Effects of β4 integrin expression on microRNA patterns in breast cancer

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Summary
The integrin α6β4 is defined as an adhesion receptor for laminins. Referred to as ‘β4’, this integrin plays a key role in the progression of various carcinomas through its ability to orchestrate key signal transduction events and promote cell motility. To identify novel downstream effectors of β4 function in breast cancer, microRNAs (miRNAs) were examined because of their extensive links to tumorigenesis and their ability to regulate gene expression globally. Two breast carcinoma cell lines and a collection of invasive breast carcinomas with varying β4 expression were used to assess the effect of this integrin on miRNA expression. A novel miRNA microarray analysis termed quantitative Nuclease Protection Assay (qNPA) revealed that β4 expression can significantly alter miRNA expression and identified two miRNA families, miR-25/32/92abc/363/363-3p/367 and miR-99ab/100, that are consistently downregulated by expression of this integrin. Analysis of published Affymetrix GeneChip data identified 54 common targets of miR-92ab and others identified by our initial array analyses. The results obtained in this study provide the first example of an integrin globally impacting miRNA expression and provide evidence that select miRNA families collectively target genes important in executing β4-mediated cell motility.

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Introduction
Integrins belong to a family of heterodimeric transmembrane cell surface receptors composed of α and β subunits that mediate stable adhesions between cells and their extracellular environment (Hynes, 1999; van der Flier and Sonnenberg, 2001). The integrin α6β4, referred to as ‘β4 integrin’, is an adhesion receptor for all of the known laminins. In a homeostatic setting, β4 links the intermediate cytoskeleton to laminins in the basement membrane through structures called hemidesmosomes located on the basal surface of epithelial cells (Borradori and Sonnenberg, 1999; Lee et al., 1992). The role of this integrin evolves, however, under pathological conditions when β4 is rendered signaling competent and assumes an active role in initiating various signaling cascades and facilitating cell motility. This role is particularly striking in the context of tumorigenesis, where factors in the microenvironment of invasive carcinomas promote relocalization of β4 from hemidesmosomes to the leading edge of cells, permitting its association with F-actin in motility structures and conferring a unique signaling potential (Lipscomb and Mercurio, 2005; Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999; Santoro et al., 2003; Sehgal et al., 2006; Yang et al., 2008). Recent work from our laboratory has established an association between β4 and a “basal-like” subset of breast carcinomas, in which the expression of this integrin predicts decreased time to tumor recurrence and decreased patient survival (Lu et al., 2008). β4 regulation of the expression and function of various downstream targets underlies the ability of this integrin to promote carcinoma progression (Guo et al., 2006; Lipscomb and Mercurio, 2005; O’Connor et al., 2000; O’Connor et al., 1998; Shaw et al., 1997; Yang et al., 2004; Zahir et al., 2003). MicroRNAs (miRNAs), however, represent a class of molecules that until recently had not yet been implicated in executing β4-mediated function. Work from our laboratory identified a role for miR-29a in regulating invasion downstream of this integrin (Gerson et al., 2012).

miRNAs are non-coding single-stranded RNAs approximately 22 base pairs in length that regulate gene expression through mRNA degradation or translational inhibition (Bartel, 2004; Bartel, 2009). In mammalian cells, miRNAs most commonly function by binding well-conserved imperfect complementary sequences in the 3’ untranslated region (UTR) of their target mRNA to block translation (Bartel, 2004; Bartel, 2009). Our work is the only to date that suggests a role for integrins in the regulation of this small class of RNAs. On the basis of our
previous observations, as well as the growing role of miRNAs in tumorigenesis (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006) and their ability to regulate gene expression, we explored the effect of β4 integrin on global miRNA expression using a novel array approach termed quantitative Nuclease Protection Assay (qNPA). The results obtained in this study demonstrate that β4 expression modulates families of miRNAs, and highlight a potential role for these miRNAs in executing β4-mediated cell motility.

Results

β4 status correlates with miRNA expression patterns

Two breast carcinoma cell lines and a collection of invasive breast carcinomas with varying β4 status were examined to assay the effect of this integrin on miRNA expression. MCF10CA1a cells were selected, because they are a highly aggressive breast carcinoma cell line in which β4 integrin is endogenously expressed. Expression of the integrin was transiently depleted using siRNA (Fig. 1A). MDA-MB-435 breast carcinoma cells, which express α6β4 endogenously but lack α6β4, were also chosen. Expression of the β4 subunit results in preferential heterodimerization of the α6 subunit with β4 (Hemler et al., 1989; Shaw et al., 1996). Stable subclones were generated expressing wild type β4 (referred to as β4 transfectants); mock transfectants were also generated (Fig. 1B). As the final component of our analysis, a subset of breast carcinoma specimens was analyzed to substantiate cell line observations and establish a link between β4 and miRNAs in vivo. Specifically, twenty invasive ductal breast carcinomas were examined, half of which were positive for β4 expression, as established previously in our laboratory (Lu et al., 2008).

