The Promyelocytic Leukemia Zinc Finger–MicroRNA-221/-222 Pathway Controls Melanoma Progression through Multiple Oncogenic Mechanisms

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Abstract

The incidence of cutaneous melanoma is steadily increasing. Although several molecular abnormalities have been associated with melanoma progression, the mechanisms underlying the differential gene expression are still largely unknown and targeted therapies are not yet available. Noncoding small RNAs, termed microRNAs (miR), have been recently reported to play important roles in major cellular processes, including those involved in cancer development and progression. We have identified the promyelocytic leukemia zinc finger (PLZF) transcription factor as a repressor of miR-221 and miR-222 by direct binding to their putative regulatory region. Specifically, PLZF silencing in melanomas unblocks miR-221 and miR-222, which in turn controls the progression of the neoplasia through down-modulation of p27Kip1/CDKN1B and c-KIT receptor, leading to enhanced proliferation and differentiation blockade of the melanoma cells, respectively. *In vitro* and *in vivo* functional studies, including the use of antisense "antagomir" oligonucleotides, confirmed the key role of miR-221/-222 in regulating the progression of human melanoma; this suggests that targeted therapies suppressing miR-221/-222 may prove beneficial in advanced melanoma. [Cancer Res 2008;68(8):2745–54]

Introduction

Cutaneous melanoma is an aggressive neoplasm refractory to traditional therapies, especially at the metastatic stage. Furthermore, its incidence is continuously increasing during the last decade (1). Melanomas develop through a multistep process that from normal melanocytes proceeds to nevi and to radial and vertical growth phase tumors (2). Although several molecular abnormalities have been associated with melanoma progression, as the loss of AP-2 transcription factor (3) or the high mutation rate of the *B-RAF* oncogene (4), the mechanisms underlying the differential gene expression are still largely unknown and the conventional histologic classification remains the best prognostic factor (5).

A new class of small regulatory RNA sequences, termed microRNAs (miR), has recently been identified. Although relatively few miR targets have been experimentally validated, growing evidence indicates that miRs play important roles in major cellular processes (e.g., proliferation and differentiation, apoptosis, and angiogenesis) and, as a consequence, their abnormal expression may contribute to cancer development/progression (6, 7).

MiR-221 and miR-222 are clustered on the X chromosome and possibly transcribed in a common precursor suggestive of a coordinate functional role. They have been reported to be overexpressed in pancreatic cancer (8), papillary thyroid carcinoma (9), glioblastoma (10, 11), and prostate carcinoma (12). Considering that, in some cases, miR-221 and miR-222 exert their function through c-KIT receptor (9, 13), in view also of c-KIT down-regulation in the majority of invasive and metastatic melanomas (14), we tested whether miR-221 and miR-222 might be directly involved in melanoma pathogenesis. We show that the promyelocytic leukemia zinc finger (PLZF), previously reported as a tumor suppressor down-modulated in melanomas (15), is an upstream negative regulator of miR-221 and miR-222 expression. Moreover, we provide evidences of miR-221 and miR-222 capabilities to regulate two distinct but functionally convergent pathways of melanocyte transformation through the cyclin-dependent kinase inhibitor 1B (p27Kip1/CDKN1B) on one side and c-KIT and its downstream genes on the other.

Materials and Methods

Cell lines culture and transduction. The human melanoma cell lines used in the current study were stabilized from surgical specimens obtained from primary or metastatic tumors at the Istituto Nazionale Tumori in Milan (Italy). Cell lines were characterized for growth in soft agar and, whenever possible, their metastatic potential was evaluated into athymic nude mice. We included in Supplementary Table S1 the stage and a reference for each analyzed cell line. Normal human epidermal melanocytes from the foreskin were obtained from Promocell.

The PLZF cDNA encompassing its complete coding sequence was cloned into the retroviral vector LXSN as described (15). "Control" cell lines are always empty vector transduced. Overexpression of miR-221 and miR-222 was obtained in melanoma cells by using a lentiviral vector system according to standard techniques (13).

MiR-221 and miR-222 silencing by antagomir treatment. Chemically modified antisense oligonucleotides (antagomir) have been used to inhibit miR expression *in vitro* and *in vivo* (16, 17). The sequences of antagomir-221 and antagomir-222 used are as follows: 5′P-GAAACCCAGCAGACAAUGUAGCU-3′-Chl and 5′P-GAGACCCAGUAGCCAGAUGUAGCU-3′-Chl, respectively; all the bases were 2′-OMe modified. Antagomir oligonucleotides, deprotected, desalted, and purified by high-performance liquid

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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chromatography (Dharmacon), were transfected at doses ranging from 50 to 250 nmol/L by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's procedures. As controls, an unrelated antagomir (specifically the antagomir targeting miR-133a that is not expressed in melanomas) and a FITC-conjugated oligonucleotide targeting the luciferase sequence (FITC oligonucleotide to antagomir ratio, 1:10) were transfected as well. Transfection efficiencies were analyzed by fluorescence-activated cell sorting. Cell growth was monitored on days 1, 2, and 3 after transfection, and RNAs and proteins were extracted for further analysis.

To study any possible miR-dependent effect on p27 protein stability, the expression levels of p27 were analyzed through a cycloheximide treatment (60 μg/mL) in parental versus antagomir-transfected melanoma cells. Total protein extracts were analyzed by Western blot at the indicated time points, and the normalized amounts were plotted in a regression curve.

p27 was specifically silenced by using small interfering RNA [ON-TARGET plus small interfering RNA (siRNA); Dharmacon]. Briefly, 24 h after plating, cells were transfected either with sip27 or with a siRNA control (200 nmol/L). On day 2, cells were subjected to a second round of transfections with antagomir-133, as an irrelevant control, or with antagomir-221+222 (150 nmol/L). The level of p27 was analyzed 72 h after the first transfection, and the proliferative rate was evaluated up to day 4.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from green fluorescent protein (GFP)- and PLZF-transduced 293FT cells. In each sample, 20 μg of nuclear extracts were incubated with 3.0×10^4 cpm of ^{32}P-labeled double-stranded oligonucleotide in a binding buffer containing 12% glycerol, 12 mmol/L HEPES (pH 7.9), 4 mmol/L Tris-HCl (pH 8.0), 100 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 5 mmol/L $MgCl_2$, poly(deoxyinosinic-deoxycytidylic acid), and bovine serum albumin. Reaction mixtures were incubated on ice for 45 min; the protein-DNA complexes were resolved on a 5% polyacrylamide gel. The gel was dried and exposed to a Typhoon Scanner (Amersham). For competition experiments, a 300- to 500-fold molar excess of unlabeled oligonucleotide was added. For supershift analysis, an anti-PLZF monoclonal antibody was used (Calbiochem).

As control of specificity, point mutations were inserted in the core binding sequences for PLZF, "T(A/C)(A/C)AGT". The sequences of the oligonucleotides are listed below; bold capital letters indicate the core sequences, and lowercase letters indicate the mutated bases.

