miR-214 and miR-148b Targeting Inhibits Dissemination of Melanoma and Breast Cancer

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Abstract

miR-214 and miR-148b have been proposed to antagonize the effects of each other in enabling or blocking metastasis, respectively. In this study, we provide evidence deepening their role and interrelationship in the process of metastatic dissemination. Depleting miR-214 or elevating miR-148b blocked the dissemination of melanoma or breast cancer cells, an effect that could be accentuated by dual alteration. Mechanistic investigations indicated that dual alteration suppressed passage of malignant cells through the blood vessel endothelium by reducing expression of the cell adhesion molecules ITGA5 and ALCAM. Notably, trans-endothelial migration in vitro and extravasation in vivo impaired by singly alternating miR-214 or miR-148b could be overridden by overexpression of ITGA5 or ALCAM in the same tumor cells. In clinical specimens of primary breast cancer or metastatic melanoma, we found a positive correlation between miR-214 and ITGA5 or ALCAM along with an inverse correlation of miR-214 and miR-148b in the same specimens. Our findings define an antagonistic relationship of miR-214 and miR-148b in determining the dissemination of cancer cells via tumor–endothelial cell interactions, with possible implications for microRNA-mediated therapeutic interventions aimed at blocking cancer extravasation.

Introduction

Dissemination of primary tumor cells and the consequent formation of metastasis at distant organs are the main cause of cancer-related mortality. The currently available treatments (surgery, radio-, chemo-, and targeted therapy) mainly control primary tumors, but they only exert a mild effect on metastases, mostly because of resistance mechanisms activated by tumor cells. Therefore, it is crucial to identify key regulators of metastatic dissemination and to develop new targeted therapy approaches.

MicroRNAs (miRNAs) are small noncoding, single-stranded RNAs that act as negative regulators of gene expression. Increasing evidence shows that deregulation of miRNAs is a crucial event in tumor progression. Several miRNAs, including miR-137, miR-221/222, miR-182, miR-34a, and miR-214, have been found to be involved in melanoma malignancy by regulating key genes such as c-KIT, MITF, FOXC3, ITGB3, CCND1, p27Kip1, TFAP2C, and ALCAM (1–3). Instead, let-7, miR-9, miR-10b, miR-21, miR-31, miR-146, miR-148b, miR-155, miR-200, and miR-221/222 are the main players in breast cancer dissemination (4–6). Therefore, it is essential to identify new miRNAs and understand how these small noncoding RNAs control the various steps of tumor malignancy and how they interact with one another to attempt miRNA-targeted therapies that can affect the molecular pathways involved in cancer progression. Several clinical trials based on the use of miRNA modulators in tumorigenesis have already been established (7).

We previously demonstrated that miR-214 promotes melanoma metastasis dissemination by increasing migration, invasion, extravasation, and survival of melanoma cells via a novel pathway involving the metastasis suppressor, TFAP2 transcription modulators (3). On the other hand, we found that miR-148b opposes breast cancer progression, acting directly on the integrin signaling players ITGA5, ROCK1, and PI3KCA/p110α (6). More recently, we showed that miR-214 downregulates miR-148b in tumor cells via TFAP2C with the consequent upregulation of miR-148b direct targets (2). In order to explore the relevance of miR-214 and miR-148b for miRNA-based therapeutic interventions in melanoma and breast cancer, we analyzed the dissemination of miR-214-depleted and miR-148b-overexpressing cells in mice, investigated how these small-RNAs influence cell metastatic traits and looked for the main molecular players involved in this process. We demonstrated that single or combined modulations of miR-214...
(inhibition) and miR-148b (overexpression) significantly inhibit metastatization when tumor cells cross the vessel endothelium by decreasing the expression of ITGAS and ALCAM, two adhesion molecules respectively involved in tumor-Extracellular Matrix (ECM) and cell–cell interactions.

Materials and Methods

Cell culture

MA-2 and MC-1 cells were kindly provided by L. Xu and R.O. Hynes (8) and maintained as described in refs. 2 and 3. Human HBL-100 were from ATCC and 4175-TGL and SK-MEL-28 were kindly provided, respectively, by J. Massagué (9) and L. Poliseno and maintained in standard conditions. Human umbilical vein endothelial cells (HUVEC) were kindly provided by L. Primo (with GFP) or generated by M.F. Brizzi and maintained as described in refs. 2 and 3. All used cell lines were authenticated in the last 6 months by BMR Genomics, using the CELL ID System (Promega).

