Mel-141b) were cultured for 24 h in serum-free RPMI 1640 supplemented with L-glutamine (2 mmol/L) and penicillin/streptomycin (5 units/mL). Transwell chambers (6.5 μ m in diameter, 8 μ m pore size; Vitaris) were coated with growth factor-reduced Matrigel (BD Biosciences) and equilibrated for 1 h at 37°C, 5% CO₂. Transfected human melanoma cells (1×10^5 per well) in serum-free RPMI 1640 were plated in triplicates on top of the Transwell filters. FCS-containing RPMI 1640 (10%) was added to the bottom chamber as chemoattractant. The chambers were incubated for 6 to 24 h at 37°C, 5% CO₂. Cells were fixed with 4% paraformaldehyde and nuclei were stained with Hoechst dye. Invaded melanoma cells were counted microscopically after removal of the cells on the upper side of the membrane with a cotton swab.

Statistical analysis. Analysis of data was primarily based on nonparametric statistical methods given the nonnormal distribution of sAng-2 and S100 β measurements. sAng2 and S100 β were dichotomized into two classes of elevated and nonelevated values using the 90% quantile of the healthy control group as cutoff value. As descriptive variables, we calculated proportions for categorical factors and the median with 25% or 75% quantile for quantitative factors. The cutoff value for sAng-2 was determined at 1.8 ng/mL based on the 90% quantile of healthy controls. The method of maximally selected log-rank statistics was applied to estimate an internal cutoff separating the population between favorable and unfavorable cases based on clinical

data according to Holthorn and Lausen (27) calculated with R software version 2.7.0 and R package coin version 0.6-9. The Wilcoxon rank-sum test was employed for the comparison of two groups and the Kruskal-Wallis test for the comparison of more than two groups. Correlations were examined by calculating the Spearman rank correlation coefficient for quantitative data or the χ^2 statistic for categorical data. Survival data were analyzed using the Kaplan-Meier method in combination with the log-rank test and the Cox proportional hazards model. These were based on the time from day of blood withdrawal until death or last date of follow-up (censored data). Multivariate analysis of prognostic factors for survival was also done using the proportional hazards model of Cox. An all-subset regression survival analysis was done to explore the possible effect of the factors stage, sAng2, and S100 β , that is, taking all three together in one model one at a time as well as taking only one at a time together with stage. In addition, stratified Cox regression was used to assess the prognostic effect of the two markers with stage-dependent baseline hazards. The effects were quantified using the hazard ratios with 95% confidence intervals. For all statistical tests, differences with $P < 0.05$ were considered significant.

Results

Circulating levels of sAng-2 correlate with tumor progression in melanoma patients. Circulating sAng-2 levels in healthy blood donors were determined as 1.24 ± 0.34 ng/mL (mean \pm SD; Table 1; Fig. 1A) corresponding to previously reported sAng-2 levels in healthy blood donors (21). Levels of sAng-2 were not significantly altered in patients with stage I/II melanomas versus controls (Fig. 1A). Similarly, S100 β levels were not elevated in patients with primary melanoma (AJCC stage I/II) when compared with the control group (Table 1). In contrast, sera of stage III melanoma patients with regional lymph node metastases taken within 6 weeks of final surgery had significantly elevated sAng-2 levels (Fig. 1A) with mean sAng-2 concentrations beyond the 1.8 ng/mL cutoff value marking the 90% quantile of the normal control population ($P < 0.0001$, AJCC stage III versus control; Table 1). Stage IV melanoma patients with distant metastasis had even higher sAng-2 levels with >95% of experimental values exceeding the 90% quantile of the control population ($P < 0.0001$; AJCC stage IV versus control; Table 1; Fig. 1A). Comparative analysis of sAng-2 and S100 β adjusted for tumor stage identified a statistical superiority of sAng-2 as marker of melanoma progression compared with S100 β (sAng-2: $P = 0.0016$ and S100 β : $P = 0.015$, Kruskal-Wallis test). The observed results for significance tests presented in Table 1 and Fig. 1 taken together are also significant on the multiple level of $P = 0.05$ when using the Bonferroni-Holm procedure for multiple significance testing.

