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 miR-99ab/100 within the subset of β4-regulated mRNAs, revealing several genes known to be key components of β4-regulated signaling cascades and effectors of cell motility. Gene ontology classification identified an enrichment in genes associated with cell migration within this population. Finally, gene set enrichment analysis of all β4-regulated mRNAs revealed an enrichment in targets belonging to distinct miRNA families, including 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.

Key words: Integrin β4, MicroRNA, Breast cancer, Cell motility

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 α6β1 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.

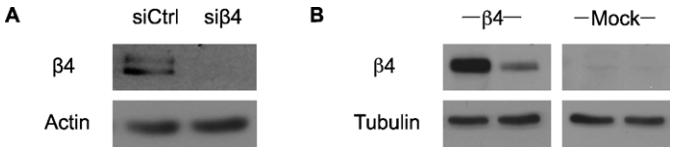

Fig. 1. β4 expression in breast carcinoma cell lines and invasive breast carcinomas. (**A**) Expression of β4 in total cell extract (50 μg) following transient knockdown of β4 at 72 hours post-transfection in MCF10CA1a cells. (**B**) Expression of β4 in total cell extract (50 μg) in MDA-MB-435/β4 and mock transfectants.

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

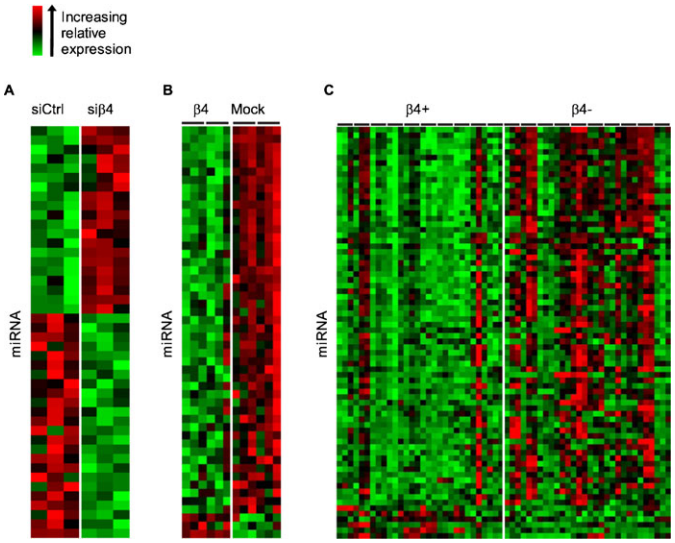

Fig. 2. β4 correlates with miRNA expression patterns. (**A**) qNPA microarray was performed in triplicate on MCF10CA1a siCtrl cells and MCF10CA1a siβ4 cells at 72 hours post-transfection. The heat map depicts the 44 miRNAs undergoing a statistically significant change in expression following transient depletion of β4 subunit in this system. (**B**) qNPA microarray was performed in triplicate on 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). The heat map depicts the 50 miRNAs undergoing a statistically significant change in expression following introduction of the β4 subunit into this system. (**C**) qNPA microarray was performed in triplicate on ten β4 positive and ten β4 negative invasive breast carcinomas. The heat map depicts the 74 miRNAs differentially expressed between tumor subsets. For all array analyses, a p-value < 0.05 and a ± 1.2-fold change cut-off was applied. Color was assigned to each miRNA based on relative expression across samples.

Table 1. Effect of β4 expression on miRNA levels.

