miR-146a Exerts Differential Effects on Melanoma Growth and Metastatization

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Abstract

Malignant melanoma is the most aggressive form of skin cancer; therefore, it is crucial to disclose its underlying molecular mechanisms. MicroRNAs (miRNAs) are small endogenous non-coding RNAs able to posttranslationally downregulate the expression of direct target genes. Using a melanoma progression model, miR-146a was identified as a key double-acting player in melanoma malignancy. In fact, miR-146a is able to enhance tumor growth, while it suppresses dissemination. It was determined that miR-146a coordinated melanoma cell growth by its direct targets lunatic fringe (LFNG) and NUMB, which operate on the NOTCH1/PTEN/Akt pathway; while inhibition of metastasis formation was linked to decreased expression of ITGAV and ROCK1. Relevantly, miR-146a expression correlated with melanoma recurrence and was enriched in both patient-derived melanoma and cutaneous metastasis specimens, while its direct targets were depleted. However, miR-146a levels drop in circulating tumor cells (CTCs), suggesting the necessity for miR-146a expression to fluctuate during tumor progression in order to favor tumor growth and allow dissemination. This study reconciles the contradictory biologic functions of miR-146a in melanoma progression and unravels distinct molecular mechanisms that need to be considered for therapeutic interventions.

Implications: miR-146a controls melanoma progression in a dual way, promoting growth and inhibiting dissemination; however, it is poorly expressed in CTCs, resulting in overall tumor spreading and distant-site colonization. Mol Cancer Res; 14(6): 548–62. ©2016 AACR.

Introduction

Malignant melanoma is the most aggressive form of skin cancer and the sixth most common cancer in the United States; it accounts for only 4% of cases but for as many as 74% of all skin cancer deaths (1). Pathogenesis of melanoma is characterized by mutations of MAPK pathway components, N-RAS, B-RAF, PTEN and p53, while metastatic transition is accompanied by gain of functions of transcription factors like NF-kB and STATs and loss of TEFAP2 family members (2, 3). Given the complexity of networks and pathways involved, disclosing the molecular mechanisms underlying melanoma formation and progression is crucial to provide novel therapeutic strategies (4, 5). MicroRNAs (miRNAs) are small endogenous noncoding RNAs able to posttranscriptionally regulate the expression of target genes via sequence-specific interactions with the 3’ untranslated regions (UTR) of cognate mRNAs (6). Given that an individual miRNA is able to inhibit multiple distinct mRNAs, they act as fundamental regulators of tumor establishment and progression in different types of cancer, including melanoma (5). miRNAs can act either as oncogenes or as tumor suppressor genes. Among others, miR-7, miR-15b, miR-182, miR-19, miR-21, miR-192, miR-221/222, and miR-214 are upregulated during melanoma growth and invasion, while let-7b, miR-145, the miR-200 family, and miR-206 are downmodulated (4, 7–9).

In this study, we evidenced that miR-146a exerts a dual role in melanoma cells, favoring tumor cell growth, while inhibiting cell invasion. However, miR-146a expression is strictly modulated across the various steps of melanoma progression; it is high in both primary and distant-site tumors, where it promotes tumor cell growth, but it drops in circulating tumor cells (CTCs), therefore favoring the escaping from the primary tumor mass. Different players operate in the various moments of tumor progression: the targeting of lunatic fringe (LFNG) and NUMB and the subsequent induction of the NOTCH1/PTEN/Akt pathway triggers tumor growth, while the inhibition of ITGAV and ROCK1 is crucial to block cell invasion. Here, we present the molecular mechanisms coordinated by miR-146a during melanoma malignancy that need to be carefully considered for any therapeutic intervention.

Materials and Methods

Vectors, primers, antibodies, reagents, RNA, protein, and human melanoma samples analysis

Detailed information about the vectors, primers, and antibodies, RNA extraction, RNA and protein analysis methods...
used in this study is available in the Supplementary Materials and Methods section. miRNA precursors and inhibitors and assays for quantitative real-time PCR (qRT-PCR) detection were purchased from Applied Biosystems. Luciferase assays and immunohistochemistry were performed using the Dual-Luciferase Reporter System (Promega) or the Anti-Mouse HRP-DAB Cell and Tissue Staining Kit, R&D Systems kits, respectively. Melanoma samples were collected from the Department of Medical Sciences, University of Torino, Torino, Italy.

**In vitro** growth, adhesion, and migration assays

For proliferation and anchorage-independent growth assays, cells were plated in 96-well plates or in 6-cm bacterial dishes (mixed with agar), and growth was followed up to 5 or 21 days, respectively. For adhesion assays, cells were seeded onto 0.5 μg/mL Vitronectin V8379 (Sigma-Aldrich) precoated 96-well plates, and adhesion was assessed 1 hour later. To measure migration, cells were seeded in the upper chamber of Transwells with or without Matrigel (Becton Dickinson) and migration to the lower side was assessed 18 hours later. For trans-endothelial migration assays, Transwell inserts were covered with a human umbilical vein endothelial cell (HUVEC)-GFP cells monolayer.

**In vivo** tumor and metastasis and extravasation assays

For all, cells were subcutaneously injected in the flank or into the tail vein of mice; for long-term experiments, tumor and/or lung colonies growth was evaluated from 4 to 6 weeks later, while for extravasation assays lung seeding was assessed 48 hours later, as described in Penna, E. and Taverna, D. (2014). *In vivo* Extravasation Assay. Bio-protocol 4(4): e1051. http://www.bio-protocol.org/e1051.