BS1 (wild-type) 5′-ACTGAGGATAA**TACAGT**TATTTTACCAAAC-3′.
BS1 (mutated) 5′-ACTGAGGATAA**atCtGT**TATTTTACCAAAC-3′.
BS2 (wild-type) 5′-GTGACATTAAA**TAAAGT**GCCACATATTTTC-3′.
BS2 (mutated) 5′-GTGACATTAA**cTgcAGT**GCCACATATTTTC-3′.
BS3 (wild-type) 5′-GTAATTCAAGG**TAAAGT**TTTCATTATTAAAG-3′.
BS3 (mutated) 5′-GTAATTCAAG**cTgcAGT**TTTCATTATTAAAG-3′.

Chromatin immunoprecipitation assay. Cells (5×10^6) from control (LXSN transduced) HeLa or Me665/1 and the corresponding PLZF retrovirally transduced cell lines were fixed in 1% formaldehyde for 10 min at room temperature. Cells were washed with ice-cold 1× PBS, scraped in 1× PBS plus protease inhibitors, and collected by centrifugation. Cell pellets, resuspended in cell lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 1% SDS] plus protease inhibitors, were then sonicated. DNA-protein complexes were immunoprecipitated using 3 μg of the following antibodies: anti-PLZF (Calbiochem) or, as an internal control, the unrelated anti–DVL-1 (Santa Cruz Biotechnology). DNA-protein cross-links were reversed by heating at 65°C overnight. The recovered DNAs were then PCR amplified with the following primer set: DIR(−514) 5′-CAGCATACATGATTCCTTGTGA-3′ and REV(−260) 5′-CTTTGGTGTTTGA-GATGTTTGG-3′, corresponding to PLZF binding site at −490 (BS1); DIR(−262) 5′-GGATCTACACTGGCTACTGAG-3′ and REV(+80) 5′-GTCA-CAAGGAATCATGTATGC-3′, PLZF binding site at −182 (BS2); and DIR(+16) 5′-CCTAGAACTTGACTCTCTCC-3′ and REV(+276) 5′-GCTGCTGGAAGGTG-TAGGTA-3′, PLZF binding site at +165 (BS3). Control amplification was carried out on input chromatin (preserved before immunoprecipitation) and on DVL-1 (mock) immunoprecipitated chromatin. HeLa and Me665/1 control cell lines, which do not express endogenous PLZF, represent the negative control. To confirm the specificity of the immunoprecipitated products, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR were also run.

Luciferase assay. A DNA fragment containing the putative regulatory region upstream to miR-222/-221 (from −555 to +336 nt) was amplified and cloned in pGL3basic (Promega). To further analyze the functional role of different binding sites, shorter constructs (from −555 to −55 nt and from −55 to +336 nt), encompassing the wild-type or mutated sequences, were also made. 293FT cells were transfected with Lipofectamine 2000 (Invitrogen) and (*a*) 150 ng of pGL3basic or of pGL3 containing the above genomic fragments, (*b*) 300 ng of pCDNA or pCDNA/PLZF plasmid, and (*c*) 10 ng of Renilla. At 48 h, cells were lysed and their luciferase activity was measured by using the FemtomasterFB 12 (Zylux). The wild-type pGL3b (−555/+336) plasmid cotransfected with the control pCDNA was considered as 100%. As controls of specificity, point mutations were inserted in the wild-type core binding sequence for PLZF, T(A/C)(A/C)AGT, by using the QuickChange site-directed mutagenesis kit (Stratagene). Mutated nucleotides are the same as indicated in the Electrophoretic Mobility Shift Assay section. Luciferase assays were also performed in Me665/1/LXSN and Me665/1/PLZF melanoma cells after Lipofectamine 2000 (Invitrogen) transfection with (*a*) 800 ng of pGL3basic or pGL3 containing the above reported genomic fragments and (*b*) 20 ng of Renilla.

Target analysis. Bioinformatic analysis was performed by using these specific programs: TargetScan,[4] PicTar,[5] and RNAhybrid.[6]

***In vivo* assay.** For the *in vivo* assays, empty vector–transduced or miR-transduced Me1402/R cells in exponential growth phase were injected s.c. at doses of 10^6 or 5×10^6 into adult athymic nude mice purchased from Charles River (Calco) and maintained at the Istituto Nazionale Tumori according to institutional guidelines. Tumor growths were monitored twice a week for at least 4 wk. Points represent the mean tumor volume (obtained by multiplying two perpendicular diameters) ±SD.

For antagomir treatment, athymic nude mice were s.c. injected with 5×10^6 cells of Me665/1 metastatic melanoma; after approximately 1 wk, when tumors became palpable, mice received intratumor antagomir-221 plus antagomir-222 (at doses ranging between 0.3 and 0.5 mg/mouse) or control saline. Tumor growths were monitored twice a week according to the above standard criteria.

Statistical analysis. Statistical and frequency distribution analysis was performed by Excel. Differences between two or three groups were compared with Student's *t* test. $P < 0.05$ or less was considered to be statistically significant.

Results

The expression of miR-221 and miR-222 correlates with melanoma progression. The progression of human melanoma toward the metastatic phenotype has been reported to be associated, among many other molecular abnormalities, with loss of expression of the tyrosine kinase receptor c-KIT; in addition, c-KIT reexpression is able to inhibit tumor growth and metastasis in nude mice (3).

Because recent studies have shown that c-KIT receptor is a major functional target of miR-221 and miR-222 (13), we evaluated by Northern blot the endogenous levels of miR-221 and miR-222 in a panel of melanoma cell lines (see Supplementary Table S1 and ref. 15), including primary vertical growth phase and metastatic melanomas, in comparison with normal human melanocytes from the foreskin. MiR-221 and miR-222 were almost undetectable in normal human melanocytes and increasingly expressed throughout a stepwise transformation process (Fig. 1*A*). To rule out any possible culture artifact, we performed *in situ* hybridization on

[4] http://www.targetscan.org/
[5] http://pictar.bio.nyu.edu/
[6] http://bibiserv.techfak.uni-bielefeld.de/