To assay global miRNA expression, a novel microarray technology termed quantitative Nuclease Protection Assay (qNPA) was utilized. MCF10CA1a cells transfected with control siRNA or siRNA to β4 were collected 72 hours post-transfection and analyzed by qNPA. Transient depletion of β4 in these cells altered the expression of 40 miRNAs (supplementary material Table S1). Two subclones of the MDA-MB-435/β4 transfectants (3A7 and 5B3) and two subclones of the MDA-MB-435/mock transfectants (6D2 and 6D7) were examined for differential miRNA expression by qNPA. Introduction of β4 into this system changed the expression of 46 miRNAs (supplementary material Table S2). Finally, ten β4 positive and ten β4 negative invasive breast carcinomas were also examined, and our analysis identified 72 miRNAs that were differentially expressed between tumor subsets (supplementary material Table S3). Statistical parameters of p-value < 0.05 and a ≥1.2-fold change cut-off were applied to all array datasets. A miRNA was excluded from the analysis if its expression was disconcordant across the three different arrays. The results from the three arrays are depicted in heat maps, in which the expression of each miRNA across samples was assigned a color value (Fig. 2). The top 30 differentially regulated miRNAs from each array are presented in Table 1. All miRNAs are normalized to the β4 null sample in each array, such that fold changes reflect the effect of the presence of β4 on any given miRNA. miRNAs are ranked by increasing fold change. Of particular interest, the major effect of β4 on miRNA expression appears to be repressive in nature.

β4 inversely correlates with the expression of select miRNA families

We next sought to correlate the results of the cell line and tumor analyses. miRNAs undergoing significant changes in expression were compared across datasets (Fig. 3A). Two miRNAs, miR-100 and miR-1244, were altered in all three arrays. While miR-100 is a well-characterized miRNA widely expressed across vertebrates, very little is known about miR-1244 (Wienholds et al., 2005). Upon closer examination of the data, we noted that several of the differentially regulated miRNAs belonged to common miRNA families. A miRNA family is commonly defined as a group of miRNAs that shares the same seed sequence (nucleotides 2–7) and therefore largely overlapping target genes. Our observation prompted us to examine the idea that specific miRNA families might be influenced by β4 expression. To address this hypothesis, all miRNA families represented in Fig. 3A were identified. We then searched for

Fig. 1. β4 expression in breast carcinoma cell lines and invasive breast carcinomas. (A) Expression of β4 in total cell extract (50 μg) following transient knockdown of β4 at 72 hours post-transfection in MCF10CA1a cells. (B) Expression of β4 in total cell extract (50 μg) in MDA-MB-435/β4 and mock transfectants.
miRNAs from each family across arrays. A miRNA family was included in the analysis if two or more family members appeared in at least two of the three different array comparisons. Conversely, miRNA families were excluded from consideration if the expression of any single family member was discordant with the expression profile of other family members within or across the three different arrays. The results of our analysis identified seven families of miRNAs that changed in at least two of the arrays and two families of miRNAs whose expression was altered in all three of the arrays (Fig. 3B; Table 2).

miRNA families target common β4-regulated genes involved in cell motility

miRNA families miR-25/32/92abc/363/363-3p/367 and miR-99ab/100 were identified by all three arrays as miRNA families whose expression is inversely correlated with β4 status. Specifically, miR-92a and miR-92b as well as miR-99a, miR-99b, and miR-100 are downregulated in the presence of β4 across systems (Table 3). To explore the implications of this observation and to validate the physiological relevance of these miRNAs downstream of β4, we analyzed the mRNA data from a published Affymetrix GeneChip performed using the MDA-MB-435/β4 model system (Chen et al., 2009). Specifically, we considered the possibility that these two families of miRNAs might be working in concert to upregulate the expression of genes important in executing β4 function. To address this idea, we compared miR-92ab and miR-99ab/100 putative targets and generated a list of overlapping genes. We then searched for these common genes within β4-regulated miRNAs. Our analysis identified 54 β4-regulated genes that are predicted targets of both miR-92ab and miR-99ab/100 miRNA families, applying a p-value <0.05 and a 1.2-fold change cut-off (supplementary material Table S4). A list of the top 30 genes is presented in Table 4 and ranked in order of fold change.

It was immediately apparent that several of these targets play critical roles in mediating cell motility, prompting us to speculate that these families of miRNAs specifically target genes involved in this biological process. Applying the AmiGo gene ontology classification database v1.8 (Ashburner et al., 2000; Carbon et al., 2009), an enrichment was detected in genes associated with the cell motility classification database v1.8 (Ashburner et al., 2000; Carbon et al., 2009).
NF1, and CDK6. Closer analysis uncovered additional genes that have been shown to provide cell motility despite having not been picked up by our gene ontology analysis. These genes include PIK3R3 (McAuliffe et al., 2010), PPMID (Wang et al., 2011), RASGRP3 (Randhawa et al., 2011; Yang, D. et al., 2010), ADAM19 (McAuliffe et al., 2010), VLDLR (Khatchadourian et al., 2007), ITGA2 (Li et al., 2011; Shen et al., 2011), SORBS3 (Kioka et al., 2010; Förster et al., 2010), HIP1 (Khatchodourian et al., 2007), PAXIP1 (Mu et al., 2008), ITGA2 (Mercurio, 2002), ARFGEF1 (Li et al., 2011; Shen et al., 2007).

