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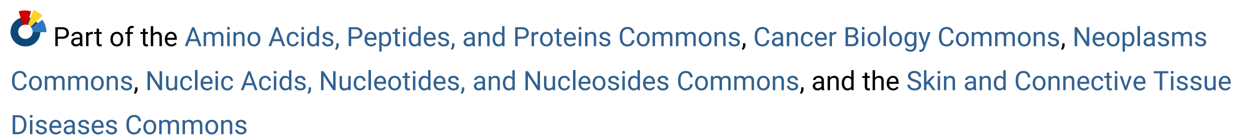
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ANALYSIS OF INTEGRIN α6β4 FUNCTION IN BREAST CARCINOMA

A Dissertation Presented

By

KRISTIN D. GERSON

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 6, 2012

CANCER BIOLOGY

ANALYSIS OF INTEGRIN α6β4 FUNCTION IN BREAST CARCINOMA

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KRISTIN D. GERSON

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ABSTRACT

The development and survival of multicellular organisms depends upon the ability of cells to move. Embryogenesis, immune surveillance, wound healing, and metastatic disease are all processes that necessitate effective cellular locomotion. Central to the process of cell motility is the family of integrins, transmembrane cell surface receptors that mediate stable adhesions between cells and their extracellular environment. Many human diseases are associated with aberrant integrin function. Carcinoma cells in particular can hijack integrins, harnessing their mechanical and signaling potential to propagate cell invasion and metastatic disease, one example being integrin α6β4. This integrin, often referred to simply as β4, is defined as an adhesion receptor for the laminin family of extracellular matrix proteins. The role of integrin β4 in potentiating carcinoma invasion is well established, during which it serves both a mechanical and signaling function.

miRNAs are short non-coding RNAs that regulate gene expression post-transcriptionally, and data describing the role of extracellular stimuli in governing their expression patterns are sparse. This observation coupled to the increasingly significant role of miRNAs in tumorigenesis prompted us to examine their function as downstream effectors of β4, an integrin closely linked to aggressive disease in breast carcinoma. The work presented in this dissertation documents the first example that integrin expression correlates with specific miRNA patterns. Moreover, integrin β4 status *in vitro* and *in vivo* is associated with decreased expression of distinct miRNA families in breast cancer,

namely miR-25/32/92abc/363/363-3p/367 and miR-99ab/100, with purported roles in cell motility. Another miRNA, miR-29a, is significantly downregulated in response to *de novo* expression of β4 in a breast carcinoma cell line, and β4-mediated repression of the miRNA is required for invasion. Another major conclusion of this study is that β4 integrin expression and ligation can regulate the expression of SPARC in breast carcinoma cells. These data reveal distinct mechanisms by which β4 promotes SPARC expression, involving both a miR-29a-mediated process and a TOR-dependent translational mechanism. Our observations establish a link between miRNA expression patterns and cell motility downstream of β4 in the context of breast cancer, and uncover a novel effector of β4-mediated invasion.

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LIST OF ABBREVIATIONS

130CAS	130Crk-associated substrate
4E-BP1	4E binding protein 1
ADMIDAS	Adjacent to metal ion-dependent adhesion site
Ago	Argonaut
BDNF	Brain-derived neurotrophic factor
BP	Bullous pemphigoid
CDK4	Cyclin-dependent kinase 4
ChIP	Chromatin immunoprecipitation
DGCR8	DiGeorge syndrome critical region 8
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ES	Enrichment score
ESC	Embryonic stem cell
FAK	Focal adhesion kinase
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
FGF2	Fibroblast growth factor 2
FNIII	Type III fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEO	Gene Expression Omnibus

GSEA	Gene set enrichment analysis
HD	Hemidesmosome
HGF	Hepatocyte growth factor
HTG	High Throughput Genomics
IcBP	Ic binding partner
I-EGF	Integrin epidermal growth factor-like
IgG	Immunoglobulin G
ILK	Integrin-linked kinase
IRS-2	Insulin receptor substrate 2
JNK	c-Jun N-terminal kinase
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MIDAS	Metal ion-dependent adhesion site
miRISC	microRNA-induced silencing complex
miRNA	microRNA
MMP	Matrix metalloproteinase
MSigDB	Molecular Signatures Database
MSP	Macrophage-stimulating protein
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
NPP	Nuclease protection probe

SFK	Src family kinase
PDGF	Platelet-derived growth factor
PI3K	Phosphatidolinosital-3 kinase
PKC	Protein kinase C
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PSI	Plexin/semaphorin/integrin
qNPA	Quantitative Nuclease Protection Assay
qPCR	Quantitative real time PCR
S6K	S6 kinase
SDL	Specificity-determining loop
S.E.	Standard error
siRNA	Small interfering RNA
SPARC	Secreted protein acidic and rich in cysteine
SynMBS	Synergistic metal ion binding site
TGF	Transforming growth factor
TOR	Target of rapamycin
TSP-180	Tumor surface protein-180
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VLA	Very late antigen
WASP	Wiskott-Aldrich syndrome proteins

CHAPTER I

INTRODUCTION

Cell Motility

The development and survival of multicellular organisms depends upon the ability of cells to move. Embryogenesis, immune surveillance, wound healing, and metastatic disease are all processes that necessitate effective cellular locomotion (1). Nearly all cells accomplish this intricate task by employing similar mechanisms. In basic terms, receptors in cellular protrusions establish connections with extracellular matrix proteins at the leading edge of cells. Stabilization of these adhesions generates traction. Contraction of the cell body followed by disassembly of adhesive contacts at the trailing edge permits translocation and cell propulsion in the forward direction. Migration, thus, requires coordinated adhesion and detachment of nucleated contacts (2).

Several adhesive complexes have been implicated in motility, including nascent adhesions, focal complexes, and focal adhesions. Both nascent adhesions and focal complexes are small dynamic structures located near the leading edge and mediate signals important for actin polymerization (3, 4). These structures exist transiently and are either rapidly disassembled or mature into focal adhesions, large sites of mechanotransduction (5). These sites serve as signaling platforms and establish connections to the actin cytoskeleton through associations with structural proteins like talin, α -actinin, and vinculin (6). Focal adhesions are present in both central and peripheral regions of the cell at the ends of long actin filament bundles (6). Formation

and turn over of adhesions is mediated by the catalytic activity of enzymes such as focal adhesion kinase (FAK) and Src family kinases (SFKs) (6). Signals from these molecules and others converge upon the RhoGTPase family of signaling molecules present in protruding motility structures. These proteins mediate actin polymerization and novel adhesion formation (7). Regulation of cytoskeletal organization and cell morphology is coupled to activation of signaling cascades that drive changes in gene expression and cell survival. Central to these processes is the family of transmembrane glycoproteins called integrins.

The Integrin Family

Overview

Integrins belong to a family of heterodimeric transmembrane cell surface receptors that mediate stable adhesions between cells and their extracellular environment. They are expressed across metozoa and likely evolved prior to the Cambrian explosion as a requirement for multicellularity, permitting adhesion of cells to basement membranes essential for the development of multilayered organisms (8, 9). Their history of discovery nearly thirty years ago was protracted by both technical limitations and conceptual challenges—cell biologists in search of *the* fibronectin receptor were confounded by our current understanding that integrins bind multiple ligands, and that most ligands are recognized by several integrin family members. Ultimately, the use of monoclonal antibodies, affinity chromatography, and crosslinking uncovered this novel family of cell surface receptors. Richard O. Hynes, a British cell biologist at the forefront

of these discoveries, coined the term integrin, a name reflecting the role of these integral membrane proteins in maintaining the integrity of connections between the cytoskeleton and extracellular matrix (9, 10).

These adhesion receptors link the actin cytoskeleton (with the exception of integrin α6β4) to components in the extracellular matrix, including laminin, collagen, fibronectin, vitronectin, and fibrinogen (11). A role for integrins has also been established in mediating cell-cell adhesion. Eighteen α subunits and 8 β subunits have been identified in mammalian cells. Despite the potential for a sizeable number of heterodimers, selectivity of many α subunits in their association with a single β subunit limits the family of receptors to only 24 unique heterodimeric pairs. Ligand specificities and phenotypes from knockout studies in mice indicate that each integrin has a unique nonredundant function (8). Integrins are composed of one α and one β subunit, both of which are single pass transmembrane proteins linked together non-covalently through large ectodomains. Functional studies demonstrate that truncated integrin subunits lacking either the transmembrane or cytoplasmic domains still form heterodimers (12). Many cytoplasmic domains have alternative splice variants and, with the exception of the β4 subunit, all are relatively short (13).

Structure

In simple structural terms, integrins consist of a globular extracellular head formed by both subunits from which two stalks extend and penetrate the plasma membrane. Both α and β subunits are extensively disulfide bonded. The ectodomain of each subunit is architecturally complex. At their N-terminus, all α subunits contain seven

repeats of cysteine-rich segments folded into a seven-blade β -propeller (14). This domain constitutes the bulk of the α head domain and mediates the essential interface with the β subunit (15). Half of α subunits contain an insert (I) or von Willebrand factor A domain, which is positioned within the β-propeller if present (16). This α I domain spans 200 amino acids in length and houses a divalent cation (Mg^{++}) ligated by three loops of secondary amino acid structure, which constitute the metal ion-dependent adhesion site (MIDAS) (17). The MIDAS is critical for metal binding and, thus, integrin function, as divalent cations are universally required for integrins to bind their cognate ligands (15). C-terminal to the head is the leg of the α subunit composed of three β-sandwich domains: the thigh constitutes the upper leg, while calf-1 and calf-2 domains make up the lower leg. A small Ca^{++} binding loop is located between the thigh and calf-1 domains. This position is referred to as the genu (French for knee) and is the pivot point for α subunit extension (15).

The β subunits are structurally more complex. The β head consists of PSI (plexin/semaphorin/integrin), hybrid, and β I domains. The β I domain is situated in the PSI domain, which is located within the hybrid domain. Spanning about 240 amino acids in length, this highly conserved β I domain is analogous to α I domain but is composed of two additional segments: the specificity-determining loop (SDL) involved in ligand binding and an interface domain that interacts with the β-propeller of the α subunit (15). The β I domain contains a MIDAS that binds negatively charged residues, which in turn bind the Mg^{++} in the α I domain (17). Two adjacent metal binding sites termed synergistic metal ion binding site (SynMBS) and the adjacent to metal ion-dependent

adhesion site (ADMIDAS) are present and both bind Ca^{++} (15). C-terminal to the hybrid domain is the leg of the β subunit, a cysteine-rich segment containing four integrin epidermal growth factor-like (I-EGF) domains, a β-ankle, and a β-tail domain. The knee is located between I-EGF domains 1 and 2.

The transmembrane domains of integrin subunits are believed to associate via a ridge-in-groove packing model involving an α-helical interface in the resting state (15). A salt bridge linking the two subunits has also been proposed (18). Cytoplasmic domains are believed to associate very weakly with one another if at all.

Bidirectional Signaling

Integrin ectodomains are thought to equilibrate between three conformational states: bent conformation with a closed headpiece, intermediate extended conformation with a closed headpiece, and extended conformation with an open headpiece. Such conformations roughly correspond to low affinity, primed and activated, and ligand-bound activated integrin states, respectively (19). In the closed conformation, the ectodomain of the integrin is bent and juxtaposed to the plasma membrane. This confirmation is stabilized tenuously by interactions between the α and β legs, the head domain and both lower legs, and the α and β transmembrane domains (17, 20). Conformational changes producing destabilization of these interactions can be induced upon association of effector molecules, such as talin, with the cytoplasmic tail, which link the integrin to the cellular cytoskeleton. Mutations in the cytotail can also destabilize the bent conformation (18, 21-23). These events cause separation of the legs within the transmembrane segment, extension of the head in a switchblade-like motion, and swing-

out of the hybrid domain (17). This conformational change primes or activates the integrin for ligand binding. The concept of integrin priming is known as inside-out signaling, because intracellular events induce conformational changes of integrin ectodomains to facilitate ligand-binding with greater affinity (19).

