# Platelet-derived growth factor C and calpain-3 are modulators of human melanoma cell invasiveness

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**Abstract.** The molecular mechanisms responsible for the elevated metastatic potential of malignant melanoma are still not fully understood. In order to shed light on the molecules involved in the acquisition by melanoma of a highly aggressive phenotype, we compared the gene expression profiles of two cell clones derived from the human cutaneous metastatic melanoma cell line M14: a highly invasive clone (M14C2/MK18) and a clone (M14C2/C4) with low ability to invade the extracellular matrix (ECM). The highly invasive phenotype of M14C2/MK18 cells was correlated with overexpression of neuropilin-1, activation of a vascular endothelial growth factor (VEGF)-A/VEGFR-2 autocrine loop and secretion of matrix metalloprotease-2. Moreover, in an *in vivo* murine model, M14C2/MK18 cells displayed a higher growth rate as compared with M14C2/C4 cells, even though *in vitro* both clones possessed comparable proliferative potential. Microarray analysis in M14C2/MK18 cells showed a strong upregulation of platelet-derived growth factor (PDGF)-C, a cytokine that contributes to angiogenesis, and downregulation of calpain-3, a calcium-dependent thiol-protease that regulates specific signalling cascade components. Inhibition of PDGF-C with a specific antibody resulted in a significant decrease in ECM invasion by M14C2/MK18 cells, confirming the involvement of PDGF-C in melanoma cell invasiveness. Moreover, the PDGF-C transcript was found to be upregulated in a high percentage of human melanoma cell lines (17/20), whereas only low PDGF-C levels were detected in a few melanocytic cultures (2/6). By contrast, inhibition of calpain-3 activity in M14C2/C4 control cells, using a specific chemical inhibitor, markedly increased ECM invasion, strongly suggesting that downregulation of calpain-3 plays a role in the acquisition of a highly invasive phenotype. The results indicate that PDGF-C upregulation and calpain-3 downregulation are involved in the aggressiveness of malignant melanoma and suggest that modulators of these proteins or their downstream effectors may synergise with VEGF-A therapies in combating tumour-associated angiogenesis and melanoma spread.

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## Introduction

Cutaneous melanoma is an extremely aggressive skin cancer with high metastatic potential. Indeed, melanoma metastatisation to distant organs is the primary cause of human skin cancer-related deaths. Although the majority of melanomas are detected at an early stage (<1 mm Breslow thickness) and surgically cured, melanomas diagnosed at later stages are associated with poor survival rates, since they are refractory to most of the available therapies (1). The molecular mechanisms associated with the acquisition of a metastatic phenotype by melanoma cells are not very well defined and, therefore, great effort is being given to identify molecular determinants involved in the metastatic switch that may either cause or contribute to the aggressiveness of melanoma.

At the Markers and Tissue Resources for Melanoma Meeting held in 2005 at Gaithersburg (MD, USA), a list of melanoma biomarkers was generated (2). The list included biomarkers associated with melanocytic tumour progression, potential markers with prognostic significance and putative therapeutic targets. These markers were established using two criteria: differential expression during melanocytic tumour progression and detection in routinely processed formalin-fixed paraffin-embedded tissues by means of a reliable method. More recently, a melanocytic tumour progression tissue microarray (TMA) was developed in order to evaluate these candidate biomarkers by immunohistochemical analysis (3). The TMA includes samples from benign nevi, primary cutaneous melanomas and melanoma metastases (lymph node and visceral metastases). Nevertheless, TMA does not include cutaneous melanoma metastases nor does it distinguish between the different types of metastases.

The majority of human melanoma cell lines, derived from primary or metastatic lesions, secrete the vascular endothelial growth factor (VEGF)-A and express the receptors VEGFR-1, VEGFR-2 and neuropilins (NRP) (4). The simultaneous presence of the growth factor and its receptors in melanoma cells allows the establishment of an autocrine loop, sustained by the interaction of VEGF-A mainly with VEGFR-2, promoting the ability of melanoma cells to migrate and invade the extracellular matrix (ECM) (5). We also demonstrated the existence in cutaneous melanomas and lymph node metastases of a differential VEGFR expression pattern (6,7), which seems to confer distinct invasive properties to the different types of metastases.

In an attempt to identify new players involved in melanoma progression that may favour tumour spreading to distant sites, we compared the gene expression profiles of two cell clones derived from the human cutaneous metastatic melanoma cell line M14: a highly invasive clone (M14C2/MK18) and a clone (M14C2/C4) with low ability to invade the ECM. The highly invasive phenotype of M14C2/MK18 cells was correlated with NRP-1 expression, activation of a VEGF-A/VEGFR-2 autocrine loop and secretion of matrix metalloprotease-2 (MMP-2), and resulted in a high *in vivo* growth rate in a murine model. The results indicated that platelet-derived growth factor (PDGF)-C upregulation and calpain-3 downregulation were directly involved in M14C2/MK18 melanoma cell invasiveness.

## Materials and methods

*Reagents*. Cell culture media and reagents were purchased from Lonza (Basel, Switzerland); fetal bovine serum (FBS) was from Euroclone (Pero, Italy), heparin, insulin/transferrin/selenium supplement (ITS) and gelatin were from Sigma-Aldrich (St. Louis, MO, USA) and fatty acid-free bovine serum albumin (BSA) was from Roche (Mannheim, Germany). VEGF-A, PlGF and polyclonal goat anti-VEGF-A antibodies used in ELISA (AF-293 and BAF-293) were from R&D Systems (Abingdon, UK). Control goat and mouse IgGs were from Sigma-Aldrich. The VEGFR tyrosine kinase inhibitor 4-[(4'-chloro-2'-fluoro) phenylamino]-6,7-dimethoxyquinazoline (VEGFRin) (8) and the calpain inhibitor N-Acetyl-Leu-Leu-Met (ALLM) (9) were from Merck (Darmstadt, Germany).

*Cell lines*. The origin and culture conditions of M14C2/C4 and M14C2/MK18 subclones, other human melanoma cell lines, melanocyte and human umbilical vein endothelial cell (HUVEC) cultures, were previously described (4,7).