Interestingly, several genes also play distinct roles in β4-mediated signaling cascades, including PIK3R3, a regulatory subunit of the PI3K complex, as well as PTPN11, also known as SHP-2. Such observations are intriguing given that β4 signals through the PI3K signaling cascade to increase cell migration and invasion (Shaw et al., 1997). Furthermore, it was recently established that the tyrosine phosphatase SHP-2 binds to the cytoplasmic tail of β4 and plays a key role in activating downstream signaling events critical for cell invasion (Meredek et al., 2007; Yang, X. et al., 2010). These data provide compelling evidence that β4 regulation of cell migration is executed in part by miR-92ab and miR-99ab/100 miRNA families through upregulation of genes both directly involved in cell migration as well as those important for preceding signal transduction events.

β4-regulated mRNAs are enriched in putative targets of miRNA families
To extend our analysis, we next conducted gene set enrichment analyses to determine whether β4-regulated mRNAs were enriched for targets belonging to these two miRNA families. A significant enrichment was detected (p=0.028) for putative miR-92ab targets in this population of genes; however, our analysis did not identify an enrichment for miR-99ab/100 predicted targets (Fig. 4A). While this finding suggests that the miR-99ab/100 family likely does not target a large population of β4-regulated genes, it does not negate the possibility that these miRNAs function downstream of β4 to regulate the expression of select target genes involved in executing β4 function. Work published from our laboratory has also established there to be no enrichment for predicted targets of miR-93, a miRNA selected as a negative control on the basis that it was expressed at robust levels in all samples from the qNPA arrays but did not change in response to expression of β4 (Gerson et al., 2012). As part of this analysis, lists of leading edge genes were generated, a compilation of mRNAs that contribute to the detected enrichment for miR-92ab (supplementary material Table S5).

Based on our findings, we were curious to determine whether other predicted targets for families of miRNAs were also enriched in this population of β4-regulated mRNAs. To explore this idea using an unbiased approach, we employed the Broad Institute’s Molecular Signatures Database (MSigDB) C3:MIR Database, composed of gene sets sharing a 3′-UTR miRNA binding motif (Subramanian et al., 2005). Interestingly, a comparison of this dataset to our β4-regulated mRNAs identified an enrichment for several of the miRNA families depicted in Fig. 3B and Table 2, including miR-15abc/16/16abc/195/322/424/497/1907, miR-23abc/23b-3p, miR-27abc/27a-3p, and miR-30b/30a/5p/30d/30e in response to expression of β4 (Gerson et al., 2012). As part of this analysis, lists of leading edge genes were generated, a compilation of mRNAs that contribute to the detected enrichment for miR-92ab (supplementary material Table S5).

Discussion
We conclude from this study that integrin expression correlates with specific patterns of miRNA expression and that β4 integrin status affects the expression of specific families of miRNAs. Manipulation of β4 expression in two breast cancer cell lines provided in vitro model systems for analysis, while a collection of invasive breast carcinoma specimens established an in vivo link to the cell line data. The novel qNPA array technology identified two miRNA families, miR-25/32/92abc/363/363-3p/367 and miR-99ab/100, as undergoing repression in the presence of β4 across all systems. An analysis of published Affymetrix GeneChip data (Chen et al., 2009) identified 54 common putative targets of these two miRNA families within β4-regulated genes. Many of these identified genes are established mediators of cell adhesion, cell motility, and signal transduction. Statistical analysis established that this population is enriched in genes involved in cell migration. These data reveal previously
recognized β4 targets, which could contribute to the ability of β4 to promote carcinoma progression. Finally, gene set enrichment analysis detected an enrichment in predicted targets of several miRNA families, including miR-92ab, within β4-regulated genes, substantiating the physiological relevance of our findings with respect to the effect of β4 on the expression of distinct miRNA families.

Although the fields of integrin and miRNA biology have been extensively linked to cancer initiation and progression, the connection between these two disciplines has remained elusive. Our novel observation that a specific integrin correlates with miRNA expression has implications for development and disease, especially tumorigenesis. Along these lines, tyrosine kinase receptors, such as EGFR, have also been shown to regulate miRNA expression (Avraham et al., 2010). Our data support the hypothesis that cells utilize this small class of RNAs to respond to external cues in their microenvironment, employing surface receptors like integrins as intermediates in the delivery of key information. An interesting observation that emerged from the results of the miRNA microarray analysis involves the predominantly repressive effect of β4 on global miRNA expression. This is consistent with published data describing global downregulation of miRNA expression in cancers (Gaur et al., 2007; Lu et al., 2005). Differential expression of the endogenous miRNA processing machinery represents a potential explanation for the repressive patterns of miRNA expression that we observed, as recent reports have highlighted the importance of miRNA processing genes in the regulation of miRNA biogenesis and function (Cheng et al., 2009; Van der Auwera et al., 2010). We examined the expression of dicer,
Table 4. Predicted targets of miR-92ab and miR-99ab/100 families among β4-regulated genes.