Circulating tumor cells isolation

To isolate circulating tumor cells (CTCs), blood was collected from heart-punctured mice and put in culture plates with melanoma cells medium for 3 days; subsequently, attached cells were washed and cultured in fresh medium for at least 4 days. Puromycin was added to the medium to select human melanoma cells. Cells were then washed thoroughly to remove nonadherent cells, fixed with 2.5% glutaraldehyde and stained with 0.1% crystal violet or total RNA was isolated.

Ingenuity Pathway Analysis (IPA) system

The Ingenuity Pathway knowledge Base (http://www.ingenuity.com) is currently the world’s largest database of knowledge on biological networks, with annotations organized by experts. We exploited this database to look for Canonical Pathways and Gene Ontology functions characterizing miR-146a putative target genes.

Statistical analyses

Data are presented as mean ± standard error of the mean and two-tailed Student t test or one- or two-tailed Mann–Whitney test were used for comparison, with *, P < 0.05; **, P < 0.01; ***, P < 0.001 considered to be statistically significant. n.s. indicates a not statistically significant P value.

Results

miR-146a expression is upregulated during melanoma progression and enhances *in vitro* and *in vivo* cell and tumor growth

With the goal of identifying microRNAs involved in malignant melanoma progression, we performed miRNA profiling on a human melanoma cellular model consisting of a poorly aggressive B-RAF V600E-mutated parental cell line (A375P) and its four variants MA-1, MC-1, MA-2, and MC-2 derived from one (MA-1 and MC-1) or two (MA-2 and MC-2) subsequent rounds of injections in mice and showing progressively increased metastatic potential (10). Ninety-eight significantly downmodulated or upmodulated miRNAs have been identified when invasive (MA-1, MC-1, MA-2, and MC-2) versus noninvasive (A375P) cells were used (Supplementary Fig. S1 and Supplementary Table S1). Among the upregulated miRNAs, we focused on miR-146a and, with the intent to validate microarray results, we analyzed its expression in our melanoma model and in the unrelated B-RAF V600E-mutated 1300-Mel and SK-Mel-28 or B-RAF-wild-type WK-Mel melanoma cell lines by qRT-PCR analysis. Interestingly, only B-RAF–mutated cell lines showed high miR-146a expression (Fig. 1A). Relevantly, when patient-derived melanoma samples were evaluated, miR-146a expression changed accordingly, being low in *in situ* melanomas, while increasing in invasive samples as well as in cutaneous metastases (qRT-PCR, Fig. 1B). Here, it is relevant to refer to ref. 11 for melanoma classification, which considers thickness (mm), ulceration, and mitotic status as main parameters to distinguish more or less aggressive melanomas. Moreover, it is important to point out that the selection of the tumor areas was performed on hematoxylin and eosin (H&E) archival slides originally used for histopathologic diagnosis. The pathologist marked the areas of tumor enrichment on the slide from histologic specimens and recorded the percentage and absolute number of cancer cells. In this way, a similar number of tumor cells were evaluated for all kinds of tumors. Furthermore, when we explored the GSE62372 dataset (12), composed of 92 primary melanomas, we observed that miR-146a expression significantly correlated with tumor thickness and recurrence (Supplementary Fig. S2A–S2B).

Based on all the evidence, we aimed to test whether miR-146a involvement in cell and tumor growth; therefore, we silenced miR-146a in MA-2 cells by stable lentiviral transduction of miR-146a-specific antisense sponges, compared with empty vectors (pLentiGFP-sponge or -EV), or in MC-1 cells by transient transfection of miR-146a antisense inhibitors compared with negative controls (anti-146a or anti-Cntrl). Sponge efficacy was evaluated, as shown in Supplementary Fig. S3. Conversely, we induced miR-146a expression in MA-2 and 1300-Mel cells by stable lentiviral transduction of miR-146a (pLemIR-146a), or upon transient transfections of pre-miR-146a (pre-146a), compared with empty controls (pLemIR-EV, pre-Cntrl). The efficacy of miR-146a modulations was tested by qRT-PCR (Supplementary Fig. S4A–S4E). Interestingly, miR-146a stable or transient downmodulation (pLentiGFP-sponge and anti-146a) in MA-2 and MC-1 cells strongly impaired cell proliferation up to 5 days as well as cell anchorage-independent growth in soft agar, *in vitro* (Fig. 1C and D, a–b and Supplementary Fig. S5A, top, and Supplementary Fig. S5B, a–b). Conversely, miR-146a overexpression (pLemIR-146a and pre-146a) in MA-2 and 1300-Mel cells reverted these effects, as it increased both growth rate...
Figure 1.
miR-146a expression is upregulated during melanoma progression and enhances in vitro and in vivo cell and tumor growth. A, miR-146a expression in A375P, MA-1, MC-1, MA-2, MC-2, 1300-Mel, Sk-Mel-28, and WK-Mel cells was assessed by qRT-PCR. Results are shown as fold changes (mean ± SEM) relative to A375P cells, normalized on U44 RNA levels. (Continued on the following page.)
and colony-forming ability (Fig. 1C and D, c–d and Supplementary Fig. S5A, bottom). Relevantly, we observed that miR-146a affects cell proliferation by enhancing growth rather than inhibiting cell death; in fact, it resulted irrelevant in cell apoptosis and anoikis studies (Supplementary Fig. S5C). More importantly, miR-146a inhibition strongly impaired primary tumor growth, as assessed by subcutaneous injections of miR-146a-modulated MA-2 cells into the flank of NSG mice; compared with control cells, miR-146a–downmodulated (pLentiGFP-sponge) tumors resulted significantly smaller, 5 weeks after injection. Instead, miR-146a–overexpressing (pLe-miR-146a) cells generated bigger tumor masses than empty controls (Fig. 1E). Moreover, proliferating cell nuclear antigen (PCNA) staining of primary tumors and distant lung colonies (5 weeks after subcutaneous injections of MA-2 cells) revealed that miR-146a–downmodulated cells were less able to proliferate in vivo, both in primary and in disseminated masses, compared with controls (pLentiGFP-sponge vs. -EV; Fig. 1F, a–b and c–d). In conclusion, all these results reveal the crucial involvement of miR-146a in primary melanoma and distant metastasis growth as a proproliferative player.

