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miR-31 is a broad regulator of β 1-integrin expression and function in cancer cells

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Abstract

Integrins are adhesion receptors involved in bidirectional signaling that are crucial for various cellular responses during normal homeostasis and pathological conditions such as cancer progression and metastasis. Aberrant expression of noncoding microRNAs has been implicated in the deregulation of integrin expression and activity, leading to the development and progression of cancer tumors, including their acquisition of the metastatic phenotype. miR-31 is a key regulator of several critical genes involved in the invasion-metastasis cascade in cancer. Using diverse cell based, genetic, biochemical, flow cytometry and functional analyses, we report that miR-31 is a master regulator of integrins as it targets multiple α subunit partners (α 2, α 5 and α V) of β 1 integrins and also β 3 integrins. We found that expression of miR-31 in cancer cells resulted in a significant repression of these integrin subunits both at the mRNA and protein levels. Loss of expression of α 2, α 5, α V and β 3 was a direct consequence of miR-31 targeting conserved seed sequences in the 3'UTR of these integrin subunits leading to their posttranscriptional repression, which was reflected in their diminished surface expression in live cells. The biological consequence of decreased the cell surface of these integrins was a significant inhibition of cell spreading in a ligand-dependent manner. While different reports have shown that a single integrin can be regulated by several microRNAs, here we show that a single microRNA, miR-31, is able to specifically target several integrin subunits to regulate key aspects of cancer cell invasion and metastasis.

Keywords

miR-31; Integrins; cell adhesion and spreading; cancer cell invasion

INTRODUCTION

As a large family of cell-cell and cell-extracellular matrix (ECM) receptors, integrin α/β heterodimers play prominent roles during embryonic development and postnatal physiology and pathology¹. In addition to their adhesive functions, integrins mediate bidirectional signaling across the cell membrane that regulates numerous cellular responses, including motility, differentiation, growth and gene expression^{2,3,4,5-7}. In this context, alterations in integrin expression and function impart phenotypic properties to transformed cells that have significant impact on cancer progression and metastasis^{8,9}.

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microRNAs, short non-coding, single-stranded RNAs that regulate protein translation and messenger RNA (mRNA) degradation of their target genes¹⁰, have been shown to be involved in a large number of biological processes. It is estimated that about one-third of all human mRNAs are regulated by microRNAs¹¹; and, thus far, more than 1000 microRNAs have been identified in humans with the potential to control more than 30,000 target genes. The broad spectrum of genes that can be targeted by a single microRNA is attributed to the high level of conserved target motifs, seed sequences, within the 3' untranslated regions (UTR) of the affected genes, thus making them powerful regulators of gene expression in numerous and complex cellular responses, including cancer cell invasion and metastasis¹²⁻¹⁵. microRNAs have also been found to regulate integrin activity in such complex biological processes, such as angiogenesis, cell migration and invasion (reviewed in¹⁶). Recently, we and others have identified and characterized miR-31 as a master regulator of different steps in the invasion-metastasis-cascade by virtue of its capacity to modulate key metastasis-promoter genes, including WAVE3 and integrin $\alpha 5$ subunit^{17, 18}. In this study, we show that in addition to $\alpha 5$, miR-31 directly targets seed sequences in the 3'UTRs of two other integrin alpha subunits, $\alpha 2$ and αV , and the $\beta 3$ subunit. We also find that the miR-31-mediated suppression of the integrin alpha subunits indirectly suppresses cell surface expression of their $\beta 1$ subunit partner and show that suppressed expression of these integrins reduces cell spreading on specific target ECM ligands of these affected integrins.

MATERIALS AND METHODS

Reagents

All monoclonal antibodies (mAbs) to integrins were from Millipore (Bedford, MA) except for anti- $\beta 5$ integrin, which was from eBioscience (San Diego, CA) and $\beta 8$ integrin, which was from R&D Systems (Minneapolis, MN). UV Live Dead dye and all secondary antibodies were from Molecular Probes (Eugene, OR). Other reagents were from Sigma (St. Louis, MO).

Flow cytometry

MDA-MB-231 cells expressing GFP-pBABE vector or GFP-miR-31, a kind gift from Dr. Robert Weinberg¹⁸, were propagated in DMEM culture medium supplemented with 10% FBS and puromycin (5 $\mu\text{g}/\text{ml}$). Sub-confluent cells were detached by trypsinization and washed with Hank's Balanced Salt Solution with Ca^{2+} , Mg^{2+} and 0.1% BSA. Cells were processed for flow cytometry as described¹⁹. Alexa Fluor 647 tagged goat anti-mouse antibody was used to detect all integrin mouse mAbs, including the $\beta 1$ activation specific HUTS-4, except for rat anti- $\alpha 6$ integrin mAb, which was detected with Alexa Fluor 647 tagged anti-rat antibody. All primary and secondary antibodies were used at 10 $\mu\text{g}/\text{ml}$. As a positive control for integrin activation, 3.5 mM MnCl_2 was used to treat the cells for 5 min at 37°C in a humidified tissue culture incubator. All data were acquired in a BD LSR II instrument and analyzed with FlowJo 7.6.3 (Treestar, Ashland, OR) software. Binding of only live, GFP⁺ cells to different antibodies was used in the analyses. Non-specific binding due to secondary antibody alone to empty vector transfected cells was subtracted from all flow cytometric data. The resultant fluorescence intensity values were then plotted graphically.

Cell Culture, transfections and immunoblotting

The established human cancer cell lines were obtained from American Type Culture Collection (ATCC) and cultured as previously described²⁰. Growth and morphology of each cell line were routinely monitored and key features were found to be consistent with prior description of each cell line. No further genetic analyses or fingerprinting were

performed. Transient transfections and immunoblotting analyses were performed as described previously²⁰⁻²³.