In α subunits that express the I domain, this structure functions as the major ligand binding domain, while the β I domain regulates ligand-binding activity of the α I domain. In integrins lacking the α I domain, the β I domain MIDAS directly binds the ligand. During cell adhesion or migration, tensile forces transmitted from a ligand-bound integrin are resisted by the cytoskeleton and associated adapter molecules bound to the cytoplasmic tail. Such resistance stabilizes the headpiece and favors an extended conformation over bent or closed positions (17). Ligand-binding affinity and adhesiveness of integrins are generally enhanced by increasing concentrations of extracellular Mn^{++} and decreasing concentrations of extracellular Ca^{++} (17). Binding of the large multivalent ligands promotes lateral association of integrin heterodimers into oligomers on the cell surface known as clustering. Close proximity of integrin cytoplasmic domains results in kinase recruitment and activation of intracellular signaling cascades, often referred to as outside-in signaling (19).

The bi-directional signaling capacity intrinsic to integrins results in a wide range of biological consequences. For example, inside-out signaling is critical for establishing adhesive strength between integrins and their extracellular environment, permitting transfer of tensile force required for integrin-mediated cell adhesion and extracellular matrix remodeling. Outside-in signaling, on the other hand, drives activation of signaling

cascades involved in cellular processes such as cytoskeletal organization, gene expression, and cell differentiation. Though distinct processes, these two directions of integrin signaling are closely linked and often converge upon complex cellular processes, a key example being the coordination of cell motility.

The Cancer Connection

Integrins regulate cell migration in variety of physiological and pathological contexts. Many diseases, including autoimmune disorders and cancer, are associated with aberrant integrin function. Carcinoma cells in particular can hijack integrins, harnessing their mechanical and signaling potential to propagate cell invasion and metastatic disease. In this context, cells can move singularly or as sheets of cells linked together by cell adhesion molecules (24). The morphology of a single migrating cell is mesenchymal, arising from a presumed epithelial-to-mesenchymal transition (EMT) occurring in response to stimuli from the tumor microenvironment of carcinoma cells. Specifically, downregulation of molecules that establish cell-cell adhesions, such as cadherins, induces changes in cytoskeletal organization and signaling pathways that allow neoplastic cells to dissociate from the primary tumor (1).

Dissemination of malignant cells and subsequent metastasis depend upon coordination of migratory and proteolytic processes. Four cell protrusions at the leading edge of motile cells have been described: lamellipodia, filipodia, invadopodia, and blebs (7). Tumor cells are unique in their ability to form invadopodia, sites of rapid actin polymerization and associated proteins, including Wiskott-Aldrich syndrome proteins (WASP), Rho GTPases, SFKs, and the actin nucleating Arp 2/3 complex (6). Inherent to

these actin-rich complexes is the potential to degrade surrounding matrix through proteolysis. Clearly this ability is critical for carcinoma cell invasion through basement membranes and stromal tissue and into blood vessels (25). At the forefront of these motility structures, integrins mediate the dynamics of cell morphology and adhesion in migrating cells.

The field of integrin biology has evolved since its inception nearly thirty years ago. Richard O. Hynes was credited with the discovery of integrins despite never intending to pursue a scientific career centered on cell adhesion. Rather, he began his research endeavors looking for differences on the cell surface of normal and tumor cells (9). Perhaps appropriately, fruitful extensions of his early work established a critical role for these cell surface receptors in promoting tumor progression. One such example is integrin α6β4.

The α6β4 Integrin

Discovery

In 1986, an Italian group reported the identification of a tumor associated glycoprotein complex termed TSP-180 on the surface of murine lung carcinoma cells that correlated with metastatic potential (26). Antibody characterization of the complex subsequently established preferential expression of this protein in malignant tumors relative to normal tissue in both humans and mice (27).

The following year, a group from the Netherlands described a novel noncovalent complex of glycoproteins Ic and IIa on the surface of intact platelets and postulated a role

for this complex in cell adhesion (28). The IIa subunit of this complex was ultimately determined to be identical to the β subunit of the family of human VLA (very late antigen) cell surface receptors (later recognized as integrins) by a group at Harvard (29, 30), and the complex was designated VLA-6 (31). Further characterization using antibodies to the IIa subunit, which would later come to be called β1, revealed a novel binding partner for the Ic subunit (corresponding to α6) of this complex from a mouse mammary epithelial tumor (32). This subunit was termed IcBP (or Ic binding partner) and noted to have marked similarities to the extracellular matrix protein laminin (32). The IcBP was coined β4, and additional characterization of this novel VLA subunit was carried out by the American and Dutch groups, culminating in the first published report of the heterodimeric cell surface receptor α6β4 (33).

By 1989, it became apparent that the metastatic marker TSP-180 identified by Falcioni and colleagues bore striking resemblance the newly identified superfamily of adhesion receptors called integrins, in particular to the α6β4 integrin. Collaborative efforts confirmed the speculation and established TSP-180 to be the recently identified α6β4 integrin (34). This discovery coincided with an independent publication from a group out of California providing evidence of a novel integrin family member on the surface of human epithelial cells termed αEβ4 (35). Thus began a research pursuit spanning three decades aimed at further characterizing the adhesion receptor α6β4 integrin and defining its role in development, homeostasis, and pathology.

Structure and Development

The α6β4 integrin is often referred to simply as β4, since α6 is the only partner with which it heterodimerizes. β4 integrin is conserved across the metazoan kingdom and is expressed predominantly in epithelial cells, though reports have identified the integrin on fibroblasts, thymocytes, and Schwann cells (36). β4 integrin is defined as an adhesion receptor for the laminin family of extracellular matrix proteins and joins α3β1, α6β1, and α7β1 as one of the four commonly known laminin-binding integrins. α2β1 has also been shown to bind laminin despite functioning predominantly as a collagen receptor (37). Though β4 is promiscuous in its association with various laminin isoforms, epidermal laminin-332 (previously called laminin-5) in the basement membrane is the preferred ligand for the integrin (38). Integrin biologists established early on that β4 was unique among β integrin subunits. Several defining qualities distinguish β4, including its ability to bind keratin intermediate filaments, as well as its unusually long cytoplasmic tail. While β subunit intracellular domains typically consist of 50 amino acids, the β4 cytoplasmic domain exceeds 1000 amino acids in length. Two pairs of type III fibronectin (FNIII) repeats separated by a connecting segment characterize the cytoplasmic tail. A Na^{+}-Ca^{++} exchanger (CalX) motif with unknown function is situated membrane-proximal to the first FNIII repeat. These cytoplasmic domains house multiple serine, cysteine, and tyrosine residues that are critical for β4 function.

Expression of β4 has been localized to the basal surface of epithelial cells in junctional adhesion complexes called hemidesmosomes (HDs), inert structures that link cells through their intermediate filament cytoskeleton to laminins in the basement

membrane. This adhesive function is critical for establishing epithelial integrity, as mutational studies and patient case reports link dysfunction of the integrin to pyloric atresia associated with the junctional variant of a blistering skin disease called epidermolysis bullosa (39). Knockout studies in mice corroborate these data; pups lacking expression of the integrin die shortly after birth due to detachment of the epidermis occurring in response to mechanical stress (40, 41).

Role in Hemidesmosome Organization

Further investigation has revealed that β4 plays a pivotal role in the organization of HDs. The current model of HD assembly involves an initial dephosphorylation event on the β4 cytoplasmic domain by an unknown phosphatase that induces a conformational change to expose binding sites in the FNIII repeats (38). β4 then recruits plectin, which associates via its actin binding domain with the first pair of FNIII repeats (42, 43). Reinforcement of this connection is accomplished through additional interactions of the plectin plakin domain with both the connecting segment and C-terminal end of the β4 cytoplasmic tail (44). Bullous pemphigoid (BP) 180 is then recruited and binds laminin extracellularly while associating with the third FNIII repeat and plectin intracellularly (45). Finally BP230 binds to both β4 and BP180 (45). In addition to the connections established by the β4 cytoplasmic domain, the ectodomain of α6 interacts with the tetraspanin CD151 (46). This classic or Type I HD, characteristic of basal epidermal cells, establishes connections to the intermediate filament system through both BP230 and plectin (38). Our understanding of HD organization stems largely from studies investigating the molecular consequences of human mutations in genes expressing α6, β4,

plectin, and BP180. Careful dissection of patient phenotypes and *in vitro* studies have established that β4 interaction with plectin is required for proper HD formation, and that assembly of these adhesion complexes can occur independently of the natural β4 ligand laminin-322 (42, 43, 47-50).

Role in Carcinoma Invasion

The process of invasion involves enzymatic degradation of the basement membrane followed by cell migration through the unobstructed path, thus the ability to migrate is inherent to an invasive carcinoma cell (51). It has been well established that β4 integrin can mediate carcinoma invasion, as exogenous expression of the integrin confers an invasive phenotype in both rectal and breast carcinoma cells (52, 53), while depletion of the integrin impedes chemoinvasion in metastatic breast carcinoma cells (54). Early studies linking increased expression of β4 to aggressive tumors and poor prognosis (27, 55, 56) initially proved puzzling, however, given its established role in mediating epithelial integrity coupled to the observation that most carcinomas lack HDs (57). Research during the past decade has explored this paradigm and revealed a novel role for the integrin in regulating cytoskeletal dynamics and carcinoma invasion, functions dependent upon key post-translational modifications of the β4 cytoplasmic tail.

Recent studies have explored the mechanism by which β4 transitions from a mechanical adhesion device to a signaling competent receptor in motility structures and have established a critical role for the phosphorylation of key serine residues. These phosphorylation events can occur in response to stimulation by growth factors, such as epidermal growth factor (EGF) or macrophage-stimulating protein (MSP) (58-60). For

example, EGF stimulation of carcinoma cells has been shown to induce protein kinase C (PKC)-α-mediated phosphorylation of three serine residues (S1356, S1360, S1364) on the β4 cytoplasmic tail, disrupting HDs and releasing the integrin from sites of adhesion on the basal surface of epithelial cells (59). Similar observations have been extended to keratinocytes (58, 60). A recently identified constitutively phosphorylated serine, S1424, on the cytoplasmic tail also appears to play a role in the disassembly of HDs (61). These phosphorylation events precede β4 mobilization to the leading edge in lamellae and filipodia, where the integrin engages F-actin and promotes migration of carcinoma cells. Again, the ability of β4 to promote motility is not unique to carcinoma cells, as keratinocytes employ the integrin during migration in wound healing. The mechanism by which β4 engages F-actin, however, remains undefined. Since the cytoplasmic tail of this integrin lacks a consensus actin-binding motif, the interaction is likely indirect and involves a linker protein such as plectin (62).

Biophysical analyses characterizing the microdomains of these motility structures reveal β4 residence within tetraspanin-enriched complexes and highlight a role for palmitoylation of key cysteine residues in the recruitment of β4 to these compartments in the plasma membrane. Investigation of β4 palmitoylation arose during a study of the palmitoylated tetraspanin CD151, which is known to interact closely with β4 and has been implicated in the formation of HDs. Data published from these studies provide compelling evidence that palmitoylation of the β4 cytoplasmic tail is critical for recruitment of CD151 and β4 to tetraspanin-enriched microdomains and plays a key role in promoting cell spreading and signaling (63). These observations sharply contrast

previous reports that β4 palmitoylation recruits the integrin to lipid rafts (64). Localization of β4 within these tetraspanin webs (65) likely augments its signaling function through close proximity to other palmitoylated signaling molecules.

