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Targeting of PDGF-C/NRP-1 autocrine loop as a new strategy for counteracting the invasiveness of melanoma resistant to braf inhibitors

Federica Ruffini [a,1], Claudia Ceci [a,b,1], Maria Grazia Atzori [a,1], Simona Caporali [c,2], Lauretta Levati [c], Laura Bonmassar [c], Gian Carlo Antonini Cappellini [d,3], Stefania D'Atri [c], Grazia Graziani [a,4], Pedro Miguel Lacal [c,*,4]

[a] Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy
[b] Departmental Faculty of Medicine and Surgery, Saint Camillus International University of Health Sciences, Via di Sant'Alessandro, 8, 00131 Rome, Italy
[c] Laboratory of Molecular Oncology, IDI-IRCCS, Rome, Italy
[d] Department of Oncology and Dermatological Oncology, IDI-IRCCS, Rome, Italy

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ABSTRACT

Melanoma resistance to BRAF inhibitors (BRAFi) is often accompanied by a switch from a proliferative to an invasive phenotype. Therefore, the identification of signaling molecules involved in the development of metastatic properties by resistant melanoma cells is of primary importance. We have previously demonstrated that activation of neuropilin-1 (NRP-1) by platelet-derived growth factor (PDGF)-C confers melanoma cells with an invasive behavior similar to that of BRAFi resistant tumors. Aims of the present study were to evaluate the role of PDGF-C/NRP-1 autocrine loop in the acquisition of an invasive and BRAFi-resistant phenotype by melanoma cells and the effect of its inhibition on drug resistance and extracellular matrix (ECM) invasion. Furthermore, we investigated whether PDGF-C serum levels were differentially modulated by drug treatment in metastatic melanoma patients responsive or refractory to BRAFi as single agents or in combination with MEK inhibitors (MEKi). The results indicated that human melanoma cells resistant to BRAFi express higher levels of PDGF-C and NRP-1 as compared to their susceptible counterparts. Overexpression occurs early during development of drug resistance and contributes to the invasive properties of resistant cells. Accordingly, silencing of NRP-1 or PDGF-C reduces tumor cell invasiveness. Analysis of PDGF-C in the serum collected from patients treated with BRAFi or BRAFi+MEKi, showed that in responders PDGF-C levels decrease after treatment and raise again at tumor progression. Conversely, in non-responders treatment does not affect PDGF-C serum levels. Thus, blockade of NRP-1 activation by PDGF-C might represent a new therapeutic approach to counteract the invasiveness of BRAFi-resistant melanoma.

1. Introduction

Cutaneous melanoma is an extremely aggressive tumor with a high metastatic potential. Although immune checkpoint inhibitors and, for *BRAF*-mutated melanomas, combinations of BRAF plus MEK inhibitors (BRAFi and MEKi) have significantly prolonged overall survival [1], many patients still experience disease progression or fail to respond to therapy [2]. Resistance to BRAFi is often characterized by the

Abbreviations: BRAFi, BRAF inhibitors; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; EMT, epithelial mesenchymal transition; FBS, fetal bovine serum; HOPP, heuristic online phenotype prediction; IGF1R, insulin-like growth factor-1 receptor; MEKi, MEK inhibitors; MTS, [3–(4,5 dimethylthiazol-2–yl)-5–(3–carboxymethoxyphenyl) 2–(4–sulphophenyl)-2H–tetrazolium, inner salt]; NRP-1, neuropilin-1; PDGF-C, platelet derived growth factor-C; PDGFRα, platelet-derived growth factor receptor alpha; PDGFRβ, platelet-derived growth factor receptor beta; TGFβR, transforming growth factor β receptor; VEGF-A, vascular endothelial growth factor A; VEGFR-2, vascular endothelial growth factor receptor-2.

* Corresponding author.
E-mail address: p.lacal@idi.it (P.M. Lacal).
[1] Equally contributing first authors
[2] Current address: Regional Transplant Center Lazio (CRTL), San Camillo Hospital, Rome, Italy.
[3] Current address: UOC Oncologia, Interpresidio ASL RM2, Via dei Monti Tiburtini 387, 00157 Rome, Italy
[4] Equally contributing last authors

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acquisition of a highly invasive phenotype that contributes to melanoma spreading [3,4]. However, the molecular mechanisms associated with the onset of this invasive behavior are not well defined. Therefore, the identification of molecules involved in the metastatic switch together with their validation as therapeutic targets are of primary importance for counteracting the invasiveness of tumor cells resistant to BRAFi. In this context, we have previously demonstrated that an autocrine loop represented by neuropilin-1 (NRP-1) activation after interaction with platelet derived growth factor-C (PDGF-C) strongly contributes to the invasive properties of melanoma cells [5].

PDGF-C, a member of the PDGF family of growth factors, promotes tumor growth through the activation of its receptor alpha (PDGFRα) by acting as transforming, survival and mitogenic factor for tumor cells, mitogenic and chemoattractant stimulus for cancer-associated fibroblasts and inducer of neoangiogenesis in a vascular endothelial growth factor A (VEGF-A)-independent way [6]. On the other hand, PDGF-C is also able to bind NRP-1 and, through this interaction, it enhances melanoma cell ability to invade the extra-cellular matrix (ECM) and to arrange in capillary-like tubular structures (vasculogenic mimicry) [5].

NRP-1 is a transmembrane polypeptide that mainly acts as co-receptor, amplifying the signal transmitted by different growth factors through their specific receptors, like in the case of the vascular endothelial growth factor receptor-2 (VEGFR-2) activated by VEGF-A [7]. NRP-1 expression/activation has been associated to the acquisition of chemoresistance in several tumor types after treatment with different therapeutic agents: gemcitabine or 5-fluorouracil in pancreatic cancer [8]; 5-fluorouracil, paclitaxel or cisplatin in non-small cell lung, kidney and prostate cancer [9]; doxorubicin in osteosarcoma [10]; doxorubicin/cyclophosphamide and paclitaxel or HER2-targeted drugs in breast cancer [11,12]; BRAFi in melanoma [12]; MET inhibitors in stomach and lung carcinoma [12]; temozolomide in glioblastoma [13]. Nevertheless, NRP-1 can induce signal transduction pathways after binding to VEGF-A, placenta growth factor or PDGF-C, even in the absence of their corresponding receptors [7].

In this context, aims of the present study were to investigate the role of PDGF-C/NRP-1 autocrine loop in the development of an invasive and BRAFi-resistant phenotype by melanoma cells and to evaluate the effect of its inhibition on drug resistance and ECM invasion. Furthermore, we investigated whether PDGF-C levels were differentially modulated by drug treatment in metastatic melanoma patients responsive or refractory to BRAFi/MEKi.

2. Materials and methods

2.1. Cell lines and culture conditions

The human melanoma cell line SK-Mel28 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the human melanoma M14 was obtained from the "Istituto Regina Elena" (Rome, Italy) [14]. Murine 3T3 fibroblasts, used as control for PDGFRα expression, were kindly provided by the Laboratory of Molecular and Cell Biology, IDI-IRCCS (Rome, Italy). Cells were maintained in RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 2 mM L-glutamine and 50 μg/ml gentamicin (Lonza, Basel, Switzerland), at 37 °C in a 5% CO_2 humidified atmosphere. The origin and culture conditions of the M14-derived clones were previously described [5,14,15]: M14-N and M14-K14 clones are highly invasive cell lines expressing NRP-1 and PDGF-C, while M14-C clone is devoid of NRP-1 and PDGF-C expression.

BRAFi-resistant cell lines were obtained from the BRAF-mutant human melanoma cell lines SK-Mel28 and M14 by exposing them to increasing concentrations of the kinase inhibitors (up to 1.5 μM in the case of dabrafenib, or up to 5 μM in the case of vemurafenib). SK-Mel28 cells resistant to dabrafenib (formerly defined as SK-Mel28-R and herein referred to as SK-Mel28 DR) [16,17] and M14 cells resistant to vemurafenib (M14 VR) [18] were previously obtained. SK-Mel28 cells resistant to vemurafenib and M14 cells resistant to dabrafenib were generated within this study. BRAFi-resistant cells were maintained in medium supplemented with 1 μM (M14 DR) and 1.5 μM (SK-Mel28 DR) dabrafenib or with 2.5 μM vemurafenib (M14 VR and SK-Mel28 VR). All the sensitive/resistant melanoma cell lines utilized were authenticated by STR profiling (BMR genomics, Padova, Italy) and used at low passages.

2.2. Western blot analysis

Cells were recovered from culture, washed and total cellular extracts prepared. Fifteen μg of proteins per sample were run on 10% SDS-polyacrylamide gels and polypeptides transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) by standard techniques. Membranes were pre-treated with blocking solution (2% non-fat dry milk/1% triton X100/10 mM EDTA/50 mM Tris-HCl, pH 7.5) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C in blocking solution with primary antibodies from Santa Cruz Biotechnology (Dallas, TX, USA) at the following dilutions: rabbit polyclonal anti-β-tubulin H235 (sc-9104, lot E2215) (internal standard for loading control) 1:1000, mouse monoclonal antibodies anti-NRP-1 A12 (sc-5307, lot I1014) 1:500 and anti-PDGFRα C9 (sc-398206, lot J2617) 1:1000. After washing with 0.1% Tween 20/50 mM Tris-HCl, pH 7.5, immunodetection was performed using appropriate horseradish peroxidase-linked secondary antibodies and ECL Western blot detection reagents were from GE Healthcare (Milan, Italy).

2.3. ELISA

Conditioned media from melanoma cell lines were obtained from semi-confluent cell cultures and after incubation for 24 h in 0.1% bovine serum albumin (BSA)/RPMI 1640 medium supplemented with glutamine and gentamicin but without FBS. Culture supernatants were collected and concentrated at least 10–fold in Centriplus concentrators (Millipore, Burlington, MA, USA). Cells were detached from the flasks with a PBS/EDTA solution, and the total cell number/culture was recorded to normalize cytokine secretion.

Serum was prepared from blood samples of melanoma patients, allowed to clot for 1 h at 37 C° and centrifuged at 1900xg for 15 min at 4 C°. Serum was then aliquoted and stored at − 80 °C.

Levels of PDGF-C in patients' serum and culture supernatants was determined using a human PDGF-C DuoSet ELISA kit (DY1687, R&D Systems, Minneapolis, MN, USA), in duplicate and according to the manufacturer's instructions. Optical density at 405 nm was measured in an iMark Microplate absorbance reader (Bio-Rad, Hercules, CA, USA).

2.4. Cell growth quantification and drug IC_{50} determination (MTS 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)-2 H–tetrazolium, inner salt] from Promega (Madison, WI, USA). Briefly, melanoma cells were dispensed into flat-bottom 96–well plates at the indicated concentrations and grown at 37 °C in a 5% CO_2 humidified atmosphere. For chemosensitivity assay, cells were exposed to graded concentrations of dabrafenib or vemurafenib (GSK2118436 and PLX4032, respectively; Cell Signaling, Danvers, MA, USA). BRAFi were dissolved in dimethyl sulfoxide (DMSO) and, just before use, diluted to the appropriate concentrations in complete medium with final DMSO concentration never exceeding 0.05% (v/v). Six replica wells were used for each condition in a total volume of 100 μl. After 4 days, 20 μl of MTS solution were added to each well and cells were incubated at 37 °C for 1–4 h, depending on the cell line. Absorbance was read at 490 nm (reference wavelength 655 nm) in an iMark Microplate absorbance reader (Bio-Rad). Chemosensitivity was evaluated as the concentration of the drug capable of inhibiting cell growth by 50% (IC_{50}), calculated on the regression line in which absorbance values at 490 nm were plotted against the logarithm

of drug concentration, using the CalcuSyn software. To evaluate cell doubling times, MTS assay was performed at different time-points (0, 24, 48 and 72 h) after cell seeding.

2.5. Transient siRNA transfection

The day before transfection, melanoma cells were plated in 10 cm diameter Petri dishes (0.6 $\times 10^6$ cells/dish) in complete medium. The day after, complete medium was replaced with medium without antibiotics and transfected with 20 nM siRNA directed against PDGF-C or NRP-1 (Life Technologies, Carlsbad, CA, USA) or AllStars Negative Control siRNA (siCTR; ID 1027281, Qiagen, Hilden, Germany) by using Lipofectamine RNAiMAX reagent (Invitrogen, Waltham, MA, USA). Three days after transfection, PDGF-C levels in the culture medium were evaluated by ELISA and NRP-1 expression was analyzed by western blot. Two different siRNA for each gene were tested: two selected pre-designed for PDGF-C (PDGF-Ca, ID s31862; PDGF-Cb, ID s31864); one selected pre-designed (NRP-1a, ID s225043) and one validated (NRP-1b, ID s16845) for NRP-1.

For chemosensitivity assays with siRNA-transfected melanoma cells, 3 days after transfection 1000 cells/well were seeded on 96-well plates and exposed to DMSO alone or to graded concentrations of BRAFi. Plates were incubated at 37 °C for 4 days, and cell growth evaluated by the MTS assay. Four replica wells were tested for each group.