Plasmid Construction, Site-directed Mutagenesis and 3'UTR luciferase reporter analysis

The nucleotide sequence (~300 bp) flanking the miR-31 seed sequence within the 3' untranslated region (3'UTR) of each integrin subunit under study was PCR-amplified from MDA-MB-231 genomic DNA and subcloned into the pmirGlo vector downstream of the firefly luciferase expression cassette (Promega, Madison, WI). The correct sequence and orientation were verified by sequencing. QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) was used to generate the 3'UTR mutants where the seed sequence recognized by miR-31 was deleted. pmirGlo reporter plasmids (1 µg total plasmid) were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) into the specified cancer cells and then seeded in 12-well plates (3×10^4 cells per well). Cells were collected after 48 h for assay using the Dual-Luciferase reporter assay system (Promega, Madison, WI), as described previously¹⁷. For co-transfection experiments, synthetic microRNA mimics or miR Negative Control (5 nM), were added to the transfection mix. All experiments were performed in triplicate with data averaged from at least three independent experiments.

Semi-quantitative and real-time quantitative-RT-PCR

Total RNA was extracted from established cancer cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. cDNA was generated and used as a template for semi-quantitative RT-PCR as previously described^{20, 22-24}. Expression levels of microRNAs were quantified by real-time quantitative RT-PCR using the human TaqMan MicroRNA Assays Kits (Applied Biosystems, Carlsbad, CA). The reverse transcription reaction was carried out with TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions. For each gene, quantification of expression levels was performed using the respective gene-specific primers (Table S1) and the RT² SYBR Green/Fluorescein qPCR Master Mix (SABiosciences, Frederick, MD) following the manufacturer's instructions. Quantitative RT-PCR was performed on the BioRad (Hercules, CA) iCycler PCR system where the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The cycle threshold (Ct) values were calculated with SDS 1.4 software (Bio-Rad). The expression levels of each transcript were normalized using the $2^{-\Delta\Delta Ct}$ method²⁵ relative to GAPDH. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the transcript of interest. The $\Delta\Delta Ct$ was then calculated by subtracting ΔCt of the parental cells from the control vector- or miR-31-expressing cells. Fold change in the gene expression was calculated according to the equation $2^{-\Delta\Delta Ct}$.

Immunofluorescence

MDA-MB-231 cells expressing miR-31 or the control vector were seeded onto 6-well plates coated with 10 µg/ml laminin, 10 µg/ml fibronectin, 10 µg/ml collagen or 5 times diluted growth factor-reduced Matrigel for 1 hour, fixed, permeabilized with 0.1% Triton X-100, blocked in horse serum, and stained for 30 min with Alexa 568-phalloidin to visualize actin filaments²⁶.

Cell spreading and integrin blocking

Cell spreading assays were carried out on round coverslips (Fisher Scientific, Pittsburg, PA) in 12-well plates (Falcon, Becton Dickinson&Comp., NY). Coverslips were coated 1 h with Matrigel Matrix (BD Biosciences, Bedford, MA) diluted 1:6 in PBS, 10 µg/ml collagen I, 10 µg/ml laminin or 2 µg/ml vitronectin (all from Sigma, St. Louis, MO) at 37°C. Following

three washes with PBS coverslips were blocked for 1 h with 1% BSA in PBS at 37°C. BSA (1 mg/ml) coated coverslips were used as negative controls.

MDA-MB-231-vector and MDA-MB-231-miR-31 breast cancer cells grown to ~80% confluency were detached with trypsin, washed with serum-free-DMEM, resuspended in the same medium and then plated in triplicate at a cell density of 6×10^4 cells/well. For $\alpha 2\beta 1$ integrin blocking experiments, cells were re-suspended in media containing 10 $\mu\text{g/ml}$ of the $\alpha 2\beta 1$ integrin blocking mAb, LS-C247646, (LS-Bio, Seattle, WA) and incubated 30 min at 37°C before they were seeded on coverslips coated with collagen I or Matrigel. Control cells were re-suspended with non-blocking IgG control under the same conditions. Cells were allowed to adhere and spread for 30 min and 1 h in a 37°C CO₂ incubator. At the end of the incubation period, unattached cells were removed by careful aspiration and washing two times with PBS. Adherent cells were fixed with 4% PFA for 10 min and washed twice with PBS. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 4 % bovine serum overnight at 4°C and then stained for 1 h with Alexa Fluor 568-conjugated phalloidin (Invitrogen, Molecular Probes, Eugene, OR). After washing, the coverslips were transferred on slides and mounted in immune-fluor mounting medium (MP Biomedicals, Solon, OH). Images were acquired with a 20x/0.7 or 40x/1.25 numerical aperture oil objective on a fluorescent microscope (Leica) equipped with digital camera using Image-Pro Plus version 5.1.2.59. Cells were quantified using the ImageJ software version 1.43u and the results are presented as the total surface area covered by cells, in square micrometer, averaged to 300 cells.

Oligonucleotide sequences

Sequences of the oligonucleotide primers used for genomic PCR, RT-PCR, to amplify the entire WAVE3 3'UTR or the individual seed sequences, as well as the primers used for mutagenesis were from IDT (San Diego, CA) and are listed in supplementary Table S1.

Statistical analyses

The data are presented as the means \pm standard errors of at least three independent experiments. The results were tested for significance using an unpaired Student's *t* test and *p* values of <0.05 were considered statistically significant.