A subcohort of collected serum samples of patients progressing from stage III to IV was analyzed for sAng-2 to monitor sAng-2 in individual patients during disease progression. An increase of sAng-2 levels was detected in 90% (30 of 33) of patients during the progression from stage III to IV comparing serum samples drawn within 6 weeks of first diagnosis of the disease stage. An increase of sAng-2 up to 50% was revealed in 14 of 33 patients. Eleven patients had 50% to 100% elevated Ang-2 levels and 5 patients had between 100% and 400%.

sAng-2 improves the predictive power of S100 β . S100 β is an established biomarker of melanoma progression. However, S100 β levels are only rarely elevated in early disease and in stage III melanoma (28). To probe if combinatorial S100 β and sAng-2 measurements improve the prognostic power,

we applied the established reference cutoff for S100 β (0.105 ng/mL) and the 90% cutoff for sAng-2 (1.8 ng/mL). As reported previously (28), patients with primary melanoma have S100 β levels below the cutoff, which also applied to sAng-2. Interestingly however, sAng-2 was elevated above the cutoff in 30 of 37 cases in sera of stage III melanoma patients. In contrast, but in line with the literature, only 7 of 37 samples with increased serum concentrations of S100 β were detected. Similarly, sAng-2 levels were above the 90% of the control population in 42 of 43 stage IV melanoma samples compared with 27 of 43 samples above the 0.105 ng/mL cutoff value for S100 β (Fig. 2A). Spearman rank correlation identified a strong overall correlation between sAng-2 and S100 β concentrations ($P = 0.0002$). Comparative analyses to identify stage III to IV progress using a combination of both markers revealed a 56% superiority of sAng-2 over the established marker S100 β when applying the cutoff values of 1.8 ng/mL for sAng-2 and 0.105 ng/mL for S100 β .

Circulating Ang-2 levels correlate with patient survival. To examine if sAng-2 levels correlate with patient survival, we analyzed the prognostic effect of sAng-2 serum concentrations applying the 90% of the control population as cutoff and compared these with the corresponding S100 β analysis. A strong association of elevated sAng-2 level (>1.8 ng/mL) with reduced patient survival was evident when assessing Kaplan-Meier curves by log-rank test ($P = 0.007$; Fig. 2B). Elevated serum levels of S100 β similarly showed a strong correlation with poor overall survival ($P = 0.002$; data not shown). Combination analysis of both biomarkers identified patients with elevated levels of sAng-2 (>1.8 ng/mL) as well as S100 β (>0.105 ng/mL) as strongest indicator for poor prognosis resulting in reduced patient survival (Fig. 2C). Furthermore, maximal selected log-rank statistics was used to investigate the clinical data of sAng-2 and S100 β for the location of cutoff values, which might separate the patients into favorable and unfavorable prognosis for progression-free survival. Using this calculation, we obtained the optimal cutoff for sAng-2 at 1.7 ng/mL with $P = 0.001$ as well as 0.11 ng/mL for S100 β , separating the population between favorable and unfavorable cases with $P = 0.03$. This analysis underlines the prognostic potency of Ang2. We preferred to stay with the 90% determined by the normal data as external source for the definition of the cutoff for this study. A multivariate Cox regression analysis identified tumor stage as the strongest prognostic marker for patient survival ($P = 0.01$, stage III: hazard ratio, 5.95; 95% confidence interval, 1.68-20.94, and stage IV: hazard ratio, 2.93; 95% confidence interval, 0.58-14.66) followed by S100 β ($P = 0.04$, hazard ratio, 2.27; 95% confidence interval, 1.01-5.07) and sAng-2 ($P = 0.19$, hazard ratio, 1.86; 95% confidence interval, 0.73-4.71). Furthermore, the Cox model of stage-dependent baseline hazard function confirmed the multivariate analysis (hazard ratio for Ang-2 = 1.93 and S100 β = 2.32).

Ang-2 is expressed by tumor-associated endothelial cells and melanoma cells. Ang-2 has primarily been characterized as gene product of angiogenically activated endothelial cells. To trace the source of Ang-2 production in different stages of melanomas, we probed the expression of Ang-2 by RT-PCR as well as by immunohistochemistry. In parallel to the circulating biomarker analysis, semiquantitative RT-PCR experiments revealed a robust and reproducible up-regulation of Ang-2

