IJC
International Journal of Cancer

Cilengitide downmodulates invasiveness and vasculogenic mimicry of neuropilin 1 expressing melanoma cells through the inhibition of αvβ5 integrin

Federica Ruffini[1], Grazia Graziani[2], Lauretta Levati[3], Lucio Tentori[2], Stefania D'Atri[1] and Pedro M. Lacal[1]

[1] Laboratory of Molecular Oncology, "Istituto Dermopatico dell'Immacolata"-IRCCS, Rome, Italy
[2] Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy
[3] Clinical Epidemiology Unit, "Istituto Dermopatico dell'Immacolata"-IRCCS, Rome, Italy

During melanoma progression, tumour cells show increased adhesiveness to the vascular wall, invade the extracellular matrix (ECM) and frequently form functional channels similar to vascular vessels (vasculogenic mimicry). These properties are mainly mediated by the interaction of integrins with ECM components. Since we had previously identified neuropilin 1 (NRP-1), a co-receptor of vascular endothelial growth factor A (VEGF-A), as an important determinant of melanoma aggressiveness, aims of this study were to identify the specific integrins involved in the highly invasive phenotype of NRP-1 expressing cells and to investigate their role as targets to counteract melanoma progression. Melanoma aggressiveness was evaluated *in vitro* as cell ability to migrate through an ECM layer and to form tubule-like structures using transfected cells. Integrins relevant to these processes were identified using specific blocking antibodies. The αvβ5 integrin was found to be responsible for about 80% of the capability of NRP-1 expressing cells to adhere on vitronectin. In these cells αvβ5 expression level was twice higher than in low-invasive control cells and contributed to the ability of melanoma cells to form tubule-like structures on matrigel. Cilengitide, a potent inhibitor of αv integrins activation, reduced ECM invasion, vasculogenic mimicry and secretion of VEGF-A and metalloproteinase 9 by melanoma cells. In conclusion, we demonstrated that αvβ5 integrin is involved in the highly aggressive phenotype of melanoma cells expressing NRP-1. Moreover, we identified a novel mechanism that contributes to the antimelanoma activity of the αv integrin inhibitor cilengitide based on the inhibition of vasculogenic mimicry.

Cancer Cell Biology

During disease progression, cutaneous malignant melanoma acquires a rich vascular network and increased ability to adhere to the vascular wall, which promotes the transition from the radial to the vertical growth phase.[1] The neovascularisation process is sustained by the secretion of various angiogenic cytokines [*i.e.*, vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2, placental growth factor 1 and -2, interleukin 8 and transforming growth factor 1], integrin overexpression and release of high amounts of metalloproteinases (MMPs).[2,3] Highly malignant cells, because of their ability to dedifferentiate and acquire characteristics of other cell types, form *de novo* vascular networks (vasculogenic mimicry), the presence of which predicts poor prognosis in melanoma patients.[4] This mechanism, that can contribute to new vessel formation and favour tumour growth and invasion, has been extensively studied utilizing melanoma cell lines, although it is also observed in a variety of other tumour types, including carcinomas, sarcomas, glioblastoma and astrocytoma.[4]

Integrins are heterodimeric transmembrane glycoproteins essential for tumour progression that promote processes such as cell adhesion and migration. In cells that adhere to the extracellular matrix (ECM), integrins clusterize at the cell attachment sites, called focal adhesions, promoting the reorganization of the intracellular actin cytoskeleton and stimulating the activity of signalling molecules [*e.g.*, protein kinases such as Src, focal adhesion kinase (FAK), extracellular signal-related kinase (ERK1/2), protein kinase C (PKC)].[5,6] Besides allowing mechanical adhesive interactions, focal adhesions

Key words: melanoma, integrins, cilengitide, neuropilin 1, vasculogenic mimicry

Abbreviations: BSA: bovine serum albumin; ECM: extracellular matrix; ERK1/2: extracellular signal-related kinase 1/2; EC_{50}: half maximal effective concentration; FAK: focal adhesion kinase; FBS: foetal bovine serum; IgG1: immunoglobulin G1; MMPs: metalloproteinases; MMP-2: metalloproteinase 2; MMP-9: metalloproteinase 9; MMP-14: metalloproteinase 14; NRP-1: neuropilin 1; PKC: protein kinase C; TMZ: temozolomide; VEGF-A: vascular endothelial growth factor A; VEGFR-2: VEGF receptor 2

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Italian Ministry of Health to P.M.L; **Grant number:** RC14-3.3; **Grant sponsor:** "Associazione Italiana per la Ricerca sul Cancro" (AIRC) to G.G; **Grant number:** AIRC 2013 IG 14042
DOI: 10.1002/ijc.29252
History: Received 21 Mar 2014; Accepted 17 Sep 2014; Online 4 Oct 2014

Correspondence to: Pedro M. Lacal, Laboratory of Molecular Oncology, "Istituto Dermopatico dell'Immacolata"-IRCCS, Via dei Monti di Creta 104, 00167 Rome, Italy, Tel.: +39-06-66464732, Fax: +39-06-66464705, E-mail: p.lacal@idi.it

What's new?

In melanoma, invasiveness is mediated by cell-adhesion molecules called integrins. A receptor called neuropilin-1 (NRP-1) also causes these tumors to become more aggressive. In this study, the authors identified specific αv-integrins that are involved in the switching of melanoma cells that express NRP-1 to a metastatic phenotype. The study also identified a novel mechanism by which the peptide drug cilengitide may exert anti-melanoma activity: By inhibiting the activation of αvβ5 integrin, cilengitide blocks the formation of vascular networks by the tumor cells. This should, in turn, reduce the invasiveness of the tumors.

also permit tumour cells to sense the extracellular environment, responding through the activation of intracellular signalling pathways that trigger cell survival, proliferation or migration events.[7] Therefore, changes in integrin expression or integrin-mediated functions and signals deriving from the binding to specific ligands, influence the ability of melanoma cells to interact with the extracellular environment and favour metastatic progression by promoting a switch from a proliferative to an invasive phenotype.[8] Thus, integrins represent suitable targets of therapeutic strategies to inhibit the metastatic disease. In this regard, cilengitide, a cyclic arginine–glycine–aspartic acid containing pentapeptide that potently blocks the activation of αv integrins (*e.g.*, αvβ3 and αvβ5 integrins), represents the most clinically advanced integrin-targeting therapeutics. In fact, cilengitide is currently under evaluation in a phase III study (in combination with radio-chemotherapy) for newly diagnosed glioblastoma and in phase II studies for other tumour types, including metastatic melanoma[9–11] (www.clinicaltrials.gov). However, cilengitide as single agent has shown minimal clinical efficacy in patients with metastatic melanoma, suggesting that the integrin inhibitor may prove more effective in combination therapies.[12] Indeed, preclinical studies had shown that cilengitide and the methylating agent temozolomide (TMZ), currently used for the therapy of metastatic melanoma, exerted synergistic antiproliferative effects against melanoma and endothelial cells *in vitro* and induced a statistically significant reduction of *in vivo* melanoma growth, as compared to treatment with TMZ alone.[13] In this context, a better knowledge of the molecular mechanisms by which cilengitide affects melanoma cell growth and invasion might help to potentiate its anticancer activity by combination therapies.

To identify the specific integrins activated in highly aggressive melanomas that might be targeted for the treatment of the metastatic disease, we used a cellular model represented by different clones of the human melanoma cell line M14[14]: the M14C2/MK18 clone, expressing the VEGF-A receptor 2 (VEGFR-2) and the coreceptor neuropilin 1 (NRP-1), and the M14-N clone, expressing only NRP-1.[15,16] M14C2/MK18 and M14-N clones display a highly invasive phenotype, demonstrated by their ability to migrate through an extracellular basement matrix. Studies utilizing these clones demonstrated that even though the simultaneous presence of both VEGFR-2 and NRP-1 potentiates VEGF-A secretion and the aggressiveness of melanoma cells, NRP-1 is by itself able to promote cell invasion.[15,16] Actually, NRP-1 has been detected by immunostaining in tumour specimens of different tissue origin, including melanoma.[17,18] Moreover, its expression levels have been found to directly correlate with the aggressiveness and invasive behaviour of prostate and gastrointestinal tumour cells.[19,20]

We therefore analysed the involvement of specific integrins in the invasion of ECM and in the vasculogenic mimicry by NRP-1 expressing melanoma cells. In addition, we investigated the potential activity of cilengitide as modulator of these processes.

Material and Methods

Reagents and cell lines

Cell culture media and reagents were purchased from Lonza (Basel, Switzerland), foetal bovine serum (FBS) was from Euroclone (Pero, Italy) and fatty acid-free bovine serum albumin (BSA) from Roche (Mannheim, Germany). VEGF-A and polyclonal antibodies used in ELISA assays (AF-293 and BAF-293), as well as the mouse immunoglobulin G1 (IgG1) control antibody (clone11711) used in the functional assays, were from R&D Systems (Abingdon, UK). The anti-αvβ3 (clone LM609), anti-αvβ5 (clone P1F6), anti-α2β1 (clone BHA 2.1) and anti-α5β1 (clone JBS5) monoclonal antibodies were from Chemicon International (Temecula, CA). Cilengitide was a kind gift from Merck (Merck KGaA, Darmstadt, Germany). The monoclonal antibody anti-NRP-1 (clone A12) was from Santa Cruz (Santa Cruz, CA). The concentrations of cilengitide used were as follows: 0.2–20 μg/ml in the assay of cell adhesion to vitronectin to evaluate the drug half maximal effective concentration (EC_{50}) and 20 μg/ml in all the other assays.

The origin and culture conditions of M14C2 subclones M14C2/C4, M14C2/MK18 and M14-N were previously described.[16,21] The M14C2/MK18 clone is characterised by the activation of a VEGF-A/VEGF receptor 2 (VEGFR-2) autocrine loop, overexpression of the VEGF-A coreceptor NRP-1 and secretion of high levels of metalloproteinase 2 (MMP-2)[15]; the M14-N clone expresses NRP-1 and secretes high levels of VEGF-A and MMP-2, but is defective in VEGFR-2 expression[16]; the control M14C2/C4 clone is devoid of the expression of both VEGFR-2 and NRP-1 proteins and secretes lower amounts of VEGF-A and MMP-2.[15,16]

The MW115 and MW266-4 cell lines were purchased from ATCC (Manassas, VA). Normal human fibroblasts were

kindly provided by the Laboratory of Molecular and Cell Biology, IDI-IRCCS, Rome, Italy.