To investigate whether PDGF-C and/or NRP-1 co-expression was an early event occurring during the development of resistance to BRAFi, M14 cells (500 cells/well) were plated in quadruplicate into a 96–well plate, allowed to adhere at 37 °C for 18 h. The day after, selected wells were treated with 100 nM dabrafenib or with an equivalent volume of DMSO. Every week, cells were subjected to a new cycle of drug treatment up to the fourth week and, at weeks 2 and 3, cells from selected wells were transfected with 20 nM of siCTR or siRNA directed against PDGF-C or NRP-1. At the end of the fourth week, plates were fixed with ethanol, stained with 0.5% crystal violet and photographed. For quantitative analysis of cell growth, the dye was solubilized in the presence of 10% acetic acid and absorbance at 595 nm read in an iMark Microplate absorbance reader (Bio-Rad).

2.6. Analysis of melanoma cell invasion by Boyden chamber and spheroid assays

Boyden chamber assay was performed using chambers equipped with 8–μm pore diameter polycarbonate filters (Nuclepore; Whatman Incorporated, Clifton, NJ, USA), coated with 20 μg of matrigel. Melanoma cells were suspended in invasion medium (1 μg/ml heparin/0.1% BSA in RPMI 1640) and loaded (2 $\times 10^5$ cells) into the upper compartment of the chambers. Invasion medium was added to the lower compartment of the chambers. After incubation at 37 °C in a CO_2 incubator for 4 (M14 DR and SK-Mel28 DR cells) or 2 h (M14-N cells), filters were removed from the chambers, cells fixed in ethanol for 5 min and stained in 0.5% crystal violet for 15 min. Cells attached to the upper side of the filters were removed by wiping them with a cotton swab and invaded cells, attached to the lower surface of the filters, were counted under the microscope. Twelve high-magnification microscopic fields (x100 magnification), randomly selected on triplicate filters, were scored for each experimental condition.

For spheroid invasion assay, melanoma cells (30,000 cells/ml) were suspended in RPMI-1640 containing 10% FBS supplemented with methyl cellulose (0.24% final concentration; Sigma-Aldrich), seeded in 96-well round bottom cell culture plates (100 μl/well; Corning® Costar® Ultra-Low attachment multi-well, Sigma-Aldrich) and centrifuged at 3000 rpm for 90 min [19]. Plates were then incubated for 24 h under standard culture conditions (5% CO_2, at 37 °C) to allow spheroid formation. Spheroids were collected, embedded individually in 100 μl of Matrigel (reduced growth factor basement membrane matrix, Pathclear, Cultrex, Gaithersburg, MD) in 0.1% BSA/RPMI medium and plated in wells of a 96-well flat bottom plate, previously coated with 50 μl of matrigel. After matrigel solidification at 37 °C, 100 μl of invasion medium were added and plates incubated at 37 °C for 48 h. Spheroids were visualized and photographed using a Nikon Eclipse TS100 microscope in conjunction with a Nikon DS-Fi1 high resolution camera (Melville, NY) and area measurements (in mm^2) performed using Adobe Photoshop CS6 software.

2.7. BRAF exon 15 sequencing

Genomic DNA was extracted from M14-C and M14-N melanoma clones using DNeasy Blood and Tissue Kit (Qiagen, Valentina, CA, USA) following manufacturer's instructions. PCR amplification of *BRAF* exon 15 was performed using 30 ng of DNA and the following primers: 5'-TCATAATGCTTGCTCTGATAGGA-3' (forward) and 5'-GGCCAAAAATTTAATCAGTGGA-3' (reverse). PCR conditions were set up according to the manufacturer's protocol of Invitrogen Platinum II Taq Hot-Start DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Purification of PCR products was performed using DNA Clean & concentrator-5 kit (Zymo Research, Irvine, CA, USA) and amplified DNA was sequenced in forward and reverse directions on ABI 3730 Sanger sequencing Instrument (Applied Biosystems Inc., Carlsbad, CA, USA).

2.8. Patients

Serum levels of PDGF-C were determined in 29 patients with *BRAF* V600-mutant metastatic cutaneous melanoma, consecutively enrolled for treatment with BRAFi or BRAFi+MEKi at IDI-IRCCS. Peripheral blood samples were collected before therapy administration, after two months of treatment and at disease progression. Baseline evaluation included medical history, physical examination, and radiologic tumor assessment with computer tomography or positron emission tomography scans. Dabrafenib was given at the dose of 150 mg BID, vemurafenib at the dose of 960 mg BID, dabrafenib plus trametinib at the dose of 150 mg BID and 2 mg/die, respectively, and vemurafenib plus cobimetinib at the dose of 960 mg BID and 60 mg/die, respectively, for three weeks with one week of break. All patients underwent physical examination and assessment of biochemical parameters monthly, whereas tumor response was determined with computer tomography every three months or before if required. Tumor response was classified according to RECIST 1.1 criteria.

The study was conducted in accordance with the Good Clinical Practice Guidelines and the Declaration of Helsinki and was approved by the IDI-IRCCS Ethics Committee (ID #407/1, 2013 and #407/2, 2016). A written informed consent was obtained from all patients.

2.9. Statistical analysis

For in vitro experiments, statistical analysis of the differences between pairs of groups was performed by the unpaired Student's *t* test and for multiple comparisons, the Kruskal-Wallis followed by Dunn's post hoc test was used. For the evaluation of PDGF-C serum levels, if data passed the normality test (D'Agostino test) statistical significance of differences was assessed by two-tailed Student's *t*-test. Non-normally distributed data were tested using the Mann-Whitney U-test or the Wilcoxon matched-pairs signed rank test for non-independent data. P values < 0.05 were considered statistically significant.

3. Results

3.1. Production of PDGF-C and expression of its receptors in BRAFi-resistant melanoma cells and their BRAFi-sensitive counterparts

In order to evaluate whether PDGF-C might have a role in melanoma invasiveness and resistance to BRAFi, in vitro models of *BRAF*-mutated melanoma were used. The human melanoma cell lines SK-Mel 28 and

M14 (both of which contain the *BRAF* V600E mutation), rendered resistant to the BRAFi dabrafenib or vemurafenib (Ssupplementary Table S1 and Supplementary Fig. S1) and their BRAFi sensitive counterparts were analyzed for PDGF-C secretion into cell culture supernatant and for the expression of its receptors PDGFRα and NRP-1. Results demonstrated that in both cellular models the acquisition of resistance to BRAFi was accompanied by high levels of PDGF-C secretion (Fig. 1A) and NRP-1 expression (Fig. 1B), neither of which were detected in drug-sensitive cells. On the other hand, PDGFRα was not expressed in any of the cell lines tested (Fig. 1B). Moreover, in melanoma cells rendered resistant to BRAFi, an increase of VEGF-A release was observed (Supplementary Fig. S2) in accordance with previous studies [17].

Acquisition of a stable BRAFi-resistant phenotype by melanoma cell culture is a process that has required about three months. Interestingly, naïve melanoma cells that do not express NRP-1 or secrete PDGF-C, exposed to a cytotoxic concentration of dabrafenib (100 nM) acquired the ability to express NRP-1 and to secrete PDGF-C during the first four weeks of treatment, when drug-resistant cells started to emerge and growth (Fig. 2A-C). Moreover, within this timeframe, M14 cells had already become able to invade the ECM (Fig. 2D). However, at this early stage of resistance development, in order to detect matrigel invasion it was necessary to remove the BRAFi from the culture medium for a couple of days, indicating that melanoma cells selected under the drug pressure were not yet sufficiently fit to invade the ECM. Therefore, induction of PDGF-C and NRP-1 expression and acquisition of an invasive ability seem to represent an early event during the development of a drug resistant phenotype.

A decrease of PDGF-C and NRP-1 expression was observed when dabrafenib was removed for a week from the culture after drug exposure for four weeks of M14 cells (Supplementary Fig. S3A and B), suggesting the requirement of continuous drug exposure during the acquisition of a resistant phenotype.

3.2. PDGF-C/NRP-1 autocrine loop supports an invasive phenotype in melanoma cells

According to the phenotype switching model, metastasis formation is the result of tumor transition from a proliferative to an invasive phenotype [20]. An online gene expression-based tool developed for predicting melanoma cell phenotype (i.e., Heuristic Online Phenotype Prediction, HOPP) is available and has identified a set of genes that characterize these two different melanoma phenotypes [21]. Interestingly, a previously published analysis of differential gene expression profiles in M14-derived melanoma clones indicated that cells lacking NRP-1 presented a gene expression signature corresponding to the proliferative phenotype, while those expressing NRP-1 showed the signature of an invasive phenotype [22]. Therefore, by using HOPP we evaluated PDGF-C and NRP-1 expression in 189 melanoma cell lines and short-term cultures grouped on the basis of their proliferative or invasive behavior. Confirming our hypothesis, both PDGF-C and NRP-1 were significantly up-modulated (8.1- and 8.4-fold increase, respectively) in the invasive melanoma group as compared to the highly proliferating group (Fig. 3A). Moreover, analysis of the ability to invade ECM of M14 and SK-Mel28 cells, sensitive or resistant to dabrafenib, indicated that the resistant sublines showed an increased invasive potential compared to their sensitive counterparts (Fig. 3B).

On the other hand, the quadrupling time (as a measure of proliferative ability) was not significantly changed when dabrafenib-resistant and -sensitive SKMel-28 cells were compared (35.2 ± 5.2 and 38.5 h ± 3.7, respectively), whereas it was drastically augmented in the case of dabrafenib-resistant M14 cells versus M14 sensitive cells (72.3 ± 9.2 and 28.9 h ± 4.2, respectively, $p < 0.001$).

Therefore, NRP-1 and PDGF-C expression can be regarded as markers of invasiveness. In fact, both PDGF-C and NRP-1 were included by HOPP in the list of genes that characterize the invasive phenotype [21].

3.3. Role of PDGF-C/NRP-1 autocrine loop in BRAFi-resistant melanoma cells

Studies were then designed to evaluate the involvement of the PDGF-C/NRP-1 autocrine loop in the increased invasive ability of BRAFi-resistant cells. To this end, NRP-1 or PDGF-C were transiently silenced by specific siRNAs in dabrafenib-resistant cells (M14 DR and SK-Mel28 DR) and silenced cells were analyzed for ECM invasion using the Boyden chamber assay. A marked decrease in invasiveness was observed in NRP-1 or PDGF-C silenced melanoma cells (Fig. 4A-C and E for M14 DR; Supplementary Fig. S4A-C and E for SK-Mel28 DR). These results were confirmed using a different method to evaluate cell invasiveness such as the spheroid invasion assay (Supplementary Fig. S4F and G) and by transfecting a second siRNA for each gene (Supplementary Fig. S5). Moreover, the invasive ability of PDGF-C silenced cells was in part restored by addition of exogenous PDGF-C, whereas no effect was observed when the growth factor was added to NRP-1 silenced cells, confirming the hypothesis that the PDGF-C-mediated induction of ECM invasion occurs through NRP-1 stimulation (Fig. 4D and E for M14 DR; Supplementary Fig. S4D and E for SK-Mel28 DR).

We then explored whether the PDGF-C/NRP-1 autocrine loop was also involved in the proliferative potential and response to BRAFi of

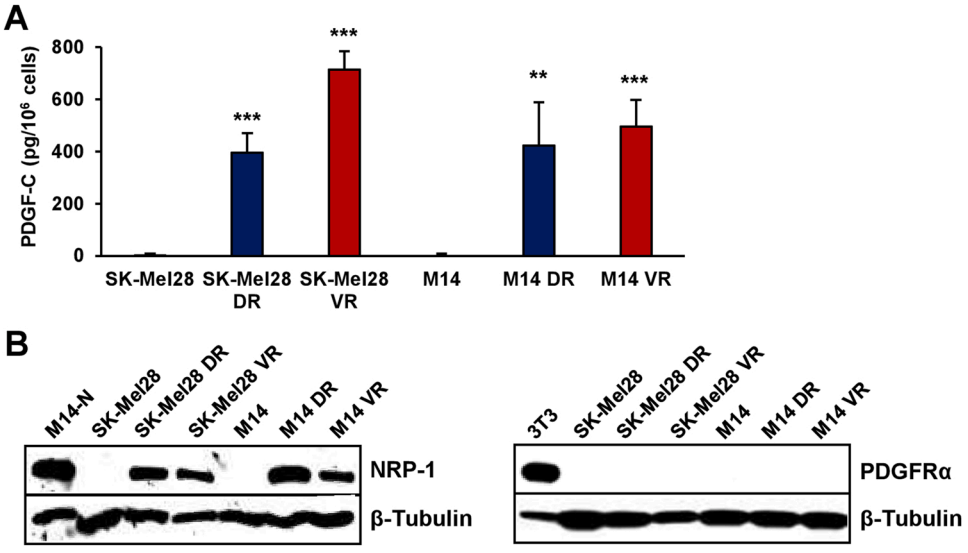

Fig. 1. Up-regulation of PDGF-C secretion and NRP-1 expression in BRAFi resistant melanoma cells, as compared to their sensitive counterparts. A) Culture supernatants of SK-Mel28 and M14 sublines, either resistant to dabrafenib (DR) or vemurafenib (VR) or sensitive to both drugs were collected in order to evaluate the levels of PDGF-C by ELISA. Values of growth factor secretion were normalized by the number of total cells harvested at the moment of supernatant collection. Results represent the arithmetic mean ± SD of four independent determinations. Statistical analysis was performed using the Student's *t*-test: $p < 0.01$ (**); $p < 0.001$ (***). B) The same cell sublines were analyzed for NRP-1 and PDGFRα expression by western blotting, using M14-N melanoma cells and 3T3 fibroblasts as positive controls for NRP-1 and PDGFRα, respectively. Results shown are representative of two independent determinations. β-tubulin levels were evaluated as loading control.