| Gene ID   | p-value | False Discovery Rate | Average β4 Intensity | Average Mock Intensity | Fold Change (β4/Mock) |
|-----------|---------|----------------------|----------------------|------------------------|-----------------------|
| EPHA3     | 4.79E−04| 1.56E−02             | 265                  | 104                    | 2.54                  |
| GOLGA5A   | 7.60E−06| 1.54E−03             | 148                  | 65                     | 2.28                  |
| ABHD2     | 4.79E−05| 4.13E−03             | 168                  | 75                     | 2.22                  |
| SGRID     | 7.74E−03| 7.84E−02             | 259                  | 124                    | 2.09                  |
| DCP2      | 3.05E−04| 1.19E−02             | 149                  | 84                     | 1.78                  |
| RMN5D1A   | 1.37E−03| 2.79E−02             | 108                  | 61                     | 1.76                  |
| WWFP2     | 3.37E−03| 4.75E−02             | 377                  | 220                    | 1.71                  |
| AMMETRI    | 1.55E−04| 7.88E−03             | 125                  | 73                     | 1.70                  |
| KLMH3      | 3.07E−03| 3.27E−03             | 759                  | 461                    | 1.65                  |
| PTPN11     | 1.96E−04| 9.26E−03             | 335                  | 210                    | 1.60                  |
| ZCH41     | 9.30E−03| 8.74E−02             | 168                  | 108                    | 1.56                  |
| ZFP106     | 3.67E−02| 2.01E−01             | 473                  | 310                    | 1.52                  |
| CTDPSL     | 6.60E−05| 4.94E−03             | 419                  | 276                    | 1.51                  |
| BAT2L2     | 2.94E−03| 4.40E−02             | 137                  | 92                     | 1.49                  |
| PIK3R4     | 3.33E−03| 4.72E−02             | 125                  | 84                     | 1.49                  |
| ZNF562     | 6.64E−04| 1.88E−02             | 48                   | 33                     | 1.47                  |
| EFNB2      | 8.31E−03| 8.19E−02             | 70                   | 48                     | 1.46                  |
| PPM1D      | 7.34E−05| 5.13E−03             | 41                   | 28                     | 1.46                  |
| SOBP       | 9.55E−03| 8.89E−02             | 40                   | 28                     | 1.46                  |
| NKR        | 2.50E−03| 4.01E−02             | 84                   | 184                    | 1.43                  |
| FOXO3      | 2.59E−03| 4.11E−02             | 262                  | 184                    | 1.42                  |
| ZNF31      | 7.34E−05| 5.13E−03             | 64                   | 45                     | 1.42                  |
| PKNOX1     | 1.47E−04| 7.69E−03             | 68                   | 49                     | 1.40                  |
| RASGRP3    | 7.45E−05| 7.64E−02             | 35                   | 25                     | 1.40                  |
| ADAM19     | 1.61E−03| 3.08E−02             | 200                  | 146                    | 1.37                  |
| GNS        | 1.64E−03| 3.11E−02             | 147                  | 107                    | 1.37                  |
| MFHAS1     | 5.10E−03| 6.04E−02             | 213                  | 155                    | 1.37                  |
| WDFY3      | 4.25E−02| 2.19E−01             | 60                   | 43                     | 1.37                  |
| WDR37      | 1.01E−02| 9.20E−02             | 199                  | 145                    | 1.37                  |
| SORB5      | 4.62E−02| 2.29E−01             | 289                  | 215                    | 1.34                  |

drosha, ago1, ago2, and trhp2 mRNAs between the β4 and mock transfectants using Affymetrix GeneChip data but observed no change that could account for the downregulated pattern of miRNA expression (data not shown).