*RT-PCR analysis*. Total cellular RNA from the different cell lines and from HUVECs was prepared using a RNeasy Midi kit (Qiagen, Hilden, Germany), following the manufacturer's directions. Three micrograms of total RNA/sample was subjected to reverse transcription by the AMV enzyme (Roche) for 60 min at 42˚C in 25 $\mu$l. Five microliters of this cDNA preparation were used for each PCR amplification reaction by 1 unit of Dynazyme II DNA polymerase (Finnzymes OY, Espoo, Finland), utilizing the following primers and annealing conditions: VEGFR-2 forward, 5'-CACAGGAAACCTGGAG AATCAGACGACAAG-3' and reverse, 5'-TGGTCGACCATG ACGATGGACAAGTA-3', which amplify a 402-bp fragment (annealing for 1 min at 58˚C); NRP-1 forward, 5'-ATGGAGAG GGGGCTGCCG-3' and reverse, 5'-CTATCGCGCTGTCG GTGTA-3', which amplify a 720-bp fragment (annealing for 30 sec at 52˚C); PDGF-C forward, 5'-GAGATGGCAGTTG GACTTAG-3' and reverse, 5'-TCAGCCACTGCACTGCA CAG-3', which amplify a 471-bp fragment (annealing for 1 min at 60˚C). The RNA integrity and the correct reverse transcription of the samples were assessed by testing each cDNA preparation for the amplification of the glyceraldehyde-phosphate dehydrogenase (GAPDH) housekeeping gene: forward, 5'-TCCCATCACCATCTTCCA-3' and reverse primer, 5'-CAT CACGCCACAGTTTCC-3', which amplify a 380-bp fragment (annealing for 30 sec at 58˚C).

*Evaluation of VEGF-A 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 and concentrated at least 10-fold in Centriplus concentrators (Amicon, Beverly, MA, USA). Cells were detached from the flasks with a solution of 1 mM EDTA in PBS (EDTA/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-Immuno™ plates (Nunc, Roskilde, Denmark) coated with goat anti-VEGF-A IgGs, as previously described (4).

*Migration and invasion assays*. The *in vitro* migration assay was performed using Boyden chambers equipped with 8-$\mu$m pore diameter polycarbonate filters (Nuclepore; Whatman, Inc., Clifton, NJ, USA), coated with 5 $\mu$g/ml gelatin (10). Briefly, melanoma cells were collected from continuous cultures, washed, suspended in migration medium (1 $\mu$g/ml heparin/0.1% BSA in RPMI-1640) and loaded ($2x10^5$ cells) into the upper compartment of the Boyden chambers. Migration medium with or without the chemotactic stimuli was added to the lower compartment of the chambers. After incubation of the chambers at 37˚C in a $CO_2$ incubator for 5 h, the filters were removed from the chambers, the cells were fixed in ethanol and stained in 0.5% crystal violet. The migrated cells, attached to the lower surface of the filters, were counted under a microscope. Twelve high-magnification microscopic fields (x200 magnification), randomly selected on triplicate filters, were scored for each experimental condition.

*In vitro* ECM cell invasion was analysed in Boyden chambers as described for the migration assay, but utilising polycarbonate filters coated with 20 $\mu$g of the basement membrane matrix Matrigel (BD Biosciences, Buccinasco, Italy) (5).

In a set of experiments, migration or invasion assays were performed in the presence of VEGFRin, the calpain inhibitor ALLM, antibodies against NRP-1 (H-286; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), PDGF-C (AF1560; R&D Systems) or MMP-2 (clone 42-5D11; Merck) or the corresponding control immunoglobulins (Igs). In the case of VEGFRin, the drug was dissolved in DMSO at a stock concentration of 20 mM, and melanoma cells were pre-incubated in the presence of 20 $\mu$M of the inhibitor for 24 h at 37˚C in a $CO_2$ incubator. ALLM was also dissolved in DMSO at a stock concentration of 50 mM, and cells were pre-incubated

in the presence of 15 or 30 $\mu$M of the inhibitor for 40 min at room temperature in a rotating wheel. The concentrations of VEGFRin or ALLM tested had no effect on cell viability. The final concentrations of DMSO used as solvent of the inhibitors did not affect cell migration. Pretreatment with the antibodies was performed for 45 min at room temperature in a rotating wheel. The cells were then loaded in the Boyden chambers without removing the antibody, ALLM or adding fresh VEGFRin.

*Western blot analysis*. Specific protein expression in the different cell lines was analysed in semi-confluent cell cultures, growing in 6-well plates. Cells were washed with PBS, lysed and boiled in 400 $\mu$l of SDS sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Secreted proteins were tested in cell culture supernatants, obtained as indicated for the evaluation of VEGF-A secretion, mixing the supernatants with an equal amount of 2X SDS sample buffer before boiling. Proteins were then separated on polyacrylamide gels and immunoblotting was performed (experimental details provided upon request), incubating the membranes overnight at 4˚C with the following primary antibodies: rabbit polyclonal antibody anti-β-tubulin (H-235, diluted to 0.2 $\mu$g/ml; Santa Cruz Biotechnology, Inc.); mouse monoclonal antibody anti-NRP-1 (A12, diluted to 1 $\mu$g/ml; Santa Cruz Biotechnology, Inc.); mouse monoclonal antibody anti-MMP-2 (clone 42-5D11, diluted to 1 $\mu$g/ml) and anti-TIMP-2 antibody (clone T2-101, diluted to 1 $\mu$g/ml; Merck); and goat polyclonal antibody anti-PDGF-C (AF1560, diluted to 1 $\mu$g/ml; R&D Systems).

*Microarray analysis of gene expression*. Gene expression profiling, utilizing GeneChip® Human Genome U133A Arrays (Affymetrix, Inc., High Wycombe, UK), and data analysis, using the Prediction Analysis of Microarrays (PAM) and the Significant Analysis of Microarrays (SAM) tests, which allow identification of significant differences between different groups, were performed as previously described (11). The genes identified as modulated in M14C2/MK18 cells corresponded to genes whose transcripts were significantly up- or down-modulated according to both PAM and SAM analyses.