miR-146a inhibits cell extravasation and metastasis formation in vivo and is poorly expressed in CTCs

Due to the fact that miR-146a is enriched during melanoma progression, we investigated its possible involvement in metastatic traits, such as cell motility, migration, and distant-site colonization. miR-146a stable (pLentiGFP-sponge) or transient (anti-146a) downmodulation in MA-2 and MC-1 cells led to a 30% to 50% increase in migrational and invasion compared with controls, as evaluated by in vitro Transwell assays in the presence or absence of Matrigel. Instead, transient (pre-146a) or stable (pLemiR-146a) overexpression in 1300-Mel, MA-2, SK-Mel-28, and WK-Mel cells reduced cell migration and invasion of about 20% to 50% compared with controls (Fig. 2A and B and Fig. S6A–S6C). miR-146a level modulation was always assessed, as in Supplementary Fig. S4. Considering that malignant cells have to detach from the primary tumor mass, enter and survive in the blood vessels and then extravasate, we evaluated miR-146a involvement in an in vitro transendothelial migration assay. For this purpose, we seeded CMRA-labeled (red) miR-146a–overexpressing or control MA-2 or WK-Mel cells in the upper chambers of fibronectin-coated Transwells, covered by a confluent GFP-transduced HUVEC monolayer, and evaluated the endothelium remodeling and melanoma cells migration (Fig. 2C; Supplementary Fig. S6D). In the absence of melanoma cells (a) the HUVECs monolayer remained intact. Notably, in the presence of miR-146a–overexpressing (pre-146a) MA-2 or WK-Mel cells, the endothelial monolayer was more compact, compared with controls (pre-Cntrl, b–c) and accordingly, miR-146a–overexpressing cells showed a 50% to 60% reduction in their ability to migrate through the endothelial layer (d–e). miR-146a’s ability to inhibit cell extravasation was reproduced in vivo, where miR-146a–overexpressing cells (pre-146a) extravasated poorly from the lung vessels to the tissue parenchyma 48 hours after tail-vein injections in CD1 nude mice, compared with control cells (pre-Cntrl; Fig. 2D, e–f). Equal lodging of the lungs was assessed 2 hours after injections (Fig. 2D, c–d). Intravascular localization of cells at 2 hours, and subsequent extravasation at 48 hours was proved by immunofluorescence analysis (Fig. 2D, a–b and Supplementary Fig. S6E). miR-146a–dependent impairment of cell extravasation resulted crucial in the inhibition of metastasis formation. In fact, as shown in Fig. 2E, a–b, miR-146a–overexpressing cells (pWPT-146a) generated a significant lower number of lung metastases compared with controls (pWPT-EV), 5 weeks after injections into the tail vein of immunocompromised mice. Efficient miR-146a overexpression was verified (Supplementary Fig. S4I). Furthermore, miR-146a was found to affect cell dissemination from primary tumors: miR-146a–downmodulated (pLentiGFP-sponge) MA-2 cells injected into the flank of NSG mice were more proficient in the dissemination from tumors to lungs, compared with controls (pLentiGFP-EV; Fig. 2E, c–d, Supplementary Fig. S7). On the contrary, lung colonization was impaired in miR-146a–overexpressing cells (pLemiR-146a vs. -EV), as shown in Fig. 2E, e–f. Taken together, all our experiments demonstrate that miR-146a favors tumor and metastasis growth but it impairs cell dissemination. Aiming to understand the dual and apparently contradictory role of miR-146a, we investigated its expression in CTCs. For this purpose, we collected CTCs from immunosuppressed mice 5 weeks after subcutaneous injections of MA-2 cells and evaluated miR-146a expression compared with primary tumors and lung metastases by qRT-PCR. Human CTCs viability was proven to be comparable with control cells, as assessed by the measurements of the survival markers BAX and BCL2 (qRT-PCR), while murine white blood cells contamination was excluded by the analysis of PITPRC1, a specific bone marrow marker (qRT-PCR; Supplementary Fig. S8A and S8B). As in Fig. 2F, miR-146a levels resulted very low in CTCs while high both in tumors and in disseminated lung colonies. Concomitantly, two other miRNAs involved in melanoma metastatization, miR-21 and miR-214, were unchanged (Supplementary Fig. S8C), indicating that miR-146a downmodulation is a specific trait of escaping tumor cells. In fact, we hypothesize that primary tumors are heterogeneous and contain cells with high or low levels of miR-146a at different percentages. While cells
expressing high levels of miR-146a have an advantage in growth, miR-146a-depleted ones show increased migration/invasion and extravasation and are responsible for distant metastasis formation. Supporting our hypothesis is the fact that we observed the formation of a significantly higher number of colonies when CTCs were derived from mice bearing miR-146a-downmodulated tumors (pLentiGFP-sponge), compared with controls (pLentiGFP-EV), as shown in Fig. 2G.