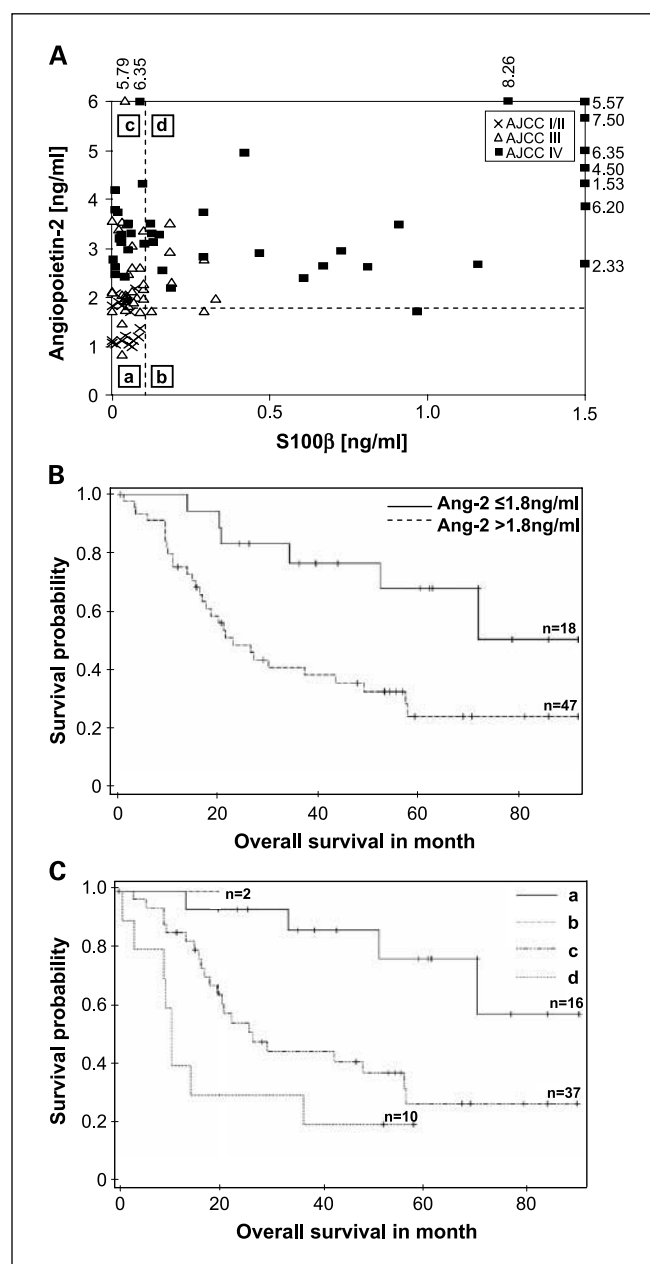


Fig. 2. Correlation of sAng-2 levels with overall survival in human melanoma patients. A, scatter plot for sAng-2 versus S100 β measurements in all serum samples ($n = 98$) from patients with malignant melanoma ($n = 65$). Dotted lines, cutoff for sAng-2 (horizontal lines) and S100 β (vertical lines). Overall correlation between Ang-2 and S100 β is $P = 0.0002$ (Spearman rank). The field denominations (a-d) indicate the corresponding Kaplan-Meier curves as shown in C. B, Kaplan-Meier curves of melanoma patients with sAng-2 concentrations exceeding of the cutoff value of 1.8 ng/mL (dotted line) or sAng-2 concentrations of <1.8 ng/mL (solid line). C, Kaplan-Meier curves of combinatorial sAng-2 and S100 β measurements in melanoma patients applying 0.105 ng/mL as cutoff for S100 β and 1.8 ng/mL as cutoff for sAng-2 (a, S100 β \leq 0.105 ng/mL and Ang-2 \leq 1.8 ng/mL; b, S100 β > 0.105 ng/mL and Ang-2 \leq 1.8 ng/mL; c, S100 β \leq 0.105 ng/mL and Ang-2 > 1.8 ng/mL; d, S100 β > 0.105 ng/mL and Ang-2 > 1.8 ng/mL).

expression in advanced tumor lesions (Fig. 3A). Corresponding double-labeling immunohistochemistry experiments for Ang-2 expression and the endothelial cell marker CD31 in the human melanomas used in the biomarker experiments identified strong Ang-2 expression in endothelial cells of intratumoral