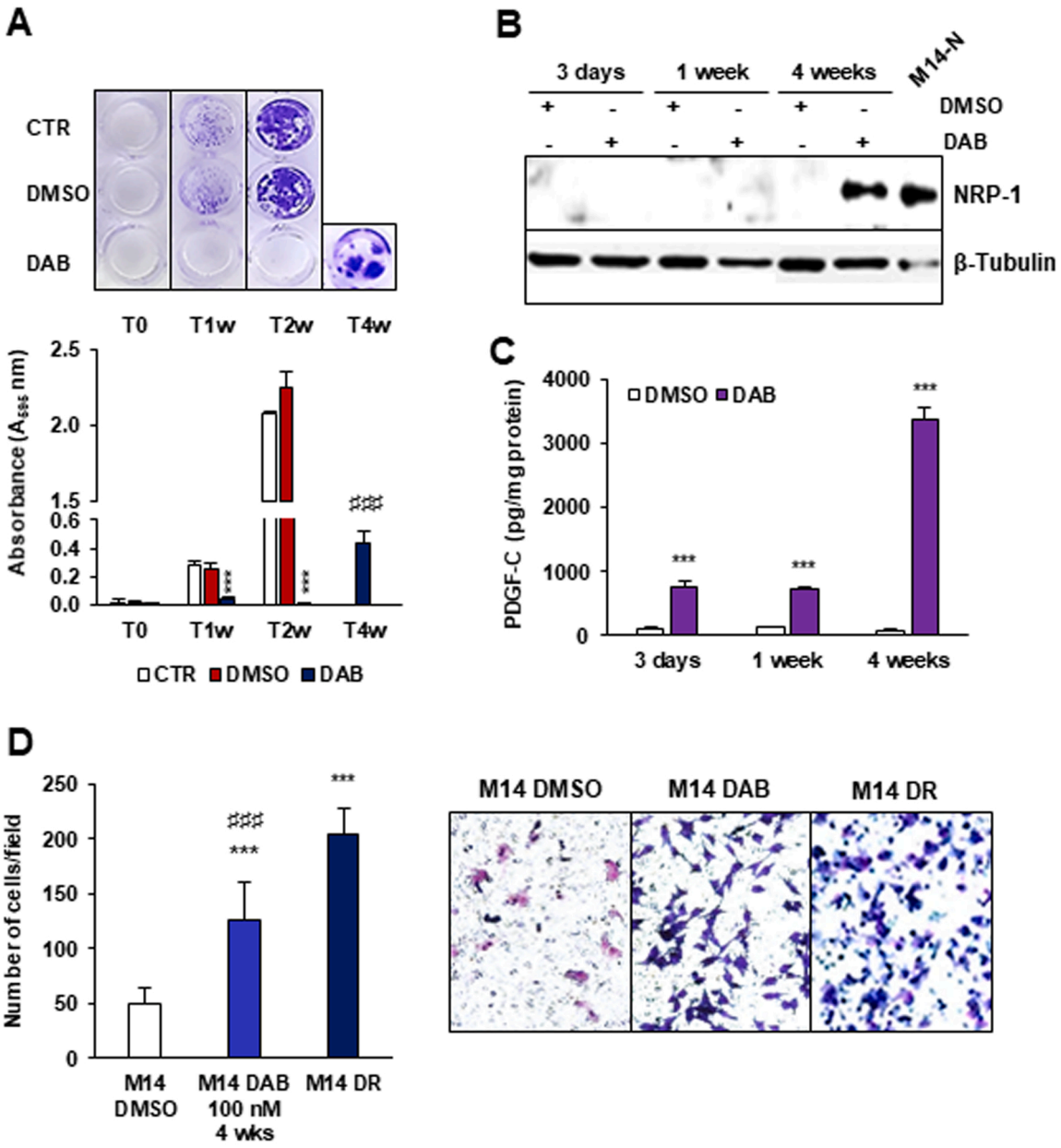

Fig. 2. NRP-1 expression and PDGF-C secretion increase early during the development of dabrafenib resistance. A) M14 cells (500 cells/well) were seeded into 96-well plates and treated with 100 nM dabrafenib (DAB), the diluent DMSO or left untreated (CTR). At the indicated times, cells were fixed, stained with crystal violet and photographed. Images from a representative experiment are shown. Histogram shows the quantitative analysis of cell growth, obtained by crystal violet solubilization and absorbance measurement at 595 nm. B,C) M14 cells were seeded into 6-well plates and treated with 100 nM dabrafenib (DAB) or with DMSO; cells and supernatants were collected at different times to analyze NRP-1 expression in the cell pellets by western blot (β-tubulin was used as loading control) (B) and PDGF-C secretion in the culture supernatants by ELISA (C). Results shown in (B) are representative of two independent determinations. D) The ability of M14 cells to invade ECM after 4 weeks treatment with 100 nM dabrafenib (DAB) or with DMSO was evaluated in Boyden chambers equipped with matrigel coated filters, as described in the methods section. The invasive ability of these cells was compared to that of M14 DR cells. Representative photographs are shown (x200 original magnification). In histograms values represent the arithmetic mean ± SD (A, n = 4; C, n = 4; D, n = 3). Statistical analysis was performed using the Student's *t*-test: in (A) and (C), $p < 0.001$ (***), DAB-treated versus DMSO-treated cells; $p < 0.001$ ($^{\#\#\#}$), DAB-treated cells for 4 weeks versus 1 week; in (D), $p < 0.001$ (***), DAB-treated or M14 DR cells versus DMSO-treated cells; $p < 0.001$ ($^{\#\#\#}$), DAB-treated cells for 4 weeks versus M14 DR cells.

resistant melanoma cells. To this end, M14 DR and SK-Mel28 DR cells were transfected with specific or control siRNAs and then left untreated or exposed to graded concentrations of dabrafenib for additional four days before analyzing cell growth. The results indicated that PDGF-C silencing did not significantly affect either cell growth or sensitivity to dabrafenib in M14 DR and SK-Mel28 DR cells (Supplementary Fig. S6A-C). In the case of NRP-1 silencing, a different behavior was observed depending on the cellular model tested: M14 DR cell growth rate and sensitivity to dabrafenib were not significantly affected, whereas proliferation of SK-Mel 28-DR cells was reduced regardless of drug exposure although their sensitivity to the BRAFi was not changed (Supplementary Fig. S6A and B). We also confirmed that, in the conditions used to test cell proliferation and chemosensitivity, silencing of PDGF-C and NRP-1 was maintained until the end of the experiment (i.e., 7 days) (Supplementary Fig. S6D and E).

The results obtained in M14 DR cells were also confirmed by treating them with a known pharmacological inhibitor of NRP-1 activation (oligopeptide EG00229) [23]. In fact, exposure to graded concentrations

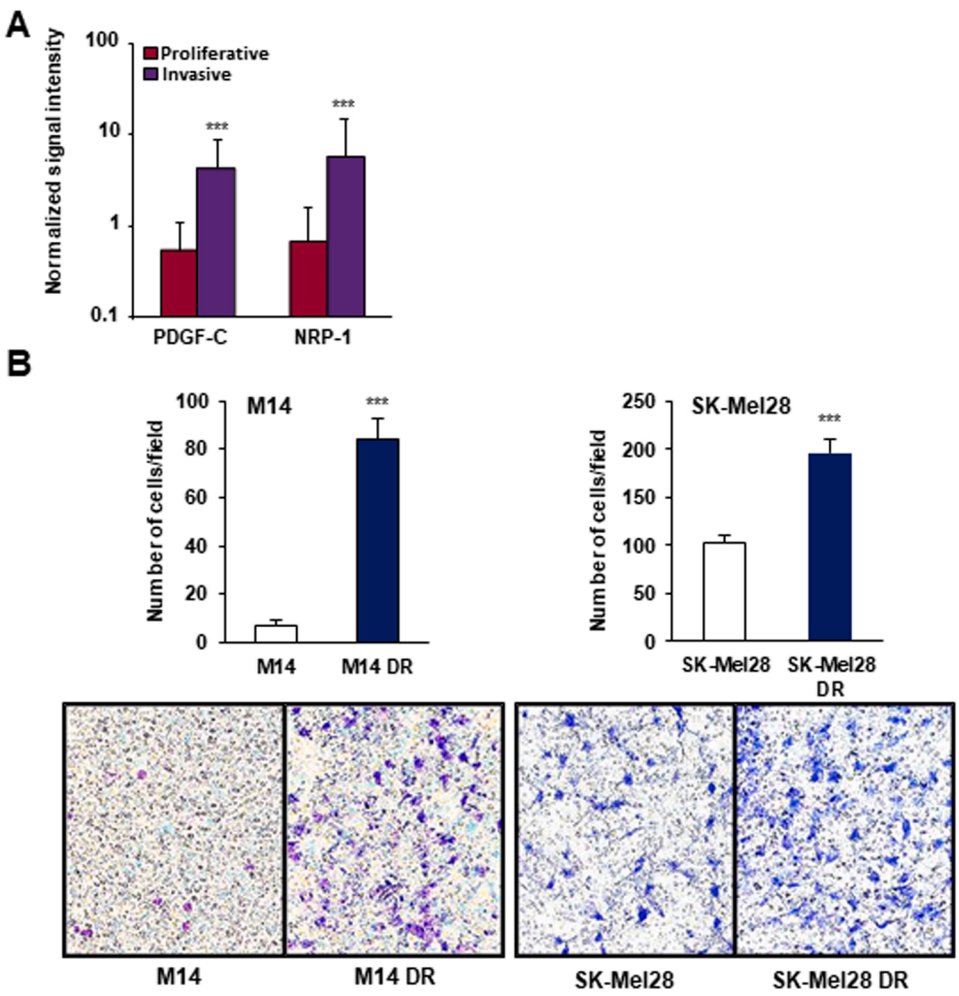

Fig. 3. BRAFi resistant melanoma sublines develop a highly invasive phenotype compared to the corresponding sensitive cells. A) Expression of PDGF-C and NRP-1 in melanoma cells with proliferative or invasive phenotypes was evaluated using a dataset of 220 melanoma cell lines and short-term cultures that includes samples defined as 100% proliferating (100) or 100% invasive (89) (the rest of the melanoma cultures showing an intermediate phenotype) [20]. Analysis of the 218718_at probeset for PDGF-C (8.1-fold, $p < 1.00E-05$) and of the 210510_s_at probeset for NRP-1 (8.4-fold, $p < 1.00E-05$) was performed by comparing the mean signal intensity value of proliferative melanomas with that of invasive melanomas. B) The ability of parental and dabrafenib resistant M14 and SK-Mel28 (DR) sublines to invade ECM was evaluated in Boyden chambers equipped with matrigel coated filters, as described in the methods section. Representative photographs are shown (x200 original magnification). Data in the histograms represent the arithmetic mean ± SD of three independent determinations. Statistical analysis was performed using the Student's *t*-test: $p < 0.001$ (***).

of EG00229, including the IC_{50} values for VEGF-A binding to NRP-1 (i.e., 8–23 μM) [23], did not affect melanoma cell proliferation (Supplementary Fig. S7A). The NRP-1 inhibitor was also tested in combination with a fixed concentration of dabrafenib corresponding to the steady-state plasma C_{max} reported in *BRAF* V600-mutated melanoma patients treated with the recommended dose of 150 mg (1.5 μg/ml, ~2.8 μM) [24,25]. Similarly to NRP-1 silencing, NRP-1 inhibition did not affect M14 DR cell sensitivity to the BRAFi (Supplementary Fig. S7A). Moreover, EG00229 markedly reduced tumor cell ability to invade the ECM in response to PDGF-C (Fig. 4F).