*In vivo tumour growth*. Tumours were induced in 5 week-old male CD1 Nu/Nu mice (10 mice/group; Charles River, Calco, Italy) by intramuscular injection of $5x10^6$ M14C2/C4 or M14C2/MK18 melanoma cells. Melanoma growth was monitored by measuring tumour nodules with callipers every 2-3 days, and the tumour volumes were calculated according to the following formula: volume = [(width)$^2$ x length]/2. The animals were euthanised when their tumours reached a volume of ~1,500 mm$^3$. All procedures involving mice and care were performed in compliance with our institutional guidelines and with international directives (Directive 2010/63/EU of the European Parliament and of the Council; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011).

*Evaluation of in vitro cell growth*. Cells ($5x10^4$/well) were re-suspended in complete medium, seeded in 24-well plates and allowed to grow at 37˚C in a $CO_2$ incubator. Selected wells were previously coated with 0.5 ml of a 200 $\mu$g/ml Matrigel solution and incubated overnight at 37˚C. Evaluation of cell growth was performed by detaching and counting the cells at the indicated times.

## Results

*Characterization of the M14C2/MK18 cell line*. We previously published the isolation of the M14C2/MK18 cell subclone obtained by transfection of an expression vector that contains the cDNA encoding for VEGFR-2 in a cell clone isolated from the metastatic cutaneous melanoma cell line M14 (7). These cells also express NRP-1 and MMP-2 (Fig. 1A and E), molecules that are involved in ECM invasion by melanoma cells. Indeed, ECM invasion by M14C2/MK18 cells in the absence of a stimulus was ~40-fold more efficient than that observed in control cells (parental cells or cells from the M14C2/C4 clone, transfected with the pcDNA3 empty vector) (Fig. 1B). Exposure to a stimulus not related to VEGF-A or NRP-1 (i.e., ITS, at 5 $\mu$g/ml), further increased ECM invasion by M14C2/MK18 cells and stimulated invasion by parental and M14C2/C4 cells. Moreover, M14C2/MK18 cells, which do not express VEGFR-1, showed a specific chemotactic response to VEGF-A mediated by VEGFR-2 (Fig. 1C), as demonstrated by its down-modulation after treatment with a VEGFR-2 inhibitor and by the lack of response to PlGF, a VEGF-A-related growth factor that does not bind to VEGFR-2 (Fig. 1D). M14C2/MK18 cells, already described as melanoma cells expressing VEGFR-2 (7,12), showed an increased ability to secrete VEGF-A (5.35±0.89 ng/$10^6$ cells) as compared with M14C2/C4 cells or with the parental cell clone (0.46±0.05 and 0.63±0.06 ng/$10^6$ cells, respectively). High levels of the fully processed form of MMP-2 were also found in the supernatant of M14C2/MK18 cells, whereas M14C2/C4 cells showed barely detectable amounts of this protein (Fig. 1E). By contrast, the tissue inhibitor of metalloproteases (TIMP-2), used as a loading control, was expressed at similar levels in the supernatants of both cell clones (Fig. 1E). TIMP-2 is involved in docking pro-MMP-2 to the cell surface, where the pro-enzyme is activated by membrane-bound MMPs and by an additional TIMP-2 molecule (13,14). Therefore, the balance between the amount of MMP-2 and TIMP-2 determines the extent of tissue remodeling and ECM invasion. Indeed, blockage of MMP-2 proteolytic activity with a specific antibody down-modulated the invasiveness of M14C2/MK18 cells (Fig. 1F). Altogether, these results demonstrated that M14C2/MK18 cells displayed *in vitro* highly invasive characteristics.

We next analysed whether the aggressive phenotype showed by M14C2/MK18 cells *in vitro* was a characteristic maintained by these cells when growing in an *in vivo* model. Thus, cells were intramuscularly injected in CD1 Nu/Nu mice, and the volume of the tumour nodules was monitored. Six days after injection, 70% of the animals inoculated with M14C2/MK18 cells had already developed measurable tumours, as compared to only 10% of the mice injected with the M14C2/C4 cells (Fig. 2A). Only on day 11 after challenge the number of mice showing measurable M14C2/C4 nodules was identical to that of animals injected with M14C2/MK18 cells. Moreover, from day 13 onward M14C2/MK18 tumour volume was significantly higher with respect to that of control cells (Fig. 2B).