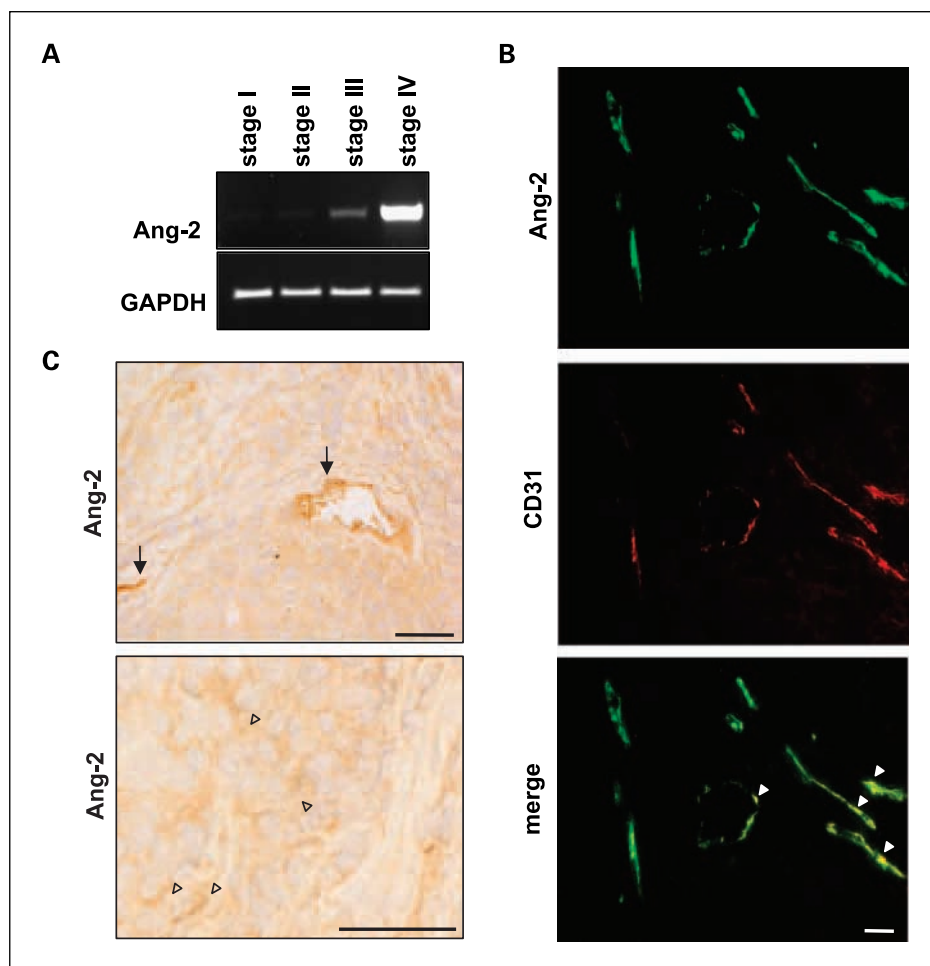
microvessels (Fig. 3B, *arrowheads*). For more sensitive detection and better visualization of the cellular context, we also analyzed Ang-2 expression employing light microscopic immunoperoxidase detection techniques. These experiments confirmed intense endothelial cell expression of Ang-2 (Fig. 3C, *arrows*) but intriguingly also identified weaker but consistently detectable expression of Ang-2 within melanoma cells of advanced-stage tumors (Fig. 3C, *arrowheads*). To validate Ang-2 protein expression by isolated melanoma cells, we analyzed supernatants of the human melanoma cell lines MA-Mel-48a, MA-Mel-141b, and MA-Mel-142 for Ang-2. All three melanoma cell lines constitutively secreted Ang-2 protein into their supernatants. Stimulation with either VEGF or basic fibroblast growth factor did not affect Ang-2 levels in the supernatants of cultured tumor cells (Supplementary Fig. S1).

Primary tumor-derived melanoma cells engage an autocrine Ang-2/Tie2 activation loop. The surprising identification of Ang-2 expression by the tumor cells of human melanomas prompted further experiments to address the question if tumor cell-derived Ang-2 acts in a paracrine manner on endothelial cells or if tumor cell-derived Ang-2 affects the properties of tumor cells themselves in an autocrine manner. We analyzed toward this end the melanoma cell lines MA-Mel-48a, MA-Mel-141b, and MA-Mel-142. These cell lines were selected because the corresponding patients' sera exhibited strongly elevated sAng-2 levels. Additionally, we studied the melanoma cells