RESULTS

miR-31 targets several integrins subunits

Previous reports have shown that microRNA miR-31 targets the integrin $\alpha 5$ subunit (ITGA5) by interacting with a perfectly matched seed sequence in the 3'UTR of the ITGA5 transcript^{17, 18}. We sought to determine whether miR-31 could also target other integrin subunits. By *in silico* analyses, we found that the seed sequence of miR-31 was conserved in the 3'UTR of three other integrin subunits; $\alpha 2$, αV and $\beta 3$ (ITGA2, ITGAV and ITGB3, respectively, Figure 1A). Since binding of microRNAs to their specific seed sequences often represses gene expression, we examined the effect of miR-31 on the expression levels of these candidate target integrin subunits. The analysis was performed in MDA-MB-231 cells because these breast cancer cells have very low levels of endogenous miR-31^{17, 18}. Stable expression of miR-31 in these cells had no effect on proliferation compared to control vector transfected cells (Fig. S1). Overexpression of miR-31 in MDA-MB-231 cells resulted in ~50% decrease in the levels of $\alpha 2$, $\alpha 5$, αV and $\beta 3$. This suppression was found both at the mRNA as observed by semi-quantitative RT-PCR (Fig. 1B) and qRT-PCR (Fig. 1C) and the cellular protein levels as determined by Western blots (Fig. 1D and Fig. S2). All these four integrin subunits contain target sites for miR-31 in their respective 3'UTRs (Fig. 1A), suggesting that the silencing of their expression is consequent to miR-31 targeting of the

3'UTR in their transcripts. Integrin subunit $\beta 5$, which lacks a miR-31 seed sequence, was not affected by miR-31 expression in these cells. We also found that miR-31 overexpression resulted in ~2-fold decrease in $\beta 1$ subunit (ITGB1) mRNA and protein expression levels compared to the parental cells or the vector-transfected cells (Fig. 1, all panels), even though $\beta 1$ does not contain a target sequence for miR-31 in its 3'UTR. This result could be a secondary to effect of miR-31 on the expression levels of $\alpha 2$, $\alpha 5$ and αV , alpha subunits partners of $\beta 1$; which could lead to degradation of surplus $\beta 1$ subunit. The effect of miR-31 on the expression of these specific integrin subunits, $\alpha 2$, $\alpha 5$ and αV , and $\beta 1$, was also confirmed in LNCaP prostate cancer cells, and $\beta 5$, which lacks the miR-31 target sequence, was again unaffected (Fig. S3).

Surface expression of α -subunits partners of $\beta 1$ integrins are reduced due to miR-31

Since integrin mediated cellular responses depend upon the expression at the cell surface, as a next step, we monitored the surface expression patterns of the different α -subunits that commonly form heterodimers with $\beta 1$ subunit in cells stably expressing either the empty control vector or miR-31 by flow cytometry. In accord with our qRT-PCR, semi-quantitative RT-PCR and western blot results (Fig. 1), flow cytometry analyses revealed that miR-31 significantly reduced the surface expression of $\alpha 2$ ($p < 0.05$), $\alpha 5$ ($p < 0.05$) and αV ($p < 0.001$) subunits (Fig. 2). Consistent with the observed reduction in expression of $\beta 1$ integrin subunit detected at the mRNA and protein levels, there was also a reduction in surface expression of the $\beta 1$ subunit partner of these α subunits on the surface of the miR-31 expressing cells.

$\beta 3$ integrin is a target of miR-31

αV integrin subunit is known to complex with several β integrins, including $\beta 1$, $\beta 3$, $\beta 5$ and $\beta 6$ integrins. Since our results show miR-31 can affect $\beta 1$ indirectly, we evaluated whether other common partners of αV , $\beta 3$, $\beta 5$, $\beta 6$ and $\beta 8$, were also targeted by miR-31. Only one of these 4 integrin β subunits, the 3' UTR of $\beta 3$, was found to harbor the seed sequence that could be targeted by miR-31 (Fig. 1). Consistent with this prediction, we found $\beta 3$ surface levels were significantly reduced ($p < 0.05$) in cells expressing high levels of miR-31 compared to vector control (Fig. 3A), which is concordant with the RT-PCR and western blotting analyses (Fig. 1). Surface expression of $\beta 5$ subunit was also reduced in the presence of miR-31, probably as an indirect effect since there is no seed sequence of miR-31 in the 3'UTR of $\beta 5$ (Fig. 3A). Reduced expression levels of either αV or $\beta 3$ subunits resulted in a significant reduction of cell surface expression of the $\alpha V\beta 3$ and the $\alpha V\beta 5$ heterodimers using antibodies that specifically recognize these integrins heterodimers (Fig. 3B). Thus, miR-31 can simultaneously regulate the expression of multiple α and β integrin subunits in a cellular environment.

Reduced expression of $\alpha 2$, $\alpha 5$ and αV subunits by miR-31 results in decreased expression of activated $\beta 1$ integrin on the cell surface

Based on the above results, we assessed whether miR-31-mediated suppression also affected the activation state of integrins expressed on the surface of the MDA-MB-231 cells. This analysis was performed using HUTS-4, a mAb that recognizes the active conformation of $\beta 1$ integrins upon dimerization with one of its α subunit partner²⁷. miR-31 transfectants showed a significantly reduction in their capacity to spontaneously bind HUTS-4 compared to vector control ($p < 0.001$), and this difference remained significant in the presence of Mn^{2+} ($p < 0.05$), which activates integrins upon binding to their extracellular domain (Fig 4A). However, this decrease in HUTS-4 binding was proportional to the decrease in the surface expression of $\beta 1$ integrins in these cells (Fig. 4B). Thus, when the activation status of these integrins is expressed as a ratio of the total $\beta 1$ surface expression, measured in HUTS-4 binding, no significant differences were found between the vector or miR-31

expressants in either unstimulated (resting) cells or cells in which integrins were activated with Mn^{2+} (Fig. 4B).

miR-31 directly targets the 3'UTR of ITGA2, ITGA5, ITGAV and ITGB3 integrins and represses their expression