MCF10CA1a		MDA-MB-435		Tumors	
miRNA	*Fold Change (siCtrl/siβ4)*	*miRNA*	*Fold Change (β4/Mock)*	*miRNA*	*Fold Change (β4+/β4−)*
hsa-miR-187	−3.04	hsa-miR-29a	−5.56	hsa-miR-92b	−3.02
hsa-miR-574-5p	−2.19	hsa-miR-886-5p	−5.26	hsa-miR-145	−2.89
hsa-miR-146a	−2.00	hsa-miR-125b	−3.03	hsa-miR-191	−2.79
hsa-miR-216b	−1.95	hsa-miR-100	−2.94	hsa-miR-193b	−2.67
hsa-miR-127-5p	−1.95	hsa-miR-342-3p	−2.70	hsa-miR-423-3p	−2.52
hsa-miR-516b	−1.88	hsa-miR-22	−2.33	hsa-miR-342-3p	−2.40
hsa-miR-190	−1.85	hsa-miR-27a	−2.27	hsa-miR-24	−2.37
hsa-miR-616	−1.63	hsa-miR-23a	−2.17	hsa-miR-99b	−2.37
hsa-miR-100	−1.60	hsa-miR-130a	−2.04	hsa-miR-574-3p	−2.34
hsa-miR-1233	−1.59	hsa-miR-15b	−2.00	hsa-miR-16	−2.30
hsa-miR-222	−1.57	hsa-miR-16	−1.96	hsa-miR-27a	−2.27
hsa-miR-1275	−1.55	hsa-miR-182	−1.92	hsa-miR-103	−2.22
hsa-miR-637	−1.54	hsa-miR-24	−1.92	hsa-let-7a	−2.18
hsa-miR-221	−1.52	hsa-miR-222	−1.92	hsa-miR-320b	−2.15
hsa-miR-31	−1.51	hsa-let-7f	−1.82	hsa-let-7f	−2.13
hsa-miR-768-5p	−1.49	hsa-miR-92b	−1.67	hsa-miR-199a-5p	−2.13
hsa-miR-296-5p	−1.48	hsa-miR-185	−1.67	hsa-let-7b	−2.03
hsa-miR-1207-5p	−1.48	hsa-miR-30c	−1.61	hsa-miR-149	−2.02
hsa-miR-1244	−1.47	hsa-miR-1244	−1.61	hsa-miR-1291	−2.01
hsa-miR-92a	−1.47	hsa-miR-151-5p	−1.59	hsa-miR-92a	−2.00
hsa-miR-194	1.50	hsa-miR-1260	−1.56	hsa-miR-214	−1.96
hsa-miR-548c-5p	1.55	hsa-miR-20b	−1.54	hsa-miR-93	−1.91
hsa-miR-609	1.56	hsa-miR-30b	−1.52	hsa-miR-143	−1.90
hsa-miR-421	1.61	hsa-miR-606	−1.47	hsa-miR-1259	−1.88
hsa-miR-330-3p	1.61	hsa-let-7b	−1.47	hsa-miR-193a-5p	−1.86
hsa-miR-105	1.64	hsa-miR-1201	−1.47	hsa-miR-200c	−1.83
hsa-miR-33b	1.65	hsa-miR-23b	−1.45	hsa-miR-107	−1.81
hsa-miR-218	1.68	hsa-miR-574-3p	−1.43	hsa-miR-195	−1.81
hsa-miR-18a	1.73	hsa-let-7a	−1.43	hsa-miR-484	−1.77
hsa-miR-422a	1.75	hsa-miR-765	−1.39	hsa-miR-423-5p	−1.68
hsa-miR-1284	1.79	hsa-miR-30a	−1.39	hsa-miR-23a	−1.67
hsa-miR-559	1.81	hsa-miR-181a	−1.39	hsa-miR-125a-5p	−1.64
hsa-miR-33a	1.88	hsa-miR-345	−1.37	hsa-miR-22	−1.59
hsa-miR-331-5p	1.91	hsa-miR-663b	−1.37	hsa-miR-30d	−1.58
hsa-miR-632	1.93	hsa-miR-486-5p	−1.37	hsa-miR-620	−1.58
hsa-miR-375	1.96	hsa-miR-19b	−1.37	hsa-miR-675	−1.57
hsa-miR-301b	1.97	hsa-miR-720	−1.33	hsa-miR-125b	−1.55
hsa-miR-891b	2.16	hsa-miR-1296	−1.33	hsa-miR-197	−1.55
hsa-miR-936	2.35	hsa-miR-15a	−1.32	hsa-miR-606	−1.53
hsa-miR-622	2.76	hsa-miR-768-3p	1.59	hsa-miR-650	1.79

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 disconcordant 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 mRNAs. 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 accession term “cell motility” (GO:0048870) within this population of genes compared to all β4-upregulated genes using the hypergeometric probability ($p=0.048$). Six genes were identified and include *EPHA3*, *ABHD2*, *PTPN11*, *EFNB2*,