Reagents and antibodies

miR precursors and inhibitors. Pre-miR miRNA Precursor Negative Control #1, Pre-miR miRNA Precursor Hsa-miR-214 (PM12124), Pre-miR miRNA Precursor hsa-miR-148b (PM10264), Anti-miR miRNA Inhibitor Negative Control #1, Anti-miR miRNA Inhibitor Hsa-miR-214 (AM12124), Anti-miR miRNA Inhibitor hsa-miR-148b (AM10264) (all from Applied Biosystems). TaqMan MicroRNA assays for miRNA detection: Hsa-miR-214 ID 002306, Hsa-miR-148b ID 000471, U6 snRNA ID001973, U44 snRNA ID001904 (all from Applied Biosystems). Primary antibodies: anti-PKSCA #4255 (Cell Signaling Technology), anti-GFP ab290 (Abcam), anti-ITGAS pAb RM10 kindly provided by G. Tarone, anti-CD166/ALCAM mAb MOG/07 (Novoceastra Laboratories), anti-ROCK-1 pAb H-85, anti-hsp90 mAb F-8, anti-GAPDH pAb V-18, anti-ACTIN 1-19 pAb (all from Santa Cruz Biotechnology), anti-tubulin mAb B5-1-2 (Sigma), anti-CD31 pAb (Becton Dickinson). Secondary antibodies: HRP-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, donkey anti-goat IgG (all from Santa Cruz Biotechnology), goat anti-rat IgG Alexa-Fluor-568 and goat anti-rat IgG Alexa-Fluor-488 (Molecular Probes, Invitrogen Life Technologies). siRNAs: si-ITGAS (Hs_ITGAS_5 siRNA), si-ALCAM (Hs_ALCAM_5 siRNA), and All Stars Negative Control siRNA were purchased from Qiagen.

Sponge design and recombinant vector preparations

miR-214 sponges were described in ref. 3. miR-148b-specific sponge sequences containing eight miRNA binding sites interrupted by 15-nts spacers were designed to be perfectly complementary to the miR-148b seed region, with a bulge position 9–12 to prevent undesired cleavage of the sponge RNA. Sponges were synthesized by DNA 2.0, cloned into pl241 plasmids, excised using flanking HindIII sites, blunt-ended and subcloned into blunted BamHI and SalI sites, downstream of EGFP into pLenti-CMV-GFP-Puro (658-5) vector (Addgene), giving rise to pLenti148-sponge/B. Nucleotides (in Supplementary Table S1) were verified by sequencing. pLemiR-empty, pLemiR-214, and pLemiR-148b expression vectors were described in refs. 3 and 6. The same miR-148b expression cassette was also subcloned in a p lentiv/ V5 expression vector (kindly provided by C.M. DiPersio, Albany Medical College, Albany, NY).

Transient transfections, vectors, and generation of stable cell lines

To obtain transient anti-miR or pre-miR or siRNA expression, cells were plated at 50% confluency and immediately transfected using HiPerFect Transfection Reagent (Qiagen), with 100 nmol/L anti-miR, 75 nmol/L pre-miR, or 100 nmol/L siRNA. For transient cDNA overexpression, cells were plated at 90% confluency and transfected 24 hours later using Lipofectamine2000 reagent (In vitrogen Life Technologies). For ITGAS or ALCAM overexpression pEGFP-N3-ITGAS (6) or plVX-ALCAM (2) expression vectors were used. All stable cell lines were generated via lentiviral infection. Sponge vectors were obtained as described above. The pLKO.1-shALCAM lentiviral vector was from Open Biosystems (RH53979). Lentiviruses were produced by calcium phosphate transfection of 20-μg vector plasmid together with 15-μg packaging (pCMVdR8.74) and 6-μg envelope (pMD2.G-VSVG) plasmids in 293T cells, according to Trono’s lab protocol (http://tronolab.epfl.ch), and supernatants were harvested 48 hours after transfection.

Protein or RNA isolation, immunoblotting, qRT-PCRs for miRNA detection, and proliferation assays

Total protein or RNA extracts, immunoblotting, qRT-PCRs, and proliferation assays were performed as described in refs. 2 and 3.