Our observation that family members miR-92a and miR-92b are consistently downregulated in the presence β4 in our arrays is interesting considering the defined role of miR-92a as an “oncomir” (Olive et al., 2010). miR-92b belongs to the miR-17–92 cluster, a group of six miRNAs generated from a single polycistrionic transcript that includes miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a. This cluster confers potent oncogenic potential and is overexpressed in a variety of cancers, often the result of genomic amplification (Olive et al., 2010). These findings are seemingly at odds with our observation that miR-92a inversely correlates with the expression of β4, an integrin with a well-established role in potentiating carcinoma cell migration, invasion, and survival. Recent data, however, have identified a role for miRNAs from this family as tumor suppressors (O’Donnell et al., 2005), highlighting the importance of cellular and molecular context in determining the role of specific miRNAs in tumorigenesis. Interestingly, an analysis of the arrays failed to identify consistent downregulation of other members from this miRNA cluster with the exception of miR-19b, which was repressed in two of the three arrays (data not shown). miR-92b, despite sharing the same seed sequence and common putative mRNA targets with miR-92a, is transcribed from an independent genomic locus and is less well characterized from a functional standpoint. Its intergenic location near the THBS3 gene, which is known to share a common promoter with MTX1, prompted us to examine both thrombospondin 3 and metataxin 1 mRNA expression using our Affymetrix GeneChip data from the MDA-MB-435/β4 cells. Conveniently, miR-92b was downregulated in this particular miRNA array; however, no detectable changes were observed in the expression of either thrombospondin 3 or metataxin 1 mRNA levels in this system (data not shown). This finding, along with the paucity of other downregulated miRNAs from the miR-17–92 cluster, suggest changes in miR-92a and miR-92b expression are not mediated at a transcriptional level, rather the presence of this integrin likely affects the stability of these previously transcribed miRNAs. Our hypothesis is intriguing in light of recent data linking miRNA decay to changes in cell adhesion (Kim et al., 2011), as well as the general notion that global miRNA expression is typically downregulated in cancer (Gaur et al., 2007; Lu et al., 2005).

The role of miR-99a, miR-99b, and miR-100, the other miRNA family identified by our array, in tumorigenesis appears to be controversial. However, downregulation of members of this miRNA family has been linked to breast carcinoma, hepatocellular carcinoma, prostate carcinoma, nasopharyngeal carcinoma, oral carcinomas, hepatoblastoma, and ovarian carcinoma (Cairo et al., 2010; Henson et al., 2009; Lobert et al., 2011; Nam et al., 2008; Petrelli et al., 2012; Shi et al., 2010; Sun et al., 2011; Wong et al., 2008). All three miRNAs are transcribed from independent genomic loci with clustered miRNAs. miR-99a is co-transcribed with let-7c, miR-99b is co-transcribed with let-7e and miR-125a, and miR-100 is an intergenic miRNA co-transcribed with let-7a. Again using the Affymetrix GeneChip data from the MDA-MB-435/β4 cells, we detected no change in the expression of genes surrounding the miR-100 cluster despite downregulation of miR-100 in this system (data not shown). However, we noted that all of the other co-transcribed clustered miRNAs were repressed across arrays (Table 2). In fact, let-7a, let-7c, and let-7e belong to the let-7/98/4458/4500 miRNA family and miR-125a belongs to the miR-
Moreover, these data contribute to our understanding of P4 function in the context of signal transduction, implying that this integrin not only activates signaling cascades through phosphorylation events but it also may alter the expression of key molecules involved in these complex processes by regulating miRNAs. Future studies aimed at exploring the mechanism of regulation of miR-25/32/92abc/363/363-3p/36 and miR-99ab/100 miRNA families in the presence of P4, as well as the role of putative targets in mediating cell motility downstream of this integrin, will provide further insight into the role of P4 function in promoting carcinoma progression.

Materials and Methods

Cell lines, antibodies, and reagents
MDA-MB-435 cells (Price et al., 1990) were obtained from the Lombardi Cancer Center (Georgetown University, Washington, DC, USA). MCF10C1a1a cells (Miller et al., 1993) were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI, USA). MDA-MB-435 cell lines were maintained in low glucose DMEM medium ( Gibco, Carlsbad, CA, USA) supplemented with 10 mM HEPES, 5% fetal bovine serum, and 1% streptomycin and penicillin. MCF10C1a1a cells were maintained in DMEM/F12 1:1 medium ( Gibco, Carlsbad, CA, USA) supplemented with 10 mM HEPES, 5% horse serum, and 1% streptomycin and penicillin. All cell lines were grown at 37°C in an incubator supplied with 5% CO2.

siRNA experiments
MCF10C1a1a cells were transfected with 20 nM On-TARGETplus SMARTpool siRNA targeting P4 (Dharmacon, Lafayette, CO, USA) at 50% confluency using DharmaFECT 4 transfection reagent (Dharmacon, Lafayette, CO, USA). A non-targeting siRNA pool (Dharmacon, Lafayette, CO, USA) was used as a control for these experiments. At 72 h post-transfection, cells were harvested for protein as described below.

Immunoblotting
Cells were solubilized on ice for 10 min in Triton X-100 lysis buffer (Boston Bioproducts, Ashland, MA, USA) containing 50 mM Tris buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitors (Complete mini tab; Roche Applied Science, Indianapolis, IN, USA) (Lysis Buffer A). Nuclei were removed by centrifugation at 16,100 g for 10 min. Concentrations of total cell lysate were assayed by Bradford method. Lysates (50 μg) were separated by electrophoresis through 10% SDS-PAGE and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 5% non-fat milk in Tris-buffered saline/Tween 20 for 1 h and blotted with the antibody to P4 (1:4,000) or tubulin (1:10,000) overnight at 4°C. Proteins were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) after incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies.