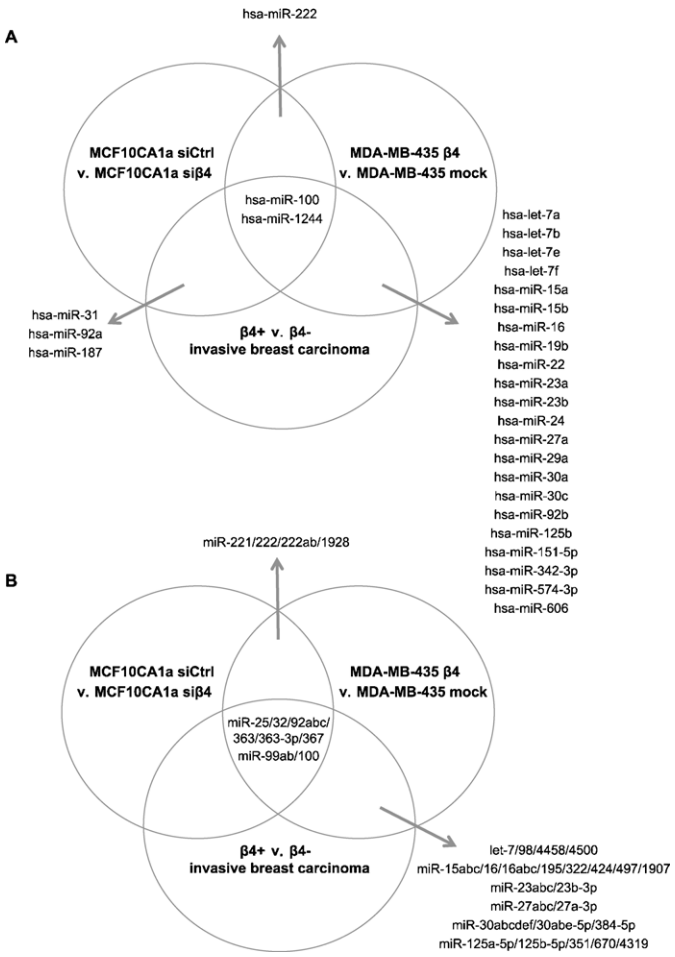

Fig. 3. β4 inversely correlates with the expression of select miRNA families. (**A**) Venn diagram of overlapping miRNAs that undergo differential expression in response to β4 across all three arrays. (**B**) Venn diagram of overlapping miRNA families that undergo differential expression in response to β4 across all three arrays.

NF1, and *CDK6*. Closer analysis uncovered additional genes that have been shown to promote cell motility despite having not been picked up by our gene ontology analysis. These genes include *PIK3R3* (McAuliffe et al., 2010), *PPM1D* (Wang et al., 2011), *RASGRP3* (Randhawa et al., 2011; Yang, D. et al., 2010), *ADAM19* (Wildeboer et al., 2006), *SORBS3* (Kioka et al., 2010; Mizutani et al., 2007), *ITSN1* (Ma et al., 2011), *MECP2* (Degano et al., 2009; Yaqinuddin et al., 2008), *VLDLR* (Förster et al., 2010), *HIP1* (Khatchadourian 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 *PIKR3*, 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 (Merdek 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-30abcdef/30abe-5p/384-5p (Fig. 4B). While these miRNA families were differentially regulated in only two of the three arrays, these data still provide compelling evidence that β4 status correlates with expression patterns of these miRNA families and suggests a role for them in mediating the expression of β4-regulated genes.

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

Table 2. Effect of β4 expression on miRNA families.

miRNA Family	Effect of β4 on Expression	Differentially Expressed miRNA Family Members		
		MDA-MB-435	*MCF10CA1a*	*Tumors*
let-7/98/4458/4500	↓	let-7a let-7b let-7e let-7f		let-7a let-7b let-7c let-7e let-7f
miR-15abc/16/16abc/195/322/424/497/1907	↓	miR-15a miR-15b miR-16		miR-15a miR-15b miR-16 miR-195
miR-23abc/23b-3p	↓	miR-23a miR-23b		miR-23a miR-23b
miR-27abc/27a-3p	↓	miR-27a miR-27b		miR-27a
miR-30abcdef/30abe-5p/384-5p	↓	miR-30a miR-30b miR-30c		miR-30a miR-30c miR-30d
miR-25/32/92abc/363/363-3p/367	↓	miR-92b	miR-92a	miR-92a miR-92b
miR-99ab/100	↓	miR-100	miR-100	miR-99a miR-99b miR-100
miR-125a-5p/125b-5p/351/670/4319	↓	miR-125b		miR-125a-5p miR-125b
miR-221/222/222ab/1928	↓	miR-222	miR-221 miR-222	

Table 3. Effect of β4 expression on miR-92ab and miR-99ab/100 family members.