Migration, invasion, and transendothelial migration

Transwell assays

To measure migration and matrigel invasion, 7.5 × 104 MA-2 or MC-1, 3 × 104 HBL-100, and 5 × 104 4175-TGL or SK-MEL-28 were seeded in serum-free medium in the upper chambers of cell culture transwells with 8.0-μm pore size membrane (BD Biosciences), precoated or not with 4 μg/well growth factor-reduced matrigel (BD Biosciences) or in BioCoat Matrigel Invasion Chambers (Becton Dickinson). The lower chambers were filled with complete growth medium. After 18 hours, the migrated cells on the lower side of the membrane were fixed in 2.5% glutaraldehyde, stained with 0.1% crystal violet and photographed using an Olympus IX70 microscope. For transendothelial migration assay, 104 HUVECs were seeded in complete medium in the upper part of Transwell inserts with 5.0-μm pore size membrane (Costar, Corning Inc.) coated by fibronectin at 5 μg/cm2 or 0.1% gelatin, and grown till confluence. Then, 5 × 104 cells labeled with CellTracker Orange CMRA or Green CMFDA (Molecular Probes, Invitrogen Life Technologies) were seeded onto the HUVECs monolayer. Twenty hours later, HUVECs and nonmigrated cells were removed and the red or green fluorescent cells that migrated on the lower side of the membrane were fixed in 4% paraformaldehyde and photographed using Zeiss Axiovert200M microscope. Migration, invasion, and transendothelial migration were evaluated by measuring the area occupied by migrated cells using the ImageJ software (http://rsbweb.nih.gov/ij/).

ALCAM localization imaging

A total of 2 × 104 HUVEC-GFP were seeded on coverslips coated with fibronectin at 5 μg/cm2, and grown till confluence. Then, 5 × 104 ALCAM-overexpressing MA-2 cells (stably transduced with plVX-ALCAM expressing vector) were seeded on the HUVEC-GFP monolayer. Twenty-four hours later, the cocultures were fixed in cold methanol and immunostained for ALCAM protein. Briefly, samples were blocked with 5% BSA,
incubated with anti-CD166/ALCAM mAb MOG/07 (Novocastra Laboratories, 1:100 dilution) for 2 hours, then with anti-mouse IgG Alexa-Fluor-568 for 45 minutes and finally mounted on microscope slides for analyses and photos, performed with Confocal Leica SP5 microscope.

**In vivo tumor growth and metastasis assays**

All experiments performed with live animals complied with ethical care. For tumor growth, 5 × 10^6 MC-1 cells (in PBS) were subcutaneously injected into the flanks of 8- to 12-week-old NOD/SCID/IL2R_null (NSG) immunocompromised mice, animals dissected 4 weeks later and proteins extracted from tumors. For experimental metastasis assays, 5 × 10^5 MA-2 or MC-1 or SK-MEL-28 or 3 × 10^5 4175-TGL cells were injected into the tail vein of 8- to 12-week-old SCID or NOD/SCID/IL2R_null (NSG) immunocompromised mice and the animals were dissected 7 or 4 weeks later, respectively. Green or red fluorescent lung metastases were evaluated and photographed in fresh lungs *in toto* using a Leica MZ16F fluorescence stereomicroscope. The number of metastases was measured on photographs using the ImageJ software (http://rsbweb.nih.gov/ij/). Micrometastases were evaluated on paraffin-embedded and haematoxylin and eosin (H&E)-stained slides, scanned with Panoramic Desk (3DHistech, Euroclone).

**In vivo extravasation assay**

A total of 1.5 × 10^6 MC-1 or MA-2 or SK-MEL-28 cells, previously labeled with CellTracker Orange CMRA (Molecular Probes, Invitrogen Life Technologies), were injected into the tail vein of 4- to 6-week-old female NSG mice (Charles River Laboratories). Two or 48 hours later, mice were sacrificed, and 4% paraformaldehyde was injected into the trachea. Lungs were dissected and photographed *in toto* using a Leica MZ16F fluorescence stereomicroscope and red fluorescence was quantified 48 hours following injections using the ImageJ software (http://rsbweb.nih.gov/ij/). Lungs were embedded in OCT (Kilik, BioOptica), frozen, cryostat-cut in 6-μm-thick sections. Localization of tumor cells, inside/outside the vessels, was evaluated on sections at a Zeiss AxiosObserver microscope with the ApoTome Module (3), following blood vessels staining with an anti-CD31 primary antibody in immunofluorescence.

**Human tumor correlation analyses**

Normalized expression values for mRNA and miRNA in breast cancer were downloaded from the European Genome-phenome Archive (EGAS00000000122, EGAD00010004343, and EGAD00010004348; refs. 10, 11, and 1302 samples were used. Instead, for melanomas, expression values were downloaded from The Cancer Genome Atlas (TCGA, https://tga-data.nci.nih.gov/tga/)) and 352 samples were used. All analyses were performed with R using the packages stats (lm) and ggplot2 (12). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (URL https://www.R-project.org/).