miR-146a affects different tumor progression-related genes and pathways

Because miRNAs biologic functions are exerted through negative regulation of cognate target genes, we sought for miR-146a targets potentially involved in melanoma establishment and spreading by using TargetScan 6.1 (13) to predict its targets. In this way, we identified 223 conserved predicted genes containing one or more putative binding sites for miR-146a (Supplementary Table S2), later on used to identify miR-146a-altered pathways and functions by employing the IPA software (www.ingenuity.com). Notably, the most statistically significant Canonical Pathway that emerged was Notch Signaling, where 3 out of 38 genes in the pathway were putative miR-146a targets (NUMB, LFNG, and NOTCH2; P = 6.77e−03; Supplementary Fig. S9A). NUMB is an adaptor protein that triggers NOTCH degradation (14), while lunatic fringe (LFNG) is a beta-1,3-N-acetylgalcosaminyltransferase that leads to elongation of O-linked fucose residues on NOTCH extracellular domain, causing inhibition of signaling by the Serratex/JAGGED class of ligands (15). Instead, NOTCH2 is highly expressed in human melanomas and favors tumor formation and invasion (16), making its targeting by miR-146a unlikely. On the other hand, searching for putative targets involved in tumor invasion, IPA identified 22 candidate genes (Supplementary Fig. S9B); among them, NUMB and integrin alpha-V (ITGAV) are key players in tumor cell invasion (17). Interestingly, ROCK1 is targeted by miR-146a in prostate cancer cells, thereby inhibiting cell proliferation and invasion (18), suggesting that this gene could be a fundamental driver of melanoma cell spreading. With all that in mind, we focused on the role of LFNG, NUMB, ITGAV, and ROCK1.

miR-146a stable (pLentiGFP-sponge) or transient (anti-146a) inhibition in MA-2 and 1300-Mel cells (efficient downmodulation was verified in Supplementary Fig. S4) led to increased LFNG, ROCK1, ITGAV, and NUMB protein expression in a range going from +15% to +300% compared with controls; conversely, transient miR-146a overexpression (pre-146a) reduced protein levels of LFNG, ROCK1, ITGAV, and NUMB by about 15% to 75%, 48 hours after transfection compared with controls, as assessed by Western blot (WB) analysis in Fig. 3A; Supplementary Fig. S10A. mRNA levels of these genes were also decreased by about 50% to 70% compared with controls, as assessed by qRT-PCR, 48 hours after transient miR-146a overexpression in MA-2 cells (pre-146a compared with pre-Cntrl, Fig. 3B), while miR-146a downmodulation in MA-2 cells led to opposite effects (Supplementary Fig. S10B). Notably, analysis of 352 melanoma metastatic samples in The Cancer Genome Atlas (TCGA) dataset (http://cancergenome.nih.gov/) showed a significant negative correlation between miR-146a and LFNG, ROCK1, ITGAV, and NUMB expression (Supplementary Fig. S11), suggesting the relevance of miR-146a and its targets in melanoma dissemination. To assess if LFNG, ITGAV, ROCK1, and NUMB fluctuations in miR-146a–modulated cells directly depend on the binding between miR-146a seed and the complementary sequence present on the 3′-UTRs of these genes, their 3′-UTRs were cloned into reporter vectors and luciferase assays performed in MA-2 cells transfected with miR-146a precursors or negative controls (pre-146a or pre-Cntrl). As shown in Fig. 3C, miR-146a significantly impaired luciferase expression in the presence of LFNG, ITGAV, ROCK1, and NUMB 3′-UTRs. Being completely novel target genes, miR-146a specificity on repression of LFNG and ITGAV was further validated by inserting three point mutations into their 3′-UTR miR-146a complementary sequences (LFNG-mut, ITGAV-mut). Binding site mutations completely abrogated miR-146a–dependent inhibition of luciferase expression. Strong evidence of the relevance of LFNG, ROCK1, and NUMB...
in melanoma progression came from qRT-PCR analysis of their expression in patient-derived samples: all the genes showed less expression in invasive human melanomas and in cutaneous metastases than in in situ tumors (Fig. 3D). Instead, ITGAV was not found statistically significant here (Fig. 3D), maybe because not enough samples were analyzed.
miR-146a controls cell and tumor growth, but not cell movement, through LFNG and NUMB

To characterize LFNG and NUMB involvement in miR-146a–driven phenotypes, MA-2, 1300-Mel and WK-Mel cells were transiently transfected either with a control or a LFNG or/and NUMB specific siRNAs (siRNA-Cntrl, siRNA-LFNG, or siRNA-NUMB or siRNA-LFNG + siRNA-NUMB), leading to efficient silencing of LFNG and NUMB expression, as verified by qRT-PCR and WB analysis (Supplementary Fig. S12A–S1C and S13A). Downmodulation of NUMB and mainly of LFNG increased
in vitro cell proliferation up to 4 days in culture, if compared with controls, resembling miR-146a overexpression (Fig. 4A; Supplementary Fig. S14A, black triangles or black squares vs. black circles). Concomitant downmodulation of NUMB increased cell growth at a higher extent, suggesting an additive role for LFNG and NUMB in the regulation of cell proliferation (Fig. 4A; Supplementary Fig. S14A, white squares and dashed line). Furthermore, as depicted in Fig. 4B, LFNG transient downmodulation (siRNA-LFNG, b) in MA-2 cells increased colony formation in soft-agar assays, compared with controls (siRNA-Cntrl, a). Most relevantly, LFNG plus NUMB involvement in in vivo tumor growth was assessed upon subcutaneous injections in the flank of immunocompromised mice of MA-2 cells stably downmodulated for LFNG + NUMB (pLKO-shLFNG + shNUMB) or LFNG only (pLKO-shLFNG) or NUMB only (pLKO-shNUMB) and compared with controls (pLKO-EV) or pLKO-EV + EV or pLKO-EV, as verified by qRT-PCR and WB in Supplementary Fig. S12D and S13B. 4 weeks after injections, LFNG and NUMB-downmodulated cells, in combination or as single modulations, generated significantly bigger tumors compared with all controls (Fig. 4C), phenocopying miR-146a overexpression. Notably, even if the effect of LFNG silencing was more pronounced compared with NUMB silencing, an additive effect in tumor growth was not observed for LFNG + NUMB-downmodulated cells, suggesting that, in vivo, LFNG acts as the main player.