Cell adhesion to ECM components

Cell adhesion was tested on 96-well plates previously coated with the commercial basement membrane matrix matrigel (20 μg/ml; BD Biosciences, Buccinasco, Italy), laminin (10 μg/ml, Sigma-Aldrich, Saint Louis, MO), Collagen I (10 μg/ml, Roche), Collagen IV (10 μg/ml, BD Biosciences), fibronectin (10 μg/ml, Sigma-Aldrich) or vitronectin (5 μg/ml, Sigma-Aldrich) and blocked with 3% BSA. Cells were seeded into the wells (4×10^4 cells/well) and incubated at 37°C for 40 min. Nonadherent cells were, then, washed out and attached cells were fixed in ethanol and stained with crystal violet. The adhesion efficiency was determined by quantitative dye extraction and spectrophotometric measurement of the absorbance at 595 nm in a Microplate reader 3550-UV (Bio-Rad, Hercules, CA).

Chemotaxis and ECM cell invasion assays

In vitro invasion assays were performed using Boyden chambers equipped with 8-μm pore diameter polycarbonate filters (Nuclepore; Whatman Incorporated, Clifton, NJ), coated with 20 μg of matrigel.[14] Briefly, melanoma cells were collected from continuous cultures, washed, suspended in migration medium (1 μg/ml heparin/0.1% BSA in RPMI 1640), and loaded (2×10^5 cells) into the upper compartment of the Boyden chambers. Migration medium was also added to the lower compartment of the chambers. For each experimental condition, three Boyden chambers were set up. After incubation of the Boyden chambers at 37°C in a CO_2 incubator for 3 h, the filters were removed from the chambers and cells were fixed in ethanol for 5 min and stained in 0.5% crystal violet for 15 min. Nonmigrating cells were removed from the upper surface of the filter by wiping with a cotton swab and the migrated cells, attached to the lower surface of the filters, were counted under the microscope. Twelve high-magnification microscopic fields (×200 magnification), randomly selected on triplicate filters, were scored for each experimental condition. Where indicated, invasion assays were performed in the presence of antibodies directed against selected integrins or in the presence of cilengitide, after pre-incubation of the cells with these reagents for 30 min at room temperature, in a rotating wheel.

The chemotactic response of melanoma cells to different stimuli was analysed as described for the invasion assays, using polycarbonate filters coated with 5 μg/ml gelatin solution and allowing the cells to migrate for 18 h, as previously reported.[22]

Flow cytometry analysis of integrin expression

Cells were harvested, washed with PBS and incubated at 4°C for 30 min with each of the following monoclonal antibodies (2 μg/10^6 cells): anti-αvβ3 (clone LM609), anti-αvβ5 (clone P1F6), anti-α2β1 (clone BHA 2.1), anti-α5β1 (clone JBS5) or mouse IgG1 control (clone 11711). Cells were then washed with PBS, incubated with a secondary goat anti-mouse IgG (Fc specific)-FITC antibody (Sigma-Aldrich) and analysed using a FACScan flow cytometer (Becton & Dickinson).

Evaluation of VEGF-A and MMP secretion

Semi-confluent melanoma cell cultures were incubated in 0.1% BSA/RPMI 1640 medium without FBS for 24 h. Culture supernatants were then collected, centrifuged at 600*g* for 10 min to remove cells in suspension, concentrated at least ten-fold in Centriplus concentrators (Amicon, Beverly, MA) and frozen at −20°C till use. Cells were detached from the flasks with a solution of 1 mM EDTA in PBS and the total cell number/culture was recorded. Quantification of the amount of VEGF-A in the concentrated supernatants was performed using Maxisorp Nunc immunoplates (Nunc, Roskilde, Denmark) coated with goat anti-VEGF-A IgGs, as previously described.[23] The amount of MMP-2 and MMP-9 in the supernatants were determined using human Quantikine immunoassay kits (R&D Systems), whereas the amount of MMP-14 in supernatants and cell extracts was measured using an ELISA kit from MyBioSource (San Diego, CA), according to the manufacturer's directions. The activity of MMP-2 and MMP-9 was tested using QuickZyme activity assays (QuickZyme Biosciences, Leiden, The Netherlands).

Differentiation of melanoma cells in tubule-like structures

For the analysis of tubule-like structures on matrigel, the matrix was allowed to thaw overnight at 4°C and diluted 1:3 in serum-free RPMI 1640 medium. Diluted matrigel (100 μl) was layered onto 24-well plates that were then incubated at 37°C for 30 min, until matrigel solidification, as described.[24] Melanoma cells were suspended in complete culture medium (1×10^5 cells in 0.5 ml), dispensed onto the solidified matrix and incubated at 37°C in a 5% CO_2 environment. At the indicated times, the plates were photographed using a Leica inverted microscope and a Canon digital camera PowerShot G5. The formation of tubule-like structures was quantified by counting the number of cell intersections in ten different microscopic fields per group (×50 magnification).

ITGB5 gene knockdown

M14-N cells were reverse transfected with either siRNA targeting the integrin β5 gene *ITGB5* (ID 11201, Life Technologies, Carlsbad, CA) or nontargeting siRNA (Silencer Negative Control #1 siRNA, Life Technologies) to a final concentration of 20 nM, using lipofectamine RNAiMAX in OptiMEM serum-free medium (Life Technologies), according to the manufacturer's instructions. Cells were harvested 72 h after transfection and subjected to further analyses.

Cell proliferation assay

Cell proliferation was evaluated in 96-well plates using the tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulphophenyl)-2H-tetrazolium,

inner salt] from Promega (Madison, WI), as previously described.[22]

Statistics

Results are expressed as the arithmetic mean ± standard deviation (SD) and significance of the differences was tested by unpaired, two-tailed Student's *t*-test; *p* values <0.05 (*), <0.01 (**) and <0.001 (***) were considered significant.

Results

Involvement of specific integrins in the adhesiveness and invasiveness of highly aggressive human melanoma cells expressing VEGFR-2 and NRP-1

We had previously reported the *in vitro* highly invasive phenotype and the *in vivo* aggressiveness of a cell clone originated from the human melanoma cell line M14 (M14C2/MK18) that expresses VEGFR-2 and NRP-1 and secretes high levels of VEGF-A and MMP-2.[15] In order to investigate the involvement of specific integrins in the aggressiveness of these cells, we initially analysed cell adhesion to matrigel and to single ECM components, known to be ligands for specific integrins. The results demonstrated that M14C2/MK18 cells adhered more efficiently than the noninvasive control cells (the M14C2/C4 cell clone lacking both VEGFR-2 and NRP-1 expression) to matrigel (Fig. 1*a*), Collagen IV, one of the main components of matrigel, or ECM components not present in matrigel, such as Collagen I and vitronectin (Fig. 1*b*). Both melanoma cell clones adhered to fibronectin (not present in matrigel) to a similar extent, whereas none of them adhered to laminin, the other main component of matrigel (Fig. 1*b*). The integrin contribution to the aggressive phenotype of M14C2/MK18 cells was confirmed by the finding of a constitutive activation of FAK, a kinase involved in the integrin-induced signal transduction pathway. In M14C2/C4 control cells the phosphorylated/active form of this kinase was, instead, detected only after induction of cell adhesion (Supporting information Fig. S1).

The integrins involved in the interaction of M14C2/MK18 cells with the different components of the ECM were then identified performing the adhesion assays in the presence of antibodies recognizing specific integrins known to bind particular ECM components.[25] Adhesion to Collagens I and IV was markedly inhibited by treatment of melanoma cells with blocking antibodies directed against α2β1 integrin (Fig. 1*c*). The adhesion of these cells to vitronectin was strongly reduced by an antibody against the αvβ5 integrin, whereas it was only modestly affected by an antibody against αvβ3, the other main integrin interacting with vitronectin (Fig. 1*c*). Blocking antibodies against α5β1 integrin, the major fibronectin receptor, strongly interfered with the binding to this ECM component and to a minor extent to Collagen IV.

To test whether the differences in adhesion to Collagens I and IV and to vitronectin between M14C2/MK18 and M14C2/C4 clones were due to differences in the amount of α2β1 and αvβ5 active complexes on the cell surface, the integrin expression was evaluated by flow cytometry analysis (Fig. 1*d* and Supporting information Fig. S2*a*). The results showed that the expression of α2β1 and αvβ5 integrins in M14C2/MK18 cells was, respectively, fourfold and twofold higher than in the control cells. Conversely, the expression of αvβ3 expression in M14C2/MK18 was reduced by about a 40% (Fig. 1*d*). These data might explain the relevance of αvβ5 in M14C2/K18 cell adhesion to vitronectin and the modest inhibitory effect of the anti-αvβ3 antibody on the adhesion to vitronectin.

We next investigated the specific role played by each of the above described integrins in the ability of M14C2/MK18 cells to adhere to and to migrate through a layer of matrigel as a measure of melanoma aggressiveness. Addition during the assays of specific anti-integrin antibodies demonstrated that inhibition of α2β1 integrin reduced cell adhesion by 50% (Fig. 1*e*) and cell invasion by 25% (Fig. 1*f*). These results are consistent with the fact that Collagen IV is one of the principal matrigel components and are in agreement with the previously suggested involvement of α2β1 integrin in melanoma progression.[8,26] The blockage of the αvβ3 or αvβ5 integrins, which are both vitronectin receptors, did not affect cell adhesion to matrigel, since vitronectin is not a matrigel component (Fig. 1*e*). Nevertheless, the anti-αvβ3 or anti-αvβ5 integrin antibodies inhibited cell invasiveness through a matrigel monolayer by about 30% (Fig. 1*f*), suggesting a role for these integrins in melanoma cell invasion by a mechanism different from cell adhesion to ECM components. Moreover, no changes in either adhesion or invasiveness of M14C2/MK18 cells were observed in the presence of anti-α5β1 integrin antibodies (Figs. 1*e* and 1*f*), indicating that this integrin is not directly involved in these melanoma properties.