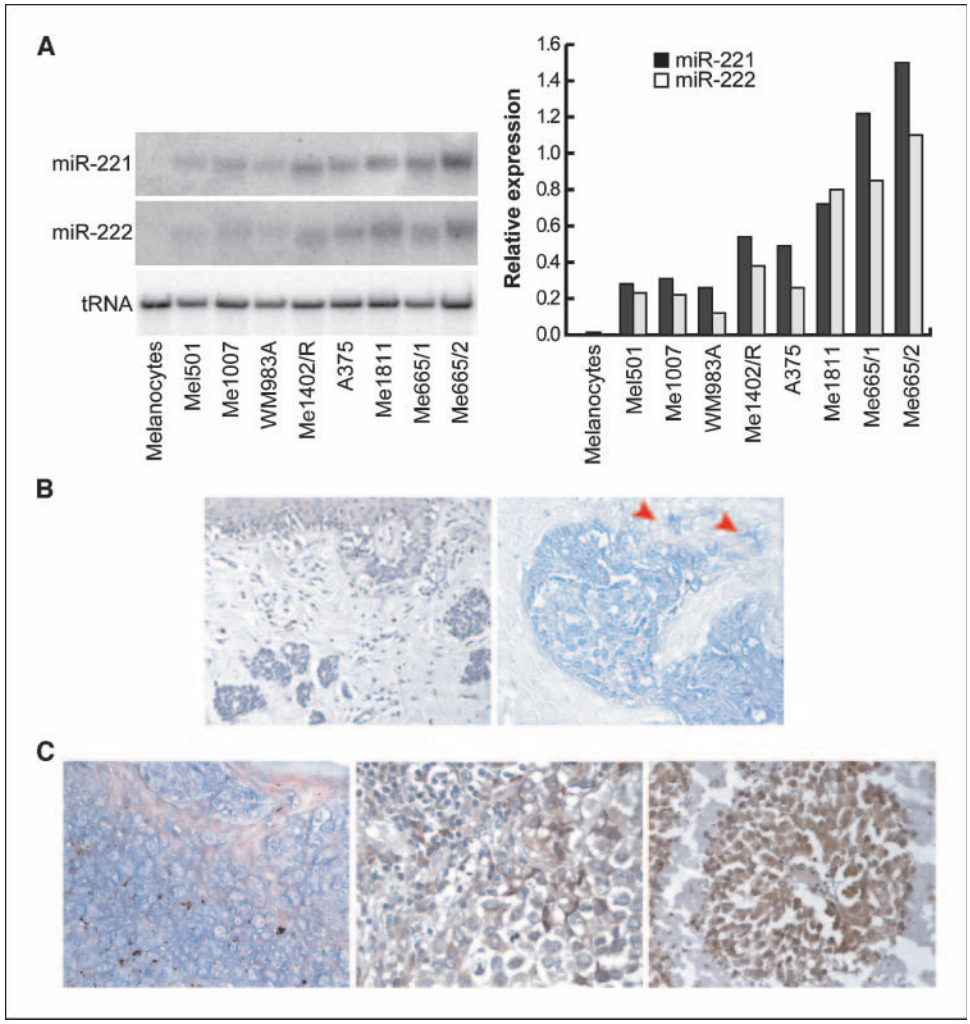

Figure 1. MiR-221 and miR-222 expression in normal human melanocytes and melanoma cell lines. *A,* Northern blot analysis and relative expression values obtained by densitometric analysis. *B* and *C,* representative *in situ* hybridization analyses of miR-221. *B,* compound (*left*) and dysplastic (*right*) nevi. *C,* cutaneous melanoma (*left*) and lymph node (*middle*) and lung (*right*) metastases. *Arrowheads,* light positivity of atypical melanocytes. Melanoma cells (*C*) show a strong positive signal.

primary samples, including compound and dysplastic nevi, s.c. melanomas, and lymph node or lung metastases. In the compound nevus, the intraepithelial thecae as well as the intradermic nests are totally negative for miR-221 and miR-222 (Fig. 1*B, left*; data not shown). Looking at the residual dysplastic component of a nodular melanoma (Fig. 1*B, right*), some positivity was visible in the superficially spreading atypical melanocytes, whereas a strong expression of both miRs was detected in cutaneous melanomas (Fig. 1*C, left*) and, even greater, in lung and lymph node metastases (Fig. 1*C, middle* and *right*; data not shown). In all cases, the expression levels of miR-221 and miR-222 were superimposable.

PLZF directly regulates miR-222/-221 transcription. Little is known about the regulation of miRNAs (18, 19). Based on our previous results showing the loss of the tumor suppressor gene *PLZF* in melanomas and its functional role, when reexpressed, in the induction of a more differentiated, melanocyte-like, phenotype (15),[7] we hypothesized a possible "PLZF→miR-221/222→target genes" signal transduction pathway. We therefore analyzed the expression level of miR-221 and miR-222 in five PLZF-transduced melanoma cell lines corresponding to different stages of progression (15) compared with empty vector–transduced controls. A clear down-regulation of both miRs was observed in PLZF-transduced cells (Fig. 2*A*; data not shown). We then investigated the ~1 kb sequence upstream to the miR-222/-221 genomic cluster searching for a putative regulatory region. By using the MatInspector software,[8] we found 5′ to miR-222/-221 two putative consensus sequences for PLZF transcription factor indicated as BS1 and BS2; a third site, named BS3, was localized in the intragenic region between the two miR sequences (Fig. 2*B*). Cotransfections performed in the 293FT cell line of the whole sequence (from −555 to +336 nt) or of the 5′ (from −555 to −55 nt) or 3′ (from −55 to +336 nt) genomic fragments, in the presence of the empty vector (pCDNA) or of the vector driving the expression of the human PLZF cDNA, revealed that PLZF induced 40% to 50% reduction of the luciferase activity, likely binding the miR regulatory sequences (Fig. 2*C*). The introduction of point mutations in each core binding site restored the luciferase levels (Fig. 2*C*). A similar suppressive function was obtained in PLZF-transduced Me665/1 melanoma cells. The exception was the barely detected effect obtained on the BS3 site, which is reasonably attributable to the poorer transfection efficiency obtained in melanoma cells, but also to an actual lower activity.

To assess the ability of the three binding sites to actually bind PLZF nuclear protein, we performed EMSA by comparing 293FT/

[7] Unpublished results.