Because NUMB was depicted by IPA and by the literature as a cell invasion–related molecule, we investigated if miR-146a-dependent targeting of NUMB plus LFNG could inhibit melanoma progression. However, in vitro Transwell and transendothelial migration of MA-2 cells through a HUVEC monolayer was completely unaffected by LFNG + NUMB co-downmodulation (siRNA-LFNG + siRNA-NUMB), compared with controls (siRNA-Cntrl), as shown in Fig. 4D and E. Furthermore, NUMB does not affect in vivo cell extravasation, as evaluated 48 hours after tail-vein injection into immunocompromised mice of NUMB-downmodulated cells (siRNA-NUMB, compared with siRNA-Cntrl), or lung metastasis formation, 6 weeks after tail-vein injection into mice of NUMB-overexpressing MA-2 cells (pLenti-NUMB) compared with controls (pLenti-EV; Supplementary Fig. S14B and C). NUMB cDNA efficient overexpression was verified in Supplementary Fig. S13D. Collectively, we evidenced that two NOTCH pathway genes, LFNG and NUMB, are pivotal in miR-146a–driven enhancement of cell growth and primary tumor formation, considering LFNG as the main player. However, these two genes are irrelevant for cell invasion and metastatization.

miR-146a–dependent activation of NOTCH1 via LFNG + NUMB induces cell growth through the Akt/PTEN pathway. Because LFNG + NUMB modulation in melanoma cells affects tumor growth, we investigated whether miR-146a can activate NOTCH signaling in melanoma cells through the double action of these two players. Reporter assays performed using a NOTCH1-dependent promoter located upstream of a luciferase cassette confirmed that miR-146a overexpression (pre-146a) in MA-2 cells enhances luciferase expression of about fourfold compared with control (pre-Cntrl), as shown in Fig. 5A, light gray bars. LFNG or NUMB downmodulation (siRNA-LFNG or siRNA-NUMB) increased luciferase expression to the same extent, compared with control (siRNA-Cntrl). Interestingly, LFNG plus NUMB codownmodulation (siRNA-LFNG + siRNA-NUMB) strongly induced NOTCH activity, to a level comparable with miR-146a alone, revealing again an additive effect of the double gene targeting (Fig. 5A, dark gray bars). NOTCH1 constitutively active cleaved form (pCNA-hNIC, overexpression verified in Supplementary Fig. S13E) was used as a positive control (patterned bars). Furthermore, NOTCH1 transcriptional targets hairy and enhancer of Split (HES)-1 and -5, and Cyclin D1 (CCND1) were found strongly induced in MA-2 cells upon miR-146a overexpression (pre-146a vs. pre-Cntrl), or significantly reduced by miR-146a inhibition (anti-146a vs. anti-Cntrl); accordingly, their expression also increased upon LFNG or NUMB stable downmodulation (pLKO-shLFNG or pLKO-shNUMB, compared with pLKO-EV), but the effect of LFNG inhibition was stronger, as assessed by qRT-PCR (Fig. 5B; Supplementary Fig. S15A), NOTCH1 and Cyclin D1 protein modulations were assessed by WB in Supplementary Fig. S15B. Based on this evidence, we asked

Figure 5.

miR-146a–dependent activation of NOTCH1 via LFNG + NUMB induces cell growth through the Akt/PTEN pathway. A, luciferase assays were performed in MA-2 cells cotransfected with a vector containing a NOTCH1-dependent promoter cloned upstream of the luciferase coding sequence, together with miR-146a precursors or negative controls (pre-146a or pre-Cntrl), LFNG, and/or NUMB siRNAs or controls (siRNA-LFNG, siRNA-NUMB, siRNA-LFNG + siRNA-NUMB, and siRNA-Cntrl), or Notch intracellular domain–overexpressing or empty vectors (pCNA-NIC or -EV). Results are shown as mean ± SEM of Firefly luciferase activity relative to controls, normalized on Renilla luciferase activity. Three independent experiments were performed and representative results are shown. B, HES1 and Cyclin D1 (CCND1) mRNA levels were measured by qRT-PCR in MA-2 cells 48 hours after transfection with miR-146a precursors or inhibitors or their negative controls (pre- and anti-146a or control), or in MA-2 cells stably transduced with LFNG or NUMB shRNAs, compared with empty vectors (pLKO-shLFNG and pLKO-shNUMB vs. pLKO-EV). Results are shown as fold changes (mean ± SEM) relative to controls, normalized on GAPDH mRNA levels. Three experiments with independent RNA preparations were performed and representative results are shown. C, in vitro proliferation assays were performed in MA-2 cells starting from 12 hours after transfection with NOTCH1 or HES1 siRNAs, compared with controls (siRNA-NOTCH1, siRNA-HES1 vs. siRNA-Cntrl). Results are shown as mean ± SEM of the optical density (OD); three independent experiments were performed in sextuplicate and representative results are shown. D, Akt phosphorylation status at Thr308 or Ser473 was assessed in MA-2, WK-Mel, and 1300-Mel cells 48 hours after transfection with miR-146a precursors (pre-146a or pre-Cntrl), or in MA-2 cells stably transduced with miR-146a–overexpressing or control lentivirus vectors (pLemir-146a or -EV). Phosphorylated protein modulations were calculated relative to controls, normalized on the total amount of proteins and on hsp90 or alpha-tubulin loading controls and expressed as percentages. E and F, PTEN protein and mRNA levels were assessed by WB (E) and qRT-PCR (F) in MA-2 and 1300-Mel cells 48 hours after transfection with miR-146a precursors or inhibitors or their negative controls (pre- and anti-146a or control), upon stable miR-146a overexpression or empty controls (pLemir-146a or -EV) or 48 hours after LFNG or NUMB transient downmodulation (siRNA-LFNG or siRNA-NUMB vs. siRNA-Cntrl). Protein modulations in E were calculated relative to controls, normalized on hsp90 or alpha-tubulin loading controls and expressed as percentages; results in F are shown as fold changes (mean ± SEM) relative to controls, normalized on GAPDH mRNA levels. Three experiments with independent protein and RNA preparations were performed and representative results are shown. G, in vitro proliferation was assessed in MA-2 cells from 12 hours after transfection with miR-146a precursors or controls (pre-146a vs. pre-Cntrl), combined with a daily treatment with the Calbiochem Akt inhibitor VIII 62847-09-3 (Akti) or controls (dimethyl sulfoxide, DMSO). Results are shown as mean ± SEM of the optical density (OD); three independent experiments were performed in triplicate, and representative results are shown. The two-tailed Student t test was used for statistical analyses considering *P < 0.05; **P < 0.01; ***P < 0.001; ns, no significant difference.