**Statistical analysis**

The results are shown as mean ± SD or as mean ± SEM, as indicated, and a two-tailed Student t test was used for comparison. *P < 0.05; **P < 0.01; ***P < 0.001 were considered to be statistically significant. ns indicates a nonstatistically significant P value.

### Results

**miR-214 depletion and miR-148b overexpression inhibit melanoma and breast cancer metastasis formation in mice**

High levels of miR-214 are found in malignant melanomas and breast tumors and promote metastatization in mice by regulating a complex network of players, in a negative or positive manner, including the downregulation of the antimetastatic miR-148b. Here, we evaluated the potential therapeutic value of miR-214 depletion and miR-148b overexpression in tumor progression in mice.

Specific miR-214 or miR-148b sponges A or B were bio-informatically designed, cloned at the 3’ end of a Green Fluorescent Protein (GFP) expression cassette in lentivirus vectors as in Supplementary Table S1, Supplementary Fig. S1A, and ref. 3 and tested in MC-1 or MA-2 melanoma cells. Specifically, cells were transduced with pLenti-214-spongeA/B or pLenti-148b-spongeA/B or pLenti-empty (control) vectors together with precursors for miR-214 or miR-148b or controls (pre-miR-214 or pre-miR-148b or pre-control), and GFP levels were evaluated in cells at the microscope (Supplementary Fig. S1B and S1C), by Western Blot-WB (Supplementary Fig. S1D and S1E), or FACS analysis (Supplementary Figs. S1F and S1G). As shown, all sponges were able to inhibit GFP expression, suggesting miR-214 or miR-148b binding to their complementary sequences.

MC-1 or MA-2 melanoma or 4175-TGL breast cancer cells were then transduced with lentiviruses expressing miR-214-sponges (pLenti-214-spongeA/B) or miR-148b (pLenti-iV5-148b, pLenti-148b) or with empty controls (pLenti-empty, pLenti-iV5-empty, pLenti-empty). Alternatively, MA-2 or SK-MEL-28 melanoma or 4175-TGL breast cancer cells were transduced with lentiviruses expressing miR-148b-sponges (pLenti-148b-spongeA/B) or miR-214 (pLenti-214) or with empty controls (pLenti-empty, pLenti-empty) to verify the mechanism in the opposite directions. miR-214 and miR-148b levels were evaluated by qRT-PCR analyses as shown in Supplementary Figs. S2 to S5. Cells with the expected miR-214 or miR-148b modulations were injected in the tail vein of immunocompromised mice, and metastasis dissemination was evaluated 4 to 7 weeks later by measuring the number/area of lung metastasis in H&E-stained lung or liver sections or the fluorescent (green or red) tumor cells present in the whole organs (Fig. 1A–C; Supplementary Figs. S6A–S6D and S7A–S7D). Single miR-214 downmodulation or miR-148b overexpression significantly blocked metastasis dissemination for MC-1 or MA-2 melanoma or 4175-TGL breast cancer cells compared with controls. Relevantly, simultaneous depletion of miR-214 and increased levels of miR-148b further blocked tumor spreading of melanoma and breast tumor cells compared with single modulations, suggesting a combined action. In parallel, single miR-214 overexpression or miR-148b downmodulation, or simultaneous double targeting, favored melanoma or breast cancer cell dissemination compared with controls.

In conclusion, we can consider miR-214 and miR-148b as promising targets for miRNA-based therapeutic interventions in tumor progression.