MCF10CA1a Array					
miRNA	***p-value***	***False Discovery Rate***	***siCtrl Average Intensity***	***siβ4 Average Intensity***	***Fold Change (siCtrl/siβ4)***
hsa-miR-92a	4.87E−02	6.07E−01	14599	21407	−1.47
hsa-miR-100	1.75E−02	5.30E−01	15424	24711	−1.60
MDA-MB-435 Array					
miRNA	***p-value***	***False Discovery Rate***	***Average β4 Intensity***	***Average Mock Intensity***	***Fold Change (β4/Mock)***
hsa-miR-92b	4.0E−06	3.5E−04	2837	4700	−1.67
hsa-miR-100	5.0E−07	5.9E−05	2625	7732	−2.94
Tumor Array					
miRNA	***p-value***	***False Discovery Rate***	***Average β4 Positive Intensity***	***Average β4 Negative Intensity***	***Fold Change (β4+/β4−)***
hsa-miR-92a	1.16E−02	8.31E−02	3498	6989	−2.00
hsa-miR-92b	4.50E−06	1.06E−03	1125	3400	−3.02
hsa-miR-99a	2.72E−02	1.42E−01	393	551	−1.40
hsa-miR-99b	4.65E−04	1.22E−02	926	2190	−2.37
hsa-miR-100	1.74E−02	1.09E−01	272	338	−1.24

unrecognized 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
GOLGA8A	7.60E−06	1.54E−03	148	65	2.28
ABHD2	4.79E−05	4.13E−03	168	75	2.22
SGCD	7.74E−03	7.84E−02	259	124	2.09
DCP2	3.05E−04	1.19E−02	149	84	1.78
RMND5A	1.37E−03	2.79E−02	108	61	1.76
WWP2	3.37E−03	4.75E−02	377	220	1.71
AMMECR1	1.55E−04	7.88E−03	125	73	1.70
KLHDC3	3.07E−05	3.27E−03	759	461	1.65
PTPN11	1.96E−04	9.26E−03	335	210	1.60
ZC3HAV1	9.30E−03	8.74E−02	168	108	1.56
ZFP106	3.67E−02	2.01E−01	473	310	1.52
CTDSPL	6.60E−05	4.94E−03	419	276	1.51
BAT2L2	2.94E−03	4.40E−02	137	92	1.49
PIK3R3	3.33E−03	4.72E−02	125	84	1.49
ZNF652	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
NKTR	2.50E−03	4.01E−02	84	59	1.43
FOXO3	2.59E−03	4.11E−02	262	184	1.42
ZNF331	7.34E−05	5.13E−03	64	45	1.42
PKNOX1	1.47E−04	7.69E−03	68	49	1.40
RASGRP3	7.45E−03	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
SORBS3	4.62E−02	2.29E−01	289	215	1.34

drosha, ago1, ago2, and trpb2 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-92a belongs to the miR-17-92 cluster, a group of six miRNAs generated from a single polycistronic 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-