line A375 as well as blood endothelial cells isolated from human dermal microvascular endothelial cells of human foreskin, which served as negative and positive controls for the immunohistochemical detection of Ang-2, respectively. Ang-2 expression was detected in the cytoplasm of the three melanoma cell lines MA-Mel-48a, MA-Mel-141b, and MA-Mel-142, whereas the melanoma cell line A375 did not express Ang-2 (Fig. 4A). These findings were validated by corresponding RT-PCR analysis (Fig. 4B). All three human melanoma-derived cell lines expressed Ang-2 mRNA albeit at a lower level than cultured endothelial cells, which matched the difference in expression level between tumor cells and endothelial cells as observed by immunohistochemistry *in vivo*. Further RT-PCR analysis revealed the human melanoma-derived primary tumor cell lines MA-Mel-48a, MA-Mel-141b, and MA-Mel-142 express Tie2, VEGF, and VEGFR-2 but not VEGFR-1 or Tie1 (Fig. 4B).

The detection of both Ang-2 and Tie2 by melanoma cells prompted the hypothesis that melanoma cell-derived Ang-2 may act in an autocrine manner. We consequently pursued siRNA-based Ang-2 loss-of-function experiments and probed the effect of Ang-2 silencing on melanoma cell migration and invasion. Silencing of Ang-2 expression in human melanoma cells was verified by RT-PCR comparing Ang-2-silenced and control-transfected cells. Tumor cell migration was studied in a wound closure assay (Fig. 5A). Comparison of Ang-2-silenced

Fig. 3. Expression and localization of Ang-2 in biopsies of human malignant melanomas. **A**, semiquantitative RT-PCR detection of Ang-2 mRNA in different stages of melanoma progression. GAPDH was used as a loading control. **B**, immunofluorescence double staining for Ang-2 (*red*) and CD31 (*green*) to validate endothelial Ang-2 expression. *Arrowheads*, colocalization of the endothelial cell marker CD31 and Ang-2. **C**, Ang-2 immunoperoxidase staining of melanoma sections revealing prominent Ang-2 expression in tumor-associated endothelial cells (*arrows*) and weaker but consistently detectable Ang-2 expression in melanoma cells (*arrowheads*). Bar, 50 μ m.