Tumor samples
A total of 20 cases of invasive ductal breast carcinomas were gross dissected by the Department of Pathology at the University of Massachusetts Medical School, Worcester, MA, USA. Ethics approval was not necessary because samples were discarded, anonymous, de-identified breast cancer specimens provided by the UMass Cancer Center Tissue Bank, which collects fresh tumor samples from patients with triplenegative breast cancer that is not eligible for routine surgical intervention. All samples were snap-frozen in liquid nitrogen immediately after excision. Tumor samples were used to study 1050 mature miRNAs in human, rat, and mouse based upon the Sanger miRBase release 9.1. The qNPA-based miRNA microarrays comprised DNA oligo capture probes that were synthesized directly on the slide surface (Roche NimbleGen, Madison, WI, USA) which are complementary to, and capture, biotinylated miRNA-specific nucleic acid protection probes. Each microarray slide has 21 synthesized arrays, each representing all of the 1050 miRNAs plus
housekeeper genes, in separate wells that mimics standard SBS 96-well format plates. Arrays were run 24-4 from front to back (The Gel Company, San Francisco, CA, USA), permitting 24 samples to be tested per slide.

Sample preparation
For cell line analysis, cell lysates were prepared at a final concentration of 25,000 cells per reaction in 25 μl of Lysis Buffer (HTG). For formalin-fixed paraffin-embedded (FFPE) samples, FFPE tissue was snapped off of slides into a clean eppendorf tube. Tissues were lysed in 100 μl of Lysis Buffer in >1600 μl of Denaturation Oil at 95°C for 15–20 min followed by digestion with 1:20 proteinase K (Ambion, Austin, TX, USA). Proteinase K digested FFPE lysate was distributed into 25 μl aliquots for each technical replicate and processed by regular qNPA procedure. Three technical replicate samples were used for assaying miRNA expression.

qNPA procedure and quantification
qNPA was performed using 16–28bp complementary and 5’ biotinylated Nuclease Protection Probes (NPPs) matching all the unique human, rat, and mouse miRNA sequences from miRBase release 9.1. Nuclease Protection Probes were added at a final concentration of 31.5 pm. Samples were overlaid with 70 μl of Denaturation Oil (HTG) and heated to 95°C for 10–15 min followed by 16–24 h hybridization in a 37°C incubator to allow formation of NPP-miRNA duplexes. S1 nuclease was then added to degrade all non-hybridized NPPs, leaving behind NPP-miRNA duplexes. Base hydrolysis treatment of the NPP-miRNA complexes at 95°C followed; resulting in dissociation of the duplex, hydrolysis of the target miRNA, and free single-stranded NPPs present in amounts stoichiometric to those of miRNA present in the sample. These free single-stranded NPPs were available for capture and detection on the array. Base treatment was followed by neutralization using Neutralization solution (HTG) containing 1:200 proteinase K (Ambion, Austin, TX, USA). The resulting qNPA lysate was then hybridized to the qNPA miRNA microarrays for 16–24 h in a 50°C incubator for quantification of the NPPs. After the NPP hybridization, qNPA Microarrays were washed rigorously with 1× wash buffer (HTG). Microarrays were then hybridized with Avidin-peroxidase (1:600) and Nimblegen alignment oligos (500 pM) in Detection buffer. After a 3-min room temperature incubation, TSA-Plus Cy3 reaction was stopped by washing the arrays in wash buffer. Finally, Microarrays were spun dry and scanned at 5 μm resolution using a GenePix 4200AL microarray slide scanner (Molecular Devices, Sunnyvale, CA, USA). Probe intensities were extracted from TIFF images using NimbleScan 2.5 software (Roche NimbleGen) for further analysis.

Statistical analysis
Microarrays for each sample were performed in triplicate (technical replicates). For each array, human mRNA raw expression values were extracted, converted to log base 2, and intra-array mRNA replicates (spot replicates) averaged. Arrays were then normalized against the 25th percentile of median expression values on each array. BRB-ArrayTools v4.1.0 was used for all analyses (Simon et al., 2007). Differentially expressed miRNAs were selected using a random variance t-test p-value less than 0.05 and an absolute fold change greater than 1.2. miRNAs were eliminated from consideration after adjustment for multiple testing using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995). The FDR was calculated during analysis using the software Bioconductor (Gentleman et al., 2004). Gene ontology enrichment analysis was performed using the Gene Ontology (GO) database (Ashburner et al., 2000; Gene Ontology Project, 2004). GO Enrichment analysis was performed using the Gene Ontology (GO) database (Ashburner et al., 2000; Gene Ontology Project, 2004).