We also evaluated the sensitivity to dabrafenib and vemurafenib of BRAFi-naïve M14 cell clones that differ in the ability to invade ECM and in the expression of PDGF-C and NRP-1 [5,14]. Interestingly, cell lines expressing high levels of PDGF-C and NRP-1 (M14-N and M14K14 cells) were found 1000-fold more resistant to dabrafenib and 10 or 15-fold more resistant to vemurafenib than M14-C cells (negative for both polypeptides), even though they had never been pre-exposed to BRAFi (Table 1 and Supplementary Fig. S8). Dabrafenib and vemurafenib IC_{50}s calculated for M14-N and M14K14 cells were comparable to those obtained for melanoma cells with an acquired resistance phenotype to BRAFi (Supplementary Table S1). Resistance of these clones was not due to the loss of the *BRAF* V600 mutation (Supplementary Fig. S9).

Gene silencing experiments were then performed to analyze the influence of PDGF-C or NRP-1 expression in M14-N cells on their innate BRAFi-resistance and ability to invade the ECM. In accordance with previous studies performed using neutralizing antibodies against PDGF-C or NRP-1 [5], the corresponding gene silencing markedly reduced ECM invasiveness (Fig. 5A-C). Results also indicated that, in this cellular model, only NRP-1 silencing reduced the proliferative potential of M14-N cells, whereas down-regulation of either genes significantly increased the sensitivity to dabrafenib (Fig. 5D and E). The crucial role of NRP-1 in melanoma invasive behavior was confirmed by exposing M14-N cells to the NRP-1 inhibitor EG00229, since this agent significantly hampered ECM invasion induced by PDGF-C (Fig. 5F). Regarding the influence of EG00229 on M14-N cell growth and response to the BRAFi, the NRP-1 inhibitor did not affect per se cell proliferation and increased melanoma cell sensitivity to dabrafenib only at the highest concentration tested (Supplementary Fig. S7B).

3.4. Evaluation of PDGF-C levels in the serum of melanoma patients treated with BRAFi or BRAFi plus MEKi

Based on our in vitro results, we investigated whether PDGF-C serum levels might represent an indicator of clinical response in melanoma patients receiving BRAFi. PDGF-C levels were evaluated in the serum of 29 patients diagnosed with *BRAF* V600 mutant cutaneous metastatic melanoma and consecutively treated with BRAFi as single agents or in combination with MEKi (see patients' characteristics in Supplementary Tables S2 and S3). Serum samples were collected before treatment start (T0), after two months of treatment (T2) and, in responders' patients, at time of disease progression (TP). As best response, 6 patients presented progressive disease (PD), 3 stable disease (SD), 18 partial response (PR) and 2 complete response (CR) (Supplementary Tables S2 and S3). Patients experiencing PR or CR constituted the group of responders, whereas patients with PD or SD as best response were included in the group of non-responders.

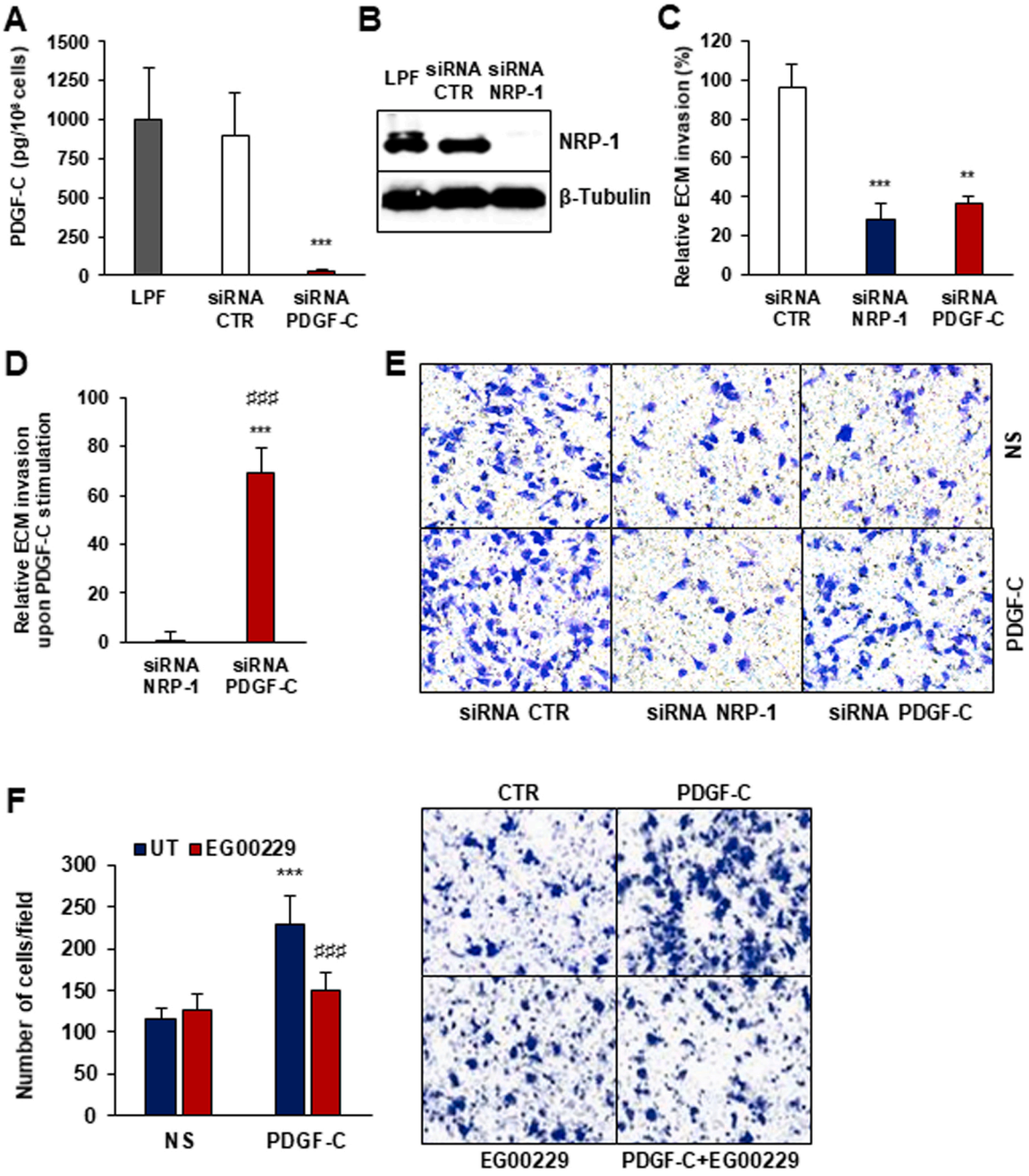

Fig. 4. Effect of PDGF-C and NRP-1 silencing on ECM invasion by M14 DR cells. Cells were transfected in 6 cm diameter Petri dishes with oligonucleotide siRNAs targeting NRP-1 (ID# s16845), PDGF-C (ID# s31864) or with the AllStars siRNA as negative control oligonucleotide (siRNA CTR), as described in the methods section. Three days after transfection, cells were harvested and analyzed for PDGF-C secretion by ELISA (A) or for NRP-1 expression by western blot (B). In both cases, lipofectamine-treated (mock-transfected) cells were used as reference (LPF). The ability of transfected cells to invade the ECM, in the absence of any stimulus (C) or in response to 20 ng/ml PDGF-C (D), was measured by a Boyden chamber assay. Results are expressed as percentage of cell invasion compared to non-stimulated, mock-transfected cells (C), or to the corresponding non-stimulated transfected cells (D). (E) Representative photographs showing invaded cells on polycarbonate filters, corresponding to the experimental conditions described in panels C and D (x200 original magnification). F) Effect of the NRP-1 inhibitor EG00229 on the invasiveness of M14 DR cells, evaluated by a Boyden chambers assay. M14 DR cells were untreated (UT) or pre-treated with EG00229 (15 μM) for 30 min in a rotating wheel at room temperature and then loaded in the upper compartment of Boyden chambers. Migration medium (0.1% BSA/1 mg/ml heparin/RPMI) (NS) or PDGF-C (20 ng/ml), utilized as stimulus, were included in the lower compartment of the chambers. Representative photographs of invaded cells on the polycarbonate filters are shown (x200 original magnification). Results shown in (B) are representative of two independent determinations. In histograms, data represent the arithmetic mean ± SD (A, n = 4; C, n = 3; D, n = 6; F, n = 6). Statistical analysis was performed using Student's *t*-test, comparing the following experimental groups: siRNA transfected cells vs. mock-transfected cells, $p < 0.01$ (**) and $p < 0.001$ (***) (A and C); siRNA PDGF-C transfected cells stimulated with PDGF-C vs. non-stimulated cells ($^{¤¤¤}$, $p < 0.001$) and cells transfected with siRNA specific PDGF-C stimulated with PDGF-C vs. siRNA NRP-1 transfected cells stimulated with PDGF-C (***, $p < 0.001$) (D); cells stimulated by PDGF-C vs. non-stimulated untreated cells (***, $p < 0.001$) and PDGF-C stimulated cells treated with EG00229 vs. untreated cells (UT) ($^{###}$, $p < 0.001$) (F).

Table 1
BRAFi susceptibility profile of M14 cells lacking (M14-C) or expressing (M14-N and M14K14) NRP-1 and PDGF-C.

Cell line	IC_{50} for dabrafenib[a]	IC_{50} for vemurafenib[a]
M14-C	0.015 ± 0.001 μM	1.00 ± 0.04 μM
M14-N	18.19 ± 0.97 μM* **	15.41 ± 4.52 μM*
M14K14	23.00 ± 3.97 μM* **	10.29 ± 3.19 μM* **

[a] Cells were incubated with graded concentrations of the indicated BRAFi or with DMSO alone for four days. Proliferation was assessed by the MTS assay and IC_{50} values were calculated as described in the Methods section. Each value represents the arithmetic mean ± SD of three independent experiments and statistical analysis was performed using the Student's *t*-test: $p < 0.05$ (*), $p < 0.001$ (***) for each drug-resistant subline versus their corresponding sensitive partners.

PDGF-C levels were detectable in all serum samples tested. In responders, PDGF-C was significantly reduced in T2 respect to T0 and increased at TP compared to T2 (Fig. 6). In particular, an overall mean decrease of − 248.9 pg/ml (95% CI= −152.4, −345.4) was observed in T2, with a significant difference in the before-after comparison (Student *t*-test, $p < 0.0001$). Furthermore, the statistically significant increase of PDGF-C levels observed at TP in comparison with T2, corresponded to an overall mean increase of + 184.6 pg/ml (95% CI= 93.33, 275.8; Student *t*-test, $p = 0.0004$). Levels of the growth factor in the serum of responders at TP were not significantly different to baseline levels (Student *t*-test, $p < 0.3398$). On the other hand, in non-responders, no significant variations were observed when PDGF-C baseline levels were compared to those measured at T2 (Wilcoxon matched-pairs signed rank test, $p = 0.25$) (Fig. 6). When comparing baseline levels in responders and non-responders, the observed difference was not statistically significant: mean baseline values were 893.2 and 1074 pg/ml for responders and non-responders, respectively (Mann-Whitney U test, $p = 0.3892$). Nevertheless, the different sample size (20 responders and 9 non-responders) does not allow a reliable statistical analysis. Serum levels of PDGF-C detected in samples collected from each patient are reported in the Supplementary Tables S2 and S3.

Overall, the results of this analysis suggest that in melanoma patients PDGF-C serum levels correlated with tumor burden and BRAFi/MEKi treatment failure.

3.5. Evaluation of mRNAs coding for NRP-1, PDGF-C and markers of epithelial-mesenchymal transition (EMT) in the tumor of melanoma patients treated with BRAFi during disease progression by using a gene expression database

The Gene Expression Omnibus (GEO) database GSE50509 was used to analyze the expression of the genes of interest in tumor samples derived from melanoma patients (29 tumors from 21 patients) before starting the therapy with BRAFi (16 with dabrafenib and 5 with vemurafenib) and after disease progression. At first, NRP-1 and PDGF-C mRNA expression was analyzed to investigate the in vivo relevance of the data obtained in our in vitro studies (Fig. 7A). Results indicated a significant increase of NRP-1 expression at tumor progression, with an overall mean increase of + 19.20 (mean expression values were 53.79 and 73.86 for Pre and Prog tumors, respectively; 95% CI= 8.42, 31.72; Wilcoxon matched-pairs signed rank test, $p = 0.0012$). PDGF-C expression remained at higher levels than those of NRP-1, with no significant change between pre- and post-progression values (mean expression values were 84.69 and 80.97 for Pre and Prog tumors, respectively; Wilcoxon matched-pairs signed rank test, $p = 0.5258$).