**miR-214 inhibition and miR-148b overexpression control-specific tumor cell metastatic traits**

To understand which metastatic traits of melanoma (MA-2, MC-1, and SK-MEL-28) and normal or tumor breast (HBL-100 and 4175-TGL) cells were affected by miR-214 and miR-148b
Figure 1. miR-214 depletion and miR-148b overexpression inhibit metastasis formation in mice. Lung or liver colony formation in immunodeficient mice, 7 (A) or 4 (B and C) weeks after tail-vein injection of MC-1 (A) or 4175-TGL (B and C) cells. Cells were transduced with controls (pLenti-empty, pLenti4/V5-empty) or miR-214-sponge (pLenti-214-spongeB) or miR-148b-overexpressing (pLenti4/V5-148b) vectors, single or in combination, as indicated in the panels. Representative pictures of H&E-stained sections (A, B, a–d, bar, 500 μm; C, a–d, bar, 100 μm) are shown. Graphs (bottom of each figure) represent quantitated results as mean ± SEM H&E-stained colonies number (A) or as a percentage of metastatic/total areas (B) in lungs or as mean metastases/field in liver, referring to the indicated number of mice (n). Two independent experiments were performed and representative results are shown.
modulations, we evaluated proliferation, migration, invasion through matrigel and transendothelial migration on a HUVEC monolayer in vitro. Cells were either transduced with lentiviruses for the depletion (pLenti214-spongeB, pLenti148b-spongeB) or overexpression (pLemiR-214, pLenti4/V5-148b) of miR-214 or miR-148b or with empty controls (pLemiR-empty, pLenti-empty, pLenti4/V5-empty) or transiently transfected with miRNA precursors or inhibitors (pre-miR-214, anti-miR-148b, pre/anti-control). miR-214 and miR-148b modulations were evaluated by qRT-PCR assays as shown in Supplementary Figs. S2 to S5. Single or double alterations of miR-214 and miR-148b did not significantly affect proliferation compared with controls in any tested cell line (Fig. 2A and B, and Supplementary Fig. S8). Instead, modulations of miR-214 or miR-148b significantly affected migration, cell movement across a HUVEC monolayer (transendothelial migration) and invasion in matrigel compared with controls (Fig. 3A–F, Supplementary Fig. S9A–F). Precisely, in all assays, cell movement was impaired in miR-214-depleted and miR-148b-overexpressing cells. Opposite results were observed when miR-214 was highly expressed in cells while miR-148b expression was reduced. Relevantly, in transendothelial migration assays, but generally not in migration or invasion analyses, simultaneous miR-214 inhibition and miR-148b overexpression almost always led to combined effects, like for in vivo metastasis (Fig. 1), suggesting a combinatorial, specific effect of miR-214 and miR-148b at the level of tumor–endothelial cell interactions.

**miR-214 depletion and miR-148b overexpression impair tumor cell extravasation in mice**

The effect of single or combined sponge-induced miR-214 depletion and miR-148b overexpression was investigated on in vivo cell extravasation of tumor cells. CMRA-labeled MC-1 or SK-MEL-28 cells stably expressing miR-214-sponge (pLenti214-spongeB) or miR-148b (pLenti4/V5-148b) or control vectors (pLenti-empty or pLenti4/V5-empty) were injected in the tail vein of immunocompromised mice (Fig. 4A–I, and graph, Supplementary Figs. S10a–S10h and graph). Lodging to the lung vasculature was evaluated 2 hours after injection (Fig. 4A–D, Supplementary Fig. S10a–S10b), and no difference was observed among modified cells. Instead, a strong decrease in early (48 hours after injection) lung colonization was observed following single or combined sponge-driven miR-214 downmodulation and miR-148b overexpression compared with controls in MC-1 (Fig. 4E–H) or SK-MEL-28 (Supplementary Fig. S10c–S10d) cells. Note that cells were localized inside blood vessels or associated with them at 2 hours, as shown in Supplementary Figs. S10e–S10f. Instead, cells were found in the lung parenchyma at 48 hours (Fig. 4I–L, Supplementary Fig. S10g–S10h). As for metastasis dissemination (Fig. 1) and transendothelial migration (Fig. 3), simultaneous miR-214 inhibition and miR-148b overexpression led to combined effects, suggesting, once more, a combinatorial, specific effect of miR-214 and miR-148b at the level of tumor–endothelial cell interactions.

**Depletion of miR-214 and overexpression of miR-148b affect the adhesion molecules ITGA5 and ALCAM**

To identify the molecular players involved in reduced cancer dissemination/extravasation by miR-214 depletion and miR-148b overexpression in tumor cells, expression of ITGA5 and ALCAM, two validated miR-148b direct targets, known to be highly relevant for cancer cell dissemination, was analyzed in cell cultures or in mouse subcutaneous tumors. Single or combined miR-214 depletion and miR-148b overexpression in melanoma (MA-2, MC-1, SK-MEL-28) and breast cancer (4175-TGL) cells were obtained following stable transduction of lentivirus vectors for the expression of miR-214 sponges (pLenti214-spongeB) or miR-148b (pLenti4/V5-148b) or empty controls (pLenti-empty, pLenti4/V5-empty). miR-214 and miR-148b alterations were evaluated by qRT-PCR analyses as in Supplementary Figs. S2 to S5. Important reduction of ITGA5 or ALCAM expression was observed for single or combined miRNA-modulations. Combined inhibitions of ITGA5 and ALCAM by dual miR-214/miR-148b interventions were rarely observed compared with single alterations (Fig. 5A–D, and Supplementary Fig. S11A–E). Considering the relevance of ITGA5 and ALCAM expression impairment for the inhibition of transendothelial migration following simultaneous miR-214/miR-148b alterations, as presented below (see the next paragraph), we can speculate that the effects of combined miR-214/miR-148b changes occur specifically when tumor cells get in contact with endothelial cells. Alternatively, supplementary alterations of gene expression could be involved in the passage of tumor cells through the blood vessels. Opposite stable or transient modulations were obtained in some of the cells (MA-2, 4175-TGL) listed above and in HBL-100