References
Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful method for multiple testing. J. R. Stat. Soc. B 57, 289-300.
Benner, A. S. and Sonenberg, N. (2009). Structure and function of heme biosomes: more than simple adhesion complexes. J. Invest. Dermatol. 112, 411-418.
Cairns, S., Wang, J., de Reyniers, A., Duroure, K., Dahan, D., Redon, M. J., Fabre, M., McClelland, M., Wang, X. W., Croce, C. M. et al. (2010). Stem cell-like microRNA signature driven by Myc in aggressive liver cancer. Nat. Rev. Clin. Oncol. 7, 204-217.
Calin, G. A. and Croce, C. M. (2006). MicroRNA signatures in human cancers. Nat. Rev. Cancer 6, 857-866.
Carbon, S., Ireland, A., Mangill, C. J., Shu, S., Marshall, B., Lewis, S. and the AmiGO Hub and the Web Presence Working Group, (2009). AmiGO online access to ontology and annotation data. Bioinformatics 25, 288-289.
Chen, M., Sinha, M., Luxon, B. A., Bresnick, A. R. and O’Connor, K. L. (2009). Integrin α4β1 regulates the expression of genes associated with cell motility, invasion, and metastasis, including S100A4/metaspin. J. Biol. Chem. 284, 1484-1494.
Cheng, C., Fu, X., Alves, P. and Gerstein, M. (2009). miRNA expression profiles show differential regulatory effects of microRNAs between estrogen receptor-positive and estrogen receptor-negative breast cancer subtypes. BMC Cancer 9, 5.
Degano, A. L., Pasterkamp, R. J. and Ronnett, G. V. (2007). MicroRNA deficiency disrupts axonal guidance, fasciculation, and targeting by altering Semaphorin 3F signaling. Mol. Cell. Neurosci. 24, 234-253.
Esquela-Kerscher, A. and Slack, F. J. (2006). Oncorni - microRNAs with a role in cancer. Nat. Rev. Cancer 6, 259-269.
Förster, E., Bock, H. H., Herz, J., Chai, X., Frotscher, M. and Zhao, S. (2010). Integrating AML data in relation to clinical features. Int. J. Hematol. 91, 151-158.
Gaur, A., Jewell, D. A., Liang, Y., Ridzon, D., Moore, J. H., Chen, C., Ambros, V. R., Calin, G. A. and Croce, C. M. (2006). Integrin α4β1 regulates SARC protein to promote invasion. J. Biol. Chem. 281, 9035-9044.
Guen, W., Palyaveva, Y., Pepe, A., Yoshinaka, T., Muller, W. J., Inghirami, G. and Giancotti, F. G. (2006). Integrin α4β1 and metastasis, including S100A4/metastasin. Cell 126, 489-502.
Rabinovitz, L., Toker, A. and Mercurio, A. M. (1999). Protein kinase C-dependent mobilization of the \( \eta_6 \) integrin from hemidesmosomes and its association with actin-rich cell protrusions drives the chemotactic migration of carcinoma cells. J. Cell Biol. 146, 1147-1160.

Randhawa, P. K., Rylova, S., Heinz, J. Y., Kiser, S., Fried, J. H., Dunworth, W. P., Andjelkovic, M., L. L. Barber, A. T., Chappell, J. C., Roberts, D. M. et al. (2011). The Ras activator RasGRP3 mediates diabetes-induced embryonic defects and affects endothelial cell migration. Circ. Res. 108, 1199-1208.

Santerre, M. M., Gaudino, G. and Marchillos, P. C. (2003). The MSP receptor regulates \( \eta_6 \) and \( \eta_11 \) integrins via \( 14-3-3 \) proteins in keratinocyte migration. Dev. Cell 5, 257-271.

Seghal, B. U., DeBlase, P. J., Matzno, S., Chew, T. L., Claiborne, J. N., Hopkinson, S. B., Russell, A., Marinovich, M. P. and Jones, J. C. (2006). Integrin \( \gamma 4 \) regulates motile behavior of keratinocytes by determining laminin-332 organization. J. Biol. Chem. 281, 53847-53854.

Shaw, L. M., Lotz, M. and Mercurio, A. M. (1993). Inside-out integrin signaling in macrophages. Analysis of the role of the \( \alpha 6 \) and \( \beta 1 \) integrin isoforms in laminin adhesion by cDNA expression in an \( \eta 6 \) integrin-deficient macrophage cell line. J. Biol. Chem. 268, 11401-11408.

Shaw, L. M., Chao, C., Wewer, U. M. and Mercurio, A. M. (1996). Function of the integrin \( \eta 6 \) in metastatic breast carcinoma cells assessed by expression of a dominant-negative receptor. Cancer Res. 56, 959-963.

Shaw, L. M., Rabinovitz, L., Wang, H. H., Toker, A. and Mercurio, A. M. (1997). Activation of phosphoinositide 3-kinase by the \( \eta 6 \) integrin promotes carcinoma invasion. Cell 91, 949-960.

Shen, X., Hong, M. S., Moss, J. and Vaughan, M. (2007). BIG1, a brefeldin A-inhibited guanine nucleotide-exchange (BIG) 1 and BIG1 protein on cell periphery and directed migration during wound healing. Proc. Natl. Acad. Sci. USA 104, 19228-19233.