We previously demonstrated that activation of a PDGF-C/NRP-1 autocrine loop in melanoma cells results in an increased expression of proteins involved in EMT, a process that contributes to melanoma aggressiveness [26]. Thus, we evaluated the expression pattern of two important EMT markers, E-cadherin and N-cadherin, in the same GSE50509 database. Indeed, activation of signal transduction pathways during the EMT process results in reduced E-cadherin expression, leading to disassembly of the inter-cellular adhesion complexes characteristic of epithelial cells, and increased expression of N-cadherin, with consequent promotion of tumor cell mobility and spreading [27]. Results indicated that at disease progression E-cadherin expression significantly decreased with an overall mean decrease of − 279.8 (mean expression values were 1249 and 969.4 for Pre and Prog tumors, respectively; 95% CI= −691.7, 132.2; Wilcoxon matched-pairs signed rank test, $p = 0.0193$), whereas N-cadherin slightly increased although changes before and after BRAFi treatment did not reach statistical significance (mean expression values were 619.7 and 665.2 for Pre and Prog tumors; Wilcoxon matched-pairs signed rank test, $p = 0.5362$), likely due to the limited number of samples (Fig. 7B).

Several additional markers have been described as indicators of transition from a proliferative to a more invasive phenotype: lower levels of claudin 1, occludin and β-catenin together with higher levels of vimentin and matrix metalloproteinase 2 (MMP2). Expression of these markers was also evaluated in the GSE50509 database (Supplementary Fig. S10A and B). Claudin 1 expression decreased at disease progression with an overall mean decrease of − 53.8 (mean expression values were 330.8 and 277 for Pre and Prog tumors; 95% CI= −332.2, 224.6; Wilcoxon matched-pairs signed rank test, $p = 0.0585$) and occludin expression was extremely low (mean expression values were 1.84 and 0.27 for Pre and Prog tumors, respectively). Moreover, β-catenin expression was markedly and significantly reduced at tumor progression with an overall mean decrease of − 187.6 (mean expression values were 848.5 and 660.9 for Pre and Prog tumors, respectively; 95% CI= −383.9, 8.611; Wilcoxon matched-pairs signed rank test, $p = 0.0322$). On the other hand, vimentin expression was maintained at high levels before therapy and at disease progression (mean expression values were 12,747 and 12,061 for Pre and Prog tumors; Wilcoxon matched-pairs signed rank test, $p = 0.4169$), whereas MMP2 expression was higher at disease progression although the increase did not reach statistical significance (overall mean increase 26.69; mean expression values were 57.76 and 84.45 for Pre and Prog tumors; 95% CI= −20.49, 73.87; Wilcoxon matched-pairs signed rank test, $p = 0.5117$).

Multiple transcription factors are involved in the switch from a proliferative to an invasive phenotype, including Snai1/Snail, Snai2/Slug, the zinc finger E-box-binding homeobox (ZEB) 1 and 2 and the microphthalmia-associated transcription factor (MITF). Data referring to the mRNA expression of these transcription factors in the GSE50509 database showed undetectable expression for Snai1/Snail and ZEB1 at both time points. Regarding Snai2/Slug and ZEB2, which are known to induce melanocytic differentiation rather than invasiveness [28,29], a reduction in the expression of both transcription factors was observed at disease progression. In the case of Snai2/Slug, the overall mean decrease was − 85.59 and mean expression values were 515.3 and 429.7 for Pre and Prog tumors, respectively (Wilcoxon matched-pairs signed rank test, $p = 0.247$); for ZEB2, the overall mean decrease was − 95.79 and mean expression values were 364.2 and 268.5 for Pre and Prog tumors, respectively (Wilcoxon matched-pairs signed rank test, $p = 0.015$) (Supplementary Fig. S10C).

MITF expression controls melanoma phenotype switching and melanocyte development. Indeed, high levels of this transcription factor correlate with melanoma cell proliferation, whereas low levels determine an invasive phenotype [30]. Analysis of MTIF expression in the GSE50509 database showed a statistically significant down-regulation at melanoma progression, which may favor tumor invasiveness and drug resistance (overall mean decrease −251.6; mean expression values were 837.8 and 586.2 for Pre and Prog tumors; 95% CI= −457.9, −45.16; Wilcoxon matched-pairs signed rank test, $p = 0.0203$) (Supplementary Fig. S10C).

Overall, the increase of NRP-1 expression at tumor progression was paralleled by down-regulation of E-cadherin, β-catenin and repressors of genes involved in melanoma cell invasiveness together with high levels of mesenchymal markers (i.e., N-cadherin, vimentin). Further analysis will be required in the future to confirm the indicated variations in a larger number of patients.

4. Discussion

Herein we demonstrated for the first time that NRP-1 activation by PDGF-C contributes to the invasive behavior of melanoma cells resistant to BRAFi, favoring their metastatic spreading. Indeed, acquisition of resistance to BRAFi by *BRAF* V600 mutant cells is accompanied by high PDGF-C secretion and NRP-1 expression that result in increased ability to invade the ECM. Accordingly, silencing of either of these molecules

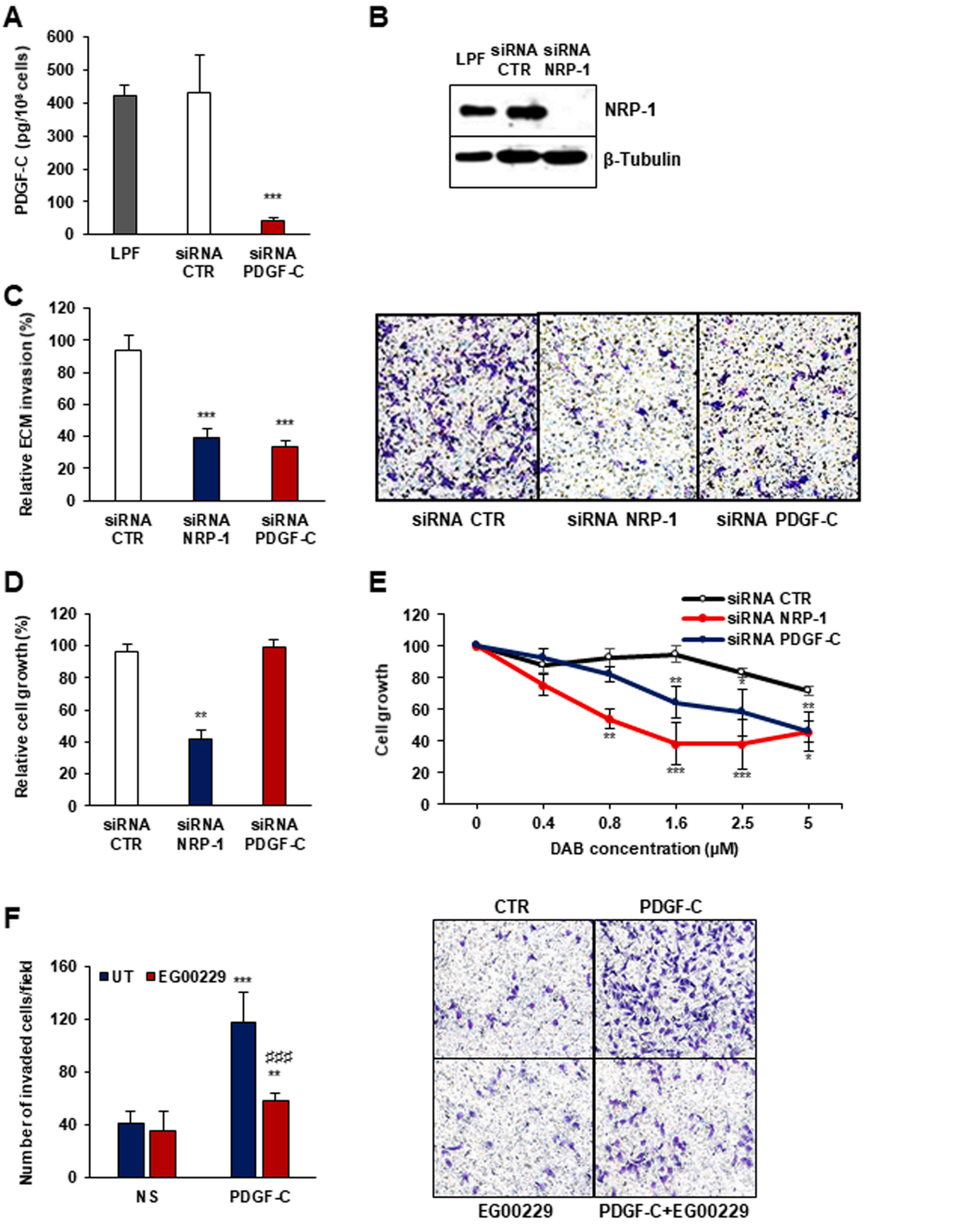

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Fig. 5. Effect of NRP-1 or PDGF-C silencing on chemosensitivity and invasiveness of a M14 cell clone expressing NRP-1/PDGF-C with innate resistance to BRAFi. M14-N cells were transfected in 10 cm diameter Petri dishes (0.6×10^6 cells/plate) with specific siRNAs for NRP-1 (ID# s16845), PDGF-C (ID# s31864) or with a negative control siRNA (siRNA CTR). Three days after transfection, culture supernatants were analyzed for PDGF-C secretion by ELISA (A) and cells tested for NRP-1 expression by western blot (B), ECM invasion by a Boyden chambers assay (C) or growth in the presence or absence of dabrafenib by MTS assay (D,E). Results shown in (B) are representative of two independent determinations. Results of ECM invasion are expressed as percentage of cell invasion respect to lipofectamine treated (mock-transfected) cells (LPF) and representative photographs of invaded cells on the polycarbonate filters are shown (x200 original magnification) (C). MTS assay was performed by seeding 1000 cells in 96-well plates and selected wells cultured for 4 days in the absence (D) or in the presence of graded concentrations of dabrafenib (DAB) or the diluent (DMSO) (E). Results were normalized to those of DMSO treated cells and then to those of lipofectamine treated cells for each drug concentration. Values in the graph are expressed as percentage of cell growth respect to untreated cells (E). In histograms, data represent the arithmetic mean ± SD (A, n = 4; C, n = 4; D, n = 4; E, n = 3; F, n = 5). Statistical analysis was performed using the Student's *t*-test, comparing the following experimental groups: siRNA transfected cells vs. mock-transfected cells, $p < 0.001$ (***) (A and C); cells transfected with siRNA specific for NRP-1 vs. siRNA CTR transfected cells, $p < 0.01$ (**) (D); NRP-1 and PDGF-C silenced cells vs. siRNA CTR transfected cells at each dabrafenib concentration tested, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***); cells stimulated with PDGF-C vs. non-stimulated untreated cells, $p < 0.01$ (**) and $p < 0.001$ (***), and PDGF-C stimulated cells treated with EG00229 vs. untreated cells (UT) ($^{\#\#\#}$, $p < 0.001$) (F).

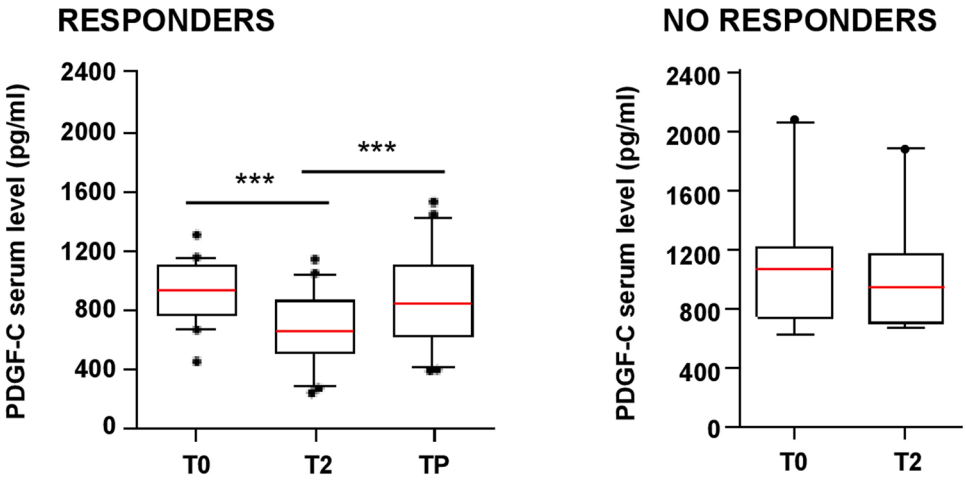

Fig. 6. Box-and-whisker diagrams of PDGF-C serum levels in melanoma responder and non-responder patients treated with BRAFi or BRAFi+MEKi. PDGF-C serum levels were measured in 20 responder and 9 non-responder melanoma patients before therapy commencement (T0), after two months of treatment (T2) and, in the case of responders, at disease progression (TP). The edges of each box represent the 75th and 25th percentile and whiskers indicate the 90% and 10% of the values obtained for each group. The horizontal red bar within each box indicates the mean and the points out of the intervals indicate the value for outliers. In the case of data from responder patients, which passed the normality test, statistical analysis was performed by the Student's *t*-test: $p < 0.001$ (***). Data from non-responder patients, since non-normally distributed, were analyzed by Wilcoxon matched-pairs signed rank test.