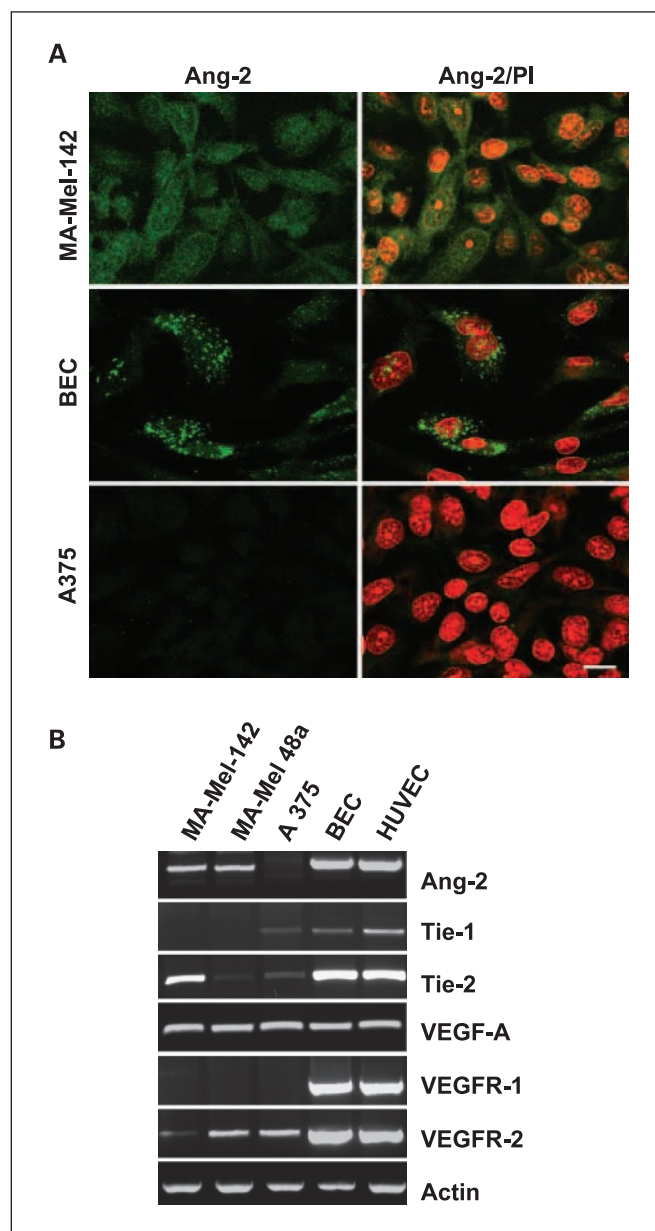


Fig. 4. Ang-2 expression in cultured melanoma and endothelial cells. *A*, human A375 melanoma cells, MA-Mel-142 cells (established from stage IV human melanoma), and human primary blood endothelial cells were costained for Ang-2 (green) and counterstained with propidium iodide (red) to visualize nuclei. Prominent cytoplasmic expression of Ang-2 is detectable in MA-Mel-142 cells (top). Granular expression of Ang-2 in endothelial cells reflects storage in Weibel-Palade bodies (center; ref. 19). A375 melanoma cells do not express detectable levels of Ang-2. Bar, 20 μ m. *B*, semiquantitative RT-PCR analysis of components of the angiopoietin/Tie and VEGF/VEGFR signaling pathways in culture melanoma cells and in endothelial cells. Actin using was used as a loading control.

and control siRNA-transfected cells revealed a pronounced reduction of tumor cell migration at 24 and 36 h ($P = 0.0001$ for both time points compared with control; Fig. 5B). Next, we studied the contribution of an autocrine Ang-2/Tie2 loop to tumor cell invasion employing Transwell invasion chambers. Invasion of Ang-2-silenced tumor cells was strongly reduced after 6 h as well as after 24 h ($P = 0.0001$ for both time points compared with control; Fig. 5C). Robust down-regulation of Ang-2 was observed up to 3 days (Fig. 5D).

Discussion

Malignant melanoma is a neoplastic disease of increasing incidence with poor prognosis and limited therapeutic options, particularly in advanced tumors. The 5-year survival rate of advanced tumors with distant metastases is still as low as 5% to 10% (26). Early detection and surgical excision therefore continue to be the key variables for long-term survival and eventual cure. The identification of early stage III disease is still challenging and consequently among the primary objectives of cancer follow-up programs. Plasma and serum biomarkers are heavily explored toward this end to identify and validate robust diagnostic and prognostic markers of disease progression. The most intensely studied biomarker of malignant melanoma is S100 β , which is a reliable indicator of tumor burden, disease progression, and therapy response in patients with advanced stages of melanoma (28–31). S100 β is elevated only in a subset of stage III patients (29) stressing the need to identify additional biomarkers of melanoma progression that may be independent of S100 β and thereby enhance its prognostic power.

Melanoma progression is critically linked to the induction of angiogenesis. Correspondingly, the assessment of intratumoral microvessel densities has been established as an independent prognostic marker for multiple solid tumors, including melanomas (15, 17). Similarly, circulating biomarkers of angiogenesis, such as the angiogenic factors VEGF, basic fibroblast growth factor, interleukin-8, and angiogenin, have been determined in melanoma patients (32). Population studies have clearly associated elevated levels of these biomarkers with ongoing angiogenesis. Yet, all hitherto analyzed angiogenesis biomarkers have little prognostic power for individual tumor patients (33, 34).

The angiopoietin/Tie system regulates later stages of the angiogenic cascade controlling endothelial cell survival and vessel maturation (10). Ang-1 acts as the agonistic ligand of Tie2 thereby maintaining vascular quiescence. In contrast, Ang-2 primarily functions as antagonistic ligand of Tie2, although context-dependent agonistic effects of Ang-2 have also been described. Ang-2-mediated vessel destabilization primes the responsiveness of endothelial cells toward angiogenic cytokines. Ang-2 thereby acts in tumors primarily as promoter of angiogenesis, which has made it an attractive target for therapeutic intervention (35). Most intriguingly, Ang-2, unlike Ang-1, is almost exclusively produced by endothelial cells themselves suggesting an autocrine mode of action (18, 19). Endothelial cell secretion of Ang-2 is therefore readily detectable in the circulation, which has in recent years stimulated the exploitation of circulating sAng-2 in the blood as biomarker of endothelial cell activation (24, 36). Elevated sAng-2 levels have been identified in several tumors, including lung cancer (22), colorectal cancer (23), and hepatocellular carcinoma (37).