Lipscomb, E. A. and Mercurio, A. M. (2005). Mobilization and activation of a signaling competent \( \eta_6 \) integrin underlies its contribution to carcinoma progression. Cancer Metastasis Rev. 24, 413-423.

Lobert, S., Jefferson, B. and Morris, K. (2007). Structural abnormalities in spermatids together with reduced sperm count and motility underlying the reproductive defect in HIP1\(-/\-) mice. J. Biol. Chem. 282, 343-359.

Kim, Y. K., Yeo, J., Ha, M., Kim, B. and Kim, V. N. (2011). Cell adhesion-dependent control of microRNA decay. Mol. Cell. 43, 1005-1014.

Kikuzawa, K., Itou, T., Yamashita, H., Uekawa, K., Umemoto, T., Mototsuji, S., Imai, T., Takahashi, K., Watanabe, H., Yamada, M. et al. (2010). Crucial role of vinexin for keratinocyte migration in vitro and epidermal wound healing in vivo. Exp. Cell Res. 316, 1728-1738.

Lee, E. C., Lottz, M. B., Steele, G. D. and Jr and Mercurio, A. M. (1992). The integrin alpha 6 beta 4 is a laminin receptor. J. Cell Biol. 117, 671-678.

Li, C. C., Kuo, J. C., Waterman, C. M., Kiyama, R., Moss, J. and Vaughan, M. (1996). Function of the \( \eta_6 \) integrin in carcinoma cell migration on laminin by expression of a dominant-negative receptor. J. Biol. Chem. 271, 11401-11408.

Liu, C., Kuo, J. C., Waterman, C. M., Kiyama, R., Moss, J. and Vaughan, M. (2011). Effects of brefeldin A-inhibited guanine nucleotide-exchange (BIG) 1 and BIG1 protein on cell periphery and directed migration during wound healing. Proc. Natl. Acad. Sci. USA 108, 19228-19233.

Lippens, S. T., Alajez, N., Badovinac, H., Bozanic, J. H., Buss, P., Lo, K. W., Ng, R., Walderlon, J. et al. (2010). Significance of PI3K \( \gamma 1 \) reduction by miR-100 in human nasopharyngeal cancer. Int. J. Cancer 126, 2036-2048.

Lincoln, S., Alam, L., Li, M. C., Ngan, M., Menendez, S. and Zhao, Y. (2007). Analysis of gene expression data using BRB-ArrayTools. Cancer Inform. 3, 11-17.

Subramanian, A., Tamayo, P., Mothia, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S. et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA 102, 15545-15550.

Subramanian, A., Kunein, H. G., Jacqu, A., Takenesi, P. et al. (2007). GSEA-P: a desktop application for Gene Set Enrichment Analysis. Bioinformatics 23, 3251-3253.

Sun, S., Lee, Y. S., Malhotra, H., Kim, H. K., Mateic, M., Evans, C., Jensen, R. V., Moskaluk, C. A. and Dutta, A. (2011). miR-99 family of MicroRNAs suppress the expression of prostate-specific antigen and prostate cancer cell proliferation. Cancer Res. 71, 1313-1324.

Van der Auwer, L., Limaye, R., van Dam, P., Vermeulen, B. P., Dirix, L. Y. and Van Laere, S. J. (2010). Integrated miRNA and mRNA expression profiling of the inflammatory breast cancer subtype. Br. J. Cancer 103, 532-541.

van der Flier, L. and Sonnenberg, A. (2001). Function and interactions of integrins. Curr. Top. Cell. Regul. 305, 285-298.

Wang, P., Ruo, J., Yang, H., Zhao, H., Yang, M. et al. (2011). PPM1D silencing by miR-100 promotes carcinoma invasion. J. Biol. Chem. 286, 28588-28596.

Yang, D., Kedzi, N., Li, L., Tao, J. Velsades, J. F., Michalowski, A. M., Töbi, B. L., Marinkovich, M. P. and Weaver, V. M. (2006). The MSP receptor regulates actin-rich cell protrusions that drive the chemotactic migration of carcinoma cells. J. Cell Biol. 174, 1749-1760.

Yang, D., Totta, U. and Shaw, L. M. (2010). SHP2 mediates the localized activation of Fyn downstream of the \( \eta 6 \) integrin to promote carcinoma invasion. Mol. Cell. Biol. 30, 5306-5317.

Yaqubuddin, A., Abbas, F., Naqi, S. Z., Bashir, M. U., Qazi, R. and Qureshi, S. A. (2008). Silencing of MBID1 and MeCP2 in prostate-cancer-derived PC3 cells produces differential gene expression profiles and cellular phenotypes. Biosci. Rep. 28, 319-326.

Zahn, M., Lakins, J. N., Russell, A., Ming, W., Chatterjee, C., Rozenberg, G., L. Marinkovich, M. P. and Weaver, V. M. (2003). Autocine laminin-5 ligation of \( \eta 6 \) integrin and activates RAC and NFX2 to mediate anchorage-independent survival of mammary tumors. J. Cell Biol. 163, 1397-1407.