We used the Firefly-Renilla dual luciferase reporter gene assay to demonstrate that the posttranscriptional repression of the $\alpha 2$, $\alpha 5$, αV and $\beta 3$ integrin subunits is a consequence of direct binding of miR-31 to target seed sequences within the 3'UTR of the transcripts for these integrin subunits. A DNA fragment, ~350 bp-long from the 3'UTR sequence of these integrins encompassing the miR-31 seed sequence, was subcloned within the 3'UTR of the firefly luciferase gene into the pmirGlo vector, and luciferase activity was measured in MDA-MB-231 breast cancer cells, which express very low or no levels of miR-31. For each construct, firefly luciferase activity was normalized to renilla luciferase activity and the ratio of miR-31 compared to control miR treatment was calculated. We found no difference in luciferase activity between MDA-MB-231 cells transfected with control vector (Glo) alone or with control vector cotransfected with either miR-31 or control microRNA (Fig. S4), demonstrating the lack of a miR-31 effect in the absence of its specific target sequence. In contrast, in cells transfected with the individual integrin 3'UTR-containing vector, overexpression of miR-31 resulted in a significant reduction in luciferase activity, ranging from 30 to 55% depending on the integrin subunit compared to the cells transfected with the same vector in the absence of miR-31 or cotransfected with control microRNA in MDA-MB-231 (Fig. 5 and S4). As a control to confirm that miR-31 is specifically targeting and binding seed sequences within these 3'UTR, we deleted the seed sequence of miR-31 in the 3'UTR of $\alpha 2$, $\alpha 5$, αV and $\beta 3$ integrin subunits. This maneuver abrogated the effect of the exogenous miR-31 on these integrin subunit reporters in the MDA-MB-231 cells (Fig. 5 and Fig. S4). Our *in silico* analyses also found a second potential seed sequence of miR-31 in the 3'UTR of ITGAV (ITGAV-2, Fig. 5), which only partially aligned to the miR-31 target sequence. However, this second seed sequence was insufficient to be regulated by miR-31; its presence had no effect on luciferase activity. As a negative control for the effect of miR-31 on the luciferase activity, the 3'UTR of ITGB5 which does not contain a seed sequence for miR-31, showed no different in renilla luciferase activity in the low or high miR-31 expressing cells. The luciferase assay for ITGB1 could not be performed because of the large size (more than 1200 bp) of its 3'UTR. Similar results were obtained when these experiments were repeated in the LNCaP prostate cancer cells (Fig. S5); namely, miR-31 repressed expression of the genes encoding for $\alpha 2$, $\alpha 5$, αV and $\beta 3$ integrin subunits but did not affect $\beta 5$ expression. These results demonstrate that the effect of miR-31 is not restricted to a single cell line but appears to extend to other cancers and show that miR-31 specifically targets and represses several integrin subunits simultaneously.

miR-31 affects cell spreading in a ligand-dependent manner

Since miR-31 overexpression leads to diminished expression of $\beta 1$ integrin and several of its α subunits partners on the cell surface (Fig. 2), we anticipated that specific integrin-mediated cellular responses might be suppressed. Accordingly, we plated control or miR-31-MDA-MB-231 cells on different ECM ligands that are recognized by various $\beta 1$ -containing integrins and examined cell spreading. The cells were allowed to spread for 30 or 60 min on collagen (an $\alpha 2\beta 1$ ligand), fibronectin (an $\alpha 5\beta 1$ ligand) or laminin (an $\alpha 6\beta 1$ ligand) or Matrigel (containing mostly laminin and collagen IV, ligands for $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins²⁸). At the two selected time points, cells were fixed and stained with phalloidin to visualize actin (Fig. 6A). We found a striking difference in cell size and actin organization between control cells and miR-31-expressing cells on collagen and Matrigel after 1 hour (Fig. 6, upper two panels). The effect of miR-31 on the ability of cells to spread on these two substrates was also observed after 30 min (Fig S6) but became more

pronounced after 1 hour. While the control cells became large and fully spread, and developed extensive stress fibers and lamellipodia at the cell periphery, the miR-31 expressing cells remained small and round on these substrata. In control experiments, a blocking mAb (LS-C247646) to $\alpha 2\beta 1$ completely inhibited spreading of both the parental and the miR-31 expressing MDA-MB-231 cells on collagen (Fig. 7), but not on Matrigel (not shown), suggesting the roles of not only $\alpha 2\beta 1$ but also other integrins that were suppressed by miR-31 expression. The difference in cell spreading between the control cells and the miR-31 expressing cells was also evident on laminin and vitronectin, but no difference was observed when cells were plated on fibronectin (Fig. 6, bottom panels).

DISCUSSION

Cell adhesion, spreading and migration on ECM proteins are critical processes for both physiological responses, such as organ development, cell-mediated immunity and wound healing, and pathological responses, such as cancer progression and metastasis. Cell adhesion and spreading are triggered by cell contact with ECM proteins such as collagen, laminin, fibronectin, vitronectin and fibrinogen. Physiological regulation of cell-matrix interactions is mediated by cell-to-ECM (inside-out) and ECM-to-cell (outside-in) signals that are themselves transmitted by various adhesion receptors on the cell surface, including integrins²⁸⁻³¹. The $\beta 1$ integrin subunit, in association with several different α integrin subunits, serve as the principal receptors for type I collagen, the most abundant extracellular matrix protein in mammals²⁸. Binding of $\beta 1$ integrin to collagen results in downstream rearrangements of the actin cytoskeleton by providing a scaffold for cytoskeletal proteins and multiple signaling molecules that are involved in the regulation of cell adhesion and spreading³². In this report we identify the regulation of expression and activity of multiple integrins by miR-31 as a mechanism for the modulation of cancer cell spreading. We and others have previously reported the targeting of integrin $\alpha 5$ subunit by miR-31^{17, 18}. In this report, we show that, in addition to $\alpha 5$, miR-31 directly targets and represses the mRNAs and proteins of two other integrin alpha subunits ($\alpha 2$ and αV). This repression is mediated by recognition of a conserved seed sequence of miR-31 in the 3'UTRs of the respective target transcripts. The suppression of these integrin alpha subunits by miR-31 was observed in a series of *in vitro* molecular and cellular assays, as well as independent experiments to verify that miR-31 regulates the expression and activity of these integrins subunits. First, we showed that overexpression of miR-31 in MDA-MB-231 breast cancer cells, which has low endogenous levels of miR-31, resulted in a significant decrease in the expression levels of multiple alpha integrins subunits along with the $\beta 1$ subunit, using both real time qt-RT-PCR and western blotting analyses. Second, we used a luciferase reporter gene assay to demonstrate that the miR-31 mediated suppression of these integrins subunits arises from direct and specific targeting of a conserved miR-31 seed sequence in their 3'UTRs, and that mutation of the seed sequence abrogates the effect of miR-31. Targeting of these α subunits by miR-31 is consistent with indirect repression of the $\beta 1$ integrin subunit which associates with $\alpha 2$, $\alpha 5$ and αV partners to form functional integrin heterodimers. The expression of $\alpha 1$, $\alpha 3$, $\alpha 4$ and $\alpha 6$ integrins remained similar between the vector and miR-31 expressing cells, showing that the effect of miR-31 is selective for specific α integrins. Interestingly, although analysis of $\beta 1$ integrin 3'UTR revealed it lacks a seed sequence for miR-31, it was significantly ($p < 0.05$) reduced at the mRNA and protein levels, which further resulted in a significant reduction in its cell surface expression. Since there was no compensatory increase in the levels of the $\alpha 1$, $\alpha 3$, $\alpha 4$ and $\alpha 6$ integrins in the miR-31 transfectants, it can be expected that there is a net decrease in the number of available α integrin subunits for $\beta 1$ to complex within these cells. Third, we showed that loss of mRNA and protein expression of these integrins downstream of miR-31 resulted in loss of both surface expression as well as activation as demonstrated by flow cytometric analyses with integrin subunit specific mAbs and the $\beta 1$ activation-specific mAb, HUTS4. Fourth, and importantly, we provided evidence