Cilengitide inhibits invasiveness and vasculogenic mimicry of melanoma cells expressing VEGFR-2 and NRP-1

Based on the finding that antibodies against αvβ3 and αvβ5 integrins were able to inhibit the invasion of matrigel by M14C2/MK18 cells, we tested the ability of the αv integrin inhibitor cilengitide to counteract melanoma cell invasiveness. Cilengitide inhibited adhesion of M14C2/MK18 cells to vitronectin in a dose-dependent manner, with an EC_{50} of 6.66 ± 1.77 μg/ml (Fig. 2*a*), whereas this agent did not affect adhesion to fibronectin (used as negative control since it mainly interacts with the α5β1 integrin). The concentration of cilengitide causing the maximal inhibitory effect on adhesion to vitronectin (*i.e.*, 20 μg/ml), strongly reduced the invasion of matrigel by M14C2/MK18 cells (Fig. 2*b*) without affecting cell viability (data not shown). Since cilengitide specifically interacts with the αvβ3 and αvβ5 integrins, our results confirmed the relevance of these integrins in melanoma invasiveness.

Previous studies have demonstrated a direct correlation between the αvβ3 and αvβ5 integrin expression and the formation by melanoma cells of tubule-like structures similar to those formed by endothelial cells under the same culture

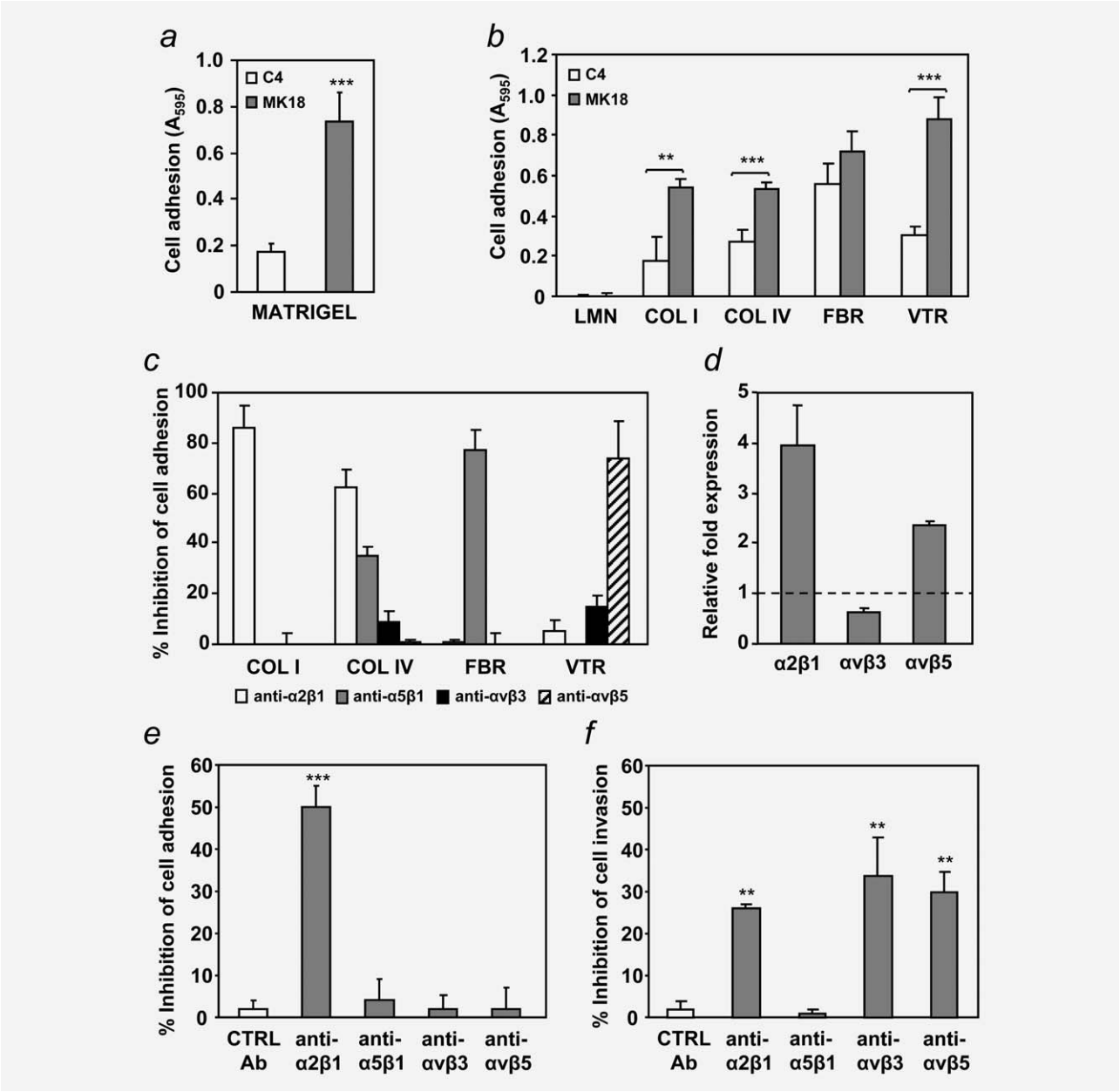

Figure 1. Identification of the integrins involved in the increased adhesiveness to ECM components and invasiveness of M14C2/MK18. (*a* and *b*) The ability of M14C2/MK18 (MK18) and control M14C2/C4 cells (C4) to adhere on matrigel (*a*) or single ECM components (*b*) was tested by plating 4×10^4 cells/well in 96-well plates for 30 min at 37°C. LMN: laminin; COL I: type I collagen; COL IV: type IV collagen; FBR: fibronectin; VTR: vitronectin. (*c*) Integrins involved in the interaction with collagens, fibronectin and vitronectin were identified by preincubating M14C2/MK18 cells with specific anti-integrin blocking antibodies (3 μg/ml) for 45 min in a rotating wheel, at room temperature, and performing the adhesion assay (20 min at 37°C) in the presence of the antibodies. COL I: type I collagen; COL IV: type IV collagen; FBR: fibronectin; VTR: vitronectin. (*d*) The expression of the integrins involved in M14C2/MK18 cell adhesion to the ECM components under study was evaluated in M14C2/MK18 cells by FACS analysis. Relative fold expression for each single integrin was calculated as the ratio between the mean fluorescence intensity value in M14C2/MK18 cells and that in M14C2/C4 control cells. (*e*) The effect of antibodies (3 μg/ml) that block specific integrins on M14C2/MK18 cell adhesion to matrigel was analysed as described in the legend to panels *a* and *b*. (*f*) The influence of anti-integrin antibodies on melanoma cell invasion through matrigel was evaluated in Boyden chambers equipped with filters coated with 20 μg matrigel, in the presence of anti-integrin blocking antibodies. Cells (2×10^5 cells per chamber) were preincubated for 45 min at room temperature with 3 μg/ml of blocking antibodies that were maintained during the assay for 4 h at 37°C. CTRL Ab, control antibody (mouse IgG1 control). In all panels data represent the arithmetic mean values ± SD of four to six independent determinations.