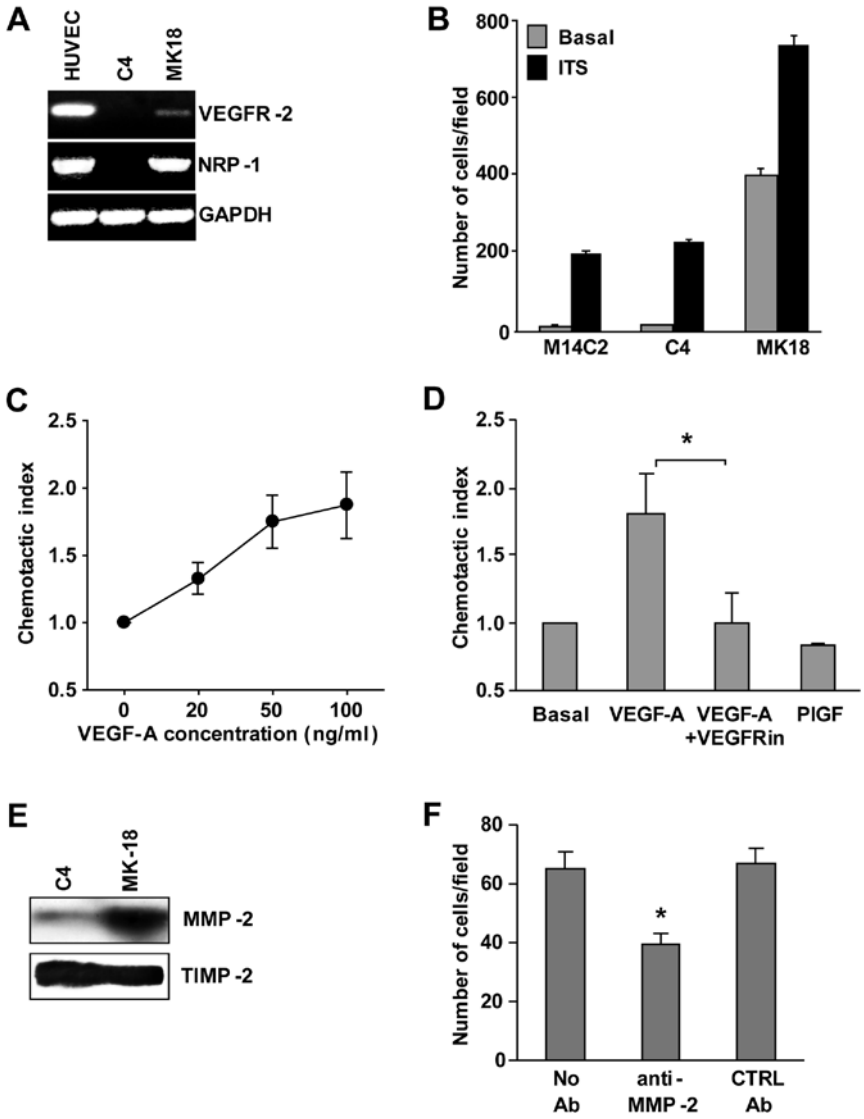


Figure 1. Characterization of M14C2/MK18 cells. (A) RT-PCR analysis of VEGFR-2 and NRP-1 expression in M14C2/MK18 and M14C2/C4 cells. As a control for RNA integrity, analysis of the GAPDH gene was also performed. HUVECs were included as positive control. Results are representative of 1 out of 3 different experiments with similar results. (B) ECM invasion by M14C2/MK18 and M14C2/C4 subclones and parental M14C2 cells was evaluated using Boyden chambers equipped with Matrigel-coated filters, allowing the cells to invade the Matrigel for 4 h, in response to migration medium (basal invasion) or migration medium supplemented with ITS (5 $\mu$g/ml). Each value represents the mean ± standard deviation (SD) of the number of cells that had migrated per microscopic field from three independent experiments. (C) Migration of M14C2/MK18 cells was evaluated using Boyden chambers equipped with polycarbonate gelatin-coated filters, allowing the cells to migrate for 5 h in response to increasing concentrations of VEGF-A. Data are expressed in terms of 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). Each value represents the mean ± SD of three independent experiments. (D) The specificity of the chemotactic response induced by VEGF-A (30 ng/ml) in M14C2/MK18 cells was evaluated as described in panel C, pre-incubating the cells (24 h at 37˚C in a $CO_2$ incubator) with culture medium in the absence or in the presence of VEGFRin (20 $\mu$M). The response to VEGF-A was also compared with the response to PlGF (50 ng/ml) that interacts only with VEGFR-1. Each value represents the mean ± SD of three independent experiments. *P<0.05, according to Student's t-test analysis. (E) Secretion of MMP-2 into the cell culture supernatant from M14C2/MK18 and M14C2/C4 subclones was evaluated by western blot analysis. Fifty microliters of concentrated cell supernatant, corresponding to an equal number of cultured cells, was loaded in a 6-12% SDS-polyacrylamide gel gradient. As loading control, TIMP-2 protein secretion in the supernatant from both cell lines was also evaluated. Results are representative of one out of three independent experiments giving comparable results. (F) The influence of anti-MMP-2 antibody treatment (3 $\mu$g/ml) on the ability of M14C2/MK18 cells to spontaneously invade the ECM was analysed using Boyden chambers equipped with Matrigel-coated filters, allowing cells to invade for 2 h. As a negative control, a non-specific antibody (mouse control IgG, CTRL Ab) was used. Each value represents the mean ± SD of the number of cells that had migrated per microscopic field from three independent experiments. *P<0.05, according to Student's t-test analysis, comparing the invasion of cells treated with anti-MMP-2 antibodies with that of cells untreated or treated with control antibodies.

Notably, the augmented *in vivo* growth of M14C2/MK18 cells was not due to an elevated *in vitro* proliferation rate. In fact, M14C2/MK18 and M14C2/C4 cells showed comparable proliferation rates, even when cells were allowed to grow on a basement membrane matrix (Fig. 2C). Therefore, the highly invasive characteristics of M14C2/MK18 cells seemed to favour tumour formation and growth also *in vivo*, likely as a result of the interaction with the stromal compartment.

*Differential gene expression in M14C2/MK18 cells as compared with M14C2/C4 cells*. In an attempt to identify the molecules responsible for the elevated invasiveness of