Mobilization of β4 to motility structures precedes initiation of signaling events that occur in response to ligand binding and association with other growth factor receptors. As aforementioned, the unusually long cytoplasmic domain of β4 integrin distinguishes it among integrins, prompting curiosity as to its biological role. Clearly one function involves its ability to serve as a signaling platform, initiating various signaling cascades that mediate chemotactic responses involved both in maintaining normal tissue homeostasis, such as in wound healing, as well as in promoting carcinoma cell motility during tumor progression. At the nexus of these intracellular signaling events is phosphatidolinosital-3 kinase (PI3K), the most critical mediator of β4-regulated carcinoma invasion. Much effort has been invested in defining the mechanisms by which this lipid kinase orchestrates signaling events downstream of the integrin, culminating in the identification of key tyrosine residues on the β4 cytoplasmic tail essential for executing its function. Specifically, six tyrosine residues (Y1257, Y1422, Y1440, Y1494, Y1526 and Y1642) have been reported to participate in β4-mediated signaling events (38, 66). Tyrosine 1494 has emerged as the master regulator of β4 phosphorylation and signaling, as mutational analyses have demonstrated that phenylalanine substitution at this site reduces overall tyrosine phosphorylation and impedes β4-mediated functions, including carcinoma cell survival, migration and invasion, as well as anchorage independent growth, tumor development, and

angiogenesis (67, 68). Mechanistic work revealed that Y1494 is required for phosphorylation of the insulin receptor substrate 2 (IRS-2), which subsequently binds to the p85 regulatory subunit of PI3K and activates signaling in response to β4 ligation (67). Furthermore, this tyrosine residue is located within a consensus binding motif for the SH2 domain of tyrosine phosphatase SHP-2, which binds β4 and activates SFKs upstream of PI3K, events that are also required for the invasive phenotype of carcinoma cells (69-71).

The role of targets downstream of PI3K in promoting carcinoma invasion has been well established. The pioneering study establishing that β4 signals through this pathway also identified a positive role for the Rho GTPase Rac downstream of PI3K in chemoinvasion (53). This small G protein has also been shown to regulate the migratory behavior of keratinocytes (50). Subsequent studies characterized the functions of distinct Akt isoforms, highlighting a role for Akt2 in promoting carcinoma motility (72, 73). Extensions of this work have investigated various NFAT (nuclear factor of activated T cells) family members and defined roles for both NFAT1 and NFAT5 in promoting carcinoma invasion, in part through increased transcription of motility factors autotaxin/ENPP2 and S100A4/metastatin (72-77).

Other β4-mediated signaling molecules facilitate carcinoma invasion independently of the PI3K cascade. Specifically, β4 promotes the formation of lamellae and cell motility in carcinoma cells through inhibition of intracellular cAMP levels, which are repressed by a cAMP-specific phosphodiesterase (78). A related study also demonstrated a role for cAMP metabolism in the RhoA-mediated cell motility

downstream of β4 occurring independently of Rho GTPase family member Rac1 (79). Finally, MSP-dependent phosphorylation of the β4 cytoplasmic tail has been shown to induce p38 and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling to promote wound healing in keratinocytes (58). Though these studies were conducted using immortalized skin cells, it is feasible that this mechanism of cell motility could contribute to the progression of invasive carcinomas.

In addition to its regulation of pro-invasive factors, β4 has also been shown to directly transmit mechanical forces from the acto-myosin system that can presumably propel carcinoma cells during the invasive process (80). Using traction force detection assays, Rabinovitz *et al*. demonstrated that the integrin transmits forces on either laminin-111 or antibody to the α6 subunit. Compression forces generated by the integrin remodel the basement membrane, a process dependent upon activation of both PI3K and RhoA; these two signaling pathways have been implicated in β4-dependent carcinoma invasion (53, 79). An important conclusion stemming from this work involves the observation that β4 can function independently of other integrins to impact extracellular matrix organization and drive chemoinvasion.

Despite an abundance of literature establishing a functional role for the integrin in promoting cell motility, β4 is not an island. Association with growth factor receptors is believed to augment β4-mediated signaling and carcinoma invasion. Initial reports documenting β4 interaction with growth factor receptors described cooperative associations with members of the EGF family of receptors, including EGFR, ErbB2, and ErbB3 (81-84). Of note, these early studies relied heavily on the results of co-

immunoprecipitation experiments, which are often difficult to accurately interpret due either to the transient nature of β4 interactions with other molecules or the fact that β4 resides in protein-rich microdomains, and molecules pulled down by such assays may not physically interact with the integrin (85). Nonetheless, Falcioni and colleagues demonstrated β4 association with the orphan ErbB2 receptor in breast carcinoma cells, and a role was subsequently established for this association in the activation of PI3K signaling and carcinoma invasion (83). It has also been established by various groups that interactions between β4 and EGFR promote HD disassembly and β4-mediated carcinoma cell invasion, suggesting a role for effector signaling molecules downstream of the integrin including Fyn and Rho (81, 86, 87). β4 association with c-Met has also been documented, and data from this work describe a novel role for β4 as a signaling adapter molecule that enhances hepatocyte growth factor (HGF)-induced carcinoma invasion (88). The physical interaction between these two cell surface receptors remains controversial, however, and more recent evidence suggests that both can facilitate carcinoma invasion independently of one another (89). The first biophysical evidence linking β4 to a growth factor receptor arose from investigation of human epidermal wound healing as briefly described above, in which MSP stimulation of Ron was shown to induce PI3K-mediated phosphorylation of both Ron and the β4 cytoplasmic tail. These phosphorylation events in turn generate binding sites that permit formation of a heterotrimeric complex in which β4 associates with Ron presumably via 14-3-3 proteins. Formation of this complex displaces β4 from HDs to lamellae and facilitates keratinocyte migration and wound closure (58). Although these studies were not conducted in a

cancer model, such interactions parallel events observed in carcinoma cells with respect to growth factor-induced disassembly of HDs and relocalization of β4 to motility structures at the leading edge. Similar mechanisms could, thus, be at play in the progression of invasive carcinoma cells.

Trafficking

Integrin trafficking plays a critical role in chemoinvasion, as well as cell adhesion, spreading, and migration (90). This process is characterized by integrin delivery to the cell surface, receptor internalization, and recycling of the internalized integrin. As such, this cycle mediates disassembly of adhesive complexes, matrix turnover, and the formation of new focal contacts on the leading edge of migrating cells (90). Little is known about β4 trafficking, though studies conducted during the past decade have shed some light on the subject. One group reported that hypoxia promotes the invasion of breast carcinoma cells through stabilization of microtubules and increased trafficking of β4 to the cell surface, a process mediated by small G protein Rab family member Rab-11 (91). Another group demonstrated that arrestin family member ARRDC3 interacts with the β4 subunit to induce integrin internalization, ubiquitination, and subsequent degradation (92). Moreover, expression of ARRDC3 is downregulated in breast carcinomas, consistent with the observation that β4 plays a role in promoting aggressive disease (92).

Role in Other Biological Functions

In addition to its ability to promote cell migration and invasion, β4 has been linked to tumor cell survival, anchorage independence, and tumor initiation. A functional

link between the integrin and survival was born from the observation that breast carcinoma cells expressing β4 could evade apoptosis under serum- and matrix-deprivation conditions, and that this survival mechanism was dependent upon β4 activation of the PI3K signaling pathway (93). Interestingly, this phenomenon only occurs in cells expressing mutant p53, as the wild-type tumor suppressor induces caspase-3-dependent cleavage and inactivation of Akt in response to β4 expression and ligation (94). Along these lines, mammalian target of rapamycin (mTOR) has been shown to function downstream of PI3K to promote β4-mediated survival via a mechanism involving upregulation of vascular endothelial growth factor (VEGF) cap-dependent translation (95). A continuation of this work demonstrated that β4 regulates tumor cell survival *in vivo* dependent upon VEGF (96). Recent data has shown that β4 upregulates ErbB3 expression and formation of the ErbB2/ErbB3 heterodimer, which is required for β4-mediated activation of PI3K and breast carcinoma cell evasion of apoptosis (84). Utilization of three-dimensional model systems has further contributed to our understanding of β4 function in carcinoma survival and demonstrated that β4-induced polarity of breast carcinoma cells promotes evasion of apoptosis via an NFκB-dependent mechanism (97). An extension of this work uncovered a laminin-332 autocrine loop, by which cells secrete their own extracellular matrix protein leading to β4-dependent activation of Rac and NFκB that promotes anchorage-independent carcinoma cell survival (98).

Recent attention has been directed toward the ability of β4 to promote tumor initiation, a phenomenon studied largely in the context of squamous cell carcinoma. Data

from a murine model of Ras-driven invasive epidermal carcinoma identified a role for β4 and laminin-332 in promoting tumor formation (99). Another murine model using targeted expression of β4 to the suprabasal layer of the epidermis demonstrated that the integrin suppresses transforming growth factor (TGF)-β-mediated growth inhibition, resulting in increased formation of both benign and malignant tumors induced by chemical carcinogenesis (100). These data mesh with observations from mouse models of mammary tumorigenesis demonstrating that β4 and downstream effector VEGF can promote tumor initiation (96), and that depletion of β4 in a breast carcinoma cell line reduces tumor uptake (101). Moreover, another group established a role for β4 signaling in mediating tumor initiation in a mouse model of ErbB2-induced mammary carcinoma (102).

Ligand Independence

While ligand binding is central to the activation of integrin signaling and downstream effects on cell behavior, data from the field provide compelling evidence that β4 can function in a ligand-independent manner. Early studies revealed that expression of β4 in a rectal carcinoma cell line endogenously devoid of the integrin promotes growth arrest, invasion, and cell spreading independent of adhesion to laminin (52, 103). Furthermore, β4 has been shown to promote migration in a breast carcinoma cell line on a collagen matrix (78). Such studies do not negate the possibility, though, that ligation occurs in response to endogenous laminins secreted by carcinoma cells. More compelling evidence relies on data generated from carcinoma cells expressing a trunctated β4, which lacks the extracellular binding domain but retains signaling capacity

and confers an invasive potential equivalent to that of wild-type β4 (88). Several hypotheses have been proposed to explain these observations, including the possibility that clustering of β4 cytoplasmic domains initiates signaling or that constitutive activation of adhesion-dependent signaling pathways in transformed cells confers a survival advantage for tumor cells in the absence of their natural ligand (78, 88, 103, 104).

microRNAs

Overview

In 1993, Victor Ambros identified a defective gene responsible for a mutant phenotype known as the "bag of worms" in the nematode worm *C. elegans*. The unfortunate developmental defect was characterized by the accumulation of fertilized eggs that ultimately hatch within the mutant worm. Surprisingly, the gene did not encode a protein but a short RNA named *lin-4* that was shown to negatively regulate the expression of another gene called *lin-14* by binding to the 3' untranslated region (UTR) of its transcript to block translation (105, 106). Nearly a decade later, the scientific community recognized that small snippets of RNA, termed microRNAs (miRNAs), could regulate cellular processes, a discovery that transformed our understanding of genetics, development, and human disease.

miRNAs are short single-stranded non-coding RNAs that mediate post-transcriptional gene expression. This class of regulatory molecules recognizes and binds complementary sequences on target mRNAs to induce transcript degradation or

translational inhibition. Hundreds of miRNAs have been identified and extensive research conducted during the past two decades has characterized their biogenesis, regulation of target genes, and contributions to disease.