Figure 2. miR-214 depletion and miR-148b overexpression do not affect proliferation. A and B, proliferation of MC-1 (A) or 4175-TGL (B) cells stably transduced with control (pLenti-empty, pLenti4/V5-empty) or miR-214-sponge (pLenti214-spongeB) or miR-148b-overexpressing (pLenti4/V5-148b) vectors, single or in combination, as indicated in the graphs. Results are indicated as mean ± SD of the proliferation ratio versus plated cells, measured by optical density at 0–72 hours. At least two independent experiments (with triplicates) were performed, and representative results are shown.

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miR-214 and miR-148b Targeting in Tumor Progression

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miR-214 depletion and miR-148b overexpression inhibit transendothelial migration. Transwell migration assays were used to evaluate migration (through a porous membrane) or transendothelial migration (through an HUVEC monolayer on top of a porous membrane) for MC-1 (A, B), 4175-TGL (C and D), or SK-MEL-28 (E and F) cells stably transduced with control (pLenti-empty, pLenti4/V5-empty) or miR-214-sponge (pLenti-214-spongeB) or miR-148b-overexpressing (pLenti4/V5-148b) vectors, single or in combination, as indicated in the panels. For migration, results are indicated as a ratio of mean ± SEM of the area covered by migrated versus plated tumor cells; for transendothelial migration, results are shown as mean ± SEM of the area covered by tumor migrated cells. At least two independent experiments (with triplicates) were performed, and representative results are shown.
normal breast cells to further evaluate this mechanism. Here, cells were transduced with pLenti-148b-spongeA/B or pLemiR-214 or the relative empty controls (pLenti-empty, pLenti4/V5-empty) or miR-214-sponge (pLenti-214-spongeB) or miR-148b-overexpressing (pLenti4/V5-148b) vectors, single or in combination, as indicated in the panels. Representative pictures of whole red fluorescent lungs at 2 hours or 48 hours after injection (A-H) and representative fields of murine lung sections, 48 hours after injection (I-L), stained for CD31 and counterstained with DAPI are shown; bar, 800 μm. Results are indicated in the graphs (bottom) as mean ± SEM of the number of extravasated cells at 48 hours for n = 4 mice per group. White arrows, extravasated cells.

Two independent experiments were performed, and representative results are shown.

Impairment of tumor dissemination by miR-214 depletion and miR-148b overexpression depends on ITGA5 and ALCAM expression inhibition

In order to understand if reduction of ITGA5 or ALCAM was essential for miR-214/miR-148b-driven extravasation inhibition, we modulated ITGA5 or ALCAM levels in the presence of miR-214 depletion and miR-148b overexpression.

First, ITGA5 or ALCAM expression was inhibited by RNA interference in a transient (si-ITGA5 or si-ALCAM) or stable (sh-ALCAM) manner in MA-2 melanoma or 4175-TGL breast cancer cells and transendothelial migration or extravasation evaluated by comparison with empty (si-control, sh-control) cells. As shown in Supplementary Fig. S12, reduced levels of ITGA5 or ALCAM significantly impaired transendothelial migration (Supplementary Fig. S12A and S12B) or lung extravasation, 48 hours after injection (tail vein) in immunocompromised mice (Supplementary Fig. S12C and S12D) compared with controls. Modulations of ITGA5 or ALCAM were evaluated by Western blot analysis (Supplementary Fig. S12). Tumor–endothelial cell contacts are shown in a confocal microscope image (Supplementary Fig. S12E) in which red (ALCAM staining) MA-2 tumor cells were in contact with green (GFP expression) HUVECs. All these results suggest the relevance of ITGA5 and ALCAM in the control of tumor cell transendothelial migration or extravasation during tumor progression. At this point, ITGA5 or ALCAM were overexpressed in MC-1 or 4175-TGL cells previously transduced with pLenti-214-spongeB or pLenti4/V5-148b or pLenti-empty/pLenti4/V5-empty lentivirus vectors, in single or dual combinations, and cells used to evaluate transendothelial migration. Modulations of ITGA5 or ALCAM were evaluated by Western blot analysis (Fig. 6A–F). In all conditions, we observed increased transendothelial migration, compared with controls, when ITGA5 or ALCAM was overexpressed in cells. Thus, suggesting that repression of transendothelial migration, driven by miR-214-depletion and miR-148b-upregulation, depends on the reduction of these adhesion molecules in tumor cells. In fact, transendothelial migration inhibition was rescued when ITGA5 or ALCAM levels were increased.
Taken together, our results prove that the negative targeting of miR-214 and/or the positive modulation of miR-148b impairs dissemination by acting on ITGA5 or ALCAM, two main players for tumor cell extravasation.