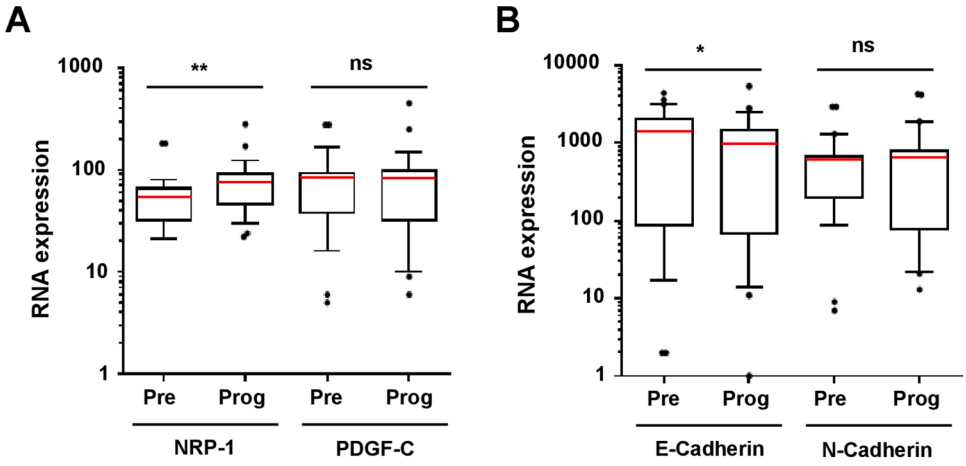

Fig. 7. Box-and-whisker diagrams of mRNA expression for NRP-1, PDGF-C and the EMT markers E-cadherin and N-cadherin in tumors from melanomas patients before therapy with BRAFi and at progression. Expression of mRNA coding for NRP-1 and PDGF-C (A) or N-cadherin and E-cadherin (B) in tumors from melanoma patients was evaluated using a GEO database of gene expression (GSE50509 database) that contains data from tumor samples collected from patients before commencing treatment with a BRAFi (Pre) and at time of tumour progression (Prog). The edges of each box represent the 75th and 25th percentile and whiskers indicate the 90% and 10% of the values obtained for each group. The horizontal red bar within each box represents the mean and the points out of the intervals indicate the value for outliers. Statistical analysis was performed by Wilcoxon matched-pairs signed rank test: $p < 0.05$ (*); $p < 0.01$ (**); ns, not significant.

has the opposite effect.

High NRP-1 expression in melanoma has been associated with disease progression and reduced survival [31] and, for its prevalent role in tumorigenesis, has been tested as a therapeutic target [32]. In particular, the biodistribution of a human monoclonal antibody (MNRP1685A) targeting the VEGF-A-binding domain of NRP-1 was tested in tumor-bearing mice [26]. However, due to the widespread NRP-1 expression in normal tissues, the antibody interfered with physiological functions, presented a reduced uptake by tumor cells and was rapidly eliminated [33]. Results of two phase 1 trials with MNRP1685A indicated that the antibody, administered as single agent, had a modest clinical activity [34]. Moreover, its combination with the anti-VEGF-A monoclonal antibody bevacizumab plus chemotherapy induced severe toxicity that did not support further testing of the combined treatment [35].

Most of the approaches targeting NRP-1 investigated so far were

based on the blockage of its ability to bind VEGF-A or to interact with VEGFR-2. Therefore, in an attempt to shed light on other possible mechanisms of NRP-1 activation whose targeting could be more efficient in counteracting tumor progression, we evaluated NRP-1 interaction with PDGF-C, a member of the PDGF family that shares with VEGF-A a high structural homology [36]. A number of functions have been attributed to PDGF-C that influence processes such as activation and mobilization of monocyte/macrophages [37,38], neurovascular cross-talk [39–42] and disease progression in glioblastoma [43,44], mesothelioma [45] and breast cancer [46–49]. In the case of melanoma, PDGF-C expression accelerated tumor growth through the recruitment and activation of different subsets of cancer-associated fibroblasts [50]. Moreover, inhibition of PDGF-C has been considered an efficient strategy to counteract resistance to anti-VEGF-A therapies or to synergistically inhibit pathological angiogenesis [43,44,51]. These functions have been usually analyzed considering PDGF-C ability to activate its cognate receptor PDGFRα.

Nevertheless, we recently demonstrated that PDGF-C can also directly bind and activate NRP-1 without the involvement of PDGFRα [5], and herein we demonstrated that, in melanoma cells rendered resistant to BRAFi, increased levels of both proteins result in the activation of an autocrine loop that contributes to the invasive properties characteristic of these cells. In fact, silencing of PDGF-C or NRP-1 significantly reduced melanoma ability to invade ECM that was restored by exogenously adding the growth factor only in PDGF-C silenced cells and not in NRP-1 silenced cells.

NRP-1 is known to be also involved in the acquisition of a resistant phenotype in several tumor models [9,12,13,52]. Mechanisms of resistance to BRAFi include, among others, increased expression of receptor tyrosine kinases such as PDGF receptor beta (PDGFRβ), insulin-like growth factor-1 receptor (IGF1R), transforming growth factor β receptor (TGFβR) and epidermal growth factor receptor (EGFR), which trigger signal transduction pathways alternative to BRAF [29,53–56]. The functionality of some of these receptors can be potentiated by NRP-1 as co-receptor; moreover, increased activity of several protein kinases or phosphorylation of substrates have been related to direct activation of NRP-1, independently of its co-receptor function [7].

NRP-1 activation results in the induction of a variety of cell functions [7,57]: migration, drug-resistance, cell growth, invasiveness, tumor progression. Our data demonstrate that M14 cell clones co-expressing PDGF-C/NRP-1 and characterized by an invasive phenotype were highly resistant to vemurafenib and dabrafenib, even though they had never been exposed to these BRAFi. Moreover, PDGF-C or NRP-1 silencing in the drug-naïve M14-N clone not only significantly reduced ECM invasion but also increased sensitivity to dabrafenib. On the other hand, abrogation of PDGF-C did not affect proliferation and sensitivity to dabrafenib of M14 DR and SK-Mel28 DR cell lines, whereas NRP-1 silencing inhibited proliferation only in SK-Mel28 DR cells and did not modify dabrafenib susceptibility of both M14 DR and SK-Mel28 DR cells.

A NRP-1 inhibitor, previously described in the literature (EG00229), was found to inhibit the invasiveness triggered by PDGF-C of melanoma cells with acquired (M14 DR) or innate (M14-N) resistance to BRAFi. EG00229 is a small molecule that hampers VEGF-A binding to NRP-1 b1 domain [23]. Due to the high sequence homology between PDGF-C and the VEGF-A region involved in the binding to NRP-1, it can be hypothesized that this inhibitor would affect, at least in part, also PDGF-C binding to NRP-1. Since EG00229 treatment showed an effect similar to that induced by PDGF-C silencing (i.e., no effect on tumor cell proliferation and limited influence on chemosensitivity only on M14-N cells), data obtained with the inhibitor seems to confirm that, in melanoma cells with innate BRAFi resistance, PDGF-C/NRP-1 interaction supports their invasive phenotype and, at least in part, their drug-resistant phenotype. On the other hand, in melanoma cells with acquired resistance to BRAFi, this autocrine loop, plays a role only on cell invasiveness. Additional effects of NRP-1 silencing are likely due to its ability to interact with other cytokines or growth factor receptors. Thus, the development of specific inhibitors for PDGF-C/NRP-1 activation would allow to fully evaluate the therapeutic potential of targeting this signaling pathway.

Overall, our findings suggest that in melanoma cells with intrinsic resistance to BRAFi the NRP1/PDGF-C autocrine loop promotes both the invasive and the drug-resistant phenotypes, while in melanoma cells with acquired resistance to BRAFi the influence of NRP-1 and PDGF-C expression on drug response, but not on invasiveness, is overridden by additional mechanisms of resistance. In fact, it is well-known that kinase inhibitors lose efficacy with time through adaptive resistance mechanisms that can emerge in cancer cells during treatment, including upregulation of parallel signal cascades able to promote tumor cell survival and proliferation [58]. Of note, a previous study by Rizzolio and colleagues [12], demonstrated that A375 cells with acquired resistance to a vemurafenib analogue used in preclinical studies (PLX4720) became more susceptible to this inhibitor upon NRP-1 silencing. Whether these contrasting results are due to the heterogeneity of melanoma cell lines or to the different BRAFi tested needs to be clarified in further studies.

In melanoma cells overexpressing the PDGF-C/NRP-1 autocrine loop (i.e., M14-N and M14K14 cells), we previously described [14] a signal transduction signature characterized by increased phosphorylation levels of several important players in the resistance to BRAFi [59,60]. In particular, Akt phosphorylation at S473 and T308 was 100- and 30-fold higher, respectively, compared to cells lacking PDGF-C and NRP-1 expression. The PI3K/Akt pathway is activated by growth factors through specific receptor tyrosine kinases and, under prolonged exposure to BRAFi, tumor cells overexpress receptor tyrosine kinases that sustain a continuous PI3K/Akt signaling [4]. In the BRAF mutated M14-N and M14K14 cells used in the present study, stimulation of NRP-1 by PDGF-C would keep Akt in an active state contributing to their poor response to the antiproliferative effects of BRAFi. Moreover, in these cells JNK, c-Jun, β-catenin and STAT3 phosphorylation levels were also significantly increased. JNK/c-Jun pathway is an important regulator of cell proliferation, metabolism and death, and both transcription factors have been involved in vemurafenib resistance [61,62]. Finally, it has been also demonstrated that β-catenin and STAT3 interact and cooperate in the acquisition and maintenance of resistance to vemurafenib [63]. Therefore, the activation of these signal transduction pathways (separately or in combination) by the PDGF-C/NRP-1 autocrine loop might contribute to BRAFi-resistance. Further studies are needed to shed light on the mechanisms that link NRP-1, a receptor devoid of kinase activity, to the phosphorylation and activation of effector signaling pathways which contribute to BRAFi-resistance.

Regarding the aggressive phenotype of BRAFi-resistant cells, we have also previously described that PDGF-C/NRP-1 autocrine stimulation results in the activation of integrins (αvβ3 and αvβ5), transcription factors involved in the EMT (ZEB1 and Snail) and specific signal transduction pathways (pCas130 kinase phosphorylation) [5,14,15]. All these signaling pathways contributed to the highly invasive phenotype that characterize melanoma cells co-expressing PDGF-C and NRP-1.

The results of the pilot study, performed to evaluate PDGF-C levels in the serum collected from melanoma patients refractory to BRAFi or undergoing progression after an initial response to drug treatment, indicate that PDGF-C levels correlate with tumor burden. All patients enrolled in the study were affected by stage IV/metastatic melanoma harboring the *BRAF* V600 mutation and most of them responded to BRAFi as single agents or in combination with MEKi, reporting partial or complete responses. PDGF-C levels were found to decrease in responder patients after two months of treatment and then to increase again at disease progression, whereas PDGF-C levels did not change in the serum of non-responder patients. A study using a larger cohort of patients will be required to establish whether a decrease in PDGF-C serum levels might represent a marker of response to BRAFi-based therapies. Nevertheless, our data suggest that the decline in PDGF-C likely reflects a reduction in tumor burden. Regarding the cellular origin of PDGF-C detected in the serum, besides melanoma cells also components of the

tumor microenvironment (e.g., monocytes/macrophages, vascular endothelial cells, tumor-associated fibroblasts) [64] may contribute to produce and release this growth factor favoring disease progression and spreading, especially in the case of NRP-1 expressing melanoma. In this context, we also analyzed the expression of PDGF-C and NRP-1 in the tumor tissues by using a gene expression database (GSE50509) of melanoma samples from 21 patients treated with BRAFi. Consistently with the results obtained in the serum of patients, PDGF-C was expressed in the tumor at similar levels before treatment start and at disease progression. NRP-1 expression in melanoma tissue significantly increased at disease progression supporting the activation of a PDGF-C/NRP-1 autocrine loop. Interestingly, in accordance with the previously described correlation of this autocrine loop with EMT [5], NRP-1 increase was accompanied by down-regulation of proliferative markers (i.e., E-cadherin and β-catenin) and high expression levels of mesenchymal/invasive markers (i.e., N-cadherin and vimentin), supporting melanoma progression.

5. Conclusions

Overall, our results strongly support the hypothesis that PDGF-C and NRP-1 co-expression contributes to the invasive phenotype of melanoma cells resistant to BRAFi, suggesting that blockade of PDGF-C/NRP-1 interaction might reduce the metastatic potential of tumors with acquired resistance to these kinase inhibitors. Moreover, in treatment-naïve tumors with an active PDGF-C/NRP-1 signaling pathway and innate resistance to BRAFi, inhibition of PDGF-C binding to NRP-1 could also enhance chemosensitivity. Finally, evaluation of PDGF-C levels together with analysis of NRP-1 expression in the tumor might be useful to identify metastatic melanoma patients at high risk of disease progression.