The present study identified elevated levels of sAng-2 in patients with malignant melanoma compared with healthy individuals. Moreover, circulating levels of sAng-2 strongly correlate with patients' tumor load, stage of disease, and overall patient survival. Whereas patient sera of stage I and II melanomas showed no significant increase of sAng-2 compared with healthy blood donors, sera of stage III and IV patients contained significantly elevated levels of sAng-2. Importantly,

based on the relatively small SD of sAng-2 in healthy control individuals (27.4% of the mean), we could establish a reliable 90% quantile as cutoff value that discriminated well between control individuals and stage III or IV melanoma patients. This allowed the identification of a larger number of serum samples (56%) from stage III and IV melanoma patients compared with the determination of S100 β alone, indicating that sAng-2 may be of particular use to refine S100 β clinical chemistry for the more reliable detection of stage III melanoma patients.

Analyzed sera from progressing patients over time identified increasing concentrations of sAng-2 during tumor progression, suggesting that Ang-2 may not just be a marker of tumor progression but functionally involved in melanoma progression and metastasis. To further study this hypothesis, we performed a detailed analysis of Ang-2 expression in tumor samples and in primary human tumor-derived melanoma cell lines. These experiments confirmed that Ang-2 is in tumors predominately expressed by tumor-associated endothelial cells (20). Yet, much to our surprise, Ang-2 expression was also

detected in melanoma cells themselves *in vivo* as well as *in vitro*. Expression on a per-cell basis appeared to be weaker as in endothelial cells. Yet, considering the abundance of tumor cells over endothelial cells in any given solid tumor, it appears likely that tumor cell-derived Ang-2 significantly contributed to the detected levels of circulating sAng-2.

These experiments also identified that tumor cells express the Ang-2 receptor Tie2. Expression of angiogenic growth factor receptors by tumor cells has been reported for VEGFR-2 (38) and is believed to contribute to the antitumor therapeutic efficacy of anti-VEGF/VEGFR therapies (39). Likewise, conflicting results have recently been reported if tumor cells express VEGFR-3 (40, 41). The identification of yet another angiogenic receptor tyrosine kinase expression by tumor cells of advanced-stage tumors therefore supports an emerging theme that tumor cells exploit angiogenic signaling systems of endothelial cells (that is, migration-, invasion-, and proliferation-regulating signaling systems) to support invasive and migratory signaling mechanisms related to tumor progression.