miR-214 expression correlates with ITGA5 and ALCAM levels, while it anticorrelates with miR-148b, in melanoma metastases and in primary breast tumors. Breast cancer (n = 1302; refs. 10, 11) and melanoma metastasis (TCGA, https://tcga-data.nci.nih.gov/tcga/; n = 352) datasets were used to evaluate miR-214, miR-148b, ITGA5, and ALCAM expression and to look for possible correlations or anticorrelations with one another (Fig. 7A and B). Relevantly, we found that miR-214 and miR-148b anticorrelate in melanoma metastases (P = 7.91e−05) and in primary breast tumors (P = 5.43e−08), while miR-214 significantly correlates with ITGA5 and ALCAM in both datasets. P values were the following: for melanoma metastases: ITGA5 (P = 3.09e−24) and ALCAM (P = 2.69e−09); for primary breast tumors: ITGA5 (P = 1.68e−23) and ALCAM (P = 9.27e−07). These results are therefore in line with our above-presented investigations, thus strengthening the link between miR-214, miR-148b, ITGA5, and ALCAM and its relevance in human tumor progression.

**Discussion**

We previously demonstrated the prometastatic role of miR-214 (3) and its link with the antimetastatic miR-148b (2). Here, we show that single or combined miR-214 inhibition and miR-148b overexpression in tumor cells strongly modulate metastasis formation by acting mainly during the passage across the vessel endothelium (transendothelial migration/extravasation), a metastatic trait that involves two direct miR-148b targets, the adhesion receptors ITGA5 and ALCAM, in a cell–fibronectin and cell–cell dependent manner. Our data suggest
Figure 6.
Transendothelial migration depends on ITGA5 and ALCAM expression in miR-214-depleted and/or miR-148b-overexpressing tumor cells. A–F, transendothelial migration was evaluated in MC-1 (A, C, E) or 4175-TGL (B, D, F) cells stably transduced with miR-214-sponge (pLenti-214-spongeB) or miR-148b-overexpressing (pLenti4/V5-148b) vectors, single or in combination, and, in addition, transiently transfected with recombinant vectors for the overexpression of ITGA5 or ALCAM or empty controls, as indicated in the panels. ITGA5 and ALCAM levels were evaluated by Western blot analysis, 24 hours or 48 hours following transfections. Protein modulations were calculated relative to negative controls, normalized on loading controls (GAPDH or actin) and expressed as percentages (%). Transmigration results are indicated as mean ± SEM of the area covered by tumor-migrated cells. At least two independent experiments (with triplicates) were performed, and representative results are shown.
that miR-214 and miR-148b are valuable candidates for miRNA-based targeted therapy.

miR-214 is highly expressed in malignant cutaneous and ocular melanomas (3, 13, 14) as well as in breast, osteosarcoma, ovary, pancreas, prostate, and gastric cancers (15–20). In line with these findings, upregulation of miR-214 in various tumor cells increases metastasis formation (2, 3, 21–23). On the other hand, miR-148b is poorly expressed in melanomas and in breast, pancreatic, and hepatocellular carcinomas (1, 6, 24, 25) and its modulation in tumor cell lines reveals its antimetastatic function (6, 26–28). Here, by modulating miR-214 and miR-148b, respectively, in a negative and positive manner, we show that miR-214 and miR-148b are part of a miR-ON-miR regulatory axis where miR-214 favors tumor dissemination following the downregulation of miR-148b and the consequent upregulation of miR-148b direct targets, ITGA5 and ALCAM as well as some of their downstream