that the miR-31 mediated loss of $\alpha 2$, $\alpha 5$ and αV integrins, and their $\beta 1$ partner, and $\beta 3$ integrins resulted in a significant inhibition of cell spreading in a ligand-dependent manner. The inhibition in cell spreading could reflect reduction in surface expression of total or activated integrins.

This study, together with the previously published reports from both our group¹⁷ and others^{16, 18} establish the important role of miR-31 in regulating the different aspect of cell adhesion, spreading and cancer cell invasion and metastasis and solidifies its role a master metastasis suppressor gene^{17, 18}. Natural regulation of cancer metastasis is mediated by cancer metastasis suppressor genes which either prevent the progression of tumor cells to a metastatic phenotype or reverse the metastatic phenotype. miR-31 is one of these metastasis suppressor genes, whose pleiotropic functions regulate multiple steps of the invasion-metastasis cascade by targeting a cohort of pro-metastatic targets genes all of which are known to play an important role in metastasis, including integrins. The pleiotropic effect of miR-31 is of potential interest from a translational perspective since modulation of a single miRNA can affect the function of several genes involved in cancer metastasis. Thus, these data reinforce the important role of miR-31 in the regulation of the cancer invasion-metastasis cascade and identify miR-31 as an attractive therapeutic target for blunting cancer metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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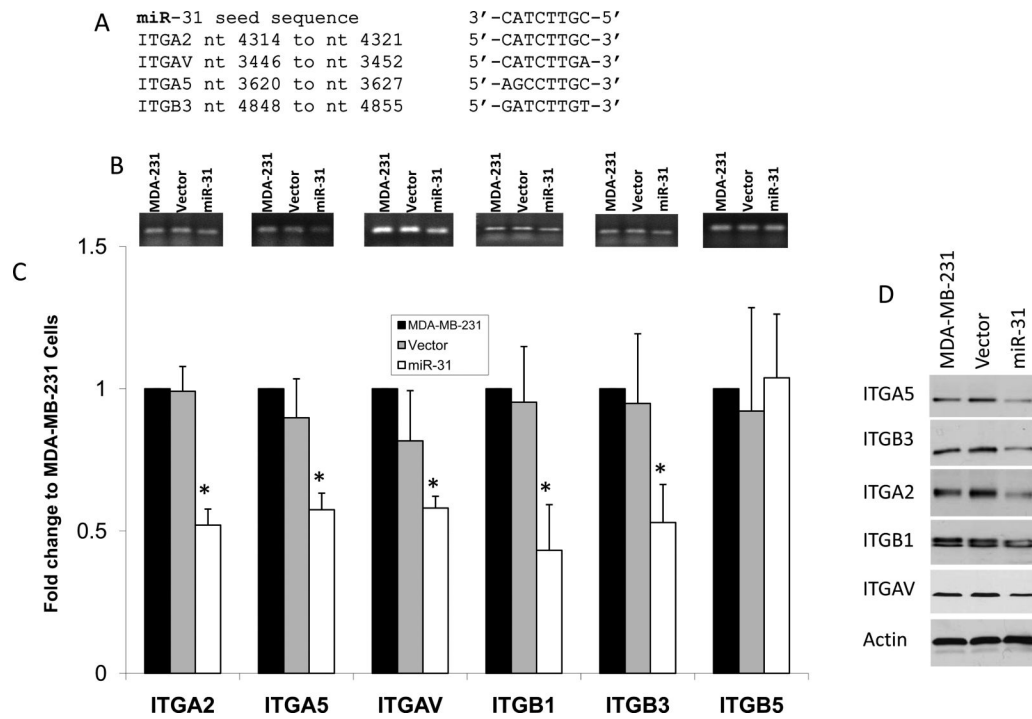


Fig. 1. miR-31 inhibits expression of specific α and β integrin subunits

(A) Alignment of miR-31 seed sequence with candidate target sequences within the 3'UTRs of the indicated integrin subunits. (B) semi-quantitative RT-PCR, (C) Real-time qt RT-PCR and (D) immunoblot of the indicated integrin subunits from MDA-MB-231 cells or MDA-MB-231 cells with stable expression of either control or miR-31 vector. Densitometry and statistical analyses of the Western Blot data is shown in Fig. S2.