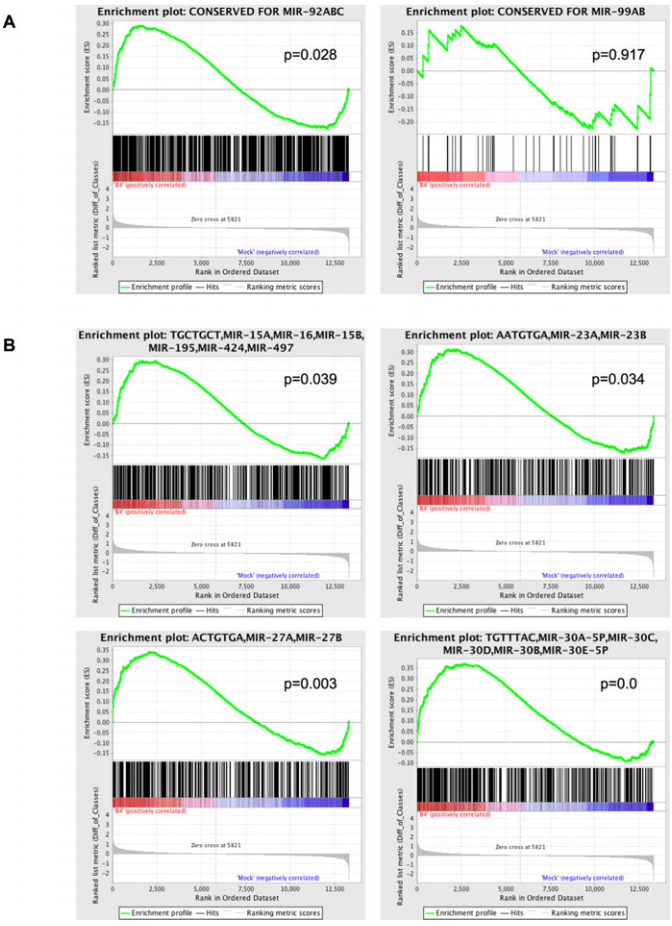

Fig. 4. β4-regulated mRNAs are enriched in putative targets of miRNA families. GeneChip derived mRNA levels were ranked from the most upregulated in β4 transfected cells to the most downregulated (x-axis, 1 to 12,300, respectively). Red shading indicates mRNA is upregulated in β4 transfectants, while blue shading indicates mRNA is downregulated. Each vertical black line represents a miRNA target. The left-to-right position of each black line indicates the relative position of the predicted target within the rank ordered mRNA list. (**A**) miR-92ab predicted target gene are enriched among mRNAs up-regulated in the β4 transfectants, as illustrated by the increasing number of black lines on the left side of each graphic and the positive running enrichment scores (ES) marked by the red lines (p=0.028). No enrichment was detected for and miR-99ab/100. (**B**) miR-15abc/16/16abc/195/322/424/497/1907 (p=0.039), miR-23abc/23b-3p (p=0/034), miR-27abc/27a-3p (p=0.003), and miR-30abcdef/30abe-5p/384-5p (p=0.0) predicted target genes are enriched among mRNAs up-regulated in the β4 transfectants.

125a-5p/125b-5p/351/670/4319 miRNA family, both of which we identified to be downregulated by β4 in two of the three arrays (Table 2). Unlike miR-92a and miR-92b, these observations suggest a complex transcriptional mechanism that induces repression of miRNAs known to be genomically and functionally linked. This observation provides compelling evidence that the relationship between β4 and the expression patterns of these miRNAs is biologically driven and highly conserved. Furthermore, this observation diminishes our negative finding that the population of β4-regulated mRNAs does not contain an enrichment for miR-99ab/100 targets.

Our observations that miR-92ab and miR-99ab/100 both target β4-regulated genes involved in cell motility and signal transduction suggests a novel miRNA-mediated mechanism by which β4 promotes carcinoma cell migration and invasion. Moreover, these data contribute to our understanding of β4 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 β4, as well as the role of putative targets in mediating cell motility downstream of this integrin, will provide further insight into the role of β4 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). MCF10CA1a 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. MCF10CA1a 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℃ in an incubator supplied with 5% CO_2. MDA-MB-435 mock transfectants (6D2 and 6D7 sublcones) and β4 transfectants (3A7 and 5B3 subclones) were generated and characterized as previously described (Shaw et al., 1997). The 505 antibody to β4 used for immunoblotting was produced by our laboratory as previously described (Shaw et al., 1993). The antibody to tubulin (Sigma, St. Louis, MO, USA) was also used for immunoblotting.

siRNA experiments

MCF10CA1a cells were transfected with 20 nM On-TARGETplus SMARTpool siRNA targeting β4 (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, 5mM 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 β4 (1:4,000) or tubulin (1:10,000) overnight at 4℃. 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 under University of Massachusetts Medical School IRB exemption (Docket # 12535, approved September 19, 2011). β4 expression was assessed as previously described (Lu et al., 2008). Formalin-fixed paraffin-embedded sections of these tumors were generated for analysis by qNPA.