Biogenesis

miRNAs may be independent transcriptional entities or located in clusters with other miRNAs. Some may be positioned in the introns of protein coding genes (107) and share transcription patterns with their host gene when found in a sense orientation (108, 109). miRNAs are transcribed from genomic DNA by RNA polymerase II into a precursor that folds back onto itself forming a characteristic stem-loop structure. Primary transcripts of clustered miRNAs contain multiple hairpins. The double-stranded RNA contained in this primary miRNA (pri-miRNA) is recognized by DiGeorge Syndrome Critical Region 8 (DGCR8), which associates with the RNase III endonuclease Drosha to form the microprocessor complex. Cleavage two helical turns into the stem releases the hairpin from the loose ends of the primary transcript, yielding a precursor miRNA (pre-miRNA) with a two-nucleotide overhang on the 3' end (110-112). Some unconventional miRNAs called mirtrons are cleaved directly out of the intron by splicing machinery and bypass the microprocessor (113-115). This pre-miRNA is then exported by the nucleocytoplasmic shuttle exportin-5 from the nucleus where the RNase III endonuclease Dicer cleaves the loop from the hairpin (116-119). The resulting double-stranded RNA is approximately 22 nucleotides in length and known as the miRNA-miRNA* duplex. This duplex unwinds, and the mature miRNA is loaded into the miRNA-induced silencing

complex (miRISC), a multiprotein complex containing members of the Argonaut (Ago) family of proteins, while the miRNA* strand is degraded (108).

Target Gene Regulation

miRNAs bind multiple targets, and genes, in turn, can be silenced by multiple miRNAs. Over 500 mature miRNAs have been identified, many of which are grouped into families based on their conserved seed region, a sequence of nucleotides (2-7) at the 5' end of miRNAs that is most critical for target recognition. Following maturation, a miRNA binds regions in the 3'UTR of target mRNAs complementary to its seed sequence. Recent evidence suggests that rare alternative mechanisms of gene regulation do occur and include miRNA binding to the 5'UTR or open reading frame of target genes or even directly to DNA to block transcription (120-122). Moreover, participation of the miRNA regions outside of the seed has also been reported to facilitate silencing of target genes (123, 124). Perfect or near perfect complementarity between the miRNA and target promotes Ago2-mediated cleavage of the transcript, the predominant mechanism of gene silencing by miRNAs in plants. Most mammalian miRNAs, however, bind imperfectly to target genes and induce translational inhibition and mRNA destabilization (123, 125).

Contributions to Tumorigenesis

A role for miRNAs in the progression of tumorigenesis has been well established. Numerous studies have documented aberrant expression of miRNAs in tumors relative to normal tissues (126). Dysregulation of miRNAs may occur in response to epigenetic changes that modify miRNA promoter methylation patterns or genetic alterations such as

chromosomal deletions. Studies have also identified defects in processing machinery, resulting in widespread effects on miRNA expression. For example, loss of Dicer function in breast cancer globally downregulates mature miRNA expression and promotes aggressive disease (127). Many miRNAs have been identified as either oncogenes (often referred to as oncomiRs) or tumor suppressors based on their biological impact. For example, members of the miR-200 family of miRNAs have been well characterized in this context and are known to be key regulators of the EMT (128), a precursor to invasion and metastasis.

Overview and Objectives

Integrins are key modulators of cell behavior. They utilize connections with the extracellular matrix to communicate information about their microenvironment, thereby inducing signal transduction events that modify cytoskeleton dynamics and cell motility. The role of integrin β4 in potentiating tumorigenesis is well established, particularly in carcinoma invasion. In this context, transformed epithelial cells infiltrate the basement membrane into local surrounding tissue, gaining access to lymph drainage and the vascular system. This process, involving complex interactions between tumor cells and the extracellular environment, is a precursor to distant metastasis and patient mortality. Integrin β4 plays both a mechanical and signaling role in this capacity. Studies on breast cancer have contributed most significantly to our understanding of how β4 contributes to the invasive process, though much remains to be seen.

The steps involved in miRNA biogenesis have been well characterized, though far less is understood about the processes governing their regulation. Specifically, data describing the role of extracellular stimuli in modifying miRNA expression patterns are sparse. Perhaps the most extensively explored example is the role of TGF-β in promoting the interaction of p68-interacting Smad proteins with the endocuclease Drosha to facilitate miRNA processing and maturation (129). Along these lines, establishment of cell-cell contacts as measured by increasing confluence of cells *in vitro* has also been shown to enhance Drosha-mediated miRNA processing (130). These observations coupled to the increasingly significant role of miRNAs in tumorigenesis necessitate additional investigation into the role of microenvironment in regulating miRNA expression and function in the context of cancer.

Our interest in the ability of integrins to potentiate carcinoma migration and invasion in breast cancer prompted us to examine the role of miRNAs as downstream effectors of β4, an integrin closely linked to aggressive disease. The work presented in the following chapters explores the role of β4 expression on miRNA patterns in the context of breast carcinoma invasion, and reveals a novel effector molecule downstream of the integrin.

CHAPTER II

EFFECTS OF β4 INTEGRIN EXPRESSION ON MICRORNA PATTERNS IN BREAST CANCER

This chapter represents work submitted as:

Effects of β4 Integrin Expression on microRNA Patterns in Breast Cancer

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Abstract

The integrin α6β4 is defined as an adhesion receptor for laminins. Referred to simply 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 the context of breast cancer, 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 migration.

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 (131, 132). 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 (133, 134). 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 HDs to the leading edge of cells, permitting its association with F-actin in motility structures and conferring a unique signaling potential (58, 85, 86, 135-137). 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 (138). β4 regulation of the expression and function of various downstream targets underlies the ability of this integrin to promote carcinoma progression (53, 63, 78, 79, 85, 98, 102). 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 (139).

miRNAs are non-coding single-stranded RNAs approximately 22 base pairs in

length that regulate gene expression through mRNA degradation or translational inhibition (108, 123). In mammalian cells, miRNAs most commonly function by binding well-conserved imperfect complementary sequences in the 3' UTR of their target mRNA to block translation (108, 123). 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 (140, 141) and their ability to regulate gene expression, we explored the effect of β4 integrin on global miRNA expression using a novel array approach termed 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. 2.1A). 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 (33, 142). Stable subclones were generated expressing wild-type β4 (referred to as β4 transfectants); mock transfectants were also generated (Fig. 2.1B). 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 (138).

To assay global miRNA expression, a novel microarray technology termed 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 44 miRNAs (Table S2.1). 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 50 miRNAs (Table S2.2). Finally, ten β4 positive and ten β4 negative invasive breast carcinomas were also examined, and our analysis identified 74 miRNAs that were differentially expressed between tumor subsets (Table S2.3). Statistical parameters of $p\text{-value} < 0.05$ and a +/-1.2-fold change cut-off were applied to all array datasets. 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. 2.2). The top 30 differentially regulated miRNAs from each array are presented in Table 2.1. 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. 2.3A). 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 (143). 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. 2.3A were identified. We then searched for 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. 2.3B and Table 2.2).

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 are 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 2.3). 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 (76). 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 (Table S2.4). A list of the top 30 genes is presented in Table 2.4 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 (144, 145), 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*, *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* (146), *PPM1D* (147), *RASGRP3* (148, 149), *ADAM19* (150), *SORBS3* (151, 152), *ITSN1* (153), *MECP2* (154, 155), *VLDLR* (156), *HIP1* (157), *PAXIP1* (158), *ITGA2* (159), *ARFGEF1* (160, 161).

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*, the gene encoding SHP-2. Such observations are intriguing given that β4 signals through the PI3K signaling cascade to increase cell migration and invasion (53). 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 (69, 162). 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. Earlier

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 (139). 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 (Table S2.5).

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 microRNA binding motif (163). Interestingly, a comparison of this dataset to our β4-regulated mRNAs identified an enrichment for several of the miRNA families depicted in Fig. 2.3B and Table 2.2, 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. 2.4B). 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 effects 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 (76) 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 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 profound implications for development and disease, especially tumorigenesis. Along these lines, tyrosine kinase receptors, such as EGFR, have also been shown to regulate miRNA expression (164). 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 (165, 166). 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 (167, 168). We examined the expression of Dicer, 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" (169). 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 (169). 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, has identified a role for miRNAs from this family as tumor suppressors (170), 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 of light recent data linking miRNA decay to changes in cell adhesion (171), as well as the general notion that global miRNA expression is typically downregulated in cancer (165, 166).

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 (172-179). 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 2.2). 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-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 2.2). 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 upregulates absolute levels of molecules involved in these

complex processes. 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 (180) were obtained from the Lombardi Cancer Center (Georgetown University, Washington, DC). MCF10CA1a cells (181) were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI). MDA-MB-435 cell lines were maintained in low glucose DMEM medium (Gibco, Carlsbad, CA) 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) 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% 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 (53). The 505 antibody to β4 used for immunoblotting was produced by our laboratory as previously described (182). The antibody to tubulin (Sigma, St. Louis, MO) was also used for immunoblotting.

siRNA Experiments: MCF10CA1a cells were transfected with 20 nM On-TARGETplus SMARTpool siRNA targeting β4 (Dharmacon) at 50% confluency using DharmaFECT 4

transfection reagent (Dharmacon). A non-targeting siRNA pool (Dharmacon) 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) 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) (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). Membranes were blocked in 5% nonfat 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°C. Proteins were detected by enhanced chemiluminescence (Pierce, Rockford, IL) 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. 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 (138). 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) 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) 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), 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˚C for 15-20 min followed by digestion with 1:20 proteinase K (Ambion, Austin, TX). 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 70µl 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 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). 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 1X 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˚C. Microarrays were washed followed by addition of TSA-Plus Cy3 reagent in amplification diluent (Perkin Elmer, Waltham, MA) 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). 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 (183). 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 (184). Heat map false-coloring of Figure 2.2 was applied using Matrix2png (http://www.chibi.ubc.ca/matrix2png) (185). 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 Tables S2.1-S2.3.

Lists of predicted targets of miRNAs used for analyses depicted in Tables 2.4 and 2.5 and gene set enrichment analyses depicted in Fig. 2.4 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 (144, 145) available through the Gene Ontology project

(www.geneontology.org). The hypergeometric probability (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. 2.4, mRNA expression data generated by Chen et. al. (76) was downloaded from the NCBI Gene Expression Omnibus (GEO), series number GSE11466. Affymetrix CEL files were processed with the robust multi-chip average (RMA) algorithm (186) 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 was loaded into the Broad Institute's Gene Set Enrichment Analysis (GSEA) software v2.06 (163, 187). 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.