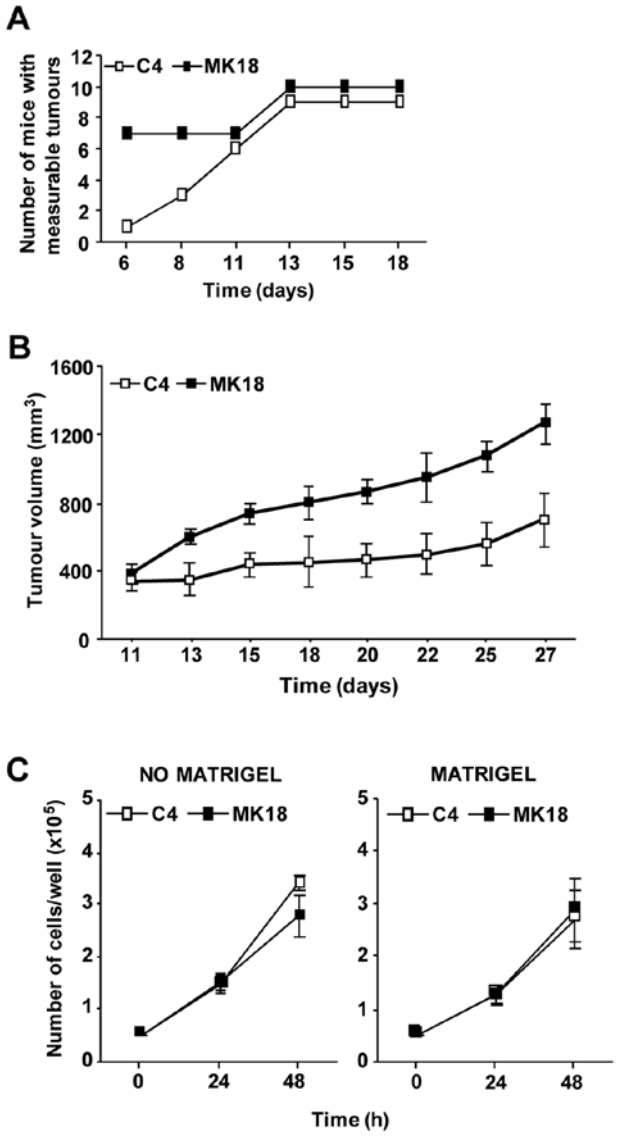


Figure 2. M14C2/MK18 cells display rapid engraftment and growth rate in the murine model. (A) M14C2/C4 or M14C2/MK18 cells ($5x10^6$) were injected in 5-week-old male CD1 Nu/Nu mice, and the number of palpable tumours per animal was scored every 2-3 days. (B) Tumour growth of the xenografts indicated in panel A was evaluated as described in Materials and methods. Statistical analysis was performed by Student's t-test analysis. From day 13 onward the differences between the volumes of M14C2/MK18 melanoma nodules and those of M14C2/C4 were consistently statistically significant ($P<0.05$). Each value represents the mean tumour volume of 10 mice for each time-point ± SD. (C) The *in vitro* cell growth of M14C2/C4 and M14C2/MK18 clones was evaluated by seeding $5x10^4$ cells in a 24-well plate, coated or not with 100 $\mu$g of Matrigel, and allowing the cells to grow at 37˚C in a $CO_2$ incubator. At the indicated times, cells were detached and counted. Results represent the means ± SD of the total number of cells/well.

M14C2/MK18 cells, differential gene expression analysis was performed in these cells and in the M14C2/C4 control cells, utilizing GeneChip Human Genome Arrays. The results showed a significant up-modulation of 18 genes (Table I) and down-modulation of 19 genes (Table II) in the M14C2/MK18 cells. The up-modulated gene transcripts included genes encoding for proteins involved in the regulation of the calcium metabolism pathway [S100 calcium binding protein A2 (S100A2), stanniocalcin (STC1), S100 calcium binding protein A3 (S100A3)], cell-extracellular matrix interactions (COL5A2, LAMB3, PCOLCE2, NRP-1) and PDGF-C, a cytokine involved in angiogenesis that displays pleiotropic effects on multiple cellular targets. Down-modulated molecules included gene products involved in melanocytic differentiation (MLANA, DCT, RAB38), in lipid and glucidic metabolism (APOE, ASAH1, GPM6B, GYG2) and CAPN3 (calpain-3), an endopeptidase endowed with numerous functions. Notably, 11 out of 19 genes found to be down-modulated in the M14C2/MK18 cells (genes highlighted in bold in Table II) are known to be under the control of the microphthalmia-associated transcription factor (MITF) (15).

*Involvement of PDGF-C and calpain-3 in melanoma cell invasiveness*. The possibility that the up-modulation of PDGF-C may play a role in the highly invasive properties of M14C2/MK18 cells was analysed using the *in vitro* ECM invasion assay. Firstly, western blot analysis confirmed the increased secretion of PDGF-C into the culture supernatant of M14C2/MK18 cells (Fig. 3A). Two immunoreactive bands of ~45 and ~22 kDa were observed, corresponding to the pro-PDGF-C and to the fully processed and active receptor binding form, respectively (15). The expression of NRP-1 polypeptide, which we recently found to promote melanoma cell invasion (16), was also confirmed by immunoblot analysis (Fig. 3A). Blockage of PDGF-C with a specific neutralizing antibody down-modulated ECM invasion by M14C2/MK18 cells to the same extent as NRP-1 inhibition (~35%). The simultaneous blockage of both polypeptides resulted in an additive effect causing a decrease in M14C2/MK18 cell invasiveness of ~70% (Fig. 3B).

We then investigated the expression of PDGF-C using RT-PCR analysis in a series of human melanoma cell lines and melanocytic cultures. The results indicated that most of the cell lines under study (17 out of 20), derived from primary or metastatic melanomas, expressed the PDGF-C transcript, while only 2 out of the 6 melanocytic primary cultures showed low PDGF-C levels (Fig. 3C). This finding suggests that PDGF-C expression may be required for melanoma development and progression.

In order to investigate the possibility that calpain-3 down-modulation, evidenced by microarray analysis, might contribute to the melanoma aggressive phenotype, M14C2/C4 cells were treated with a specific calpain inhibitor (ALLM) and analysed for their ability to invade the ECM. Interestingly, ALLM treatment significantly stimulated ECM invasion by these cells in a concentration-dependent manner (Fig. 3D).

These data demonstrated that PDGF-C upregulation and calpain-3 downregulation are both involved in the increase of M14C2/MK18 cell aggressiveness.

## Discussion

A previously isolated highly invasive cell clone (M14C2/MK18 cells) derived from a metastatic cutaneous melanoma lesion was characterised for the expression of several important determinants of melanoma aggressiveness: the activation of a VEGF-A/VEGFR-2 autocrine loop, NRP-1 expression and secretion of fully active MMP-2. These cells displayed rapid engraftment and an elevated growth rate in an *in vivo* murine model. With an intent to identify further molecules and

Table I. Upregulated genes in M14C2/MK18 cells as compared with M14C2/C4 cells.