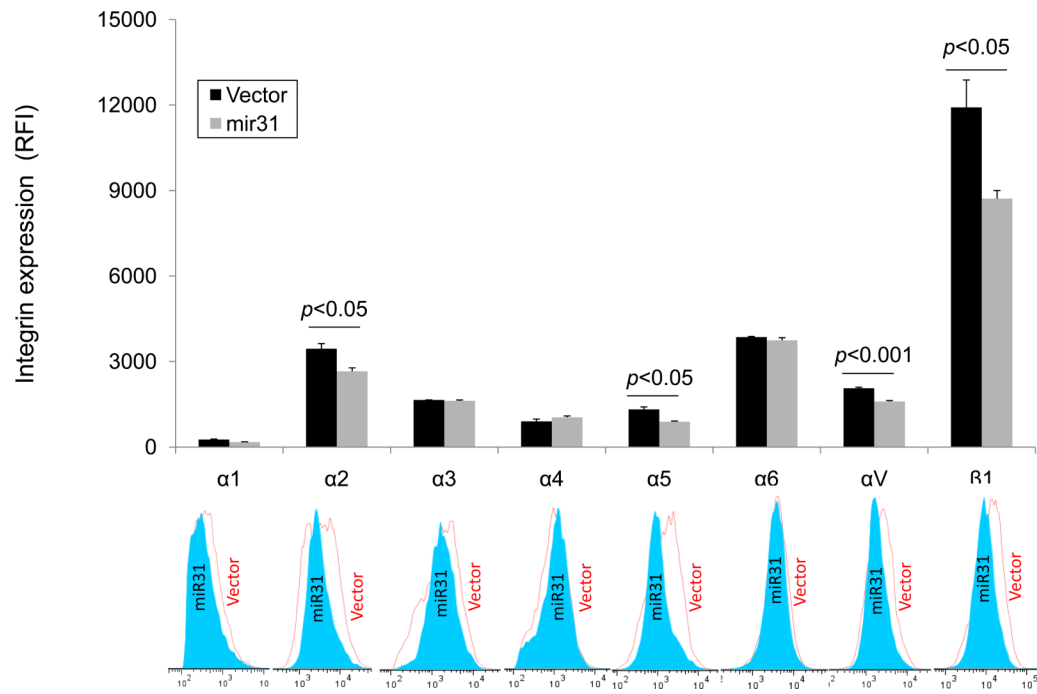


Fig. 2. Surface expression of integrin α -subunits and their β 1 subunit partner is suppressed by miR-31

Expression profiles of different integrin α subunits in MDA-MB-231 cells overexpressing GFP-pBABE vector control or GFP-miR-31. Surface expression of different α subunit partners of the β 1 integrin subunit is quantitated as the relative fluorescence intensity (RFI) with subunit specific mAbs by flow cytometry. Values are means \pm S.E. and are representative of 3 independent experiments. Representative histograms are provided below in which the empty histograms are for the vector control and the filled histograms are for miR-31 overexpressing cells.

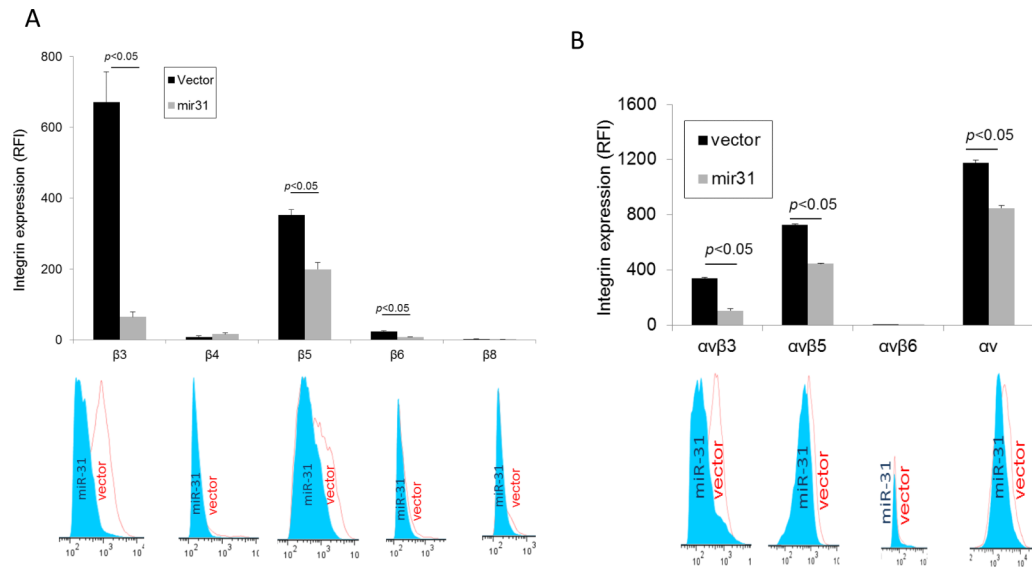


Fig. 3. Expression profile of different integrin β subunits in MDA-MB-231 cells expressing and GFP-miR-31 or control GFP-pBABE

(A) Surface expression of different β integrin subunits (other than $\beta 1$) as determined by flow cytometry with representative histograms given below. Empty histogram is for vector and filled histogram is for miR-31 overexpressing cells. Values are means \pm S.E. and are representative of 3 independent experiments. (B). Surface levels of $\alpha V \beta 3$, $\alpha V \beta 5$ and $\alpha V \beta 6$ reveal differences between vector and miR-31 cells for $\alpha V \beta 3$ and $\alpha V \beta 5$ integrins. The integrin αV level has been used as the control. Empty histogram is for vector and filled histogram is for miR-31 overexpressing cells. Values are means \pm S.E. and are representative of 3 independent experiments.