[8] http://www.genomatix.de

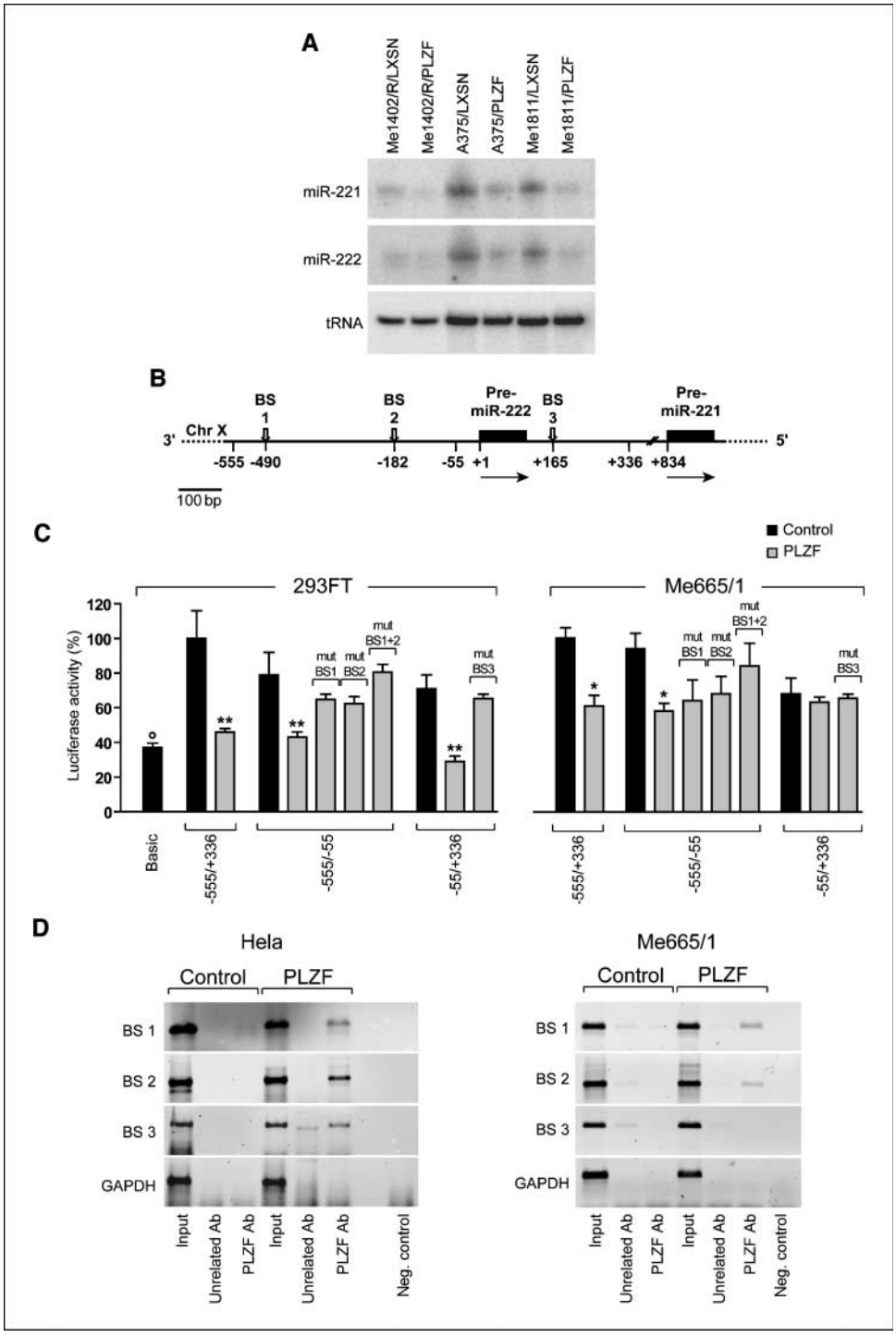

Figure 2. PLZF down-regulates miR-221 and miR-222. *A,* Northern blot analysis of empty vector– or PLZF-transduced melanoma cell lines. *B,* schematic of the genomic region upstream to pre–miR-222/-221. *BS1*, *BS2*, and *BS3,* PLZF putative binding sites; *horizontal arrows,* direction of miR transcription. *C,* luciferase assays obtained by cotransfecting different genomic fragments (as shown in *B*) in 293FT cells, transfected or not with PLZF, and in Me665/1 control or PLZF-transduced melanoma cells. As controls, mutated binding sites are included. °, $P < 0.05$ pGL3/(−555/+336) fragment versus basal activity; **, $P < 0.001$; *, $P < 0.005$ pGL3 constructs in PLZF versus empty vector–transduced cells. *D,* ChIP assays were performed with HeLa and PLZF/HeLa (*left*) and Me665/1 and PLZF/Me665/1 (*right*), subsequently analyzed by PCR. *mut,* mutated.

GFP and 293FT/PLZF nuclear protein extracts. EMSA revealed DNA-PLZF–containing complexes on BS1 and BS2 sites and, with an apparent lower affinity, on BS3; the DNA-protein complexes were competed out by wild-type oligomers and the specificity was confirmed by both supershift and site-specific mutations (see Supplementary Fig. S1 and Materials and Methods). As an internal control, we also included the PLZF binding site shown on the *TpoR* promoter region (data not shown; ref. 20). Finally, the *in vivo* interaction between these putative *cis*-regulatory elements and PLZF was investigated by chromatin immunoprecipitation (ChIP) assays performed in control HeLa and Me665/1 cell lines compared with the corresponding PLZF-transduced cells. PCR amplifications of unsheared input genomic DNA and anti-PLZF antibody-mediated reaction gave PCR products of the expected sizes, whereas the same reactions, immunoprecipitated with an irrelevant antibody, gave a barely detectable or undetectable amplified product (Fig. 2*D*), thus confirming direct and specific binding of PLZF. In agreement with the transfection results, we did not obtain PLZF binding on BS3 site when ChIP was performed in Me665/1 melanoma cell line. Accordingly, in melanomas, we always found

an apparent coregulation of miR-221 and miR-222 (see Figs. 1*A* and 2*A*).

Overexpression of miR-221/-222 results in increased tumorigenesis. To directly test the functional role of miR-221/-222 on tumorigenesis, we used a lentiviral vector to transduce the Me1402/R melanoma cell line, selected on the basis of its low but detectable levels of miR-221/-222 and of its ability to produce melanin, a function often lost in more advanced melanomas. Northern blot analysis confirmed miR overexpression in miR-transduced versus empty vector–transduced control cells (Supplementary Fig. S2*A*).

Melanoma cells overexpressing miR-221 or miR-222 showed an increase in the proliferative rate, regardless of the serum concentration (Supplementary Fig. S1*B*; data not shown). Accordingly, by cell cycle analysis on hydroxyurea-synchronized cells, miR-transduced Me1402/R cells showed a decrease of G_1 and a corresponding increase of the S and G_2-M phases. At time 0, flow cytometric analysis of DNA showed 80% to 85% of the cells in the G_1 phase; the analysis, successively performed at 2, 4, and 6 hours after hydroxyurea removal, revealed an earlier onset of DNA synthesis induced by miR-221 and miR-222 paralleled by a faster reduction of G_1 cells, contributing to the proliferative advantage (Fig. 3*A*).

As a next step, a Boyden chamber assay was used to measure the effects of miR-221 and miR-222 overexpression on cellular migration and invasion of these melanoma cells. In both assays for both miRs, we observed a significant increase in the invasive and chemotactic capabilities (Fig. 3*B*). The effects of miR-221/-222 overexpression were also evaluated on melanoma capacities of forming foci in agar semisolid medium. A significant enhancement of 10- and 5-fold of the number of foci was observed (Fig. 3*C*) for miR-221 and miR-222, respectively.

Finally, to further confirm miR-221 and miR-222 functions on melanoma tumorigenicity, we studied their role in an *in vivo* model. MiR- and empty vector–transduced Me1402/R cell lines were injected s.c. into athymic nude mice, and tumor growths followed through 4 weeks. Tumor volumes of miR-expressing melanoma cells showed a significant increase when compared with controls at all time points (Fig. 3*D*; data not shown).

Suppression of endogenous miR-221 and miR-222 by antagomir treatment. The functional effects of miR-221 and miR-222 inhibitions were also analyzed in an advanced melanoma in an attempt to reduce its malignancy. The Me665/1 metastatic cell line was treated either *in vitro* or *in vivo* with antagomir-221 and/or antagomir-222 molecules. This new class of antisense consists of RNA oligonucleotides able to efficiently and stably knock down specific miRs (16, 17). *In vitro* treatment reduced the proliferation rate of 60% to 70% with respect to cells either untreated or treated with an unrelated antagomir (i.e., the antisense sequence targeting miR-133a, whose expression is restricted to heart and skeletal muscle; Fig. 4*A, left*; ref. 17). The specificity of the down-regulation of miRs was confirmed by quantitative real-time reverse transcription-PCR (RT-PCR) and, when possible, by Northern blot (Fig. 4*A, right*; data not shown). As expected, considering the high level of homology, we observed a partial cross-reaction between antagomir-221 and antagomir-222. Moreover, Me665/1 cells transfected with antagomir-221 and/or antagomir-222 showed a decrease in both their invasion and migration abilities (Fig. 4*B*) and in the number of foci outgrowing in semisolid medium, compared with control antagomir-miR-133a–treated cells (Fig. 4*C*). Interestingly, the few small colonies derived from antagomir-221– and/or antagomir-222–transfected cells displayed a flat, nontransformed morphology (Fig. 4*C*). Finally, *in vivo*, one bolus intratumor injection of antagomirs-221+222 into athymic nude mice previously inoculated with parental Me665/1 cells inhibited tumor progression at least for the successive 7 days, a time point in which antagomir-treated nodules showed large necrotic regions (Fig. 4*D*; data not shown). Altogether, the results indicate the capacity of antagomir-221 and antagomir-222 to inhibit melanoma progression both *in vitro* and *in vivo*.