Table 2.1. Effect of β4 expression on miRNA levels

MDA-MB-435		MCF10CA1a		Tumors	
miRNA	***Fold Change (β4/Mock)***	***miRNA***	***Fold Change (siCtrl/siβ4)***	***miRNA***	***Fold Change (β4+/β4-)***
hsa-miR-29a	-5.56	hsa-miR-187	-3.04	hsa-miR-92b	-3.02
hsa-miR-886-5p	-5.26	hsa-miR-574-5p	-2.19	hsa-miR-145	-2.89
hsa-miR-29b	-5.26	hsa-miR-146a	-2.00	hsa-miR-191	-2.79
hsa-miR-125b	-3.03	hsa-miR-216b	-1.95	hsa-miR-193b	-2.67
hsa-miR-100	-2.94	hsa-miR-127-5p	-1.95	hsa-miR-423-3p	-2.52
hsa-miR-342-3p	-2.70	hsa-miR-516b	-1.88	hsa-miR-342-3p	-2.40
hsa-miR-22	-2.33	hsa-miR-190	-1.85	hsa-miR-24	-2.37
hsa-miR-27a	-2.27	hsa-miR-616	-1.63	hsa-miR-99b	-2.37
hsa-miR-23a	-2.17	hsa-miR-100	-1.60	hsa-miR-574-3p	-2.34
hsa-miR-130a	-2.04	hsa-miR-1233	-1.59	hsa-miR-16	-2.30
hsa-miR-15b	-2.00	hsa-miR-421	1.61	hsa-miR-27a	-2.27
hsa-miR-16	-1.96	hsa-miR-330-3p	1.61	hsa-miR-320a	-2.23
hsa-miR-182	-1.92	hsa-miR-105	1.64	hsa-miR-103	-2.22
hsa-miR-24	-1.92	hsa-miR-33b	1.65	hsa-let-7a	-2.18
hsa-miR-222	-1.92	hsa-miR-218	1.68	hsa-miR-320b	-2.15
hsa-let-7f	-1.82	hsa-miR-18a	1.73	hsa-let-7f	-2.13
hsa-miR-708	-1.72	hsa-miR-422a	1.75	hsa-miR-199a-5p	-2.13
hsa-miR-29c	-1.69	hsa-miR-708	1.77	hsa-let-7b	-2.03
hsa-miR-92b	-1.67	hsa-miR-1284	1.79	hsa-miR-149	-2.02
hsa-miR-185	-1.67	hsa-miR-559	1.81	hsa-miR-1291	-2.01
hsa-miR-30c	-1.61	hsa-miR-33a	1.88	hsa-miR-92a	-2.00
hsa-miR-1244	-1.61	hsa-miR-331-5p	1.91	hsa-miR-214	-1.96
hsa-miR-151-5p	-1.59	hsa-miR-29b	1.92	hsa-miR-93	-1.91
hsa-miR-1260	-1.56	hsa-miR-632	1.93	hsa-miR-143	-1.90
hsa-miR-20b	-1.54	hsa-miR-29c	1.96	hsa-miR-1259	-1.88
hsa-miR-30b	-1.52	hsa-miR-375	1.96	hsa-miR-193a-5p	-1.86
hsa-miR-606	-1.47	hsa-miR-301b	1.97	hsa-miR-200c	-1.83
hsa-let-7b	-1.47	hsa-miR-891b	2.16	hsa-miR-107	-1.81
hsa-miR-1201	-1.47	hsa-miR-936	2.35	hsa-miR-195	-1.81
hsa-miR-768-3p	1.59	hsa-miR-622	2.76	hsa-miR-650	1.79

Table 2.2. 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 2.3. Effect of β4 expression on miR-92ab and miR-99ab/100 family members

MCF10CA1a Array

miRNA	*p-value*	*FDR*[1]	*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*	*FDR*	*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*	*FDR*	*Average β4+ Intensity*	*Average β4- 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

[1] False Discovery Rate

Table 2.4. Predicted targets of miR-92ab and miR-99ab/100 families among β4-regulated genes

Gene ID	p-value	FDR[1]	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

[1] False Discovery Rate

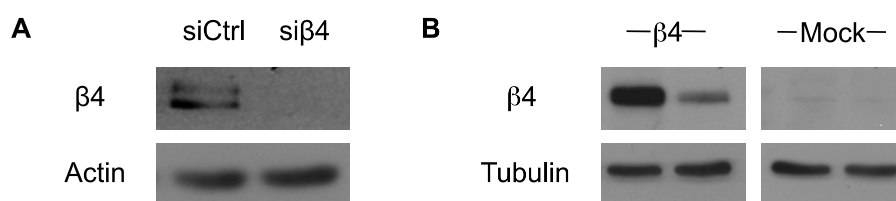


Figure 2.1. β4 expression in breast carcinoma cell lines. A, Expression of β4 in total cell extract (50 μg) following transient knockdown of β4 at 72 hours post-transfection in MCF10CA1a cells. **B**, Expression of β4 in total cell extract (50 μg) in MDA-MB-435/β4 and mock transfectants.

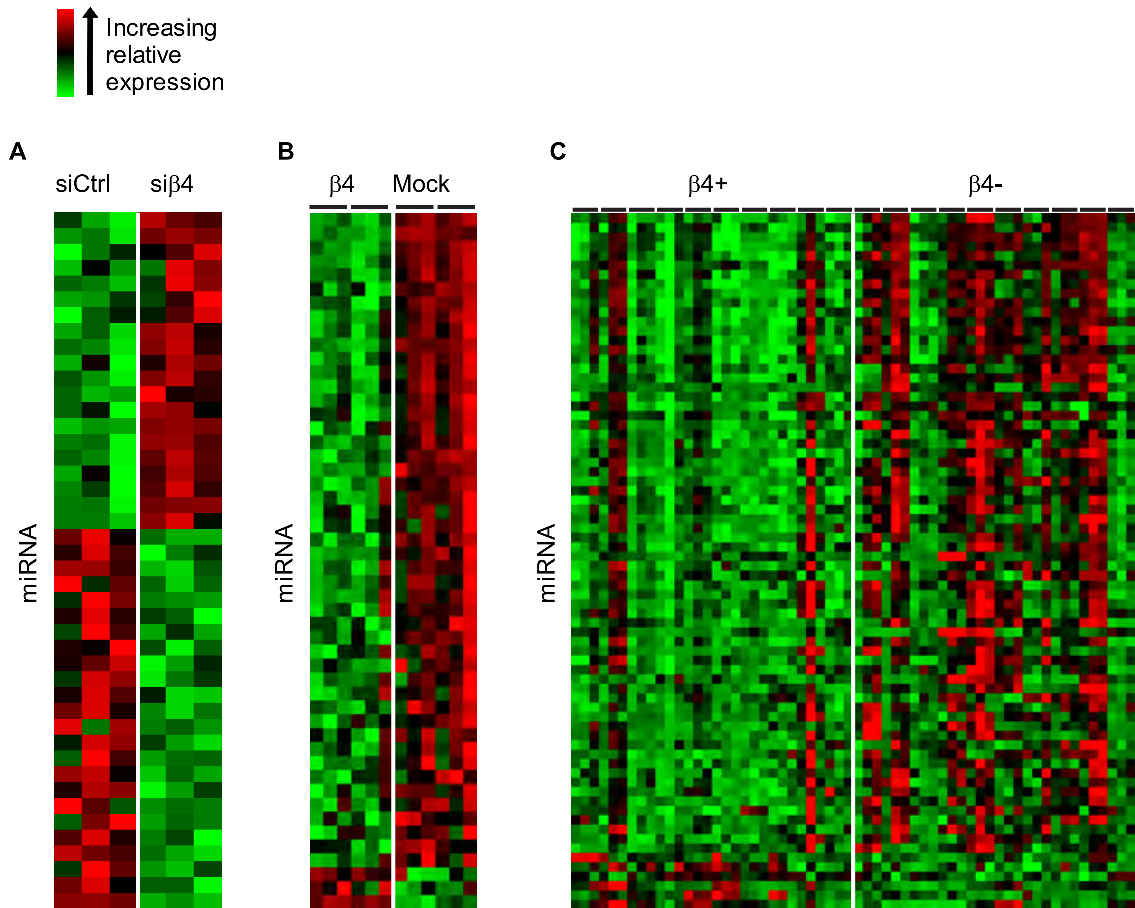

Increasing
relative
expression
A
siCtrl
siβ4
miRNA
B
β4
Mock
miRNA
C
β4+
β4-
miRNA

Figure 2.2. 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.

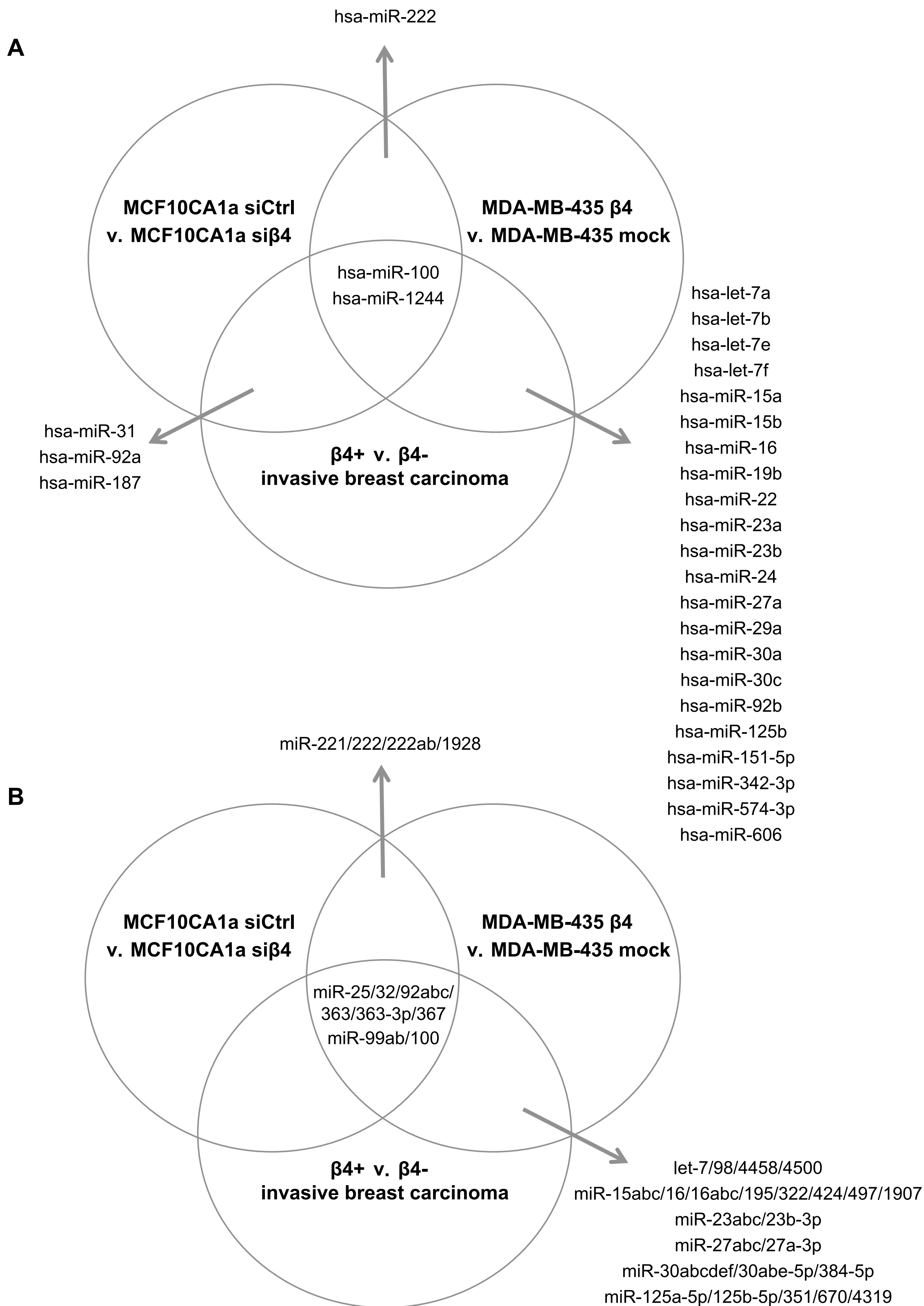
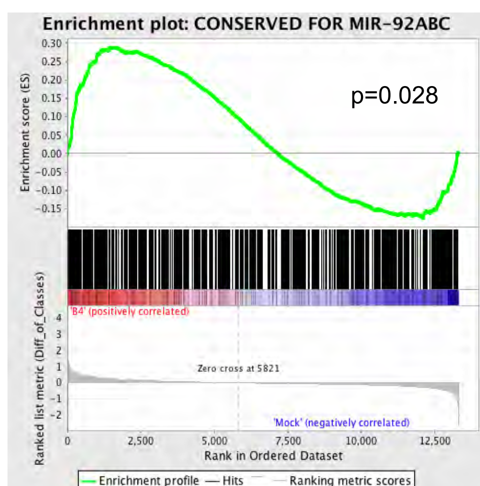
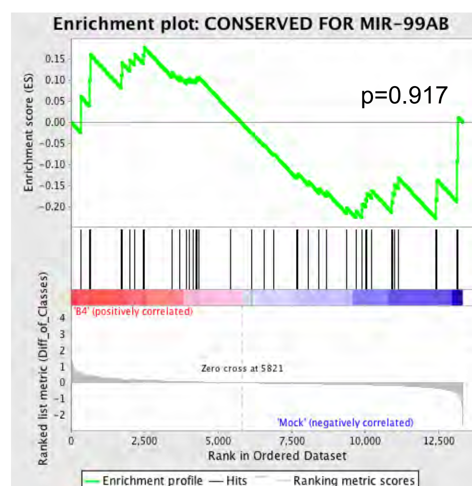