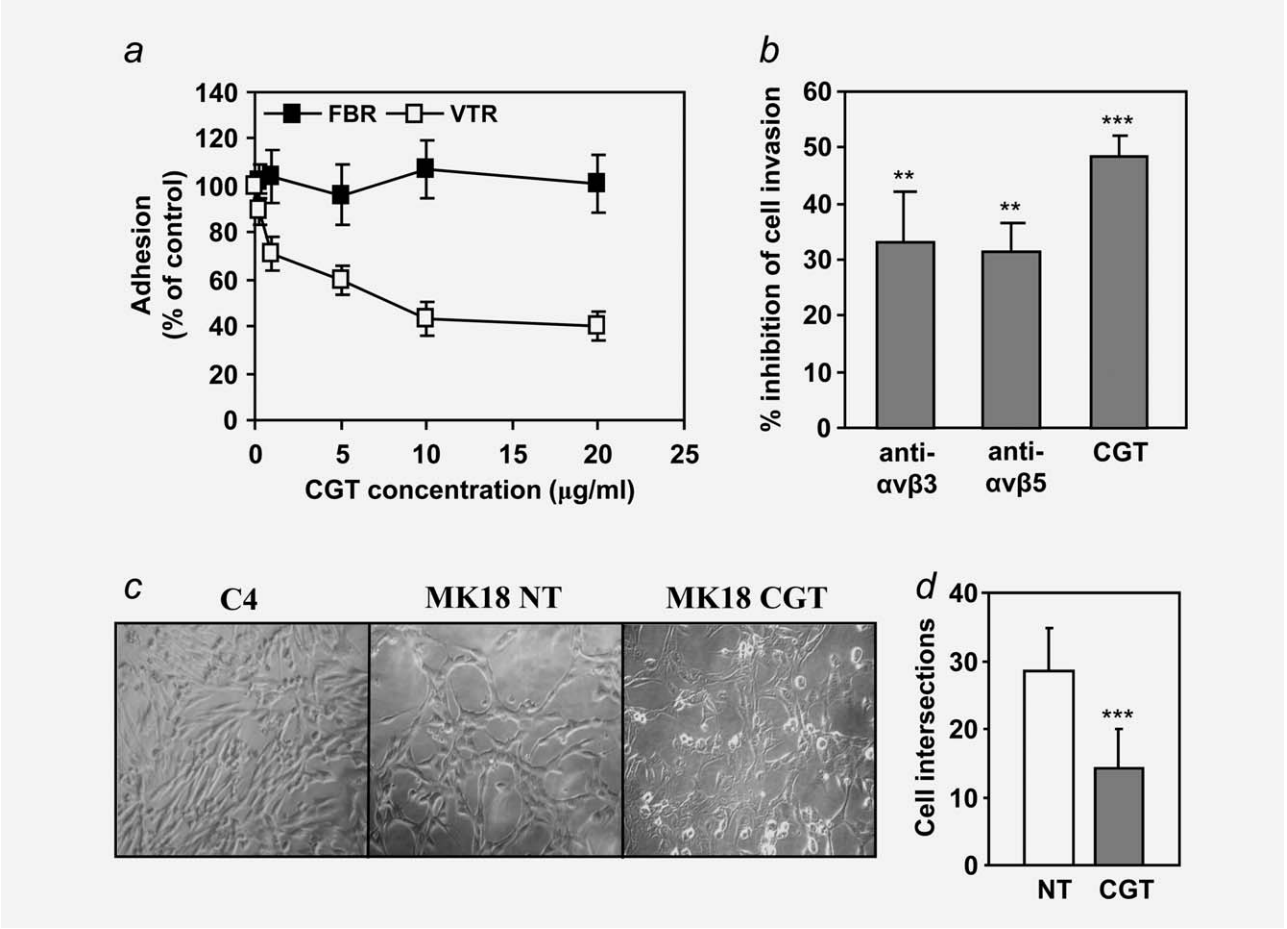

Figure 2. Effects of cilengitide on the ability of M14C2/MK18 cells to invade matrigel and to form tubule-like structures. (*a*) Dose–effect curve of the αvβ3 and αvβ5 inhibitor cilengitide (CGT) on M14C2/MK18 cell adhesion to vitronectin (VTR), or fibronectin (FBR) as a negative control. (*b*) Effect of 20 μg/ml cilengitide (CGT) on matrigel invasion by M14C2/KM18 cells, evaluated utilizing Boyden chambers as described in the legend to Figure 1*f*. The results obtained with the anti-αvβ3 or anti-αvβ5 integrin antibodies (3 μg/ml) were included for comparison. (*c*) Effect of cilengitide (CGT) on the capability of M14C2/MK18 cells (MK18) to form tubule-like structures. Cells were non-treated (NT) or pre-incubated with 20 μg/ml cilengitide for 30 min in a rotating wheel at room temperature, before seeding them on matrigel. Tubule-like structures formation was analysed after 18 h. Photographs from a representative experiment out of three are shown (×50 magnification). M14C2/C4 (C4) cells were included as a negative control because they are unable to form tubule-like structures in the same culture conditions utilized to test M14C2/MK18 cells. (*d*) The number of cell intersections of the tubule-like structures shown in panel C was counted in ten different microscopic fields for the indicated experimental groups. In all panels data represent the arithmetic mean values ± SD of three independent determinations.

conditions (vasculogenic mimicry).[27] We therefore evaluated the ability of M14C2/MK18 cells to arrange in tubular structures and the influence of cilengitide on this process. The results indicated that M14C2/MK18 cells, when cultured on matrigel, formed tubule-like structures (Fig. 2*c*) and that cilengitide significantly downmodulated this phenomenon (Figs. 2*c* and 2*d*).

The expression of αvβ5 integrin is required for the inhibitory effect exerted by cilengitide on vasculogenic mimicry and invasiveness of melanoma cells expressing NRP-1 and not VEGFR-2

Since we had previously demonstrated that the aggressiveness of M14C2/MK18 cells is prevalently due to the presence of NRP-1,[15] the expression and functionality of αvβ5 and αvβ3 integrins were also analysed in a M14 cell subclone that expresses NRP-1 but not VEGFR-2 (M14-N cells).[16] M14-N cells showed higher adhesiveness to ECM components than NRP-1 negative control cells (M14C2/C4; Fig. 3*a*). Nevertheless, the blockage of NRP-1 with an antibody that we previously reported to inhibit the matrigel invasion by both M14C2/MK18 and M14-N cell clones[15,16] did not affect their adhesion to ECM components (Supporting information Fig. S2*b*). M14-N cells had an almost identical pattern of integrin expression as M14C2/MK18 cells (Fig. 3*b* and Supporting information Fig. S2*a*) and were sensitive to the cilengitide inhibitory effect on adhesion to vitronectin (EC_{50} of 2.06 ± 0.01 μg/ml; Fig. 3*c*). Treatment with cilengitide or with the anti-αvβ5 integrin antibody resulted in a similar inhibition (about 50%) of M14-N cell

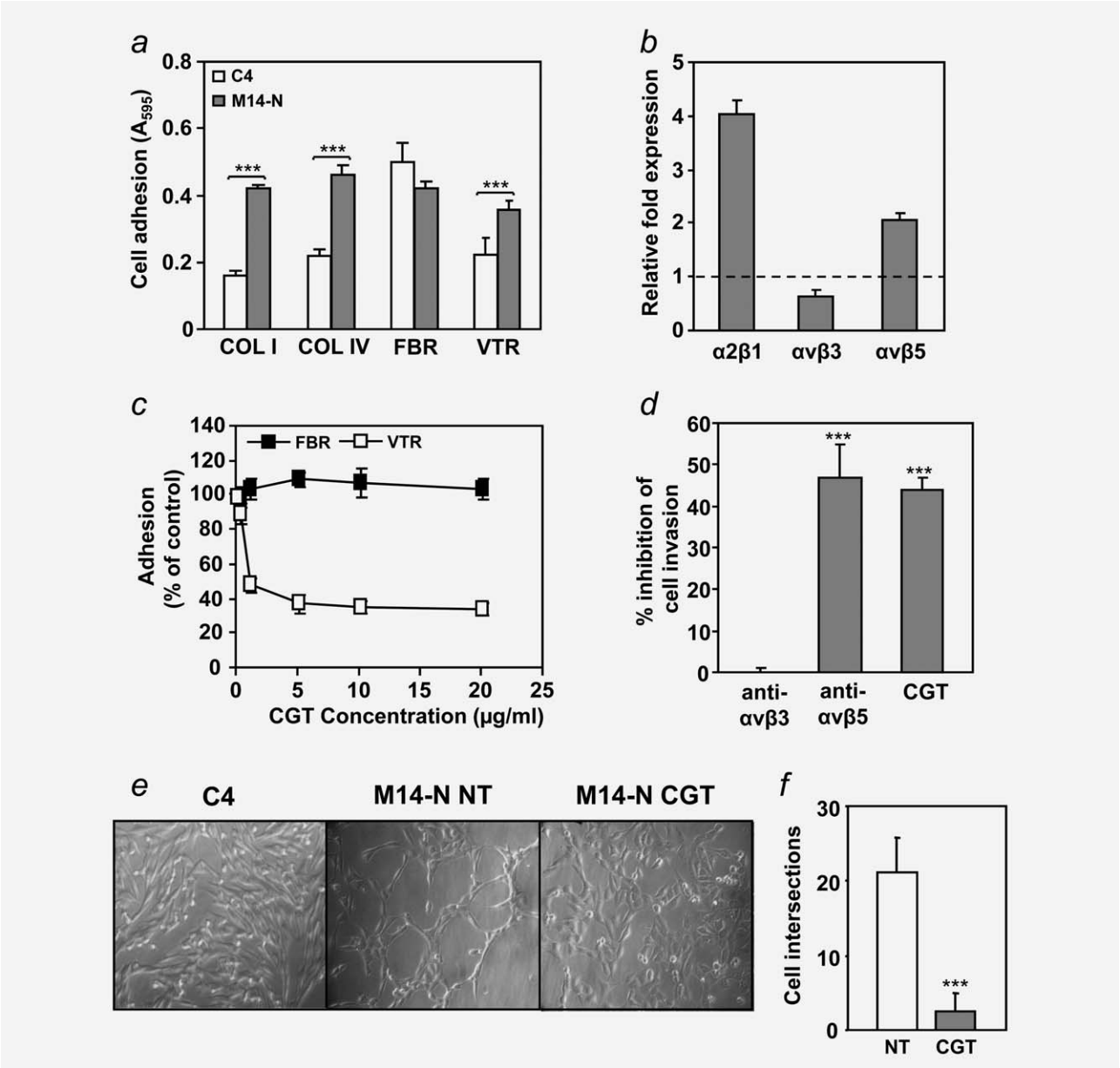

Figure 3. Effects of cilengitide on the ability of M14-N cells to invade matrigel and to form tubule-like structures. (*a*) The ability of M14-N cells to adhere on different components of the ECM was analysed and compared with that of M14C2/C4 control cells (C4). Adhesion on plates coated with single ECM components was tested by plating M14-N or M14C2/C4 cells in 96-well plates (4×10^4 cells/well) for 30 min at 37°C. COL I, type I collagen; COL IV, type IV collagen; FBR, fibronectin; VTR, vitronectin. (*b*) The expression of the integrins involved in the adhesion of M14-N cells to the ECM components analysed in panel A was evaluated by FACS analysis. Relative fold expression for each single integrin was calculated as the ratio between the mean fluorescence intensity values in M14-N cells and those in M14C2/C4 control cells. (*c*) Dose–effect curve of cilengitide (CGT) on M14-N cell adhesion to vitronectin (VTR) or fibronectin (FBR), as a negative control. (*d*) Effect of 20 μg/ml cilengitide (CGT) or antibodies that specifically block αvβ3 or αvβ5 integrins on matrigel invasion by M14-N cells, evaluated utilizing Boyden chambers as described in the legend to Figure 1*f*. (*e*) Formation of tubule-like structures by M14-N cells and effect of 20 μg/ml cilengitide on tubule-like structures formation, analysed as described in the legend to Figure 2*c*. CGT, cilengitide; NT, nontreated. Analysis was performed after 18 h. Photographs from a representative experiment out of three are shown (×50 magnification). M14C2/C4 cells (C4) were included as negative control of cells unable to form tubule-like structures in the same culture conditions utilized to test M14-N cells. (*f*) The number of cell intersections was counted in ten different microscopic fields for the indicated of the experimental groups described in panel *e*. In all panels data represent the arithmetic mean values ± SD of the results from three independent determinations.

invasiveness (Fig. 3*d*), but, unlike M14C2/MK18 cells, blockage of αvβ3 integrin with a specific antibody did not affect the invasiveness of M14-N cells (Fig. 3*d versus* Fig. 1*f*). These data suggest a prevalent role of αvβ5 signalling in the invasive phenotype of melanoma cells expressing NRP-1 and lacking VEGFR-2 and indicate that NRP-1 does not directly interact with ECM components, but it is rather involved in melanoma cell adhesion and invasiveness through integrin activation (most likely the αvβ5 integrin).