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miR-146a-dependent downregulation of ITGAV and ROCK1 affects cell motility, but not cell growth. A, *in vitro* proliferation of ROCK1-downmodulated (siRNA-ROCK1) or control (siRNA-Cntrl) MA-2 cells was evaluated starting from 12 hours after transfection. Results are shown as mean ± SEM of the area occupied by colonies, or of the optical density (OD); three independent experiments were performed, and representative results are shown. B, Transwell migration or Matrigel invasion was assayed 24 hours after transfection of MA-2 cells with ROCK1 siRNAs or their negative controls (siRNA-ROCK1 vs. siRNA-Cntrl). Results are shown as mean ± SEM of the area covered by migrated or invaded cells. Three independent experiments were performed in triplicate, and representative results are shown. C, Transendothelial migration was mimicked *in vitro* by analyzing the migration of CMRA-labeled (red) MA-2 cells through a fibronectin-coated Transwell membrane covered by a confluent monolayer of HUVEC-GFP. (Continued on the following page.)
whether miR-146a–dependent activation of NOTCH1 is crucial in promoting melanoma cell growth. In vitro proliferation was evaluated for MA-2 cells transiently downmodulated for NOTCH1 or HES1 (siRNA-NOTCH1 and siRNA-HES1), and, interestingly, inhibition of these two genes reduced cell growth, if compared with controls (siRNA-Cntrl) up to 5 days in culture, opposing miR-146a–driven phenotype (Fig. 5C). Efficient NOTCH1 and HES1 downmodulation after siRNA transfection was evaluated by qRT-PCR (Supplementary Fig. S12E-F). In vitro transendothelial migration of NOTCH1-downmodulated cells (siRNA-NOTCH1 vs. siRNA-Cntrl) was also performed, and results confirmed that the alteration of this pathway is not related to cell migration (Supplementary Fig. S15C).

Given the important role of the NOTCH1/HES1 axis in miR-146a–dependent control of cell growth, we investigated the molecular pathways responsible for these phenotypes. Interestingly, Palomero and colleagues demonstrated that, in a NOTCH1-induced T-cell acute lymphoblastic leukemia (T-ALL) model, HES1 activation leads to reduced PTEN transcription and expression, resulting in increased phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway activation and cell proliferation (19). In the same way, we found that miR-146a expression in melanoma cells affects Akt phosphorylation, as shown by WB analysis in Fig. 5D: 24 hours after transfection, miR-146a transient overexpression in MA-2 cells (pre-146a) increased Akt phosphorylation (50% and 30%, respectively) on Thr308 and Ser473, compared with controls (pre-Cntrl), while stable expression of miR-146a in MA-2 cells (pLemiR-146a) induced a 65% increase of Thr380 phosphorylation, compared with controls (pLemiR-EV). The same effect was described upon miR-146a transient overexpression in WK-Mel and 1300-Mel cells. Consistently, miR-146a transient (pre-146a) or stable (pLemiR-146a) overexpression in MA-2, 1300-Mel, and WK-Mel cells reduced PTEN protein and mRNA levels (at least 20%), compared with controls (pre-Cntrl or pLemiR-EV), respectively, while miR-146a transient downmodulation led to a 40% increase in protein expression, as assessed by WB and qRT-PCR (Fig. 5E and F and Supplementary Fig. S15D). Furthermore, LFNG and NUMB transient overmodulation in MA-2 cells (siRNA-LFNG or siRNA-NUMB, vs. siRNA-Cntrl) reduced PTEN mRNA amount by about 50% (Fig. 5F) and either HES1 or NOTCH1 transient downmodulation (siRNA-HES1 and siRNA-NOTCH1, vs. siRNA-Cntrl) increased PTEN protein expression (11% and 16%, respectively), compared with controls (siRNA-Cntrl), as shown by WB analysis in Supplementary Fig. S15E. All the evidence indicates that miR-146a–dependent inhibition of LFNG and NUMB and the subsequent activation of NOTCH1 and HES1 are responsible for PTEN downmodulation and Akt increased phosphorylation. To understand if this signaling is important for cell growth, miR-146a–overexpressing MA-2, 1300-Mel, and WK-Mel cells were treated with low concentrations (0.5 μmol/L) of an Akt inhibitor (612847-09-3), in order to restore the Akt phosphorylation status of control cells (efficacy was verified in Supplementary Fig. S13F), and analyzed for in vitro proliferation. As shown in Fig. 5G and Supplementary Fig. S15F, the proliferative advantage of miR-146a–overexpressing MA-2, 1300-Mel, and WK-Mel cells (pre-146a + Akti, black rhombi) was completely inhibited up to 5 days in culture, compared with nontreated cells (pre-146a + DMSO, white triangles). Importantly, this effect specifically occurs in miR-146a–overexpressing cells, because proliferation of control cells was poorly affected (pre-Cntrl + Akti, white squares and dashed line, and pre-Cntrl + DMSO, black triangles). This evidence indicates that miR-146a–dependent increased growth is due mainly to Akt pathway activation, making the inhibition of the specific miR-146a/Akt pathway potentially relevant for therapy. Moreover, in line with the above presented results, Akt inhibition (up to 5 μmol/L) in miR-146a-overexpressing cells (pre-146a + Akti) did not affect either cell migration or invasion in vitro (Supplementary Fig. S15G), further demonstrating that miR-146a effect on cell movement does not involve NOTCH1 pathway modulation.