A
hsa-miR-222
MCF10CA1a siCtrl v. MCF10CA1a siβ4
MDA-MB-435 β4 v. MDA-MB-435 mock
hsa-miR-100
hsa-miR-1244
hsa-miR-31
hsa-miR-92a
hsa-miR-187
β4+ v. β4- invasive breast carcinoma
hsa-let-7a
hsa-let-7b
hsa-let-7e
hsa-let-7f
hsa-miR-15a
hsa-miR-15b
hsa-miR-16
hsa-miR-19b
hsa-miR-22
hsa-miR-23a
hsa-miR-23b
hsa-miR-24
hsa-miR-27a
hsa-miR-29a
hsa-miR-30a
hsa-miR-30c
hsa-miR-92b
hsa-miR-125b
hsa-miR-151-5p
hsa-miR-342-3p
hsa-miR-574-3p
hsa-miR-606
B
miR-221/222/222ab/1928
MCF10CA1a siCtrl v. MCF10CA1a siβ4
MDA-MB-435 β4 v. MDA-MB-435 mock
miR-25/32/92abc/363/363-3p/367
miR-99ab/100
β4+ v. β4- invasive breast carcinoma
let-7/98/4458/4500
miR-15abc/16/16abc/195/322/424/497/1907
miR-23abc/23b-3p
miR-27abc/27a-3p
miR-30abcdef/30abe-5p/384-5p
miR-125a-5p/125b-5p/351/670/4319

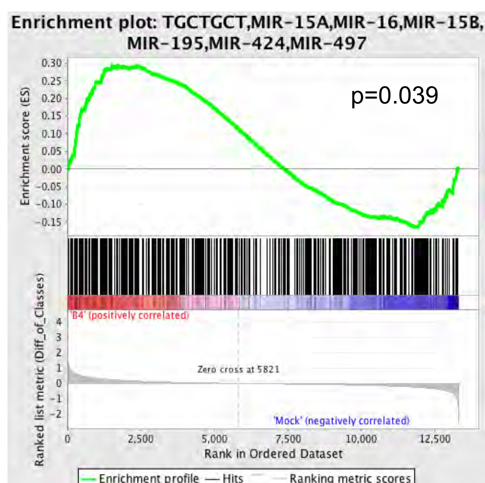
Figure 2.3. β4 inversely correlates with the expression of select miRNA families. A, Venn diagram of overlapping miRNAs that undergo differential expression in response to β4 across all three arrays. **B**, Venn diagram of overlapping miRNA families that undergo differential expression in response to β4 across all three arrays.

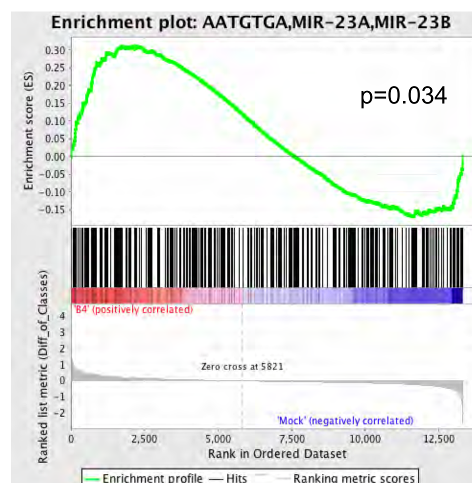
A

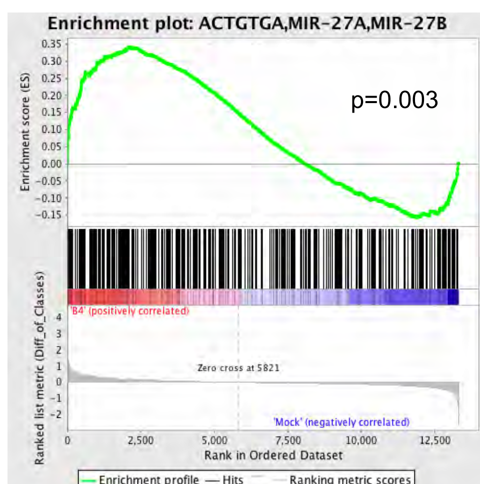




B







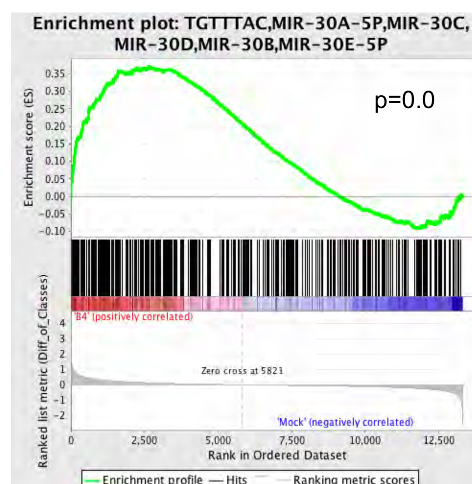


Figure 2.4. β4-regulated mRNAs are enriched in putative targets of miRNA families. GeneChip derived mRNA levels were ranked from the most upregulated in β4 transfected cells to the most downregulated (x-axis, 1 to 12,300, respectively). Red shading indicates mRNA is upregulated in β4 transfectants, while 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.

Table S2.1. MCF10CA1a microarray

miRNA	p-value	FDR	siCtrl Average Intensity	siβ4 Average Intensity	Fold Change (siCtrl/siβ4)
hsa-miR-187	1.16E-04	4.00E-02	224	682	-3.04
hsa-miR-574-5p	4.88E-04	1.12E-01	3313	7249	-2.19
hsa-miR-146a	6.25E-03	3.58E-01	5253	10509	-2.00
hsa-miR-216b	2.62E-02	5.30E-01	85	165	-1.95
hsa-miR-127-5p	6.14E-03	3.58E-01	83	162	-1.95
hsa-miR-516b	1.88E-02	5.30E-01	88	165	-1.88
hsa-miR-190	1.53E-02	5.25E-01	2193	4055	-1.85
hsa-miR-616	1.51E-02	5.25E-01	88	144	-1.63
hsa-miR-100	1.75E-02	5.30E-01	15424	24711	-1.60
hsa-miR-1233	2.94E-02	5.69E-01	2034	3227	-1.59
hsa-miR-222	2.20E-02	5.30E-01	16389	25755	-1.57
hsa-miR-1275	3.12E-02	5.69E-01	122	188	-1.55
hsa-miR-637	4.02E-02	5.89E-01	115	177	-1.54
hsa-miR-221	2.58E-02	5.30E-01	14804	22546	-1.52
hsa-miR-31	3.03E-02	5.69E-01	13659	20667	-1.51
hsa-miR-768-5p	3.72E-02	5.89E-01	19319	28784	-1.49
hsa-miR-296-5p	4.98E-02	6.07E-01	11986	17752	-1.48
hsa-miR-1207-5p	4.47E-02	6.07E-01	11006	16274	-1.48
hsa-miR-1244	3.95E-02	5.89E-01	15632	23057	-1.47
hsa-miR-92a	4.87E-02	6.07E-01	14599	21407	-1.47
hsa-miR-194	3.83E-02	5.89E-01	169	113	1.50
hsa-miR-1248	3.96E-02	5.89E-01	7343	4843	1.52
hsa-miR-548c-5p	2.42E-02	5.30E-01	152	98	1.55
hsa-miR-609	3.33E-02	5.88E-01	176	112	1.56
hsa-miR-421	2.42E-02	5.30E-01	486	301	1.61
hsa-miR-330-3p	2.60E-02	5.30E-01	892	553	1.61
hsa-miR-105	3.50E-02	5.89E-01	164	100	1.64
hsa-miR-33b	2.46E-02	5.30E-01	146	88	1.65
hsa-miR-218	1.52E-02	5.25E-01	201	120	1.68
hsa-miR-18a	1.76E-02	5.30E-01	452	261	1.73
hsa-miR-422a	1.17E-02	5.25E-01	296	170	1.75
hsa-miR-708	7.18E-03	3.80E-01	1215	688	1.77
hsa-miR-1284	2.22E-02	5.30E-01	183	103	1.79
hsa-miR-559	9.02E-03	4.43E-01	383	211	1.81
hsa-miR-33a	1.92E-02	5.30E-01	169	89	1.88
hsa-miR-331-5p	3.46E-03	3.36E-01	211	111	1.91
hsa-miR-29b	3.68E-03	3.36E-01	7537	3932	1.92
hsa-miR-632	1.40E-02	5.25E-01	183	95	1.93
hsa-miR-29c	1.24E-02	5.25E-01	409	209	1.96
hsa-miR-375	3.99E-03	3.36E-01	598	305	1.96
hsa-miR-301b	1.80E-03	2.48E-01	183	93	1.97
hsa-miR-891b	4.88E-03	3.36E-01	197	91	2.16
hsa-miR-936	1.43E-03	2.45E-01	483	206	2.35
hsa-miR-622	3.21E-05	2.21E-02	2322	843	2.76

Table S2.2. MDA-MB-435 microarray

miRNA	p-value	FDR	Average β4 Intensity	Average Mock Intensity	Fold Change (β4/Mock)
hsa-miR-29a	<1.0E-07	<1.0E-07	2410	13323	-5.56
hsa-miR-886-5p	<1.0E-07	<1.0E-07	201	1074	-5.26
hsa-miR-29b	<1.0E-07	<1.0E-07	715	3696	-5.26
hsa-miR-125b	1.0E-07	1.4E-05	722	2187	-3.03
hsa-miR-100	5.0E-07	5.9E-05	2625	7732	-2.94
hsa-miR-342-3p	3.7E-06	3.5E-04	278	749	-2.70
hsa-miR-22	6.5E-06	4.6E-04	424	979	-2.33
hsa-miR-27a	5.5E-05	2.4E-03	3808	8560	-2.27
hsa-miR-23a	1.7E-04	5.7E-03	2331	5060	-2.17
hsa-miR-130a	1.0E-07	1.4E-05	167	342	-2.04
hsa-miR-15b	2.9E-05	1.5E-03	882	1762	-2.00
hsa-miR-16	3.4E-04	1.1E-02	8831	17398	-1.96
hsa-miR-182	2.0E-05	1.1E-03	369	703	-1.92
hsa-miR-24	1.2E-04	4.3E-03	3756	7173	-1.92
hsa-miR-222	8.2E-04	2.2E-02	1163	2254	-1.92
hsa-let-7f	1.9E-03	4.2E-02	4985	9048	-1.82
hsa-miR-708	3.5E-05	1.7E-03	160	277	-1.72
hsa-miR-29c	1.4E-05	8.0E-04	222	380	-1.69
hsa-miR-92b	4.0E-06	3.5E-04	2837	4700	-1.67
hsa-miR-185	1.2E-03	2.9E-02	294	493	-1.67
hsa-miR-30c	2.7E-03	5.6E-02	556	899	-1.61
hsa-miR-1244	8.0E-03	1.3E-01	1375	2226	-1.61
hsa-miR-151-5p	3.6E-03	6.8E-02	304	483	-1.59
hsa-miR-1260	8.6E-05	3.4E-03	452	708	-1.56
hsa-miR-20b	6.0E-04	1.8E-02	301	462	-1.54
hsa-miR-30b	8.7E-03	1.3E-01	304	461	-1.52
hsa-miR-606	7.8E-03	1.3E-01	603	887	-1.47
hsa-let-7b	8.3E-03	1.3E-01	329	484	-1.47
hsa-miR-1201	9.8E-03	1.4E-01	314	459	-1.47
hsa-miR-23b	1.4E-03	3.5E-02	356	519	-1.45
hsa-miR-574-3p	6.8E-04	1.9E-02	1009	1433	-1.43
hsa-let-7a	3.3E-02	2.9E-01	365	520	-1.43
hsa-miR-765	2.5E-03	5.2E-02	214	296	-1.39
hsa-miR-30a	2.1E-02	2.2E-01	327	454	-1.39
hsa-miR-181a	3.4E-02	3.0E-01	1756	2441	-1.39
hsa-miR-345	1.9E-03	4.2E-02	289	396	-1.37
hsa-miR-663b	9.1E-03	1.4E-01	6578	9069	-1.37
hsa-miR-486-5p	2.0E-02	2.2E-01	468	646	-1.37
hsa-miR-19b	2.1E-02	2.2E-01	1675	2304	-1.37
hsa-miR-720	1.7E-02	2.0E-01	1149	1529	-1.33
hsa-miR-1296	2.4E-02	2.3E-01	187	250	-1.33
hsa-miR-15a	1.3E-02	1.7E-01	308	403	-1.32
hsa-miR-27b	1.8E-02	2.1E-01	278	359	-1.30
hsa-let-7e	8.5E-03	1.3E-01	217	276	-1.28
hsa-miR-1234	2.2E-02	2.2E-01	215	275	-1.28
hsa-miR-885-3p	1.3E-02	1.7E-01	225	286	-1.27

hsa-miR-486-3p	2.0E-02	2.2E-01	336	426	-1.27
hsa-miR-320d	2.0E-02	2.2E-01	268	208	1.28
hsa-miR-320a	2.8E-02	2.6E-01	2036	1503	1.36
hsa-miR-768-3p	1.0E-05	6.6E-04	6480	4072	1.59