M14-N cells were also able to form tubule-like structures on matrigel, and cilengitide treatment strongly reduced this property (Figs. 3*e* and 3*f*). The effect of cilengitide did not depend on the matrigel components, since it was also observed when M14-N cells were allowed to form tubule-like structures in a Collagen I matrix (Supporting information Fig. S3). Moreover, cilengitide did not significantly inhibit adhesion to matrigel or Collagen I (Supporting information Fig. S4*a*). Since cilengitide has been shown to cause tumour cell detachment and

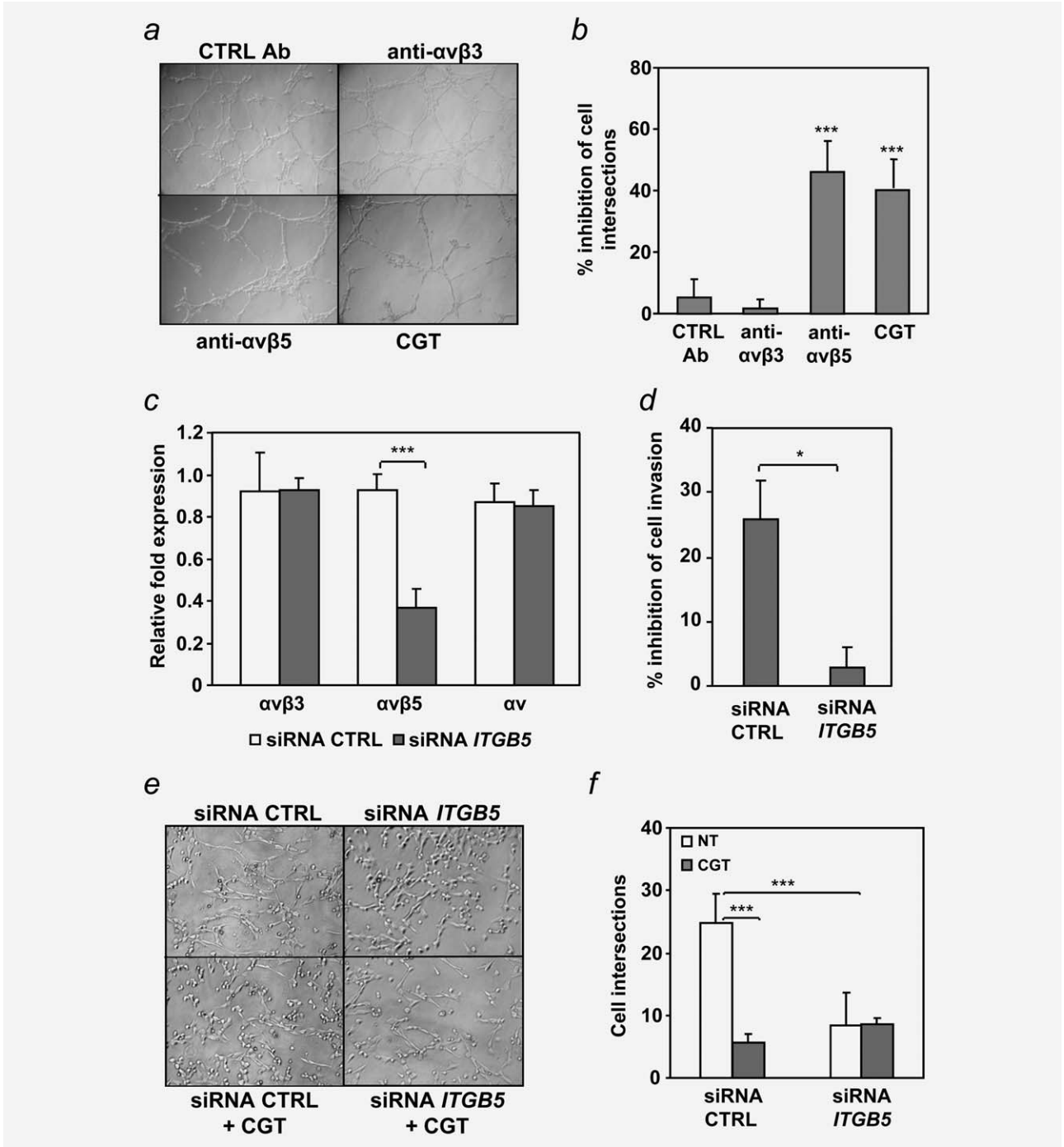

Figure 4.

death,[28,29] we have investigated whether this integrin inhibitor provoked similar effects in M14-N cells. However, in our melanoma model, cilengitide did not induce cell detachment or apoptosis (Supporting information Figs. S4*b* and S4*c*).

Further investigation on the relevance of αvβ3 and αvβ5 integrins on the vasculogenic mimicry phenotype of M14-N cells was performed by treating M14-N cells with specific neutralizing antibodies. The results demonstrated that αvβ5 integrin was required for the vasculogenic mimicry exhibited by NRP-1 positive melanoma cells, whereas αvβ3 integrin was found to be dispensable (Figs. 4*a* and 4*b*).

The role of αvβ5 integrin in the aggressive properties of M14-N cells and in the effects of cilengitide was confirmed by gene silencing experiments (Figs. 4*c* and 4*d* and Supporting information Fig. S5). In fact, M14-N cells in which a strong reduction in αvβ5 integrin expression was obtained by β5 chain silencing, showed a reduced ability to invade matrigel and to form tubule-like structures and were not sensitive to the inhibitory effect of cilengitide (Figs. 4*d*–4*f*).

Mechanisms involved in the inhibitory effects of cilengitide on M14-N cells

We had previously demonstrated that NRP-1 expressing melanoma cells produce higher levels of VEGF-A and MMP-2 as compared to NRP-1 negative syngeneic cells.[15,16] Interestingly, it has been described that MMP-2 stimulates VEGF-A secretion by melanoma cells through the activation of αvβ5 integrin.[30] Therefore, the effect of cilengitide and the role of αvβ5 integrin on the secretion of both VEGF-A and MMP-2 by M14-N cells was also evaluated. Cilengitide and *ITGB5* silencing significantly reduced the amounts of VEGF-A released in the supernatant by M14-N cells (Figs. 5*a* and 5*b*). Treatment with cilengitide did not affect MMP-2 secretion or activity (Supporting information Figs. S6*a* and S6*b*), suggesting that in our model cilengitide might inhibit the MMP-mediated activation of αvβ5 integrin which is required for VEGF-A secretion by melanoma cells.

Other possible mechanisms of action of cilengitide were also investigated. In particular, the secretion of MMPs relevant to cell invasiveness (MMP-9 and MMP-14) was assayed. The levels of MMP-9 released by M14-N cells were higher than those produced by M14C2/C4 cells and treatment with cilengitide significantly reduced MMP-9 secretion and activity in M14-N supernatant (Figs. 5*c* and 5*d*). The amount of MMP-14 secreted by M14-N cells was, instead, similar to that released by control melanoma cells (Supporting information Fig. S6*c*). Since this MMP is known to process the MMP-2 that is bound to the cell membrane,[31] we also analysed the quantity of MMP-14 present in cell extracts. The MMP-14 found in cell extracts was 100-fold higher than in supernatants and significantly more expressed in M14-N cells than in M14C2/C4 cells (Supporting information Fig. S6*d*). Moreover, cilengitide did not modulate the MMP-14 levels either in the supernatants or in the cell extracts (Supporting information Figs. S6*c* and S6*d*).

Since we and others have suggested a role for NRP-1 in endothelial or tumour cells independent of other VEGFRs,[16,32,33] we investigated whether cilengitide might modulate the response of M14-N cells to VEGF-A, using an *in vitro* chemotactic assay. Interestingly, cilengitide was found to inhibit the chemotactic response to VEGF-A, whereas it did not affect cell migration in response to human fibroblast conditioned medium (Fig. 5*e*). However, cells exposed to the integrin inhibitor showed a round-shaped morphology (Supporting information Fig. S7), likely due to a drug-mediated decrease in the phosphorylation of FAK, which is constitutively active in these cells (Fig. 5*f*).

Therefore, the mechanism by which cilengitide treatment affects invasiveness and vasculogenic mimicry of NRP-1 expressing melanoma cells seems to involve downregulation of VEGF-A production and an impaired response to this cytokine.

Effect of cilengitide on the invasiveness of melanoma cell lines expressing different levels of NRP-1

The influence of cilengitide on melanoma aggressiveness was also analysed in nontransfected cells, using two

Figure 4. The inhibitory effects of cilengitide on vaculogenic mimicry by M14-N cells involve the αvβ5 integrin. (*a*) Effect of antibodies that specifically inhibit αvβ3 or αvβ5 integrins on the capability of M14-N cells to form tubule-like structures, analysed as described in the legend to Figure 2*c*. Cells were pre-incubated with 10 μg/ml of a control antibody (mouse IgG1 control, CTRL Ab) or of the indicated anti-integrin antibodies or with 20 μg/ml of cilengitide (CGT) for 30 min at room temperature in a rotating wheel, before seeding them on the matrigel. Tubule formation was analysed after 6 h. Photographs from a representative experiment out of three are shown (×50 magnification). (*b*) The number of cell intersections was counted in ten different microscopic fields for each of the experimental groups described in panel A. The data are expressed as percentage of inhibition of cell intersections in antibody or cilengitide treated cells compared to untreated controls. (*c*) Specific downregulation of the αvβ5 integrin subunit after silencing of the *ITGB5* gene. The expression of αvβ3 and αvβ5 integrins and αv chains on the surface of M14-N cells was evaluated by FACS analysis 3 days after transfection of a specific *ITGB5* gene siRNA (siRNA *ITGB5*) or a silencer negative control (siRNA CTRL). Relative expression for each integrin was calculated as the ratio between the mean fluorescence intensity values in transfected M14-N cells and those in nontransfected control cells. The data represent the arithmetic mean ± SD of three independent determinations. (*d*) Effect of β5 integrin silencing on matrigel invasion, evaluated utilizing Boyden chambers as described in the legend to Figure 1*f*. The assay was performed 3 days after transfection with siRNA *ITGB5* or siRNA CTRL. (*e*) Effect of β5 integrin silencing on the formation of tubule-like structures by M14-N cells analysed, as described in the legend to Figure 2*c*, 18 h after seeding on matrigel. The assay was performed 3 days after transfection with siRNA *ITGB5* or siRNA CTRL. Photographs from a representative experiment out of three are shown (×50 magnification). (*f*) The number of cell intersections was counted in ten different microscopic fields for each of the experimental groups described in panel *e*. In all panels data represent the arithmetic mean values ± SD of the results from three independent determinations.