| Probe | Symbol | Description[a] | Fold-change[b] | | P-value[c] |
|---|---|---|---|---|---|
| | | | PAM | SAM | |
| 218718_at | PDGF-C | Platelet-derived growth factor-C | 2.38 | 132.27 | 0.0019 |
| 212298_at | NRP-1 | Neuropilin-1 | 2.33 | 103.09 | 0.0479 |
| 221730_at | COL5A2 | Collagen, type V, α2 | 1.91 | 42.37 | 0.0331 |
| 205404_at | HSD11B1 | Hydroxysteroid (11β) dehydrogenase 1 | 1.86 | 156.25 | 0.0003 |
| 204268_at | S100A2 | S100 calcium binding protein A2 | 1.58 | 149.25 | 0.0012 |
| 207723_s_at | KLRC3 | Killer cell lectin-like receptor subfamily C, member 3 | 1.57 | 12.31 | 0.0469 |
| 204597_x_at | STC1 | Stanniocalcin | 1.51 | 97.17 | 0.0196 |
| 201243_s_at | ATP1B1 | ATPase, $Na^+/K^+$ transporting, β1 polypeptide | 1.48 | 10.13 | 0.0061 |
| 209270_at | LAMB3 | Laminin, β3 | 1.46 | 35.71 | 0.0023 |
| 202270_at | GBP1 | Guanylate binding protein 1, interferon-inducible, 67 kDa | 1.45 | 3.74 | 0.0419 |
| 206027_at | S100A3 | S100 calcium binding protein A3 | 1.44 | 20.02 | 0.0266 |
| 204205_at | APOBEC3G | Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G | 1.41 | 12.61 | 0.0181 |
| 206067_s_at | WT1 | Wilms tumour 1 | 1.41 | 15.57 | 0.0128 |
| 208025_s_at | HMGA2 | High mobility group AT-hook 2 | 1.40 | 7.59 | 0.0007 |
| 212667_at | SPARC | Secreted protein, acidic, cysteine-rich (osteonectin) | 1.39 | 9.20 | 0.0007 |
| 212097_at | CAV1 | Caveolin 1, caveolae protein, 22 kDa | 1.33 | 12.56 | 0.0199 |
| 210510_at | NRP-1 | Neuropilin-1 | 1.28 | 10.91 | 0.0182 |
| 219295_at | PCOLCE2 | Procollagen C-endopeptidase enhancer 2 | 1.28 | 6.39 | 0.0164 |
| 204279_at | PSMB9 | Proteasome (prosome, macropain) subunit, β type, 9 | 1.23 | 4.54 | 0.0097 |

[a]The list corresponds to the genes that were upregulated in both PAM and SAM analysis; [b]Fold-change parameters are those obtained by PAM analysis; [c]P-value according to Student's t-test analysis.

mechanisms responsible for their elevated aggressiveness, the gene expression profile of M14C2/MK18 cells was analysed and compared to that of a control cell clone, derived from the same original melanoma, but with lower aggressiveness *in vitro* and *in vivo*.

The results of the microarray analysis showed a strong up-modulation of NRP-1 (known to be expressed at high levels in these cells) but also of PDGF-C expression. PDGF-C is a member of the PDGF family of growth factors secreted as a latent homodimeric form and consisting of an N-terminal CUB (Clr/Cls, urchin endothelial growth factor-like protein and bone morphogenic protein 1) domain followed by a linker sequence and a C-terminal growth factor domain (GFD) (17). The CUB domain must be proteolytically removed to permit GFD to bind and activate PDGFRs (principally PDGFRα homodimers, but PDGFRβ can also be activated via PDGFRαβ heterodimerisation) (17-19). In the adult, the angiogenic capabilities of PDGF-C are comparable to those of VEGF in different model systems (18,20) and are linked to its effects on fibroblasts, endothelial progenitor cells, bone marrow cells and mature vascular cells by promoting their recruitment, proliferation, differentiation and migration (21-23).

In breast cancer, increased PDGF-C expression correlates with lymph node metastases, higher proliferative potential, and lower rates of 7-year disease-free survival (23). A role for PDGF-C in Ewing sarcoma has also been suggested by the observation that cell lines derived from this tumour type over-express this cytokine, which promotes anchorage-independent growth (25,26). PDGF-C autocrine signalling has also been suggested to be involved in the initiation and progression of brain tumours such as glioblastoma and medulloblastoma (27,28) and in the chemoresistance of oral squamous cell carcinoma (29). PDGF-C has been correlated with melanoma progression as a paracrine factor that favours melanoma dissemination by stimulating fibroblast reactivity and fibrosis, and by inducing angiogenesis through its effect on the fibroblasts and the endothelium surrounding the tumour (23). Herein, we show, for the first time, that PDGF-C can also support melanoma cell invasiveness by autocrine activation and suggest that this mechanism may be frequently active in melanoma cells, since we also found that PDGF-C mRNA is widely expressed in melanoma cell lines. The production of the ~22 kDa fully active form of PDGF-C also requires the presence of proteases that process this cytokine. Therefore, further studies are necessary to identify the melanoma cell lines that actually produce the fully processed form of PDGF-C. In addition, PDGF-C expression may be responsible for tumour resistance to anti-angiogenic therapies targeting VEGF. In fact, levels of this cytokine are elevated in melanomas resistant to anti-VEGF therapy (30,31), likely due to the role of PDGF-C in tumour-induced vessel maturation and stabilisation (32). Therefore, the data herein shown suggest that blocking of PDGF-C may synergise with anti-VEGF-A therapies in combating tumour angiogenesis and tumour invasion.

Table II. Downregulated genes in M14C2/MK18 cells as compared with M14C2/C4 cells.