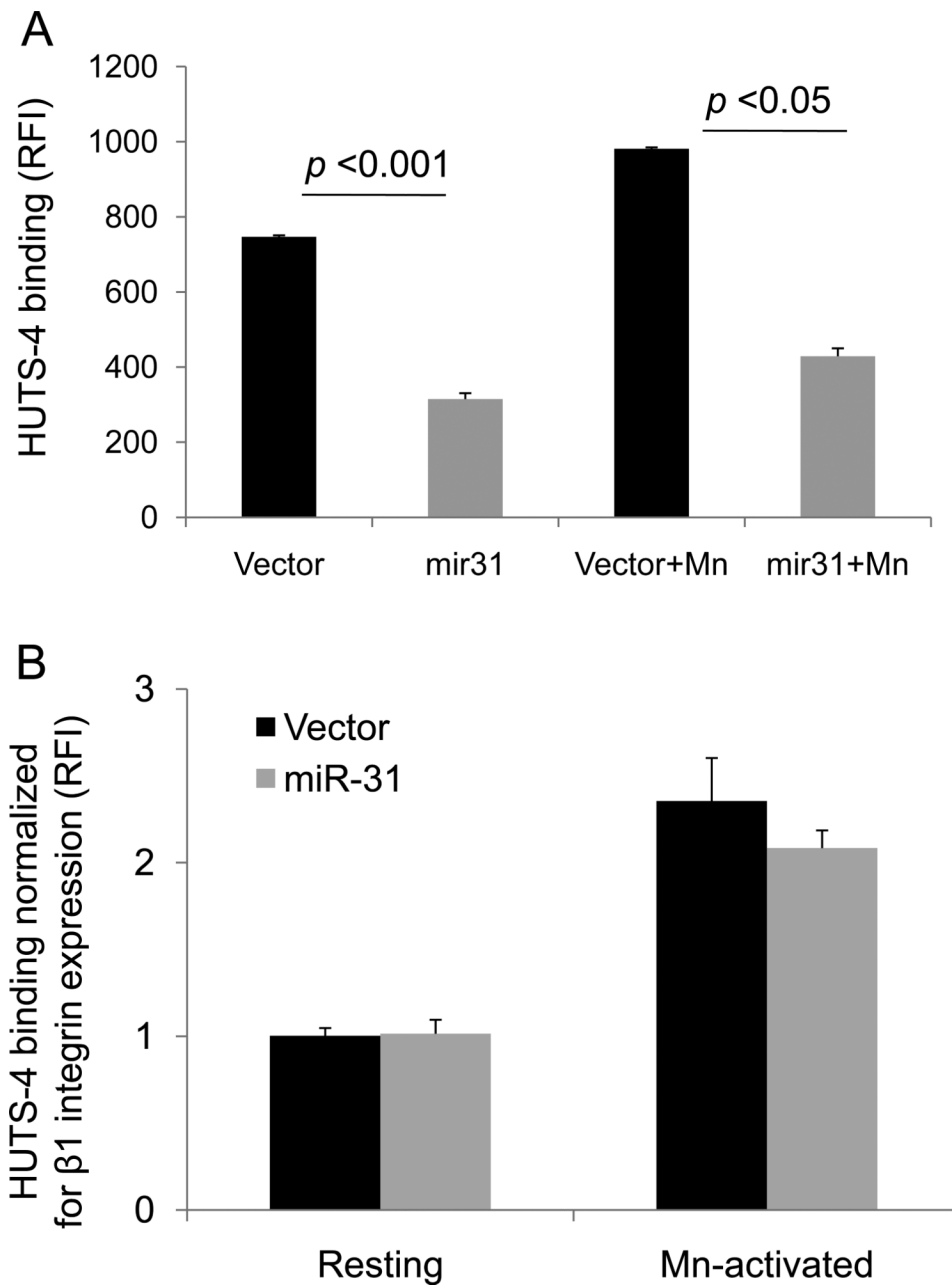


Fig. 4. Targeting of $\alpha 2$, $\alpha 5$ and αV subunits by miR-31 decreases $\beta 1$ integrin activation
 MDA-MB-231 cells stably overexpressing GFP-miR-31 show reduced $\beta 1$ integrin activation due to a quantitative reduction in $\beta 1$ integrin surface expression. (A) Activation of $\beta 1$ integrins was monitored with HUTS-4 antibody, specific for activated $\beta 1$ integrins, by flow cytometry. Spontaneous HUTS-4 binding or that induced by $MnCl_2$ are shown. Cells were either left untreated or treated with 3.5mM $MnCl_2$ and incubated with HUTS-4 antibody followed by antimouse Alexa Fluor 647 IgG to monitor $\beta 1$ integrin activation status. (B) HUTS-4 binding normalized to total $\beta 1$ integrin expression measured with mAb MAB2000 (Millipore) on control vector- or the miR-31-expressing cells in the resting and Mn^{2+} -activated states. HUTS-4 binding was done as in (A) and integrin expression was monitored using the same protocol as for HUTS-4 staining, using the expression-specific $\beta 1$ antibody. Values in both panels are means \pm S.E. and representative of 3 independent experiments.

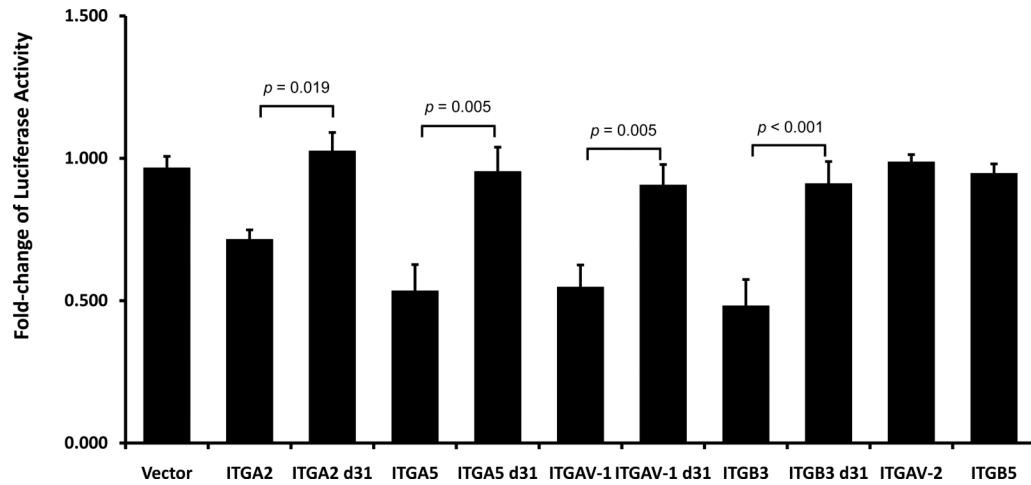


Fig. 5. miR-31 directly targets the 3'UTR of ITGA2, ITGA5, ITGAV and ITGB3 integrins and represses their expression in MDA-MB-231 cells
MDA-MB-231 cells were transfected with the luciferase reporter plasmids containing the 3'UTR of the indicated integrin subunit or with deletion of the miR31 seed sequences, and co-transfected with or without miR31. Luciferase activities were measured after 24 h. For all luciferase activity assays, renilla luciferase activity was used for normalization. The normalized firefly luciferase activity of each construct was plotted as a ratio of the control microRNA over the miR-31 treatment. The data are the mean \pm s. d. of at least 3 independent transfections.