Analysis of *miR-221* and *miR-222* target genes: c-KIT receptor. MiR-221 and miR-222 were already reported to target *c-KIT* in normal human erythropoietic (13) and endothelial (21) cells; in addition, c-KIT is known to play several critical roles in the

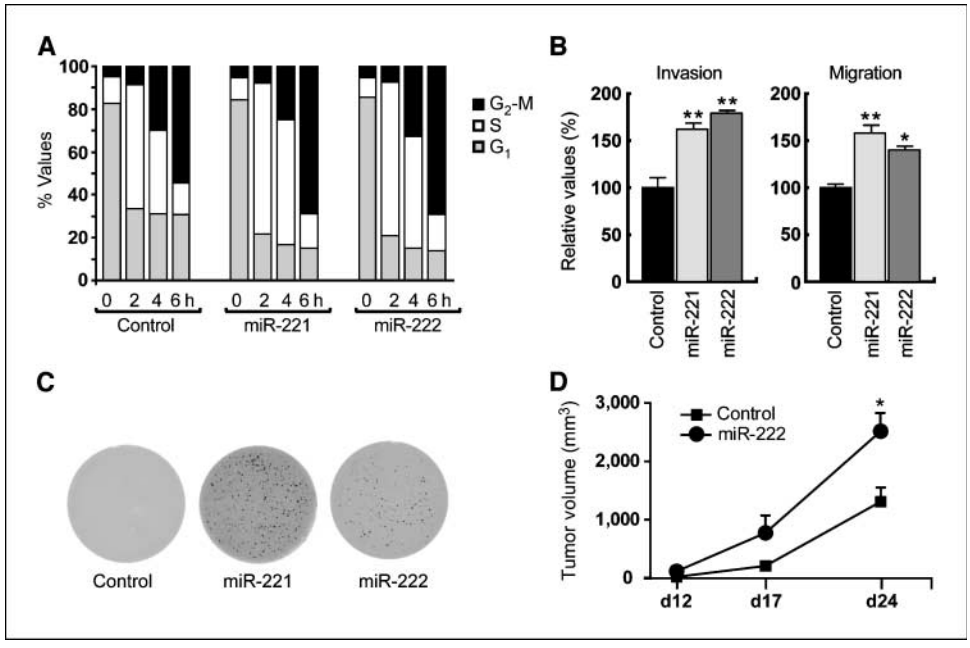

Figure 3. MiR-221 and miR-222 overexpression in Me1402/R: *In vitro* and *in vivo* functional studies. *A,* cell cycle analysis in synchronized cells. *B,* invasion and chemotaxis assays. *Columns,* mean; *bars,* SD. *C,* representative results of cell colony growth in semisolid medium. *D, in vivo* tumor growth into athymic nude mice. Tumor volumes were evaluated after s.c. injection of 5×10^6 cells. Data are representative of at least two independent experiments. *Control,* empty vector–transduced Me1402/R cell line. *, $P < 0.05$; **, $P < 0.01$.