| Probe | Symbol[d] | Description[a] | Fold-change[b] | | P-value[c] |
|---|---|---|---|---|---|
| | | | PAM | SAM | |
| 214475_x_at | **CAPN3** | Calpain-3, (p94) | -2.21 | -123.90 | 0.0072 |
| 206426_at | **MLANA** | Melan-A | -1.93 | -76.24 | 0.0393 |
| 209167_at | **GPM6B** | Glycoprotein M6B | -1.80 | -30.25 | 0.0373 |
| 216512_s_at | **DCT** | Dopachrome tautomerase | -1.76 | -130.85 | 0.0287 |
| 210944_s_at | **CAPN3** | Calpain-3, (p94) | -1.68 | -92.29 | 0.0295 |
| 219412_at | RAB38 | RAB38, member RAS oncogene family | -1.67 | -71.53 | 0.0011 |
| 211890_x_at | **CAPN3** | Calpain-3, (p94) | -1.58 | -69.13 | 0.0272 |
| 215695_s_at | **GYG2** | Glycogenin 2 | -1.58 | -15.76 | 0.0445 |
| 203382_s_at | **APOE** | Apolipoprotein E | -1.57 | -42.41 | 0.0016 |
| 203651_at | **ZFYVE16** | Zinc finger, FYVE domain containing 16 | -1.55 | -9.31 | 0.0132 |
| 216033_s_at | FYN | FYN oncogene related to SRC, FGR, YES | -1.47 | -11.29 | 0.0285 |
| 209169_at | **GPM6B** | Glycoprotein M6B | -1.41 | -30.25 | 0.0361 |
| 201534_s_at | **UBL3** | Ubiquitin-like 3 | -1.39 | -3.82 | 0.0095 |
| 214028_x_at | TDRD3 | Tudor domain containing 3 | -1.38 | -9.89 | 0.0003 |
| 213702_x_at | **ASAH1** | N-acylsphingosine amidohydrolase 1 | -1.38 | -20.27 | 0.0265 |
| 206864_s_at | HRK | Harakiri, BCL2 interacting protein (BH3 domain) | -1.32 | -8.67 | 0.0164 |
| 201362_at | **IVNS1ABP** | Influenza virus NS1A binding protein | -1.31 | -3.92 | 0.0387 |
| 209113_s_at | HMG20B | High-mobility group 20B | -1.28 | -11.18 | 0.0029 |
| 33304_at | ISG20 | Interferon stimulated gene 20 kDa | -1.28 | -10.73 | 0.0141 |
| 202295_s_at | CTSH | Cathepsin H | -1.28 | -15.28 | 0.0048 |
| 209123_at | **QDPR** | Quinoid dihydropteridine reductase | -1.25 | -7.46 | 0.0385 |
| 200924_s_at | SLC3A2 | Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 | -1.22 | -8.20 | 0.0155 |

[a]The list corresponds to the genes that were downregulated in both PAM and SAM analysis; [b]Fold-change parameters are those obtained by PAM analysis; [c]P-value according to Student's t-test analysis; [d]Genes listed in bold are under the control of the microphthalmia-associated transcription factor (MITF) (14).

On the other hand, we also found that the gene which showed the most important down-modulation in M14C2/MK18 cells was CAPN3, encoding for calpain-3. Calpains are a group of calcium-dependent thiol-proteases that respond to $Ca^{2+}$ signals by cleaving specific proteins, frequently components of signalling cascades, irreversibly modifying their function. Calpain-3 is a 94-kDa enzyme that is structurally similar to calpain-1 and -2, but has an additional N-terminal sequence of 20-30 amino acids. The function of calpains is regulated by phosphorylation, intracellular $Na^+$ concentration, calpastatin and their subcellular localization (33). In this context, three of the genes found to be highly upregulated in M14C2/MK18 cells are involved in the control of intracellular calcium concentration (STC1, S100A2 and S100A3). Their increased expression may cause a decrease in calcium levels in melanoma cells. Moreover, the elevated expression of $Na^+/K^+$ transporting ATPase β1 polypeptide (ATP1B1) would likely result in reduced intracellular $Na^+$ levels. Therefore, the upregulation of these four genes in M14C2/MK18 cells may contribute to the down-modulation, at a post-translational level, of calpain-3 activity that is maintained by a marked decrease in the CAPN3 transcript.

Calpains are involved in numerous cell functions, and consequently they are key regulators of several important pathways (34). For example, they have a role in the remodelling of cytoskeletal attachments to the plasma membrane during cell fusion and cell motility, in the proteolytic modification of molecules involved in signal transduction pathways, in the degradation of enzymes controlling cell cycle progression or in the regulation of gene expression and substrate degradation in various apoptotic pathways. A study in human biopsies showed that the expression of calpain-3 is significantly down-regulated in the most aggressive melanomas compared with benign nevi, suggesting that its downregulation may contribute to melanoma progression (35). Consistent with this hypothesis, we found that treatment of M14C2/C4 cells with a calpain inhibitor significantly triggered ECM invasion by these cells, otherwise unable to migrate across the basement matrix layer.

Notably, 11 out of the 19 genes down-modulated in M14C2/MK18 cells are known to be under the control of the microphthalmia-associated transcription factor (MITF). MITF encodes for basic helix-loop-helix-leucine-zipper transcription factors, among which the M-isoform is specifically expressed in melanocytes (36) and plays a key role in melanocyte development, survival and differentiation (37-42). When the levels of MITF polypeptide expression were evaluated during melanoma progression, results indicated an intense nuclear staining for the MITF polypeptide in 83% of nevi and 56% of primary melanomas, but only 23% of metastases (3). MITF is subjected to various