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CRediT authorship contribution statement

Federica Ruffini: Investigation, Formal analysis, Methodology; **Claudia Ceci**: Investigation, Formal analysis, Methodology; **Maria Grazia Atzori**: Investigation, Formal analysis, Methodology; **Simona Caporali**: Methodology; **Lauretta Levati**: Investigation; **Laura Bonmassar**: Resources; **Gian Carlo Antonini Cappellini**: Resources; **Stefania D’Atri**: Writing – review & editing, **Grazia Graziani**: Funding acquisition, Project administration, Supervision, Formal analysis, Writing – review & editing; **Pedro Miguel Lacal**: Funding acquisition, Project administration, Conceptualization, Supervision, Validation, Formal analysis, Visualization, Writing - original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw data will be available at the following link: https://data.mendeley.com/datasets/x9sdrkc8vm/1 (doi:10.17632/x9sdrkc8vm.1).

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Appendix A. Supporting information

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References

[1] R. Roskoski Jr., Targeting oncogenic Raf protein-serine/threonine kinases in human cancers, Pharmacol. Res. 135 (2018) 239–258, https://doi.org/10.1016/j.phrs.2018.08.013.
[2] S. Caksa, U. Baqai, A.E. Aplin, The future of targeted kinase inhibitors in melanoma, Pharmacol. Ther. 239 (2022), 108200, https://doi.org/10.1016/j.pharmthera.2022.108200.
[3] S. Sandri, F. Faião-Flores, M. Tiago, P.C. Pennacchi, R.R. Massaro, D.K. Alves-Fernandes, G.N. Berardinelli, A.F. Evangelista, V. de Lima Vazquez, R.M. Reis, S. S. Maria-Engler, Vemurafenib resistance increases melanoma invasiveness and modulates the tumor microenvironment by MMP-2 upregulation, Pharmacol. Res. 111 (2016) 523–533, https://doi.org/10.1016/j.phrs.2016.07.017.
[4] S. Caporali, E. Alvino, P.M. Lacal, L. Levati, G. Giurato, D. Memoli, E. Caprini, G. C. Antonini Cappellini, S. D’Atri, Targeting the PI3K/AKT/mTOR pathway overcomes the stimulating effect of dabrafenib on the invasive behavior of melanoma cells with acquired resistance to the BRAF inhibitor, Int. J. Oncol. 49 (2016) 1164–1174, https://doi.org/10.3892/ijo.2016.3594.
[5] F. Ruffini, L. Levati, G. Graziani, S. Caporali, M.G. Atzori, S. D’Atri, P.M. Lacal, Platelet-derived growth factor-C promotes human melanoma aggressiveness through activation of neuropilin-1, Oncotarget 8 (2017) 66833–66848, https://doi.org/10.18632/oncotarget.18706.
[6] X. Li, A. Kumar, F. Zhang, C. Lee, Y. Li, Z. Tang, P. Arjuna, VEGF-independent angiogenic pathways induced by PDGF-C, Oncotarget 1 (2010) 309–314, https://doi.org/10.18632/oncotarget.141.
[7] G. Graziani, P.M. Lacal, Neuropilin-1 as therapeutic target for malignant melanoma, Front Oncol. 5 (2015) 125, https://doi.org/10.3389/fonc.2015.00125.
[8] J.S. Wey, M.J. Gray, F. Fan, A. Belcheva, M.F. McCarty, O. Stoeltzing, R. Somcio, W. Liu, D.B. Evans, M. Klagsbrun, G.E. Gallick, L.M. Ellis, Overexpression of neuropilin-1 promotes constitutive MAPK signalling and chemoresistance in pancreatic cancer cells, Br. J. Cancer 93 (2005) 233–241, https://doi.org/10.1038/sj.bjc.6602663.
[9] H. Jia, L. Cheng, M. Tickner, A. Bagherzadeh, D. Selwood, I. Zachary, Neuropilin-1 antagonism in human carcinoma cells inhibits migration and enhances chemosensitivity, Br. J. Cancer 102 (2010) 541–552, https://doi.org/10.1038/sj.bjc.6605539.
[10] B. Yue, J.F. Ma, G. Yao, M.D. Yang, H. Cheng, G.Y. Liu, Knockdown of neuropilin-1 suppresses invasion, angiogenesis, and increases the chemosensitivity to doxorubicin in osteosarcoma cells - an in vitro study, Eur. Rev. Med. Pharmacol. Sci. 18 (2014) 1735–1741.
[11] A. Naik, A. Al-Yahyaee, N. Abdullah, J.E. Sam, N. Al-Zeheimi, M.W. Yaish, S. A. Adham, Neuropilin-1 promotes the oncogenic Tenascin-C/integrin β3 pathway and modulates chemoresistance in breast cancer cells, BMC Cancer 18 (2018) 533, https://doi.org/10.1186/s12885-018-4446-y.
[12] S. Rizzolio, G. Cagnoni, C. Battistini, S. Bonelli, C. Isella, J.A. Van Ginderachter, R. Bernards, F. Di Nicolantonio, S. Giordano, L. Tamagnone, Neuropilin-1 upregulation elicits adaptive resistance to oncogene-targeted therapies, J. Clin. Investig. 128 (2018) 3976–3990, https://doi.org/10.1172/JCI99257.
[13] R.S. Angom, S.K. Mondal, F. Wang, V.S. Madamsetty, E. Wang, S.K. Dutta, Y. Gulani, R. Sarabia-Estrada, J.N. Sarkaria, A. Quiñones-Hinojosa, D. Mukhopadhyay, Ablation of neuropilin-1 improves the therapeutic response in conventional drug-resistant glioblastoma multiforme, Oncogene 39 (2020) 7114–7126, https://doi.org/10.1038/s41388-020-01462-1.
[14] F. Ruffini, S. D’Atri, P.M. Lacal, Neuropilin-1 expression promotes invasiveness of melanoma cells through vascular endothelial growth factor receptor-2-dependent and -independent mechanisms, Int. J. Oncol. 43 (2013) 297–306, https://doi.org/10.3892/ijo.2013.1948.
[15] F. Ruffini, G. Graziani, L. Levati, L. Tentori, S. D’Atri, P.M. Lacal, Cilengitide downmodulates invasiveness and vasculogenic mimicry of neuropilin-1 expressing melanoma cells through the inhibition of αvβ5 integrin, Int. J. Cancer 136 (2015) E545–E558, https://doi.org/10.1002/ijc.29252.
[16] S. Caporali, E. Alvino, P.M. Lacal, F. Ruffini, L. Levati, L. Bonmassar, A. Scoppola, P. Marchetti, S. Mastroeni, G.C. Antonini Cappellini, S. D’Atri, Targeting the *PTTG1* oncogene impairs proliferation and invasiveness of melanoma cells sensitive or with acquired resistance to the BRAF inhibitor dabrafenib, Oncotarget 8 (2017) 113472–113493, https://doi.org/10.18632/oncotarget.23052.