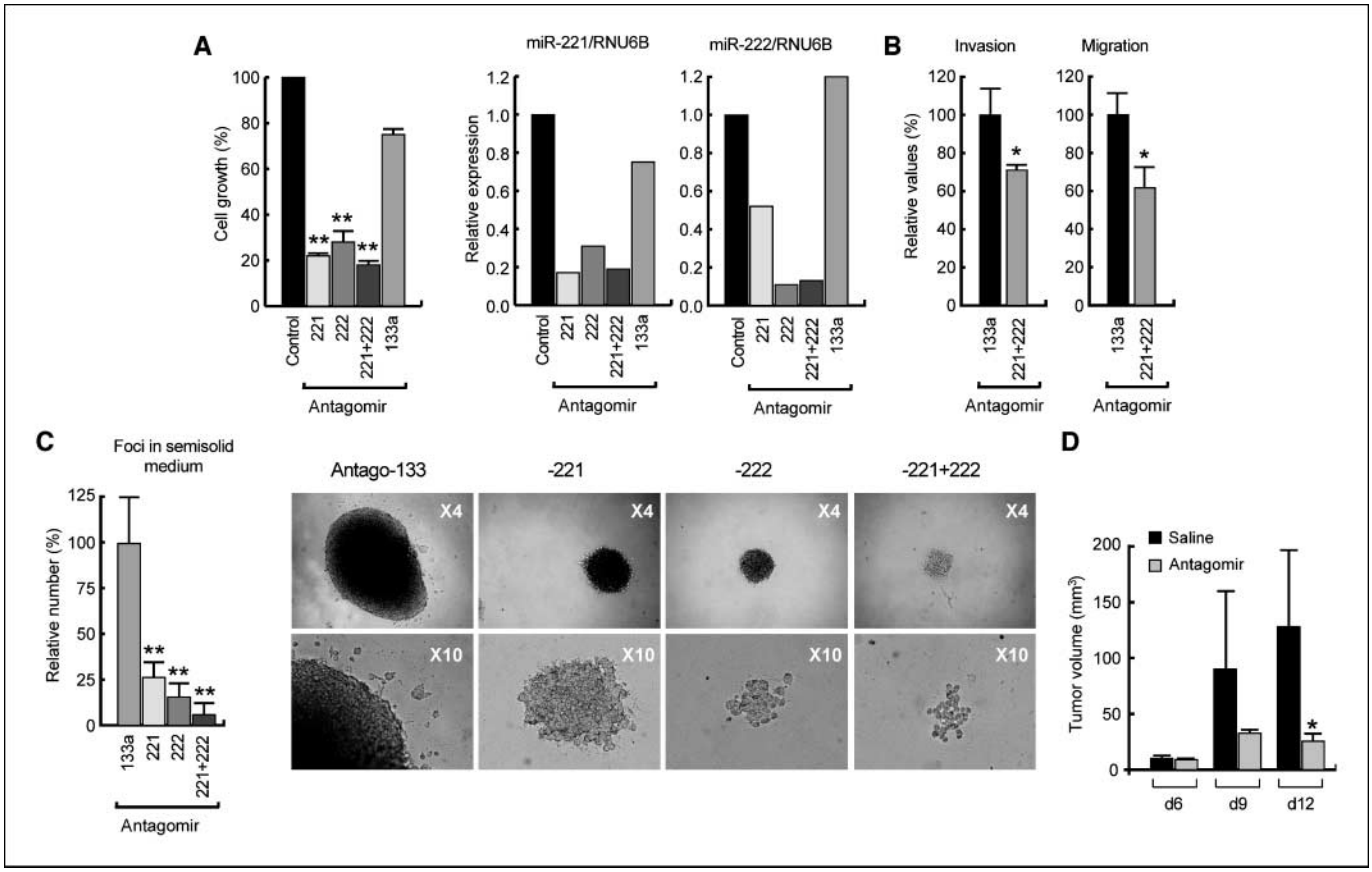

Figure 4. MiR-221 and/or miR-222 silencing by antagomir treatment in Me665/1 metastatic cell line. *A,* cell proliferation (*left*). *Columns,* mean; *bars,* SD; representative quantitative real-time PCR after antagomir treatment (*right*; dose, 200 nmol/L). RNU6B was used as internal control. *B,* invasion and chemotaxis assays. *Columns,* mean; *bars,* SD. *C,* evaluation of the foci number obtained in semisolid medium and morphologic comparison between colonies. *Columns,* mean; *bars,* SD. *D, in vivo* effects of antagomir-221+222. Athymic nude mice, ~1 wk after s.c. injection of Me665/1, received intratumor antagomirs-221+222 or control saline (n = 5 per group). Antagomir-133a was used as a control. *Columns,* mean of a minimum of two independent experiments; *bars,* SD. **, $P < 0.001$; *, $P < 0.05$.

melanocytes physiology (14). Based on these findings, we analyzed miR-221/-222 as putative upstream regulators of c-KIT receptor in the tumorigenic transformation process of melanocytes. We found a striking inverse correlation between miR-221 and miR-222 and c-KIT expression levels during melanoma progression (Fig. 5*A*). Western blot analysis showed that c-KIT expression was reduced in miR-221– and miR-222–transduced Me1402/R melanoma compared with control cell line (Fig. 5*B, left*), whereas *c-KIT* mRNA was only slightly modified (Fig. 5*B, right*). In the same cells, we observed that *MITF*, a gene downstream to the c-KIT/mitogen-activated protein kinase pathway and known to exert a multifunctional role in the complex network leading to melanogenesis (22), was moderately down-regulated (Supplementary Fig. S3; Fig. 5*C*). The reduction of MITF activity, in turn, leads to decreased levels of MITF-regulated enzymes, tyrosinase (TYR), and tyrosinase-related protein-1 (TRP-1; Fig. 5*C*; ref. 23). Also, by measuring the absorbance of miR-221– or miR-222–transduced Me1402/R versus control cell lysates, we observed a decrease in melanin pigment synthesis of 40% and 30%, respectively (data not shown). Notably, the reverse expression pattern was observed in PLZF-transduced Me1402/R (Fig. 5*B* and *C*) as well as in the antagomir-treated Me665/1 melanoma cell lines (Fig. 5*D*). In both cases, down-regulation of miRs clearly induces the up-regulation of c-KIT and of its downstream pathway, leading to melanogenesis (Supplementary Fig. S3).

Analysis of miR-221 and miR-222 target genes: p27/CDKN1B. We focused our attention on *p27,* already reported as a target of miR-221 and miR-222 in the context of prostate carcinoma and glioblastoma (11, 12). To find out whether *p27* was an actual target of miR-221 and/or miR-222 in melanoma cells, we analyzed the levels of p27 protein and mRNA in miR- or PLZF-transduced versus control empty vector–transduced Me1402/R melanoma cell line. The level of p27 was increased by PLZF overexpression (Fig. 6*A*). On the contrary, the amount of the p27 protein was strongly decreased after miR transduction, without affecting the accumulation of *p27* mRNA (Fig. 6*A*) and confirming the typical features of a miRNA target gene. In addition, we observed a strong up-regulation of p27 when the endogenously expressed miR-221 and miR-222 were inhibited by antagomir in Me665/1 metastatic melanoma cell line (Fig. 6*B*). Bioinformatics-based approaches predicted the presence of four naturally occurring putative binding sites at the *p27* 3′ untranslated region (UTR). To investigate the direct interaction between the miRs and *p27* mRNA and the relative functionalities of the putative binding sites, we separately cloned the four "seeds" downstream to the luciferase open reading frame. Interestingly, only the presence of wild-type "seed-1" caused a 70% inhibition of the luciferase activity, whereas visible but not significant effects were observed for the other three sites (Fig. 6*C*; data not shown). Mutations within the seed-1 sequence totally

abolished this repression, indicating the specificity of the action (Fig. 6*C*).

To rule out any miR-dependent effect on p27 protein stability, the expression levels of p27 were analyzed through a cycloheximide treatment in parental versus antagomir-transfected Me1402/R or Me665/1 melanoma cell lines. Although the starting amounts of p27 were obviously different in the antagomir-treated versus the untreated cells, we did not observe significant changes in the rate of p27 degradation (see Fig. 6*D, top*; data not shown). Moreover, through p27-specific silencing, we confirmed that most, if not all, of miR-221/-222 effects on cell cycling are actually mediated by this protein. Indeed, the reduction of the proliferative rate observed in antagomir-221/-222–transfected melanoma cells was abrogated by the simultaneous down-regulation of *p27* mRNA (see Fig. 6*D, bottom*).

Discussion

The multistep transformation process leading from melanocytes to metastatic melanoma cells is mainly characterized by uncontrolled proliferation, loss of CDKN, and abnormal expression of growth factors or growth factor receptors (24). Although gene expression profiling revealed that numerous molecular changes are associated with melanoma, the prognosis of this neoplasia is still based on histopathologic criteria, and molecular therapies are not yet available. To improve prognostic criteria and therapeutic tools, it is essential to study in depth the molecular oncogenic pathways implicated in melanoma transformation and progression.

This study shows that the transcription factor PLZF binds to and transcriptionally inhibits miRNA-221 and miRNA-222. Thus, the lack of PLZF in melanomas unblocks miR-221 and miR-222, which are increasingly expressed along with the disease progression and, by