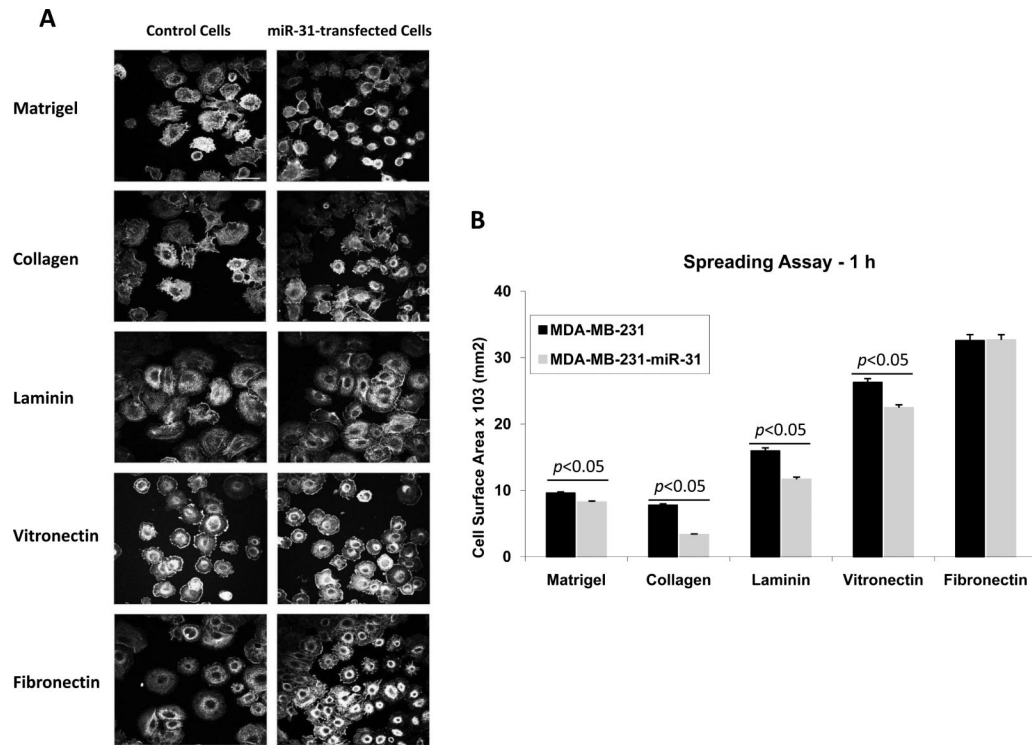


Fig. 6. miR-31 affects cell spreading in a ligand-dependent manner

Wild-type and miR-31-expressing MDA-MB-231 cells spread on collagen, Matrigel, fibronectin and laminin for 1 h, were fixed, permeabilized and stained for 30 min with Alexa 568-phalloidin to visualize actin filaments. (A) In comparison to vector control cells, miR-31-transfected cells showed significant inhibition of spreading and actin organization on collagen and Matrigel; lesser perturbation on laminin, and no difference on fibronectin. The images shown are representative micrographs of 12-15 independent scans for each integrin ligand. *Bar* = 40 μ m. (B) Quantitation of cell spreading presented as the total surface area covered by cells, in square micrometer, averaged to 300 cells.

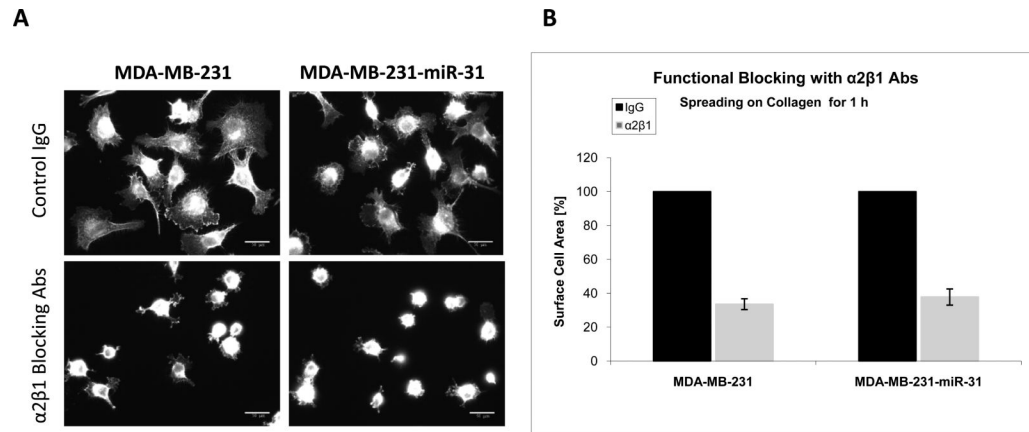


Fig. 7. A function blocking mAb to $\alpha 2\beta 1$ completely inhibits cell spreading in both the control cells and the miR31-expressing MDA-MB-231 cells

Wild-type and miR-31-expressing MDA-MB-231 cells were incubated with either an $\alpha 2\beta 1$ monoclonal blocking antibody (LS-C247646) or control IgG and spread on collagen for 1 h. Cells were then fixed, permeabilized and stained for 30 min with Alexa 568-phalloidin to visualize actin filaments. A. The images shown are representative micrographs of 12-15 independent scans for each treatment. *Bar* = 40 μm . B. Quantitation of cell spreading presented as the total surface area covered by cells, in square micrometer, from 300 cells.