Table S2.3. Tumor microarray

miRNA	p-value	FDR	Average β4 Positive Intensity	Average β4 Negative Intensity	Fold Change (β4+/β4-)
hsa-miR-92b	4.50E-06	1.06E-03	1125	3400	-3.02
hsa-miR-145	1.47E-05	1.48E-03	2472	7138	-2.89
hsa-miR-191	1.40E-06	9.90E-04	4391	12237	-2.79
hsa-miR-193b	2.32E-04	9.10E-03	2769	7384	-2.67
hsa-miR-423-3p	9.70E-06	1.14E-03	903	2279	-2.52
hsa-miR-342-3p	3.38E-03	3.98E-02	4442	10662	-2.40
hsa-miR-24	1.00E-03	2.02E-02	2570	6103	-2.37
hsa-miR-99b	4.65E-04	1.22E-02	926	2190	-2.37
hsa-miR-574-3p	2.76E-04	9.31E-03	719	1683	-2.34
hsa-miR-16	5.31E-04	1.29E-02	3219	7419	-2.30
hsa-miR-27a	4.53E-04	1.22E-02	1086	2469	-2.27
hsa-miR-320a	9.98E-05	5.43E-03	1361	3034	-2.23
hsa-miR-103	2.06E-04	8.55E-03	569	1265	-2.22
hsa-let-7a	2.06E-05	1.82E-03	439	956	-2.18
hsa-miR-320b	7.53E-05	4.84E-03	792	1698	-2.15
hsa-let-7f	1.48E-04	6.96E-03	5451	11590	-2.13
hsa-miR-199a-5p	2.76E-04	9.31E-03	429	915	-2.13
hsa-let-7b	4.60E-05	3.25E-03	423	859	-2.03
hsa-miR-149	2.03E-03	2.86E-02	262	529	-2.02
hsa-miR-1291	2.64E-04	9.31E-03	7451	14960	-2.01
hsa-miR-92a	1.16E-02	8.31E-02	3498	6989	-2.00
hsa-miR-214	2.75E-03	3.41E-02	6456	12668	-1.96
hsa-miR-93	3.50E-03	4.06E-02	578	1107	-1.91
hsa-miR-143	9.30E-06	1.14E-03	325	617	-1.90
hsa-miR-1259	5.50E-03	5.47E-02	673	1266	-1.88
hsa-miR-193a-5p	8.84E-05	5.21E-03	369	687	-1.86
hsa-miR-200c	1.42E-03	2.29E-02	433	791	-1.83
hsa-miR-107	1.31E-03	2.29E-02	435	787	-1.81
hsa-miR-195	2.80E-06	9.90E-04	318	574	-1.81
hsa-miR-484	1.30E-02	8.75E-02	1228	2170	-1.77
hsa-miR-423-5p	1.50E-03	2.30E-02	847	1424	-1.68
hsa-miR-23a	1.27E-02	8.72E-02	714	1191	-1.67
hsa-miR-125a-5p	2.72E-03	3.41E-02	348	569	-1.64
hsa-miR-22	1.30E-03	2.29E-02	345	550	-1.59
hsa-miR-30d	2.73E-03	3.41E-02	320	506	-1.58
hsa-miR-620	6.19E-03	5.76E-02	419	663	-1.58
hsa-miR-675	2.42E-02	1.37E-01	325	511	-1.57
hsa-miR-1248	1.22E-02	8.52E-02	399	623	-1.56
hsa-miR-125b	6.15E-03	5.76E-02	443	686	-1.55
hsa-miR-197	2.76E-02	1.42E-01	573	887	-1.55
hsa-miR-606	8.69E-03	7.23E-02	343	523	-1.53
hsa-miR-532-3p	3.71E-02	1.69E-01	460	700	-1.52
hsa-miR-1307	2.91E-02	1.43E-01	507	763	-1.51
hsa-miR-492	6.40E-04	1.46E-02	236	351	-1.49

hsa-miR-205	3.55E-02	1.65E-01	293	433	-1.48
hsa-miR-34a	3.34E-03	3.98E-02	353	519	-1.47
hsa-miR-19b	4.75E-02	1.90E-01	308	446	-1.45
hsa-miR-29a	1.10E-02	8.23E-02	371	537	-1.45
hsa-miR-1244	3.89E-02	1.72E-01	385	550	-1.43
hsa-miR-187	1.64E-03	2.47E-02	240	340	-1.42
hsa-miR-425	1.36E-03	2.29E-02	247	351	-1.42
hsa-miR-99a	2.72E-02	1.42E-01	393	551	-1.40
hsa-miR-744	3.93E-03	4.14E-02	283	390	-1.38
hsa-miR-151-5p	1.71E-02	1.08E-01	268	368	-1.37
hsa-miR-31	2.16E-03	2.93E-02	231	316	-1.37
hsa-miR-15b	1.19E-02	8.39E-02	251	340	-1.36
hsa-miR-127-3p	4.43E-02	1.84E-01	339	455	-1.34
hsa-miR-30a	3.22E-04	1.03E-02	220	287	-1.31
hsa-miR-324-5p	9.14E-03	7.43E-02	277	363	-1.31
hsa-let-7c	1.45E-04	6.96E-03	227	295	-1.30
hsa-miR-23b	3.32E-02	1.58E-01	305	392	-1.29
hsa-miR-26a	1.90E-02	1.15E-01	285	367	-1.29
hsa-miR-30c	2.11E-03	2.92E-02	219	282	-1.29
hsa-miR-320c	2.30E-03	3.07E-02	270	342	-1.27
hsa-let-7e	2.34E-02	1.34E-01	265	327	-1.24
hsa-miR-100	1.74E-02	1.09E-01	272	338	-1.24
hsa-miR-15a	6.32E-03	5.80E-02	229	279	-1.22
hsa-miR-340	1.16E-02	8.31E-02	214	258	-1.20
hsa-miR-661	2.76E-02	1.42E-01	256	210	1.22
hsa-miR-615-5p	1.18E-03	2.19E-02	354	288	1.23
hsa-miR-1280	1.11E-02	8.23E-02	37962	27554	1.37
hsa-miR-150	4.86E-02	1.90E-01	433	309	1.41
hsa-miR-346	1.35E-03	2.29E-02	302	212	1.43
hsa-miR-650	2.56E-02	1.41E-01	910	511	1.79

Table S2.4. Overlapping miR-92ab and miR-99ab/100 β4-regulated mRNAs

Gene ID	p-value	FDR	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
ITSN1	1.85E-03	3.32E-02	23	18	1.33
MECP2	9.82E-04	2.32E-02	198	149	1.33
VLDLR	2.81E-02	1.70E-01	80	60	1.33
HIP1	2.93E-02	1.75E-01	61	46	1.32
NIPBL	5.85E-03	6.58E-02	68	52	1.32
HLCS	3.32E-03	4.72E-02	132	101	1.31
PAXIP1	1.36E-03	2.79E-02	189	144	1.31
RNMT	3.71E-03	5.04E-02	196	150	1.31
SGSH	4.05E-03	5.29E-02	498	383	1.30
FNTA	3.31E-03	4.72E-02	290	225	1.29
MTMR1	2.35E-02	1.53E-01	60	47	1.29
SR140	2.04E-02	1.40E-01	88	68	1.29
FBXL18	2.08E-03	3.60E-02	142	111	1.28
ITGA2	9.05E-03	8.60E-02	73	57	1.28
CASK	1.03E-02	9.31E-02	161	127	1.27
AP1AR	1.16E-02	1.00E-01	92	74	1.25

ARFGEF1	4.76E-02	2.33E-01	188	150	1.25
HN1L	1.59E-03	3.07E-02	1011	811	1.25
MTF1	2.54E-02	1.60E-01	142	113	1.25
S100PBP	1.94E-02	1.36E-01	89	71	1.25
UBE2I	9.20E-03	8.69E-02	1253	1003	1.25
DYRK1A	8.43E-03	8.24E-02	541	441	1.23
NF1	7.42E-03	7.63E-02	128	104	1.23
CDK6	4.05E-02	2.13E-01	96	79	1.22

Table S2.5. miR-92ab leading edge genes

Gene Symbol	Gene Title	Rank in Gene List[1]	Rank Metric Score[2]	Running ES[3]
SOX4	SRY (sex determining region Y)-box 4	10	2.005	0.016
HAPLN1	hyaluronan and proteoglycan link protein 1	37	1.253	0.024
FRYL	furry homolog-like (Drosophila)	88	0.975	0.028
TMEM50B	transmembrane protein 50B	93	0.951	0.036
AXL	AXL receptor tyrosine kinase	124	0.855	0.040
TOB1	transducer of ERBB2, 1	129	0.843	0.047
DCP2	DCP2 decapping enzyme homolog (S. cerevisiae)	134	0.829	0.054
SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	138	0.813	0.060
DMXL1	Dmx-like 1	143	0.809	0.066
COL1A2	collagen, type I, alpha 2	153	0.795	0.072
TMF1	TATA element modulatory factor 1	163	0.778	0.078
WWP2	WW domain containing E3 ubiquitin protein ligase 2	164	0.777	0.084
CCNE2	cyclin E2	180	0.751	0.089
ITM2B	integral membrane protein 2B	183	0.749	0.095
KIF3B	kinesin family member 3B	198	0.722	0.100
ARHGEF10	Rho guanine nucleotide exchange factor (GEF) 10	215	0.702	0.105
CHD9	chromodomain helicase DNA binding protein 9	219	0.699	0.110
FMR1	fragile X mental retardation 1	228	0.687	0.115
ASPH	aspartate beta-hydroxylase	252	0.668	0.119
MTMR9	myotubularin related protein 9	261	0.662	0.124
GRAMD3	GRAM domain containing 3	277	0.649	0.128
MMP16	matrix metallopeptidase 16 (membrane-inserted)	278	0.649	0.133
ICK	intestinal cell (MAK-like) kinase	281	0.640	0.138
NFYB	nuclear transcription factor Y, beta	292	0.626	0.143
PTPRK	protein tyrosine phosphatase, receptor type, K	298	0.620	0.148
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	305	0.618	0.152
SYNJ1	synaptojanin 1	308	0.616	0.157
ATP6V1B2	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2	312	0.609	0.162
SRPK2	SFRS protein kinase 2	333	0.592	0.165
EN2	engrailed homolog 2	353	0.579	0.168
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	363	0.571	0.172
ZNF652	zinc finger protein 652	390	0.553	0.175