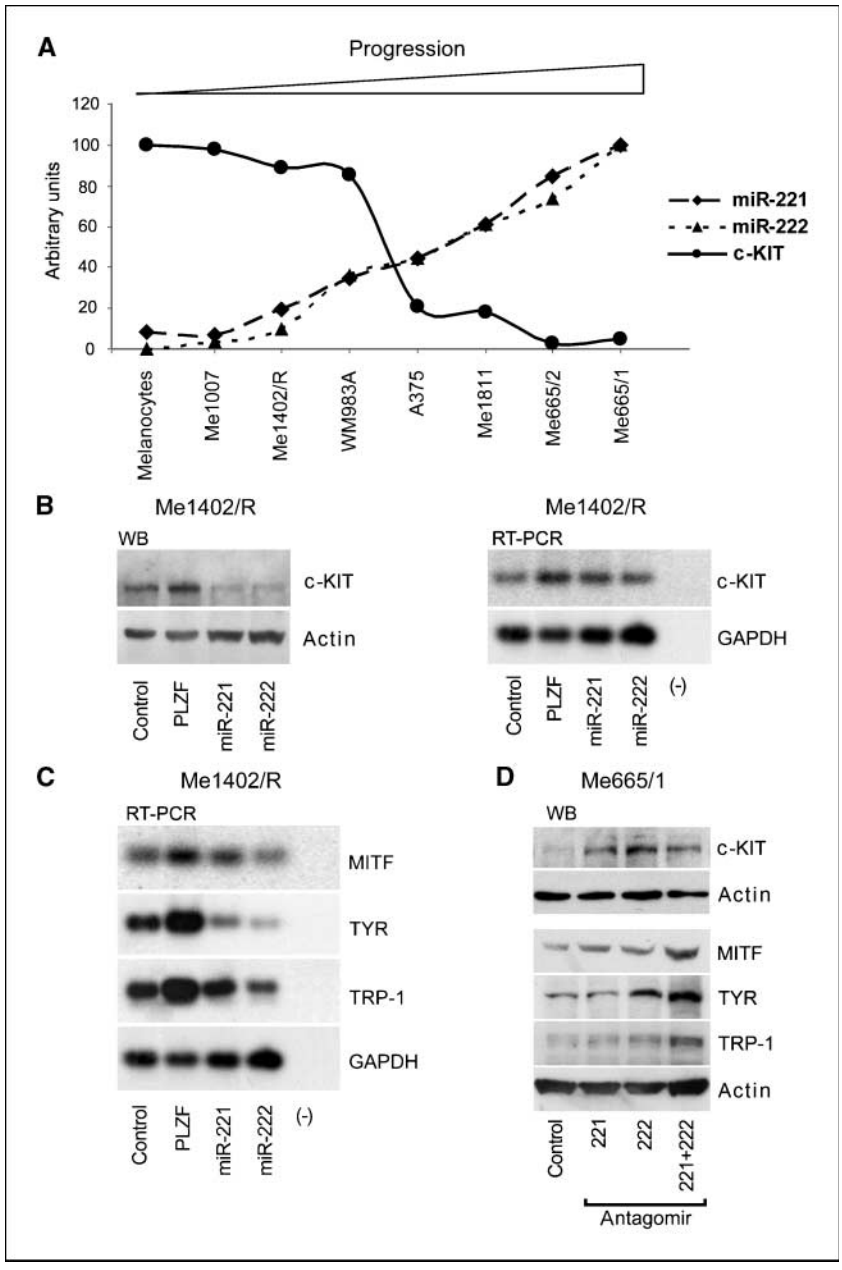

Figure 5. Analysis of miR-221 and miR-222 target genes: c-KIT. *A,* miR-221 and miR-222 expressions are inversely correlated with c-KIT protein level in melanoma progression. *B,* representative Western blot (*WB*; *left*) and RT-PCR (*right*) analyses of c-KIT in Me1402/R control versus miR-221– or miR-222–transduced cells. *C,* RT-PCR analysis of *MITF, TYR*, and *TRP-1* genes in the same cell lines. *D,* Western blot of c-KIT, MITF, TYR, and TRP-1 in antagomir-221 and/or antagomir-222 versus antagomir-133a–treated (*control*) Me665/1 melanoma metastatic cells. *GAPDH* and actin were the internal controls. Relative expression values are shown in Supplementary Fig. S3.

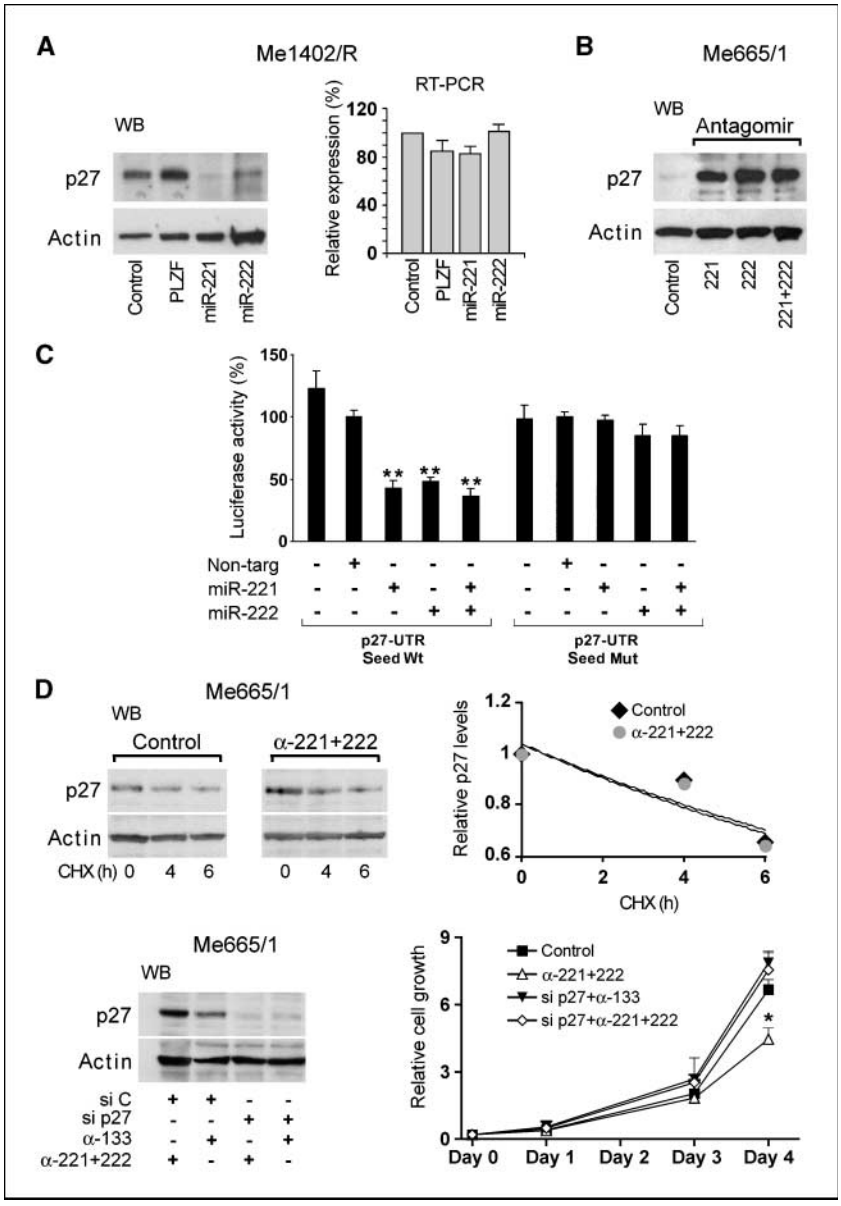

Figure 6. Analysis of miR-221 and miR-222 target genes: p27/CDKN1B. *A,* representative Western blot (*left*) and quantitative RT-PCR (*right*) analyses in Me1402/R control versus PLZF-, miR-221–, or 222–transduced cells. *B,* Western blot of antagomir-221– and/or antagomir-222–treated versus antagomir-133–treated (*control*) Me665/1 melanoma cells. Actin was used as internal control. *C,* luciferase reporter assay (*columns,* mean of minimum of 5 experiments per group; *bars,* SD) performed by cotransfecting miR-221 and/or miR-222 with a Luc reporter gene linked to p27 3′ UTR; mutated 3′ UTR sequence and a nontargeting oligomer were also included. **, $P < 0.001$. *D, top,* Me665/1 cells were transfected with either antagomir-133, as a control, or with antagomir-221+222 and treated with cycloheximide (*CHX*). Protein p27 and actin levels were evaluated at the indicated time points and the normalized amounts plotted as a regression plot. *Bottom,* Me665/1 cells were transfected with either siControl or sip27; after 24 h, cells were transfected again with antagomir-133, as a control, or with antagomir-221+222. Proteins from the different cell populations were analyzed for p27 levels, and cell proliferation was evaluated. *Wt,* wild-type; *Non-targ,* nontargeting.