qNPA™ miRNA microarrays

Design

A novel qNPA based miRNA Microarray high throughput platform from High Throughput Genomics (HTG Molecular Diagnostics, Inc.; Tuscon, AZ, USA) was used to study 1050 mature miRNAs in human, rat, and mouse based upon the Sanger miRBase release 9.1. The qNPA based miRNA microarrays comprise DNA oligo capture probes that are synthesized directly on the slide surface (Roche NimbleGen, Madison, WI, USA) which are complementary to, and capture, biotinylated miRNA-specific nuclease protection probes. Each microarray slide has 21 synthesized arrays, each representing all of the 1050 miRNAs plus

housekeeper genes, in separate wells in a design that mimics standard SBS 96-well foot print using ArraySlide 24-4 Frame gasket (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 scrapped off of slides into a clean eppendorf tube. Tissues were lysed in 100 µl of Lysis Buffer covered with 600 µl of Denaturation oil at 95℃ 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℃ for 10–15 min followed by 16–24 h hybridization in a 37℃ 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℃ followed, resulting in dissociation of the duplex, hydrolysis of the target miRNA, and free single-stranded NPPs present in amounts stoicheometric 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℃ 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 enzyme buffer (HTG) for 45 min at 37℃. Microarrays were washed followed by addition of TSA-Plus Cy3 reagent in amplification diluent (Perkin Elmer, Waltham, MA, USA) for detection. 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 miRNA raw expression values were extracted, converted to log base 2, and intra-array miRNA replicates (spot replicates) averaged. Arrays were then normalized to one another using the median miRNA expression value 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 if the average value of both β4 positive and β4 negative samples on a single microarray fell below the average background level detected on that particular microarray. Estimates of the false discovery rate (FDR) were made using the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). Heat map false-coloring of Fig. 1 was applied using Matrix2png (http://www.chibi.ubc.ca/matrix2png) (Pavlidis and Noble, 2003). miRNA values in each row were normalized to have a mean of zero and a variance of one. Coloring was applied linearly to normalized values between the 2nd and 98th percentile, while saturating color was applied below the 2nd percentile or above the 98th percentile. Gene order on the y-axis is identical to the gene order in supplementary material Tables S1–S3.

Lists of predicted targets of miRNAs used for analyses depicted in Table 4, supplementary material Tables S4 and S5, and gene set enrichment analyses depicted in Fig. 4 were obtained from publicly available algorithms TargetScan Human Release 5.1 (http://www.targetscan.org) and miRanda August 2010 Release (http://www.microrna.org). Genes involved in cell migration (GO:0016477) were identified using the AmiGo gene ontology classification database v1.8 (Ashburner et al., 2000; Carbon et al., 2009) available through the Gene Ontology project (http://www.geneontology.org). The hypergeometric probability (http://www.stattrek.com) was measured using a population size of 1487 (upregulated β4 mRNAs), sample size of 54 (common miR-92ab and miR-99ab/100 targets among β4-regulated mRNAs), successes in population of 83 (cell motility genes identified in upregulated β4 mRNAs), and successes in sample of 6 (cell motility genes identified in common miR-92ab and miR-99ab/100 targets among β4-regulated mRNAs). For miRNA gene set enrichment analysis in Fig. 4, mRNA expression data generated by Chen et. al. were downloaded from the NCBI Gene Expression Omnibus (GEO), series number GSE11466 (Chen et al., 2009). Affymetrix CEL files were processed with the robust multi-chip average (RMA) algorithm (Irizarry et al., 2003) using BRB-ArrayTools. Using total context score, the top 500 conserved targets for miR-92ab or miR-99ab/100 were compiled into gene set lists. Log base 2 mRNA data were loaded into the Broad Institute's Gene Set Enrichment Analysis (GSEA) software v2.06 (Subramanian et al., 2007; Subramanian et al., 2005). β4 phenotype was compared to mock phenotype by first collapsing the dataset to gene symbols and then using a weighted, difference of classes metric for ranking genes. Gene set permutations were performed to generate nominal p-values for each miRNA target gene set list.

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Competing Interests

Kristin Gerson, Jeffrey Shearstone, Ashraf Khan, and Arthur Mercurio declare that there are no competing interests. Bruce Seligmann works in a leadership role for, and owns stock in, HTG Molecular Diagnostics, Inc., the company that produces and markets the qNPA assay. Krishna Maddula works as a Staff Scientist for, and owns stock in, HTG Molecular Diagnostics, Inc. Bruce Seligman and Krishna Maddula have no affiliation with, nor do they consult with, the University of Massachusetts. The qNPA assay was carried out using funds from the Arizona Science Foundation through a grant to the University of Arizona, David Galbraith, PI, for which HTG Molecular Diagnostics, Inc. is the industry collaborator.

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