miR-146a–dependent downregulation of ITGAV and ROCK1 affects cell motility, but not cell growth

Because integrins are the main coordinators of tumor dissemination, and ITGAV as well as the integrin downstream player, ROCK1, are modulated by miR-146a (Fig. 3A and B; Supplementary Fig. S10), we evaluated the role of ROCK1 and ITGAV in cell adhesion/motility. We first downmodulated ROCK1 with a specific siRNA (whose efficacy was evaluated in Supplementary Figs. S12G and S13C) and observed no effect on in vitro cell proliferation, up to 4 days in culture (siRNA-ROCK, compared with siRNA-Cntrl), as shown in Fig. 6A. However, a 25% to 50% reduction in migration and invasion through Matrigel for MA-2, 1300-Mel, and WK-Mel cells, as evaluated by in vitro Transwell assays (siRNA-ROCK1 vs. siRNA-Cntrl, Fig. 6B; and Supplementary Fig. S16A). Moreover, a less pronounced remodeling (b and c) and an 80% inhibition of transendothelial migration (d and e) through a HUVEC monolayer was found (siRNA-ROCK1 vs. siRNA-Cntrl; Fig. 6C; Supplementary Fig. S16B). This in vitro evidence was further supported by an in vivo extravasation experiment, where ROCK1-downmodulated cells (siRNA-ROCK1) showed a significantly reduced ability to colonize the lung parenchyma, compared with controls (siRNA-Cntrl), 48 hours after tail-vein injections into immunocompromised mice (Fig. 6D, c–d). Equal lung lodging was verified 2 hours after injection (a–b). Furthermore, we analyzed the role of ITGAV in the miR-146a cascade involved in tumor dissemination by performing an in vitro adhesion assay on vironectin, the glycoprotein specific for the αvβ3 integrin heterodimer binding. Relevantly, we found that miR-146a–overexpressing MA-2, 1300-Mel, and WK-Mel cells (pre-146a) showed significant reduction (~30%) of vironectin adhesion, compared with controls (pre-Cntrl), 1 hour after plating (Fig. 6E; Supplementary Fig. S16C), suggesting the importance of ITGAV downmodulation in
disseminate. This hypothesis is also supported by the fact that coexist, and these are the cells that can easily escape and

However, most probably, cells that poorly express miR-146a expression. These

...ination of melanoma cells and that it affects tumor cells ability to somehow restored in distant-site colonies to favor metastatic

...ation. Concomitantly, we found that miR-146a high expression impairs migration and invasion as well as metastatic dissemination of melanoma cells and that it affects tumor cells ability to cross the vessels. In line, as anticipated above, we evidenced low miR-146a expression in CTCs, compared with tumor or metastatic masses. These results, together with the evidence that miR-146a does not affect melanoma cell survival and anoikis, let us to hypothesize that miR-146 levels need to be low when melanoma cells detach from the primary tumor mass and disseminate, in order to favor cell spreading. miR-146a expression is then somehow restored in distant-site colonies to favor metastatic growth. Putting all these data together, we believe that cell populations with different miR-146a expression are needed to obtain melanoma dissemination.

Taking advantage of bioinformatics predictions and functional analyses, we identified different relevant candidate target genes that could account for miR-146a-driven phenotypes. Looking for tumor growth-related pathways, we observed that the NOTCH pathway was significantly enriched, including three putative targets genes, LFNG, NUMB, and NOTCH2. miR-146a-dependent inhibition of NUMB and subsequent activation of the NOTCH pathway has been characterized before (23). LFNG is a O-fucose-1,3-N-acetylgalactosaminyltransferase able to glycosylate epidermal growth factor (EGF)-like repeats on the extracellular domain of NOTCH, thereby enhancing DELTA-mediated and inhibiting JAGGED-mediated NOTCH activation (33, 34). JAGGED1-dependent NOTCH activation is crucial for colon and prostate cancer cells proliferation and migration (35, 36). Zhang and colleagues demonstrated that loss of LFNG alters NOTCH signaling in the prostate, leading to intraepithelial neoplasia (37) and Xu and colleagues described that the mammary-specific deletion of Lfng induces basal-like breast cancer facilitating JAGGED/NOTCH signaling (38). Relevantly, we observed that LFNG expression is significantly lower in human invasive

Figure 7.
Summary of the results presented in this work.

miR-146a–driven impairment of cell invasion and metasatization. Taken together, these experiments suggest that the miR-146a–dependent ITGAV and ROCK1 modulations are relevant events for melanoma dissemination.