melanoma cell lines originated from the same patient and with elevated (WM115) or very low (WM266-4) NRP-1 expression (Fig. 6*a*). WM115 cells were also characterized by a higher αvβ5 integrin expression (Fig. 6*b* and Supporting information Fig. S8), a lower proliferation rate (Fig. 6*c*) and a higher invasive capability (Fig. 6*d*) as compared to WM266-4 cells. Treatment with cilengitide resulted in a significant reduction of matrigel invasion only in the case of WM115 cells (Fig. 6*d*). Moreover, WM115 cells were able to form tubule-like structures at a higher extent than WM266-4 cells, and this ability was strongly hampered by cilengitide (Figs. 6*e* and 6*f*). It can be thus concluded that melanoma cells expressing αvβ5 integrin and high levels of NRP-1 display a more aggressive phenotype and that

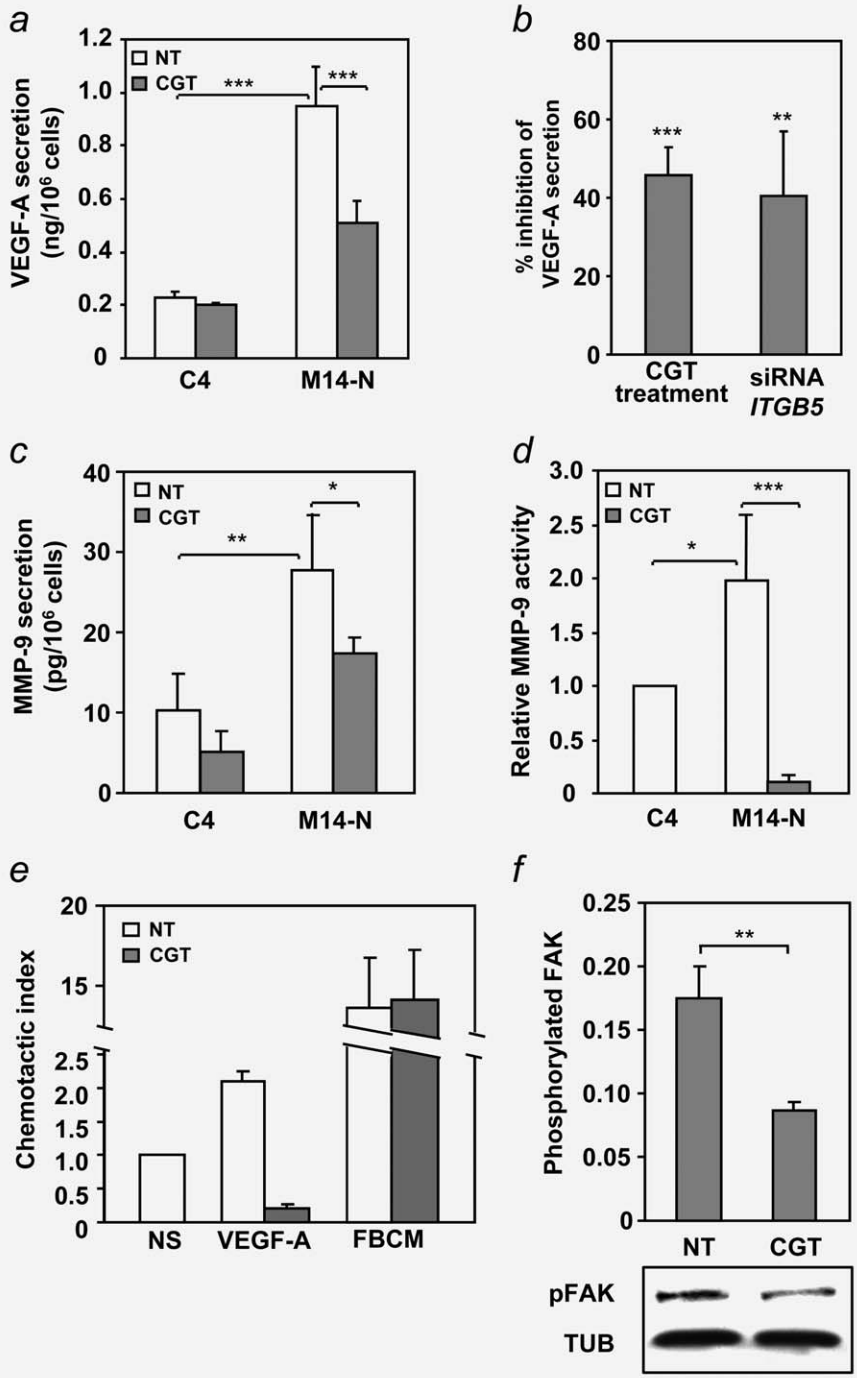

Figure 5.

cilengitide is a good candidate to revert, at least in part, the invasive behaviour of these cells.

Discussion

Integrins represent highly attractive targets for anticancer therapy since they are critical intermediaries in a wide spectrum of tumour cell activities. However, modulation of the integrin functions is a complex issue. In fact, many different integrin heterodimers are expressed in a single cell and each of them can be involved in multiple intracellular signal transduction pathways. Therefore, the identification of the particular integrin that plays a dominant role in a specific tumour type or stage appears to be crucial for the design of new therapies targeting these ECM receptors. In the present study, we demonstrate for the first time that several of the characteristics contributing to the aggressive phenotype displayed by melanoma cells expressing NRP-1 require an active αvβ5 integrin and that αvβ3 integrin activation is needed when VEGFR-2 is coexpressed with NRP-1. Moreover, we show that cilengitide is able to inhibit the *in vitro* ECM invasion, tubule-like structures formation, production of and chemotactic response to VEGF-A and secretion of MMP-9 by melanoma cells expressing NRP-1.

Adhesion of NRP-1-positive melanoma cells to collagen, and more markedly to vitronectin, was increased as compared to NRP-1 negative cells. Vitronectin is an ECM component that plays a crucial role in many biological processes, such as cell migration, adhesion and angiogenesis, through the interaction with several members of the integrin family.[34–36] Analysing the integrins involved in the augmented adhesion of melanoma cells expressing VEGFR-2 and NRP-1 or NRP-1 only, our attention was focused on αvβ3 and αvβ5 integrins, two vitronectin receptors. Neutralizing antibodies against these integrins markedly inhibited *in vitro* melanoma cell invasiveness, even though vitronectin is not a component of matrigel (the commercial ECM utilized in the *in vitro* invasion assays). Therefore, mechanisms different from cell adhesion to ECM components (such as interaction of integrins with other membrane receptors and modulation of their signal transduction pathways) are likely to underlie the role of αvβ3 and αvβ5 integrins in melanoma invasiveness. Indeed, a direct physical interaction between VEGFR-2 and the β3 extracellular domain of αvβ3 integrin has been described.[37,38] In endothelial cells, the β3 integrin subunit directly binds to NRP-1, limiting the interaction of this coreceptor with VEGFR-2 and modulating VEGF-A-mediated angiogenesis.[39] According to this study, in the presence of αvβ3 integrin, NRP-1 contributes minimally to VEGF-A-induced angiogenic processes, whereas when the β3 integrin subunit is absent or low-expressed or its function is blocked, VEGF-A-mediated responses of endothelial cells become NRP-1-dependent.[39] Indeed, in M14C2/MK18 cells (expressing both NRP-1 and VEGFR-2) the level of αvβ3 integrin on the membrane surface was found to be reduced as compared with control cells (clone M14C2/C4, lacking NRP-1 and VEGFR-2 expression) and this might emphasize the contribution of NRP-1 to the aggressiveness of melanoma cells. Nevertheless, αvβ3 integrin still influences the invasiveness of M14C2/MK18 cells, even though it is expressed at low levels and is irrelevant for cell adhesion to vitronectin.

The M14-N melanoma cells, which express NRP-1 but not VEGFR-2, displayed characteristics almost identical to those of M14C2/MK18 cells (high *in vitro* invasiveness and vasculogenic mimicry). However, blockage of αvβ3 integrin in M14-N cells with neutralizing anti-integrin antibodies did not interfere with ECM cell invasion or vasculogenic mimicry, supporting the hypothesis that αvβ3 integrin contributes to the promotion of ECM invasion by melanoma cells only in the presence of VEGFR-2. On the other hand, the results obtained using blocking antibodies against αvβ5 integrin and silencing of β5 integrin chain indicated that the aggressive properties of M14-N cells depend not only on NRP-1 but also on this integrin. These data are in agreement with those described in a human fibrosarcoma model showing that the upregulation of NRP-1 is a critical event in the vasculogenic mimicry *in vivo*[40] and with the recent demonstration that αvβ5 integrin expression is strongly upregulated in brain metastases from malignant melanoma as compared to primary tumours.[41]

Our results suggest a cross-talk between NRP-1 and αvβ5 integrin in M14-N cells that might derive from physical interactions between the two proteins or from NRP-1-dependent signal

Figure 5. Mechanisms involved in the inhibitory effects of cilengitide on M14-N cells. (*a*) The influence of the treatment with cilengitide (20 μg/ml) on VEGF-A secretion by M14-N cells and M14C2/C4 (C4) control cells was analysed measuring VEGF-A in cell culture supernatants by ELISA. NT, nontreated; CGT, cilengitide. (*b*) Comparison of the inhibitory effect on VEGF-A secretion exerted by treatment with cilengitide (20 μg/ml) or by transfection with siRNA *ITGB5* (3 days after transfection). (*c* and *d*) The influence of the treatment with cilengitide (20 μg/ml) on MMP-9 secretion (*c*) and activity (*d*) by M14-N and M14C2/C4 (C4) control cells was analysed measuring MMP-9 in cell culture supernatants by ELISA. NT, nontreated; CGT, cilengitide. The MMP-9 activity in M14-N cells was expressed as relative activity compared to that of M14C2/C4 control cells. (*e*) The influence of cilengitide (CGT) on the chemotactic response of M14-N cells to VEGF-A (50 ng/ml) or to human fibroblast conditioned media (FBCM) was analysed by cell migration assays using Boyden chambers equipped with filters coated with 5 μg/ml gelatin. The results are expressed as chemotactic index, calculated as the ratio between the number of cells per microscopic field in the experimental condition analysed and the number of cells per microscopic field in the basal condition (*i.e.*, in the absence of any stimulus, NS). Chemotactic index in the basal condition corresponds to 1. (*f*) Effect of cilengitide on constitutive FAK phosphorylation in M14-N cells. Untreated cells (NT) or cells treated for 30 min with 20 μg/ml cilengitide (CGT) were analysed by immunoblot using antibodies that recognize the phosphorylated form of FAK. The immunoblot shown is a representative experiment. The relative levels of FAK phosphorylation were calculated by densitometric analysis and normalized by β-tubulin (TUB) expression in each sample. In all panels data represent the arithmetic mean values ± SD of the results from three independent determinations.