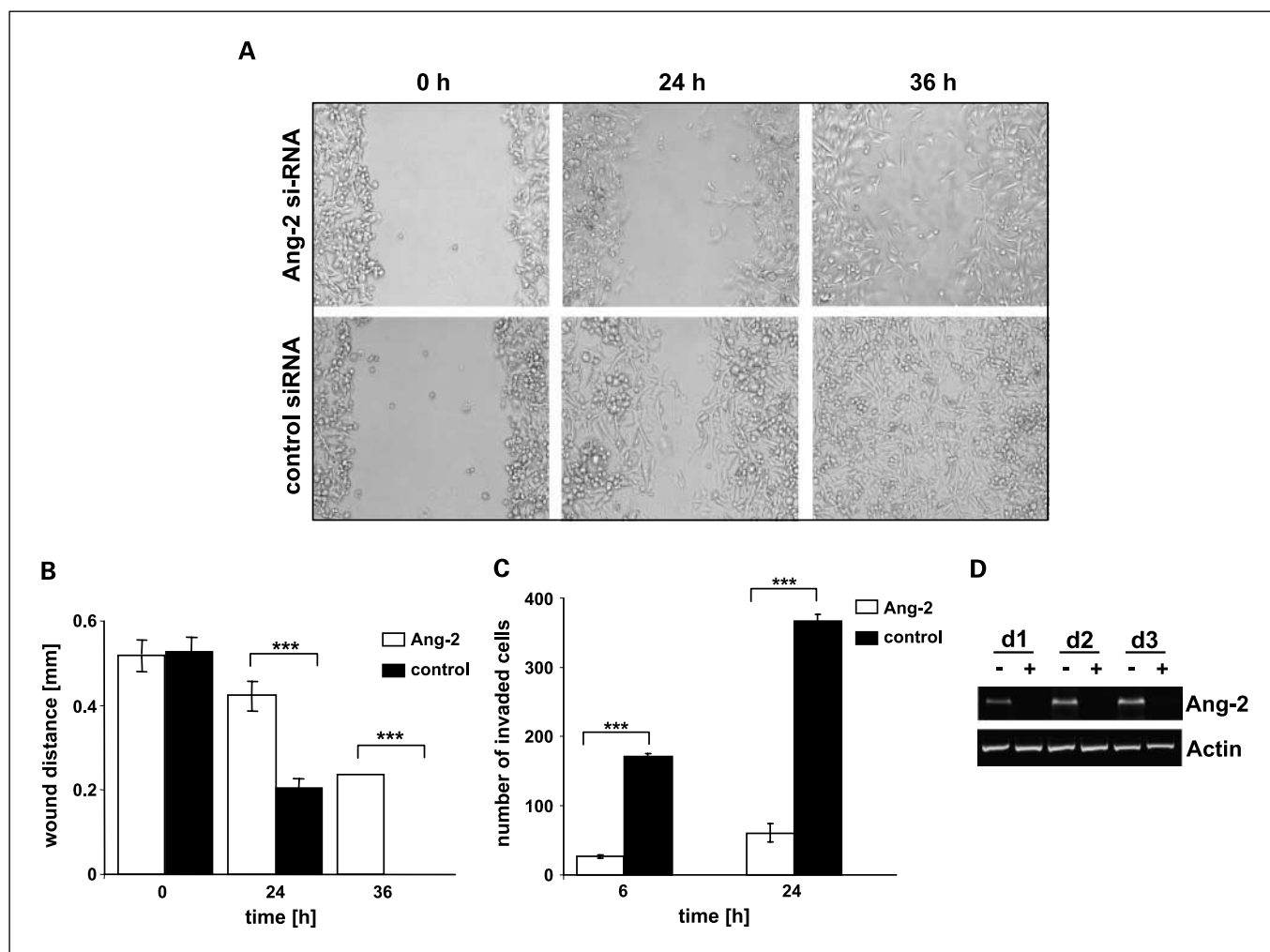


Fig. 5. Effect of autocrine, melanoma cell-derived Ang-2 on melanoma cell migration and invasion. *A*, representative wound closure assay showing the reduced lateral migration capacity of Ang-2 siRNA-transfected MA-Mel-142 melanoma cells compared with control siRNA-transfected cells. *B*, quantitative analysis of lateral migration wound closure assay. Silencing of Ang-2 expression inhibits lateral melanoma cell migration over a period of 36 h after transfection compared with control. Mean \pm SD of three independent experiments done in triplicate. ***, $P < 0.0001$. *C*, reduced tumor cell invasion after silencing of Ang-2 expression. The invasion capacity of MA-Mel-142 melanoma cells was assessed in Transwell invasion chambers following transfection with Ang-2 siRNA or control siRNA. Mean \pm SD of three independent experiments done in triplicate. ***, $P < 0.0001$. *D*, confirmation of Ang-2 silencing in MA-Mel-142 human melanoma cells by RT-PCR comparing control siRNA-transfected (-) and Ang-2 siRNA-transfected (+) cells for day 1 up to day 3 after transfection. Actin was used as a loading control.

The experiments of this study identified expression of both Ang-2 and its receptor Tie2 in melanoma cells, which strongly supported the hypothesis of an autocrine Ang-2/Tie2 loop. To test this hypothesis, we performed Ang-2 siRNA-mediated loss-of-function experiments. These experiments revealed that Ang-2 silencing in Ang-2-expressing melanoma cells strongly reduced the invasive and migratory capacity of the tumor cells.

In summary, the present study has identified Ang-2 as a marker of melanoma progression that improves the prognostic power of the established melanoma biomarker S100 β . The results of this first clinical study of sAng-2 in melanoma are promising and warrant further analysis in larger cohorts of patients for stage I to IV as well as elevated numbers of serial serum samples from progressing patients with blood withdrawal in short defined time spans. Furthermore, the identification of Ang-2 and Tie2 expression by melanoma cells in combination with the preliminary characterization of a functional autocrine Ang-2/Tie2 loop in primary human tumor-derived melanoma cells makes the angiopoietin/Tie system a

very attractive therapeutic target for human melanomas. Intense efforts are presently made to generate and validate ligand-neutralizing antibodies (35), soluble Tie receptor traps, and small molecular weight Tie2 receptor tyrosine kinase inhibitors to therapeutically interfere with angiopoietin/Tie signaling (42). The data of this study strongly warrant a concerted exploitation of the angiopoietin/Tie system as potentially bimodal anti-angiogenic and antitumorogenic target of human melanomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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