In summary, we showed that miR-146a exerts a complex role in melanoma: its expression varies through the different steps of progression, where it promotes growth but it impairs cell dissemination by targeting different and unrelated genes and pathways. Disclosing this dualism is crucial to identify new, specific, melanoma therapeutic treatments (Fig. 7).

**Discussion**

In this work, we present a new mechanism by which a small noncoding RNA, miR-146a, regulates melanoma establishment and dissemination by two parallel and opposite interventions. We proved that while high levels of miR-146a have been found in malignant melanomas and are able to promote tumor growth, at the same time, miR-146a impairs tumor cell dissemination. miR-146a opposite activities are obtained by the targeting of different genes. miR-146a direct targeting of LFNG and NUMB leads to NOTCH1 signaling activation, which coincides with increased levels of HES1, HES5, Cyclin D1 and p-Akt, decreased PTEN expression and tumor growth gain. Instead, silencing of ROCK1 and ITGAV by miR-146a favors tumor cell progression, where it promotes growth but it impairs cell dissemination. miR-146a direct targeting of LFNG and NUMB leads to NOTCH1 signaling activation, which coincides with increased levels of HES1, HES5, Cyclin D1 and p-Akt, decreased PTEN expression and tumor growth gain. Instead, silencing of ROCK1 and ITGAV by miR-146a favors tumor cell progression, where it promotes growth but it impairs cell dissemination.

Based on the above-listed findings, considering the relevance of miR-146a deregulation in tumor progression, we investigated the intervention of miR-146a in melanoma, an unfortunately still fatal neoplasia. We found that miR-146a is highly expressed in invasive human melanomas as well as in cutaneous metastases, in line with other studies (32), and that its expression correlates with tumor thickness and recurrence. Using human melanoma cells, we evidenced the protumorigenic role of miR-146a, leading to increased cell proliferation, anchorage-independent growth and primary tumor formation in mice. Interestingly, cell proliferation is favored also in distant-site metastases. Concomitantly, we found that miR-146a high expression impairs migration and invasion as well as metastatic dissemination of melanoma cells and that it affects tumor cells ability to cross the vessels. In line, as anticipated above, we evidenced low miR-146a expression in CTCs, compared with tumor or metastatic masses. These results, together with the evidence that miR-146a does not affect melanoma cell survival and anoikis, let us to hypothesize that miR-146 levels need to be low when melanoma cells detach from the primary tumor mass and disseminate, in order to favor cell spreading.

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melanomas and in cutaneous metastases, compared with in situ, showing an opposite expression than miR-146a. Accordingly, LFNG and NUMB targeting is crucial to promote cell proliferation and primary tumor growth, leading to increased NOTCH1 and HES1 expression with a strong impact on melanoma cells growth, as well as Cyclin D1 induction, which may play a role as well, considering that it drives melanoma formation (39). miR-146a-dependent activation of NOTCH1/HES1 leads to reduced PTEN expression and increased Akt phosphorylation, which drives cell growth. Activation of this pathway may also affect other features of tumor cells, like glucose intake, cell survival and protection from apoptosis (40), making the spectrum of miR-146a-dependent effects even broader.

If cell growth is driven through the activation of the NOTCH pathway, we demonstrated that cell movement and metastasis formation are independently regulated by miR-146a. Neither LFNG nor NUMB did affect melanoma cell migration and invasion in vitro, while, interestingly, two players of the integrin signaling, ITGAV and ROCK1, are affected by miR-146a. ROCK1 mediates “amoeboid,” rounded cell migration, while ITGAV is involved in “mesenchymal-like” invasion, characterized by cell polarization and membrane protruding, two invasion strategies used by melanoma cells during dissemination (41). ROCK1 is poorly expressed in invasive human melanomas and cutaneous metastases, whereas miR-146a is enriched. ROCK1 downmodulation inhibits in vitro invasion and in vivo extravasation (48 hours) of melanoma cells, resembling miR-146a-dependent effect, making it a crucial gene driving cell movement. Regarding ITGAV/miR-146a function, forced miR-146a expression in melanoma cells impairs their ability to adhere to vitronectin, the major integrin αvβ3 receptor (42). Taken together, our results indicate that miR-146a affects melanoma cell migration in multiple ways, impairing the two major strategies for invasiveness. Considering that miR146a is poorly expressed in invading melanoma cells in the blood circulation, it is likely that subsequent activation of the invasion molecules ITGAV and ROCK1 may drive efficient cell spreading.

In conclusion, we identified and characterized the multiple, antithetic roles of miR-146a in melanoma progression; in fact, while miR-146a favors tumor growth, it impairs cell metastatization and we propose that melanoma cells overcome this dualism by strongly expressing miR-146a in tumors and metastases and reducing it during invasion, resulting in ultimate efficient growth and spreading. Considering that we and others described that B-RAF V600E-bearing (but not B-RAF wild-type) aggressive melanoma cell lines show high miR-146a expression, and that miR-146a is activated in melanoma cell lines via B-RAF-dependent activation of MYC (23), it is possible that this pathway mediates miR-146a expression regulation in human tumors. Other regulators have also been described, such as NF-κB (43), V-Ets oncogene homolog 1 (ETS1; ref. 44), as well as epigenetics (45) and feedback (43) mechanisms. Autoregulation could also occur during melanoma progression; this would make the scenario even more complex if one considers miR-146a for therapeutic interventions in melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Raimo, F. Orso, E. Calautti, D. Taverna

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Orso, D. Cimino, L. Primo, P. Quaglino

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Writing, review, and/or revision of the manuscript: M. Raimo, F. Orso, M.B. Stadler, P. Quaglino, D. Taverna

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miR-146a Exerts Differential Effects on Melanoma Growth and Metastatization

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