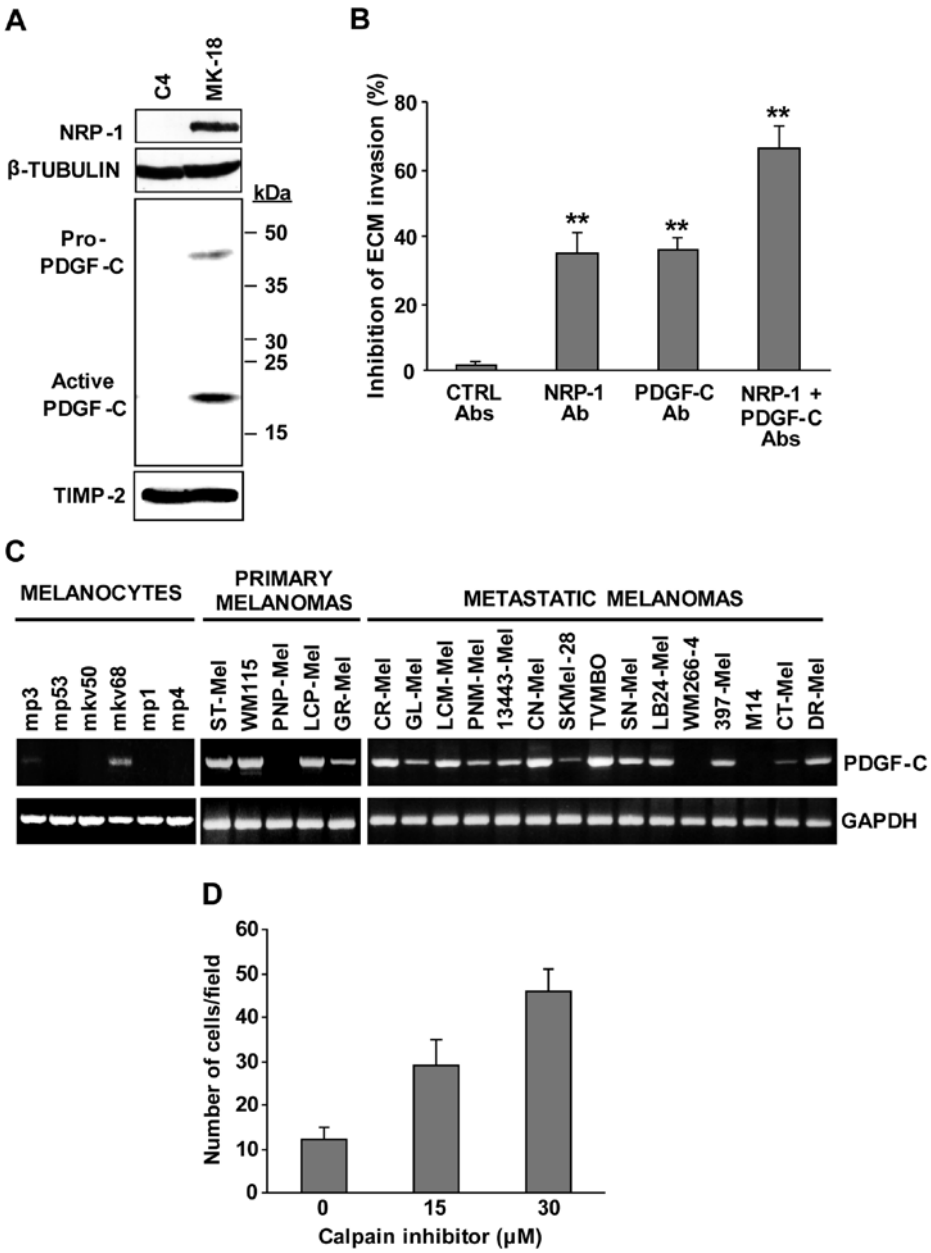


Figure 3. Involvement of PDGF-C and calpain-3 in the invasiveness of M14C2/MK18 cells. (A) The differential expression of NRP-1 and secretion of PDGF-C polypeptides in M14C2/C4 and M14C2/MK18 cells were evaluated by western blot analysis. NRP-1 detection was analysed using 80 $\mu$g of proteins per sample in a 7% SDS-polyacrylamide gel, and β-tubulin expression was assessed as loading control. The bands corresponding to the pro-PDGF-C and the fully processed, active PDGF-C forms were detected by loading 50 $\mu$l of concentrated cell supernatants, collected from an equal number of cultured cells, in a 6-12% SDS-polyacrylamide gel gradient. TIMP-2 secretion was used as loading control. Results are representative of one out of three different experiments giving comparable results. (B) The influence of antibodies that specifically block NRP-1 or PDGF-C on the ability of M14C2/MK18 cells to spontaneously invade the ECM was analysed using Boyden chambers equipped with Matrigel-coated filters. Cells were allowed to invade Matrigel in response to migration medium for 2 h. The assay was performed in the absence of antibodies or in the presence of 2.5 $\mu$g/ml of antibodies anti-NRP-1 (H-286) and/or anti-PDGF-C (AF1560), or control antibodies (mouse IgG + goat IgGs, CTRL Abs). Data are expressed as the percent inhibition of ECM invasion calculated as compared with untreated cells. Values represent the means ± SD from three independent experiments. $^{**}$P<0.01, according to Student's t-test analysis, comparing the invasion of cells treated with anti-NRP-1 or anti-PDGF-C antibodies with that of cells treated with control antibodies, or comparing the invasion of cells treated with both antibodies with that of cells treated with only anti-NRP-1 or anti-PDGF-C antibodies. (C) RT-PCR analysis of PDGF-C expression was performed in a series of human melanocyte primary cultures and in human melanoma cell lines derived from primary or metastatic melanomas. As a control of RNA integrity, analysis of GAPDH gene was performed. Results are representative of one out of three different experiments giving comparable results. (D) The influence of the treatment with the calpain inhibitor ALLM (15 and 30 $\mu$M) on the ability of M14C2/C4 cells to spontaneously invade the ECM was analysed using Boyden chambers equipped with Matrigel-coated filters and allowing the cells to invade for 3 h. Samples of untreated cells (calpain inhibitor concentration 0) were incubated with the same final concentration of DMSO, used as drug solvent, present in the sample treated with 30 $\mu$M of the inhibitor. Each value represents the mean ± SD of the number of cells migrated/microscopic field.

post-translational modifications (43), including phosphorylation at Ser73, which targets MITF for ubiquitin-dependent proteolysis (44) and increases its interaction with the repressor protein inhibitor of activated STAT3 (PIAS3). PIAS3 mediates MITF sumoylation, down-modulating its transcriptional activity (45). Moreover, PIAS3 is a substrate of calpain that negatively regu-

lates its sumoylase activity (46). Therefore, it can be hypothesised that the MITF post-translational downregulation consequent to the decrease in calpain-3 may be a determinant of M14C2/MK18 cell invasiveness. Moreover, inhibition of the calpain-3 substrate PIAS3 may contribute to maintain a low aggressive phenotype in melanoma cells, by preserving MITF activity.

In conclusion, we identified several molecules, including PDGF-C and calpain-3, that may be involved in the acquisition of an aggressive phenotype that transforms low invasive lesions into highly invasive tumours, suggesting the possibility that these proteins or their downstream effectors may represent molecular targets for more effective therapies against malignant melanoma.

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