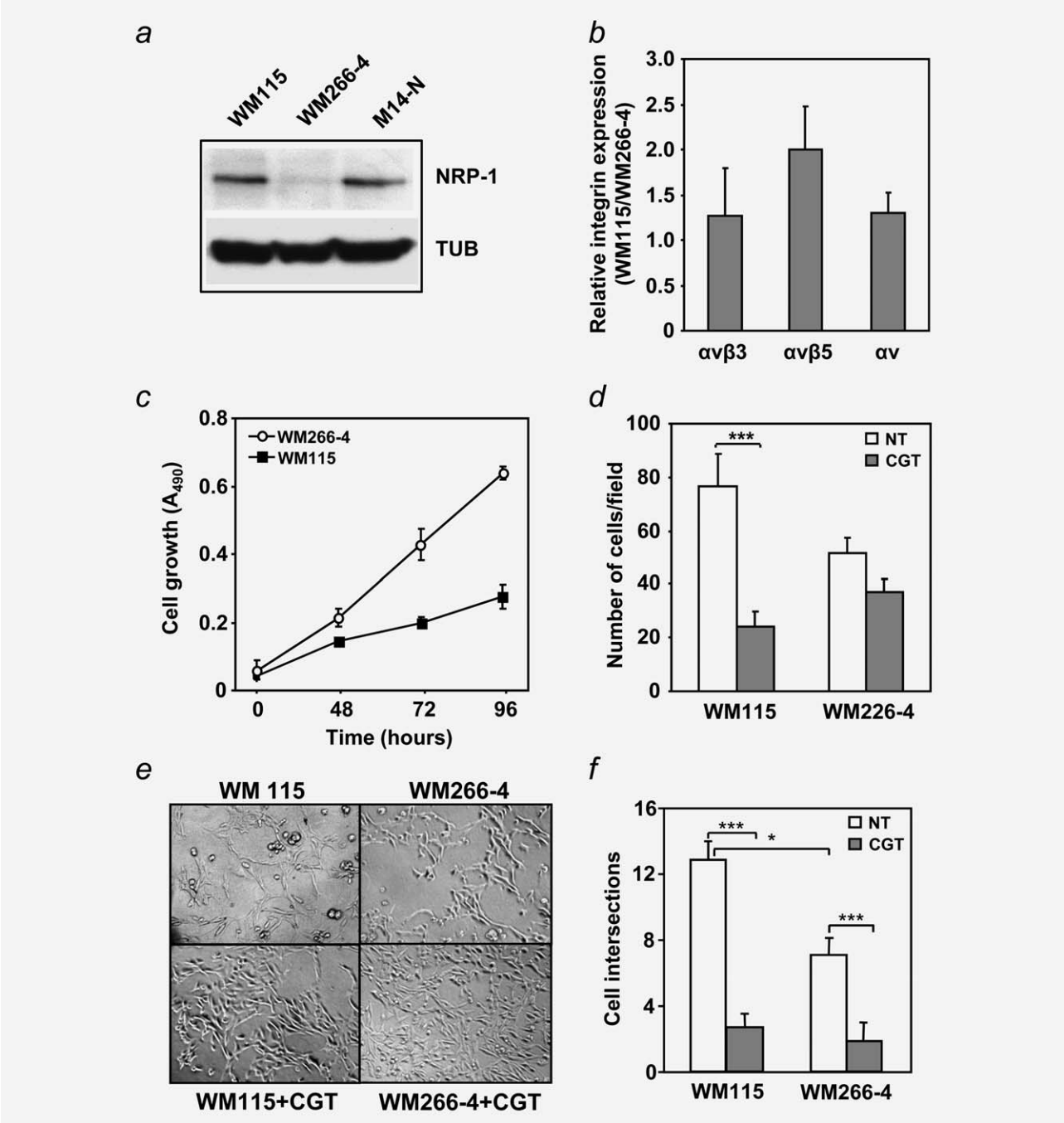

Figure 6. Effect of cilengitide on the invasiveness of melanoma cell lines expressing different levels of NRP-1. (*a*) Immunoblot analysis of NRP-1 in WM115 and WM266-4 melanoma cells, using M14-N cells as positive control and β-tubulin as loading control. (*b*) The expression of the αvβ3 and αvβ5 integrins and αv chains was evaluated in WM115 and WM266-4 cells by FACS analysis. Relative expression of each single integrin was calculated as the ratio between the mean fluorescence intensity value in WM115 cells and that in WM266-4 cells. Columns represent the arithmetic mean ± SD of the results obtained from three independent experiments. (*c*) The in vitro cell growth of WM115 and WM266-4 cells was evaluated at the indicated times by MTS assay, seeding in quintuplicate 5×10^2 cells in a 96-well plate. Results are the means ± SD of a representative experiment. (*d*) Effect of 20 μg/ml cilengitide (CGT) on matrigel invasion by WM115 and WM266-4 cells, evaluated utilizing Boyden chambers as described in the legend to Figure 1*f*. Columns represent the arithmetic mean ± SD of the results obtained from three independent experiments. (*e*) Formation of tubule-like structures by WM115 and WM266-4 cells and effect of 20 μg/ml cilengitide (CGT) on tubule-like structures formation. Analysis was performed 24 h after seeding the cells (5×10^4) on the matrigel layer, as described in the legend to Figure 2*c*. Photographs from a representative experiment out of three are shown (×50 magnification). (*f*) The number of cell intersections was counted in ten different microscopic fields for each of the experimental groups shown in panel E.

transduction pathways which favour αvβ5 integrin activation/signalling. To this regard, in cross-linking and coimmunoprecipitation experiments we did not find any evidence of a direct physical interaction between NRP-1 and αvβ5 integrin in our model (data not shown). Conversely, since we have shown that the expression of NRP-1 in M14-N cells is sufficient to promote the activation of signal transduction pathways involving several protein kinases,[16] it is likely that these protein kinases may modulate αvβ5 integrin activity. Indeed, a number of data in the literature indicate that αvβ5 integrin can be regulated by protein kinases: (*i*) several αvβ5 integrin-mediated functions (such as cell spreading, colocalization of α-actinin, tensin, vinculin, p130Cas, actin and induction of FAK tyrosine phosphorylation) require PKC activation[42]; (*ii*) the cross-talk between αvβ5 integrin and the epidermal growth factor receptor, implicated in the metastatic process of pancreatic carcinoma cells, involves signalling kinases such as Src and Rap-1[43] (*iii*) the role played by αvβ5 integrin in the vasculogenic mimicry of melanoma cells[27] is under the control of kinases, such as PKC, Akt, FAK or ERK.[44,45] Interestingly, NRP-1 expression in melanoma cells promotes an increased secretion of MMP-2.[16] Since, it has been demonstrated that MMP-2 induces the activation of αvβ5 integrin,[30] this mechanism might also account for αvβ5 integrin triggering in NRP-1 positive melanoma cells.

Cilengitide, besides counteracting the adhesion of M14-N and M14C2/MK18 cells to vitronectin, markedly decreased their invasiveness and vasculogenic mimicry. In M14-N cells, the effects of this drug are due to the inhibition of αvβ5 integrin, given that similar results were obtained treating the cells with anti-αvβ5 integrin neutralizing antibodies and by silencing of the β5 integrin chain. Actually, cilengitide did not further decrease tubule-like structure formation in β5 silenced cells. Moreover, it downmodulated the release of VEGF-A and, also in this case, αvβ5 integrin inhibition is likely to be involved, as the activation of this integrin controls the PI3K-dependent production of VEGF-A by melanoma cells.[29] Accordingly, cilengitide has shown to inhibit phosphorylation of FAK, src and Akt in endothelial and glioma cells[28] and here we demonstrate that it downmodulates FAK phosphorylation in M14-N cells. Interestingly, in drug-treated cells also the chemotactic response to VEGF-A was strongly hampered, likely as a consequence of the modulation of FAK and/or other critical proteins involved in the response to VEGF-A and essential to the maintenance of an autocrine loop that promotes melanoma invasiveness.[14] Finally, cilengitide decreased MMP-9 secretion and activity, which were higher in M14-N cells than in control melanoma cells, suggesting an important role also for this MMP in the mechanism of action of the drug. The suggested mechanisms of action of cilengitide in NRP-1 expressing melanoma cells are summarized in Supporting information Figure S9.

Noteworthy, the effect of cilengitide on melanoma aggressiveness was also confirmed using two nontransfected melanoma cell lines, originated from the same patient and representative of different malignant phenotypes: the WM115, characterised by high NRP-1 expression, high matrigel invasiveness, elevated capability to form tubule-like structures and low proliferation rate; the WM266-4, characterised by low NRP-1 expression, lower matrigel invasiveness, lower capability to form tubule-like structures and high proliferation rate. In fact, WM115 cells were significantly more susceptible than WM266-4 cells to the inhibitory effect of cilengitide on matrigel invasion and highly sensitive to the effect of the drug on vasculogenic mimicry.

Another important aspect of the studies herein presented is the possible clinical applications. Melanoma cells are able to elude the growth inhibitory effect of antiangiogenic therapies and quickly develop resistance to targeted therapies aimed at inhibiting constitutively activated kinases.[46] Thus, combination therapies including inhibitors of DNA repair, novel immunomodulators, targeted therapies or antiangiogenic agents are currently investigated to improve the outcome of melanoma patients.[47] Since vasculogenic mimicry is an alternative pathway to provide tumours with blood supply by an angiogenesis-independent mechanism, the ability of melanoma cells to produce vessel-like structures may contribute to resistance to antiangiogenic therapies and explain why these therapies have clinically failed in melanoma treatment.[48] Indeed, traditional antiangiogenic drugs have shown to be ineffective in hindering vasculogenic mimicry.[49,50] Therefore, the combination of cilengitide with anti-VEGF-A agents is expected to be synergistic and to yield substantial antineoplastic effects.