[17] S. Caporali, A. Amaro, L. Levati, E. Alvino, P.M. Lacal, S. Mastroeni, F. Ruffini, L. Bonmassar, G.C. Antonini Cappellini, N. Felli, A. Carè, U. Pfeffer, S. D'Atri, miR-126-3p down-regulation contributes to dabrafenib acquired resistance in melanoma by up-regulating ADAM9 and VEGF-A, J. Exp. Clin. Cancer Res. 38 (2019) 272, https://doi.org/10.1186/s13046-019-1238-4.
[18] M.G. Atzori, C. Ceci, F. Ruffini, M. Trapani, M.L. Barbaccia, L. Tentori, S. D'Atri, P. M. Lacal, G. Graziani, Role of VEGFR-1 in melanoma acquired resistance to the BRAF inhibitor vemurafenib, J. Cell Mol. Med. 24 (2020) 465–475, https://doi.org/10.1111/jcmm.14755.
[19] M.G. Atzori, L. Tentori, F. Ruffini, C. Ceci, L. Lisi, E. Bonanno, M. Scimeca, E. Eskilsson, T. Daubon, H. Miletic, L. Ricci Vitiani, R. Pallini, P. Navarra, R. Bjerkvig, S. D'Atri, P.M. Lacal, G. Graziani, The anti-vascular endothelial growth factor receptor-1 monoclonal antibody D16F7 inhibits invasiveness of human glioblastoma and glioblastoma stem cells, J. Exp. Clin. Cancer Res 36 (2017) 106, https://doi.org/10.1186/s13046-017-0577-2.
[20] K.S. Hoek, C.R. Goding, Cancer stem cells versus phenotype-switching in melanoma, Pigment Cell Melanoma Res 23 (2010) 746–759, https://doi.org/10.1111/j.1755-148X.2010.00757.x.
[21] D.S. Widmer, P.F. Cheng, O.M. Eichhoff, B.C. Belloni, M.C. Zipser, N.C. Schlegel, D. Javelaud, A. Mauviel, R. Dummer, K.S. Hoek, Systematic classification of melanoma cells by phenotype-specific gene expression mapping, Pigment Cell Melanoma Res 25 (2012) 343–353, https://doi.org/10.1111/j.1755-148X.2012.00986.x.
[22] F. Ruffini, L. Tentori, A.S. Dorio, D. Arcelli, G. D'Amati, S. D'Atri, G. Graziani, P. M. Lacal, Platelet-derived growth factor C and calpain-3 are modulators of human melanoma cell invasiveness, Oncol. Rep. 30 (2013) 2887–2896, https://doi.org/10.3892/or.2013.2791.
[23] A. Jarvis, C.K. Allerston, H. Jia, B. Herzog, A. Garza-Garcia, N. Winfield, K. Ellard, R. Aqil, R. Lynch, C. Chapman, B. Hartzoulakis, J. Nally, M. Stewart, L. Cheng, M. Menon, M. Tickner, S. Djordjevic, P.C. Driscoll, I. Zachary, D.L. Selwood, Small molecule inhibitors of the neuropilin-1 vascular endothelial growth factor A (VEGF-A) interaction, J. Med. Chem. 53 (2010) 2215–2226, https://doi.org/10.1021/jm901755g.
[24] D. Ouellet, E. Gibiansky, C. Leonowens, A. O'Hagan, P. Haney, J. Switzky, V. L. Goodman, Population pharmacokinetics of dabrafenib, a BRAF inhibitor: effect of dose, time, covariates, and relationship with its metabolites, J. Clin. Pharm. 54 (2014) 696–706, https://doi.org/10.1002/jcph.263.
[25] A. Puszkiel, G. Noé, A. Bellesoeur, N. Kramkimel, M.N. Paludetto, A. Thomas-Schoemann, M. Vidal, F. Goldwasser, E. Chatelut, B. Blanchet, Clinical pharmacokinetics and pharmacodynamics of Dabrafenib, Clin. Pharm. 58 (2019) 451–467, https://doi.org/10.1007/s40262-018-0703-0.
[26] D. Pedri, P. Karras, E. Landeloos, J.C. Marine, F. Rambow, Epithelial-to-mesenchymal-like transition events in melanoma, FEBS J. 289 (2022) 1352–1368, https://doi.org/10.1111/febs.16021.
[27] A. Hodorogea, A. Calinescu, M. Antohe, M. Balaban, R.I. Nedelcu, G. Turcu, D. A. Ion, I.A. Badarau, C.M. Popescu, R. Popescu, C. Popp, M. Cioplea, L. Nichita, I. Hulea, A. Brinzea, Epithelial-mesenchymal transition in skin cancers: a review, Anal. Cell Pathol. 2019 (2019), 3851576, https://doi.org/10.1155/2019/3851576.
[28] S.H. Shirley, V.R. Greene, L.M. Duncan, C.A. Torres Cabala, E.A. Grimm, D. F. Kusewitt, Slug expression during melanoma progression, Am. J. Pathol. 180 (2012) 2479–2489, https://doi.org/10.1016/j.ajpath.2012.02.014.
[29] N. Vandamme, G. Denecker, K. Bruneel, G. Blancke, Ö. Akay, J. Taminau, J. De Coninck, E. De Smedt, N. Skrypek, W. Van Loocke, J. Wouters, D. Nittner, C. Köhler, D.S. Darling, P.F. Cheng, M.I.G. Raaijmakers, M.P. Levesque, U. G. Mallya, M. Rafferty, B. Balint, W.M. Gallagher, L. Brochez, D. Huylebroeck, J. J. Haigh, V. Andries, F. Rambow, P. Van Vlierberghe, S. Goossens, J.J. van den Oord, J.C. Marine, G. Berx, The EMT transcription factor ZEB2 promotes proliferation of primary and metastatic melanoma while suppressing an invasive, mesenchymal-like phenotype, Cancer Res. 80 (2020) 2983–2995, https://doi.org/10.1158/0008-5472.CAN-19-2373.
[30] C.R. Goding, H. Arnheiter, MITF-the first 25 years, Genes Dev. 33 (2019) 983–1007, https://doi.org/10.1101/gad.324657.119.
[31] J. Lu, Y. Cheng, G. Zhang, Y. Tang, Z. Dong, K.J. McElwee, G. Li, Increased expression of neuropilin 1 in melanoma progression and its prognostic significance in patients with melanoma, Mol. Med. Rep. 12 (2015) 2668–2676, https://doi.org/10.3892/mmr.2015.3752.
[32] S. Vivekanandhan, D. Mukhopadhyay, Genetic status of KRAS influences transforming growth factor-beta (TGF-β) signaling: An insight into Neuropilin-1 (NRP1) mediated tumorigenesis, Semin Cancer Biol. 54 (2019) 72–79, https://doi.org/10.1016/j.semcancer.2018.01.014.
[33] D. Bumbaca, H. Xiang, C.A. Boswell, R.E. Port, S.L. Stainton, E.E. Mundo, S. Ulufatu, A. Bagri, F.P. Theil, P.J. Fielder, L.A. Khawli, B.Q. Shen, Maximizing tumour exposure to anti-neuropilin-1 antibody requires saturation of non-tumour tissue antigenic sinks in mice, Br. J. Pharmacol. 166 (2012) 368–377, https://doi.org/10.1111/j.1476-5381.2011.01777.x.
[34] C.D. Weekes, M. Beeram, A.W. Tolcher, K.P. Papadopoulos, L. Gore, P. Hegde, Y. Xin, R. Yu, L.M. Shih, H. Xiang, R.K. Brachmann, A. Patnaik, A phase I study of the human monoclonal anti-NRP1 antibody MNRP1685A in patients with advanced solid tumors, Investig. New Drugs 32 (2014) 653–660, https://doi.org/10.1007/s00280-014-2426-8.
[35] A. Patnaik, P.M. LoRusso, W.A. Messersmith, K.P. Papadopoulos, L. Gore, M. Beeram, V. Ramakrishnan, A.H. Kim, J.C. Beyer, L. Mason Shih, W.C. Darbonne, Y. Xin, R. Yu, H. Xiang, R.K. Brachmann, C.D. Weekes, A Phase Ib study evaluating MNRP1685A, a fully human anti-NRP1 monoclonal antibody, in combination with bevacizumab and paclitaxel in patients with advanced solid tumors, Cancer Chemother. Pharmacol. 73 (2014) 951–960, https://doi.org/10.1007/s00280-014-2426-8.
[36] L.J. Reigstad, H.M. Sande, Ø. Fluge, O. Bruland, A. Muga, J.E. Varhaug, A. Martinez, J.R. Lillehaug, Platelet-derived growth factor (PDGF)-C, a PDGF family member with a vascular endothelial growth factor-like structure, J. Biol. Chem. 278 (2003) 17114–17120, https://doi.org/10.1074/jbc.M301728200.
[37] D. Wågsäter, C. Zhu, H.M. Björck, P. Eriksson, Effects of PDGF-C and PDGF-D on monocyte migration and MMP-2 and MMP-9 expression, Atherosclerosis 202 (2009) 415–423, https://doi.org/10.1016/j.atherosclerosis.2008.04.050.
[38] D. Son, Y.R. Na, E.S. Hwang, S.H. Seok, Platelet-derived growth factor-C (PDGF-C) induces anti-apoptotic effects on macrophages through Akt and Bad phosphorylation, J. Biol. Chem. 289 (2014) 6225–6235, https://doi.org/10.1074/jbc.M113.508994.
[39] Z. Tang, P. Arjunan, C. Lee, Y. Li, A. Kumar, X. Hou, B. Wang, P. Wardega, F. Zhang, L. Dong, Y. Zhang, S.Z. Zhang, H. Ding, R.N. Fariss, K.G. Becker, J. Lennartsson, N. Nagai, Y. Cao, X. Li, Survival effect of PDGF-CC rescues neurons from apoptosis in both brain and retina by regulating GSK3beta phosphorylation, J. Exp. Med. 207 (2010) 867–880, https://doi.org/10.1084/jem.20091704.
[40] C. Lee, F. Zhang, Z. Tang, Y. Liu, X. Li, PDGF-C: a new performer in the neurovascular interplay, Trends Mol. Med. 19 (2013) 474–486, https://doi.org/10.1016/j.molmed.2013.04.006.
[41] S.A. Lewandowski, L. Fredriksson, D.A. Lawrence, U. Eriksson, Pharmacological targeting of the PDGF-CC signaling pathway for blood-brain barrier restoration in neurological disorders, Pharmacol. Ther. 167 (2016) 108–119, https://doi.org/10.1016/j.pharmthera.2016.07.016.
[42] M. Zeitelhofer, M.Z. Adzemovic, C. Moessinger, C. Stefanitsch, C. Strell, L. Muhl, L. Brundin, L. Fredriksson, T. Olsson, U. Eriksson, I. Nilsson, Blocking PDGF-CC signaling ameliorates multiple sclerosis-like neuroinflammation by inhibiting disruption of the blood-brain barrier, Sci. Rep. 10 (2020) 22383, https://doi.org/10.1038/s41598-020-79598-z.
[43] E. di Tomaso, M. Snuderl, W.S. Kamoun, D.G. Duda, P.K. Auluck, L. Fazlollahi, O. C. Andronesi, M.P. Frosch, P.Y. Wen, S.R. Plotkin, E.T. Hedley-Whyte, A. G. Sorensen, T.T. Batchelor, R.K. Jain, Glioblastoma recurrence after cediranib therapy in patients: lack of "rebound" revascularization as mode of escape, Cancer Res. 71 (2011) 19–28, https://doi.org/10.1158/0008-5472.CAN-10-2602.
[44] C.A. Clara, S.K. Marie, J.R. de Almeida, A. Wakamatsu, S.M. Oba-Shinjo, M. Uno, M. Neville, S. Rosemberg, Angiogenesis and expression of PDGF-C, VEGF, CD105 and HIF-1α in human glioblastoma, Neuropathology 34 (2014) 343–352, https://doi.org/10.1111/neup.12111.
[45] P.L. Chia, P. Russell, K. Asadi, B. Thapa, V. Gebski, C. Murone, M. Walkiewicz, U. Eriksson, A.M. Scott, T. John, Analysis of angiogenic and stromal biomarkers in a large malignant mesothelioma cohort, Lung Cancer 150 (2020) 1–8, https://doi.org/10.1016/j.lungcan.2020.09.022.
[46] N.J. Hurst Jr, A.J. Najy, C.V. Ustach, L. Movilla, H.R. Kim, Platelet-derived growth factor-C (PDGF-C) activation by serine proteases: implications for breast cancer progression, Biochem. J. 441 (2012) 909–918, https://doi.org/10.1042/BJ20111020.
[47] S. Jansson, K. Aaltonen, P.O. Bendahl, A.K. Falck, M. Karlsson, K. Pietras, L. Rydén, The PDGF pathway in breast cancer is linked to tumour aggressiveness, triple-negative subtype and early recurrence, Breast Cancer Res. Treat. 169 (2018) 231–241, https://doi.org/10.1007/s10549-018-4664-7.
[48] P. Roswall, M. Bocci, M. Bartoschek, H. Li, G. Kristiansen, S. Jansson, S. Lehn, J. Sjölund, S. Reid, C. Larsson, P. Eriksson, C. Anderberg, E. Cortez, L.H. Saal, C. Orsmark-Pietras, E. Cordero, B.K. Haller, J. Häkkinen, I.J.G. Burvenich, E. Lim, A. Orimo, M. Höglund, L. Rydén, H. Moch, A.M. Scott, U. Eriksson, K. Pietras, Microenvironmental control of breast cancer subtype elicited through paracrine platelet-derived growth factor-CC signaling, Nat. Med. 24 (2018) 463–473, https://doi.org/10.1038/nm.4494.
[49] S. Kim, D. You, Y. Jeong, S.Y. Yoon, S.A. Kim, J.E. Lee, Inhibition of platelet-derived growth factor C and their receptors additionally increases doxorubicin effects in triple-negative breast cancer cells, Eur. J. Pharmacol. 895 (2021), 173868, https://doi.org/10.1016/j.ejphar.2021.173868.
[50] C. Anderberg, H. Li, L. Fredriksson, J. Andrae, C. Betsholtz, X. Li, U. Eriksson, K. Pietras, Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts, Cancer Res 69 (2009) 369–378, https://doi.org/10.1158/0008-5472.
[51] Y. Crawford, I. Kasman, L. Yu, C. Zhong, X. Wu, Z. Modrusan, J. Kaminker, N. Ferrara, PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment, Cancer Cell 15 (2009) 21–34, https://doi.org/10.1016/j.ccr.2008.12.004.
[52] S. Rizzolio, S. Corso, S. Giordano, L. Tamagnone, Autocrine signaling of NRP1 ligand galectin-1 elicits resistance to BRAF-targeted therapy in melanoma cells, Cancers 12 (2020) 2218, https://doi.org/10.3390/cancers12082218.
[53] J. Villanueva, A. Vultur, J.T. Lee, R. Somasundaram, M. Fukunaga-Kalabis, A. K. Cipolla, B. Wubbenhorst, X. Xu, P.A. Gimotty, D. Kee, A.E. Santiago-Walker, R. Letrero, K. D'Andrea, A. Pushparajan, J.E. Hayden, K.D. Brown, S. Laquerre, G. A. McArthur, J.A. Sosman, K.L. Nathanson, M. Herlyn, Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K, Cancer Cell 18 (2010) 683–695, https://doi.org/10.1016/j.ccr.2010.11.023.
[54] H. Shi, X. Kong, A. Ribas, R.S. Lo, Combinatorial treatments that overcome PDGFRβ-driven resistance of melanoma cells to V600EB-RAF inhibition, Cancer Res 71 (2011) 5067–5074, https://doi.org/10.1158/0008-5472.CAN-11-0140.
[55] Y.K. Ng, J.Y. Lee, K.M. Supko, A. Khan, S.M. Torres, M. Berwick, J. Ho, J. M. Kirkwood, J.M. Siegfried, L.P. Stabile, Pan-erbB inhibition potentiates BRAF

inhibitors for melanoma treatment, Melanoma Res 24 (2014) 207–218, https://doi.org/10.1097/CMR.0000000000000060.
[56] J. Wang, T. Sinnberg, H. Niessner, et al., PTEN regulates IGF-1R-mediated therapy resistance in melanoma, Pigment Cell Melanoma Res 28 (2015) 572–589, https://doi.org/10.1111/pcmr.12390.
[57] S. Niland, J.A. Eble, Neuropilins in the context of tumor vasculature, Int J. Mol. Sci. 20 (2019) 639, https://doi.org/10.3390/ijms20030639.
[58] M. Fallahi-Sichani, V. Becker, B. Izar, G.J. Baker, J.R. Lin, S.A. Boswell, P. Shah, A. Rotem, L.A. Garraway, P.K. Sorger, Adaptive resistance of melanoma cells to RAF inhibition via reversible induction of a slowly dividing de-differentiated state, Mol. Syst. Biol. 13 (2017) 905, https://doi.org/10.15252/msb.20166796.
[59] J. Zhong, W. Yan, C. Wang, W. Liu, X. Lin, Z. Zou, W. Sun, Y. Chen, BRAF inhibitor resistance in melanoma: mechanisms and alternative therapeutic strategies, Curr. Treat. Options Oncol. 23 (2022) 1503–1521, https://doi.org/10.1007/s11864-022-01006-7.
[60] S.A. Luebker, S.A. Koepsell, Diverse mechanisms of BRAF inhibitor resistance in melanoma identified in clinical and preclinical studies, Front Oncol. 9 (2019) 268, https://doi.org/10.3389/fonc.2019.00268.
[61] M. Karin, E. Gallagher, From J.N.K. to pay dirt: jun kinases, their biochemistry, physiology and clinical importance, IUBMB Life 57 (2005) 283–295, https://doi.org/10.1080/15216540500097111.
[62] M. Fallahi-Sichani, N.J. Moerke, M. Niepel, T. Zhang, N.S. Gray, P.K. Sorger, Systematic analysis of BRAF(V600E) melanomas reveals a role for JNK/c-Jun pathway in adaptive resistance to drug-induced apoptosis, Mol. Syst. Biol. 11 (2015) 797, https://doi.org/10.15252/msb.20145877.
[63] T. Sinnberg, E. Makino, M.A. Krueger, A. Velic, B. Macek, U. Rothbauer, N. Groll, O. Pötz, S. Czemmel, H. Niessner, F. Meier, K. Ikenberg, C. Garbe, B. Schittek, A nexus consisting of beta-catenin and Stat3 attenuates BRAF inhibitor efficacy and mediates acquired resistance to vemurafenib, EBioMedicine 8 (2016) 132–149, https://doi.org/10.1016/j.ebiom.2016.04.037.
[64] Y. Tian, Y. Zhan, Q. Jiang, W. Lu, X. Li, Expression and function of PDGF-C in development and stem cells, Open Biol. 11 (2021), 210268, https://doi.org/10.1098/rsob.210268.