inhibiting c-KIT and p27 translation, favors the induction of a malignant phenotype. Functional studies showed that the overexpression of miR-221 or miR-222 increased the proliferative growth rate, the invasion and migration capabilities, the anchorage-independent growth, and reduced differentiation and melanogenesis, all hallmarks of oncogenic progression. The induction of a more tumorigenic phenotype by miR-221/-222 was confirmed in the athymic nude mice model. More important, suppression of endogenous miR-221/-222 by treatment with antagomir oligonucleotides strongly reduced cell growth, invasion, chemotaxis, and foci formation *in vitro. In vivo*, melanoma xenotransplants showed impaired progression when treated with antagomir-221/-222 with respect to controls, at least for the week of observation before mice were killed for tumor histology.

All these findings relate to our previous results (15),[9] showing the lack of PLZF in melanomas and the induction of a less malignant phenotype upon its reexpression *in vitro* as well as *in vivo*. Gene expression profiling of PLZF-negative versus PLZF-positive melanomas showed that PLZF controls the expression of several genes involved in tumor progression. In particular, it down-regulates tumor-promoting genes, as *integrin* $\alpha_v\beta_3$ and *matrix metalloproteinase (MMP)-9*, and conversely induces genes favoring melanoma cell differentiation, as *c-KIT* and the downstream *MITF, TYR*, and *TRP-1*. We here show that the expression of these differentiation genes is miR-221/-222 dependent, shedding new light on our previous hypothesis, suggesting that PLZF down-regulates an "unknown" repressor (15). In fact, PLZF–miR-221/-222 pathway mediates the suppression of the differentiation-associated genes. Considering the down-regulation of MMP-2 and MMP-9 in PLZF-transduced melanomas, we searched for the involvement of these metalloproteases as one of the cause underlying the increased invasiveness of miR-221/-222–overexpressing cells. RT-PCR analysis confirmed a 2- to 3-fold increase of both *MMP-2* and *MMP-9* in miR-transduced Me1402/R and a parallel decrease

[9] Unpublished.

in antagomir-221+222–transfected Me665/1 cell line (data not shown).

The enhanced expression of miR-221/-222 in melanomas activates at least two important pathways governing cell proliferation and melanogenesis through p27 and c-KIT receptor regulation, respectively (Supplementary Fig. S4; Figs. 5 and 6).

p27 plays an important function in regulating progression through the cell cycle, from G_1 to S phase, by binding to CDK/CYCLIN complexes (25). Recently, cell cycle modulators have been shown to have a predictive and prognostic value in a 10-year melanoma follow-up (26); accordingly, p27 expression is progressively lost during progression from benign nevi to metastatic cells, and its reduction is associated with a poor survival (25, 27, 28). Although p27 is a recognized tumor suppressor, inactivating point mutations are rare and p27 protein levels are mostly regulated at posttranscriptional/posttranslational level. The principal p27 regulatory mechanisms include ubiquitin-dependent degradation by the proteasome (29), functional inactivation by mislocalization into the cytoplasm, or phosphorylation events controlling p27 binding to its cellular targets (30, 31). We propose that the miR-221/-222–based mechanism, blocking p27 translation, might represent an additional oncogenic mechanism underlying the abnormal cell cycle rate of advanced melanoma and of many other tumors. Accordingly, the knockdown of miR-221 and/or miR-222 increases p27 in PC3 prostate and U87 glioblastoma cell lines (11, 12).

The c-KIT receptor is a melanocytic multifunctional player regulating melanogenesis, cell growth, migration, and survival (14). This receptor is directly targeted by miR-221/-222 in normal erythropoiesis (13) and neoangiogenesis (32), as well as in papillary thyroid carcinoma (9). In the progression of human melanoma, the loss of c-KIT is a crucial event; up to 70% of metastases lack the receptor and can, as a consequence, escape SCF/c-KIT–triggered apoptosis (33). Looking at the downstream transduction pathway leading to melanogenesis and melanocytes differentiation, we found, as a secondary target gene of miR-221 and miR-222, *MITF*, a master lineage regulator in melanocyte maturation (23) controlling, among several other functions, the main melanogenic enzymes such as TYR and TRP-1. Recent results showed that MITF is apparently able to play both antiproliferative and proproliferative functions, depending on its expression level (34). Specifically, more differentiated melanomas express higher levels of MITF and exhibit less aggressive phenotypes, whereas intermediate amounts of MITF, as in melanomas analyzed here, induce proliferation (35–37). Depletion of MITF has been recently reported to increase p27 stability (37), whereas we observed a comparable expression pattern of MITF and p27 in the analyzed melanoma cell lines. This apparent divergence might be reconciled, considering the slight miR-dependent modulation of MITF in miR-221/-222–expressing cells (see Supplementary Fig. S3) compared with the abrogation of MITF using siRNA (37). p27 expression level might derive from a dynamic equilibrium that, at least in melanoma cell lines, seems to favor p27 inhibition, possibly because of miR-221/-222 amounts and kinetics. Moreover, we did not find any effect on p27 protein stability due to miR-221/-222 and downstream targets (including MITF). Finally, we point out that MITF is, on average, expressed at significantly lower level in melanomas than in melanocytes and that increased levels of MITF seem to reduce melanoma proliferation even in the presence of mutated *B-RAF* (38).

In conclusion, we showed that PLZF negatively regulates the expression of miR-221 and miR-222. In advanced melanomas, PLZF silencing up-modulates these two miRs that, in turn, activate at least two oncogenic pathways involved in melanoma progression, through p27 and c-KIT deregulation (Supplementary Fig. S4). In particular, p27 suppression operates in many tumors, such as pancreatic cancer, glioblastoma, thyroid carcinoma (8–11), and prostate cancer (12), whereas the tumorigenic action exerted through c-KIT down-regulation is shared by few other neoplasias (9). The most common mechanism of c-KIT–based oncogenesis is actually represented by activating mutations, as in gastrointestinal stromal tumors (39). The present studies may lead to new molecular therapies in advanced melanoma, which still lacks effective treatments (40). Although PLZF reexpression might represent a valuable tool in reducing the malignant phenotype (15), the intrinsic limitations of gene therapy render this approach difficult. Based on *in vitro* and *in vivo* results, we propose the inhibition of miR-221/-222 by antagomir treatment as a more attractive and safe approach for translation into the clinical setting.

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