Figure 7.
miR-214 anticorrelates with miR-148b, while it correlates with miR-148b targets ITGA5 and ALCAM, in melanoma metastases or human primary breast tumors. The indicated datasets were used to evaluate miR-214, miR-148b, ITGA5, and ALCAM expression in melanoma metastases (A) and in primary breast tumors (B). Statistically significant negative or positive correlations are shown for miR-214 and miR-148b (anticorrelations) and for miR-214 and ITGA5 or ALCAM (correlations). Correlations of normalized expression are represented with a dot plot superimposing the regression line. The shaded area represents the 0.95 standard error confidence interval of the model predictions. Statistically significant R2 and P values and number of samples (n) are indicated.
players (8). Similarly, recent investigations underline the relevance of multiple miR connections (miR-ON-miR) or of miRs and transcription factors (TF) reciprocal regulations (miR-ON-TF-ON-miR). Examples are Lin28-let-7-miR-181 in megakaryocyte differentiation (29), miR-181b-FOS-miR-21 in glioma progression (30), miR-103/107-miR-200 in epithelial-to-mesenchymal transition (31), miR-199a/miR-214-let-7b-miR-34a/miR-762-miR-1915 in breast cancer metastasis (32). All these lines of evidence open up the possibility to target multiple players of the same pathway and give hope for combined therapeutic interventions.

In line with this hypothesis, it has recently been shown that the simultaneous systemic delivery of two small non-coding RNAs acting as tumor suppressors, miR-34 and let-7, leads to reduced non–small lung cancer growth (33).

We propose that the presented miR-214-ON-miR-148b regulatory axis controls tumor dissemination acting, in particular, when tumor cells cross the blood vessels endothelium, via the modulation of ITGA5 and ALCAM (8, 34–38). In fact, the silencing of ALCAM or ITGA5 inhibits transendothelial migration in vitro and extravasation in vivo. More relevantly, when miR-214 is depleted and/or miR-148b overexpressed, transendothelial migration suppression can be overcome by ITGA5 or ALCAM upregulation, thus suggesting that these two adhesion molecules are essential modulators of tumor–endothelial cell interactions. These results are in line with the fact that ALCAM is a cell-to-cell adhesion receptor known to play a major role in mediating the interactions between endothelial and tumor or immune cells during transendothelial migration (39–41). Moreover, ALCAM was found to be involved in cell movement (42) and in the conversion of the prometastatic pro-MMP-2 to its active form in malignancy (43). On the other hand, modulations of integrin levels were associated with various metastatic phenotypes (35, 44–46). In particular, increased ITGA5 expression has been observed in metastatic melanoma cell lines compared with primary ones (47), and survivin-dependent ITGA5 upregulation was shown to enhance motility of melanoma cells (48). In MDAMB231 breast cancer cells, ITGA5 promotes lung metastasis in both spontaneous and experimental lung metastasis models (35). In breast and ovarian cancer patients, ITGA5 expression was shown to be predictive of metastasis and poor prognosis (6, 46, 49). ITGA5 has also been linked to extravasation; in fact, it has been shown that Cav1–Rho-GTPase-dependent control of cell extravasation depends on ITGA5 levels and inhibition of this regulatory axis impairs extravasation and survival of metastatic cells (50). The fact that high levels of ITGA5 and ALCAM correlate with miR-214 and that miR-214 expression anticorrelates with miR-148b in human breast and melanoma, tumors, or metastases, further strengthens the importance of these players and their direct functional connections in tumorigenesis.

With particular relevance for targeted therapy, the evidence that single or combined miR-214 downregulation and miR-148b upregulation in tumor cells inhibit metastasis formation in mice, gives hope for an miRNA-based therapy. Due to the relevance of ALCAM and ITGA5 in the pathway presented here, one could even speculate to target these two adhesion molecules with specific antibodies, in addition to miRNA targeting, to further affect metastasis formation. Considering that the main issue of the miRNA-based targeted therapy is the in vivo delivery, it is essential to identify safe, selective, and efficient compound systemic deliveries. For this purpose, we are currently developing new tools to administer miR-214 inhibitors and miR-148b precursors to animals and test their efficacy on metastasis formation.

In conclusion, our data demonstrate that the cascade of events, including miR-214, miR-148b, ALCAM, and ITGA5, is controlling melanoma and breast cancer progression and it can be exploited for combinatorial therapeutic interventions.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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miR-214 and miR-148b Targeting Inhibits Dissemination of Melanoma and Breast Cancer

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