In conclusion, the results herein presented identify specific αv integrins that are involved in the aggressiveness of NRP-1-expressing melanoma cells: αvβ3 integrin, promoting ECM invasion in the presence of VEGFR-2; αvβ5 integrin, promoting cell adhesion to vitronectin and collaborating with NRP-1 in the development of an invasive and vasculogenic mimicry phenotype. Moreover, the involvement of αvβ5 integrin in the ability of highly aggressive melanomas to form vessel-like structures, provide the rationale for clinical testing of cilengitide in the treatment of metastatic melanoma.

Acknowledgements

This work was supported by the Italian Ministry of Health to P.M.L. (RC14-3.3) and in part by the "Associazione Italiana per la Ricerca sul Cancro" (AIRC) to G.G. (AIRC 2013 IG 14042).

References

1. Ria R, Reale A, Castrovilli A, et al. Angiogenesis and progression in human melanoma. *Dermatol Res Pract* 2010;2010:185687. doi:10.1155/2010/185687.
2. Hofmann UB, Houben R, Bröcker E-B, et al. Role of matrix metalloproteinases in melanoma cell invasion. *Biochimie* 2005;87:307–14.
3. Kuphal S, Bauer R, Bosserhoff AK. Integrin signaling in malignant melanoma. *Cancer Metastasis Rev* 2005;24:195–222.

4. Seftor RE, Hess AR, Seftor EA, et al. Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. *Am J Pathol* 2012;181:1115–25.
5. Guo W, Giancotti FG. Integrin signalling during tumour progression. *Nature Rev* 2004;5:816–26.
6. Harburger DS, Calderwood DA. Integrin signalling at a glance. *J Cell Sci* 2009;122:159–63.
7. Pinon P, Wehrle-Haller B. Integrins: versatile receptors controlling melanocyte adhesion, migration and proliferation. *Pigment Cell Mel Res* 2010; 24:282–94.
8. Bosserhoff AK. Novel biomarkers in malignant melanoma. *Clin Chim Acta* 2006;367:28–35.
9. Mas-Moruno C, Rechenmacher F, Kessler H. Cilengitide: the first anti-angiogenic small molecule drug candidate design, synthesis and clinical evaluation. *Anticancer Agents Med Chem* 2010;10: 753–68.
10. Reardon DA, Cheresh DA. A prototypic integrin inhibitor for the treatment of glioblastoma and other malignancies. *Genes Cancer* 2011;2:1159–65.
11. Reardon DA, Neyns B, Weller M, et al. Cilengitide: an RGD pentapeptide $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin inhibitor in development for glioblastoma and other malignancies. *Future Oncol* 2011;7:339–54.
12. Kim KB, Prieto V, Joseph RW, et al. A randomized phase II study of cilengitide (EMD 121974) in patients with metastatic melanoma. *Melanoma Res* 2012;22:294–301.
13. Tentori L, Dorio AS, Muzi A, et al. The integrin antagonist cilengitide increases the antitumor activity of temozolomide against malignant melanoma. *Oncol Rep* 2008;19:1039–43.
14. Lacal PM, Ruffini F, Pagani E, et al. An autocrine loop directed by the vascular endothelial growth factor promotes invasiveness of human melanoma cells. *Int J Oncol* 2005;27:1625–32.
15. Ruffini F, Tentori L, Dorio AS, et al. Platelet derived growth factor C and calpain-3 are modulators of human melanoma cell invasiveness. *Oncol Rep* 2013;30: 2887–96.
16. Ruffini F, D'Atri S, Lacal PM. Neuropilin-1 expression promotes invasiveness of melanoma cells through vascular endothelial growth factor receptor-2 dependent and independent mechanisms. *Int J Oncol* 2013;43:297–306.
17. Ellis LM. The role of neuropilins in cancer. *Mol Cancer Ther* 2006;5:1099–107.
18. Pellet-Many C, Frankel P, Jia H, et al. Neuropilins: structure, function and role in disease. *Biochem J* 2008;411:211–26.
19. Hansel DE, Wilentz RE, Yeo CJ, et al. Expression of neuropilin-1 in high-grade dysplasia, invasive cancer and metastases of the human gastrointestinal tract. *Am J Surg Pathol* 2004;28:347–56.
20. Latil A, Bieche I, Pesche S, et al. VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. *Int J Cancer* 2000;89:167–71.
21. Ruffini F, Failla CM, Orecchia A, et al. Expression of the soluble vascular endothelial growth factor receptor-1 in cutaneous melanoma: role in tumour progression. *Br J Dermatol* 2011;164: 1061–70.
22. Lacal PM, Morea V, Ruffini F, et al. Inhibition of endothelial cell migration and angiogenesis by a vascular endothelial growth factor receptor-1 derived peptide. *Eur. J. Cancer* 2008;44:1914–21.
23. Lacal PM, Failla CM, Pagani E, et al. Human melanoma cells secrete and respond to placenta growth factor and vascular endothelial growth factor. *J Invest Dermatol* 2000;115:1000–7.
24. Tentori L, Lacal PM, Muzi A, et al. Poly(ADP-ribose) polymerase (PARP) inhibition or PARP-1 gene deletion reduces angiogenesis. *Eur J Cancer* 2007;43:2124–33.
25. Jin H, Varner J. Integrins: roles in cancer development and as treatment targets. *Br J Cancer* 2004;90:561–5.
26. Kirmse R, Otto H, Ludwig T. Interdependency of cell adhesion, force generation and extracellular proteolysis in matrix remodeling. *J Cell Sci* 2011; 124:1857–66.
27. Vartanian A, Stepanova E, Grigorieva I, et al. Melanoma vasculogenic mimicry capillary-like structure formation depends on integrin and calcium signaling. *Microcirculation* 2011;18:390–9.
28. Oliveira-Ferrer L, Hauschild J, Fiedler W, et al. Cilengitide induces cellular detachment and apoptosis in endothelial and glioma cells mediated by inhibition of FAK/src/AKT pathway. *J Exp Clin Cancer Res* 2008;27:86. doi: 10.1186/1756-9966-27-86.
29. Cheng NC, van Zandwijk N, Reid G. Cilengitide inhibits attachment and invasion of malignant pleural mesothelioma cells through antagonism of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. *PLoS One* 2014;9: e90374.
30. Desch A, Strozyk EA, Bauer AT, et al. Highly invasive melanoma cells activate the vascular endothelium via an MMP-2/integrin $\alpha v\beta 5$-induced secretion of VEGF-A. *Am J Pathol* 2012; 181:693–705.
31. Lu H, Hu L, Yu L, et al. KLF8 and FAK cooperatively enrich the active MMP14 on the cell surface required for the metastatic progression of breast cancer. *Oncogene* 2014;33:2909–17.
32. Murga M, Fernandez-Capetillo O, Tosato G. Neuropilin-1 regulates attachment in human endothelial cells independently of vascular endothelial growth factor receptor-2. *Blood* 2005;105: 1992–9.
33. Snuderl M1, Batista A, Kirkpatrick ND, et al. Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma. *Cell* 2013;152:1065–76.
34. Preissner KT, Seiffert D. Role of vitronectin and its receptors in haemostasis and vascular remodeling. *Thromb Res* 1998;89:1–21.
35. Smith HW, Marshall CJ. Regulation of cell signaling by uPAR. *Nat Rev Mol Cell Biol* 2010;11: 23–36.
36. Wei Y, Eble JA, Wang Z, et al. Urokinase receptors promote beta1 integrin function through interactions with integrin alpha3beta1. *Mol Biol Cell* 2001;12:2975–86.
37. Borges E, Jan Y, Ruoslahti E. Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain. *J Biol Chem* 2000;275:39867–73.
38. Somanath PR, Malinin NL, Byzova TV. Cooperation between integrin alphavbeta3 and VEGFR2 in angiogenesis. *Angiogenesis* 2009;12:177–85.
39. Robinson SD, Reynolds LE, Kostourou V, et al. $\alpha v\beta 3$ integrin limits the contribution of neuropilin-1 to vascular endothelial growth factor-induced angiogenesis. *J Biol Chem* 2009; 284:33966–81.
40. Misra RM, Bajaj MS, Kale VP. Vasculogenic mimicry of HT1080 tumour cells in vivo: critical role of HIF-1α-neuropilin-1 axis. *PLoS One* 2012; 7:e50153. doi: 10.1371/journal.pone.0050153.
41. Vogetseder A, Thies S, Ingold B, et al. αv-Integrin isoform expression in primary human tumors and brain metastases. *Int J Cancer* 2013;133: 2362–71.
42. Lewis JM, Cheresh DA, Schwartz MA. Protein kinase C regulates $\alpha v\beta 5$-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. *J Cell Biol* 1996;134:1323–32.
43. Ricono JM, Huang M, Barnes LA, et al. Specific cross-talk between epidermal growth factor receptor and integrin alphavbeta5 promotes carcinoma cell invasion and metastasis. *Cancer Res* 2009;69: 1383–91.
44. Paulis YW, Soetekouw PM, Verheul HM, et al. Signalling pathways in vasculogenic mimicry. *Biochim Biophys Acta* 2010;1806:18–28.
45. Vartanian A, Stepanova E, Grigorieva I, et al. VEGFR1 and PKCα signaling control melanoma vasculogenic mimicry in a VEGFR2 kinase-independent manner. *Melanoma Res* 2011;21:91–8.
46. Sennino B, McDonald DM. Controlling escape from angiogenesis inhibitors. *Nat Rev Cancer* 2012;12:699–709.
47. Tentori L, Lacal PM, Graziani G. Challenging resistance mechanisms to therapies for metastatic melanoma. *Trends Pharmacol Sci* 2013;34:656–66.
48. Emmett MS, Dewing D, Pritchard-Jones RO. Angiogenesis and melanoma—from basic science to clinical trials. *Am J Cancer Res* 2011;1:852–68.
49. Rybak SM, Sanovich E, Hollingshead MG, et al. "Vasocrine" formation of tumor cell-lined vascular spaces: implications for rational design of antiangiogenic therapies. *Cancer Res* 2003;63: 2812–9.
50. Van der Schaft DW, Seftor RE, Seftor EA, et al. Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells. *J Natl Cancer Inst* 2004; 96:1473–7.