ATXN1	ataxin 1	399	0.548	0.179
AGGF1	angiogenic factor with G patch and FHA domains 1	421	0.535	0.182
LYST	lysosomal trafficking regulator	422	0.535	0.186
ARF1	ADP-ribosylation factor 1	469	0.512	0.187
CALM3	calmodulin 3 (phosphorylase kinase, delta)	520	0.484	0.187
MTF2	metal response element binding transcription factor 2	531	0.482	0.190
GALNT7	P-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc-	544	0.477	0.193
INSIG1	insulin induced gene 1	563	0.465	0.195
WDR37	WD repeat domain 37	578	0.458	0.198
MFHAS1	malignant fibrous histiocytoma amplified sequence 1	580	0.458	0.202
ADAM19	ADAM metallopeptidase domain 19 (meltrin beta)	593	0.453	0.204
MKL2	MKL/myocardin-like 2	595	0.452	0.208
MMD	monocyte to macrophage differentiation-associated	598	0.451	0.212
FBXO28	F-box protein 28	637	0.436	0.212
TBL1XR1	transducin (beta)-like 1X-linked receptor 1	655	0.431	0.214
NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	661	0.429	0.218
EXOC5	exocyst complex component 5	681	0.424	0.220
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	687	0.422	0.223
ARMC1	armadillo repeat containing 1	721	0.410	0.223
JARID2	jumonji, AT rich interactive domain 2	726	0.408	0.227
RBL2	retinoblastoma-like 2 (p130)	737	0.405	0.229
DYNLT3	dynein, light chain, Tctex-type 3	740	0.404	0.232
OTUD4	OTU domain containing 4	741	0.404	0.236
H3F3B	H3 histone, family 3B (H3.3B)	751	0.400	0.238
G3BP2	-	757	0.398	0.241
PCGF3	polycomb group ring finger 3	779	0.392	0.243
MITF	microphthalmia-associated transcription factor	791	0.388	0.245
PDE4D	phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	831	0.378	0.245
ZNF238	zinc finger protein 238	857	0.371	0.246
REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	871	0.368	0.248
PHF17	PHD finger protein 17	911	0.359	0.248
CHMP7	CHMP family, member 7	921	0.356	0.250
PAIP1	poly(A) binding protein interacting protein 1	945	0.351	0.251
CASK	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	966	0.345	0.253
QKI	quaking homolog, KH domain RNA binding (mouse)	976	0.343	0.255
CASD1	CAS1 domain containing 1	978	0.342	0.257

CPEB1	cytoplasmic polyadenylation element binding protein 1	997	0.337	0.259
EDNRB	endothelin receptor type B	999	0.336	0.262
ATXN10	ataxin 10	1000	0.336	0.264
PANK3	pantothenate kinase 3	1010	0.334	0.266
MORC3	MORC family CW-type zinc finger 3	1023	0.331	0.268
VPS4B	vacuolar protein sorting 4 homolog B (S. cerevisiae)	1028	0.330	0.271
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	1056	0.325	0.271
CNOT2	CCR4-NOT transcription complex, subunit 2	1068	0.323	0.273
IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	1098	0.317	0.273
CEP350	centrosomal protein 350kDa	1099	0.317	0.276
PHF15	PHD finger protein 15	1107	0.316	0.278
DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	1149	0.309	0.277
ZNF532	zinc finger protein 532	1151	0.308	0.280
RSBN1	round spermatid basic protein 1	1172	0.303	0.281
ZFYVE21	zinc finger, FYVE domain containing 21	1173	0.303	0.283
EIF4G2	eukaryotic translation initiation factor 4 gamma, 2	1231	0.292	0.281
CDK6	cyclin-dependent kinase 6	1254	0.287	0.282
DDHD2	DDHD domain containing 2	1284	0.282	0.282
PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1	1314	0.278	0.282
ROBO2	roundabout, axon guidance receptor, homolog 2 (Drosophila)	1341	0.274	0.282
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	1398	0.262	0.280
CCNJL	cyclin J-like	1404	0.261	0.281
CNIH	cornichon homolog (Drosophila)	1407	0.261	0.283
RANBP9	RAN binding protein 9	1422	0.259	0.284
WNT5A	wingless-type MMTV integration site family, member 5A	1425	0.259	0.286
CCNC	cyclin C	1428	0.258	0.288
TBC1D12	TBC1 domain family, member 12	1447	0.255	0.289

[1] Rank in Gene List refers to position of gene in list of β4-regulated mRNAs ranked in order of greatest change in expression
[2] Rank Metric Score is the score used to position the genes in the ranked list
[3] Running Enrichment Score (ES) reflects the degree to which the gene is overrepresented in the top of the ranked list of genes

CHAPTER III

INTEGRIN β4 REGULATES SPARC PROTEIN TO PROMOTE INVASION

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Abstract

The α6β4 integrin (referred to as 'β4' integrin) is a receptor for laminins that promotes carcinoma invasion through its ability to regulate key signaling pathways and cytoskeletal dynamics. An analysis of published Affymetrix GeneChip data to detect downstream effectors involved in β4-mediated invasion of breast carcinoma cells identified SPARC, or secreted protein acidic and rich in cysteine. This glycoprotein has been shown to play an important role in matrix remodeling and invasion. Our analysis revealed that manipulation of β4 integrin expression and signaling impacted SPARC expression, and that SPARC facilitates β4-mediated invasion. Expression of β4 in β4-deficient cells reduced the expression of a specific microRNA (miR-29a) that targets SPARC and impedes invasion. In cells that express endogenous β4, miR-29a expression is low and β4 ligation facilitates the translation of SPARC through a TOR-dependent mechanism. The results obtained in this study demonstrate that β4 can regulate SPARC expression and that SPARC is an effector of β4-mediated invasion. They also highlight a potential role for specific miRNAs in executing the functions of integrins.

Introduction

Integrins are a family of heterodimeric transmembrane cell surface receptors composed of α and β subunits that collectively link the cytoskeleton to components in the extracellular matrix or to neighboring cells (131, 132). The integrin α6β4, referred to as 'β4 integrin,' is an adhesion receptor for the laminins that plays a pivotal role in both normal tissue development and homeostasis, as well as in carcinoma progression (85,

188). β4 mediates the formation of HDs, inert structures on the basal surface of epithelial cells anchoring the intermediate cytoskeleton to laminins in the basement membrane (133, 134). Factors in the tumor microenvironment of invasive carcinomas liberate β4 from HDs and promote its relocalization to the leading edge of cells, where it becomes signaling competent and associates with F-actin in lamellae and filopodia to promote migration and invasion (58, 85, 86, 135-137). In the context of breast cancer, this integrin is associated with a "basal-like" subset of tumors, and its expression predicts decreased time to tumor recurrence as well as decreased patient survival (138). The contributions of β4 to carcinoma progression stem, in part, from its ability to regulate the expression and function of downstream effector molecules (53, 63, 78, 79, 85, 98, 102).

We conducted an analysis of published Affymetrix GeneChip data (76) and identified SPARC, or secreted glycoprotein acidic and rich in cysteine as a potential effector of β4-mediated function. SPARC plays a key role in extracellular matrix remodeling and cell motility (189). The data we obtained demonstrate that β4 expression and ligation can regulate SPARC and that SPARC is an effector of β4-mediated invasion. Interestingly, SPARC was identified as a target of miR-29a in osteoblasts (190), prompting us to examine the role of miRNAs downstream of β4 in the regulation of SPARC. miRNAs are non-coding single-stranded RNAs approximately 22 base pairs in length that regulate gene expression through mRNA degradation or translational inhibition and have been shown to play an increasingly significant role in tumorigenesis (108, 123). We identify miR-29a as a β4-regulated miRNA that can influence SPARC expression and invasion. The regulation of miR-29a by β4 is seen in cells that exhibit

high miR-29a expression; in cells that express endogenous β4, miR-29a expression is low. Finally, we provide evidence that β4 expression and ligation facilitate the translation of SPARC.

Results

β4 integrin regulates expression of SPARC

MDA-MB-435 breast carcinoma cells were utilized initially as a model system to identify β4-regulated genes that facilitate invasion. Despite some reports claiming that these cells are of melanocytic origin (191-193), several reports have refuted this claim and have provided convincing data that this is a poorly differentiated cell line of breast cancer origin (194-198). These cells express α6β1 endogenously but lack α6β4. Introduction of the β4 subunit leads to preferential heterodimerization of the α6 subunit with the β4 subunit (33, 142). Stable subclones were generated expressing wild-type β4 (referred to as β4 transfectants) or a β4 deletion mutant (referred to as β4ΔCYT transfectants) that lacks the cytoplasmic domain of the β4 subunit. This deletion impedes the signaling capacity of the integrin, and it eliminates the formation of the α6β1 heterodimer (53, 199). Mock transfectants were also generated. The β4 transfectants are significantly more invasive than either the mock or β4ΔCYT transfectants (53).

To identify potential regulators of β4-mediated invasion, we conducted an analysis of published Affymetrix GeneChip data that were obtained using the MDA-MB-435/β4 transfectants (76). SPARC, or secreted protein acidic and rich in cysteine, was identified using this approach. This secreted glycoprotein is involved in extracellular

matrix remodeling and invasion (189). SPARC mRNA and protein expression was examined to determine whether β4 differentially regulates its expression in this system. Quantitative real time PCR (qPCR) confirmed that SPARC message levels are elevated over 3-fold in the β4 transfectants compared to controls (Fig. 3.1A). Furthermore, SPARC protein expression is elevated significantly in the total cell extract and culture media of the β4 transfectants compared to either the mock or β4ΔCYT transfectants (Fig. 3.1B), providing evidence that the β4 integrin can induce SPARC expression.

β4 expression inversely correlates with miR-29a expression

SPARC was recently identified as a target of miR-29a in osteoblasts (190), prompting us to examine the role of miRNAs downstream of β4 in the regulation of SPARC. SPARC contains two conserved miR-29 predicted miRNA binding sites and one conserved miR-203 predicted binding site in its 3'UTR. These observations are relevant because of results from a miRNA microarray conducted by our laboratory to assess global miRNA expression in the MDA-MB-435/β4 system. Specifically, two subclones of the β4 transfectants (3A7 and 5B3) and two subclones of the mock transfectants (6D2 and 6D7), as well as the MDA-MB-435 parental cells, were examined using a novel microarray technology termed qNPA. The results of the array demonstrated that β4 expression repressed the expression of miR-29a and miR-29b (Fig. 3.2A). miR-29c and miR-203 levels, however, were unchanged (data not shown). We focused on miR-29a because it has been shown to target SPARC and because miR-29b undergoes rapid decay following nuclear import in cycling cells (200). The microarray data were confirmed using qPCR. The expression of β4 in MDA-MB-435 cells resulted

in an approximate 4-fold decrease in miR-29a compared to the mock transfectants. Furthermore, a subclone of the β4ΔCYT transfectants (5D5) was also examined and found to express levels of miR-29a similar to those detected in the mock transfectants (Fig. 3.2B), indicating that the cytoplasmic tail of β4 is required for repression of miR-29a.

To assess the relationship between β4 and miR-29a expression further, we examined a series of breast carcinoma cell lines with differential β4 expression. The β4-null MDA-MB-435 parental cells were compared to the β4-null SUM1315 breast carcinoma cell line, and to the β4-expressing MDA-MB-231 and SUM-159 breast carcinoma cell lines (Fig. 3.2C). Levels of miR-29a were markedly lower in cell lines expressing β4 compared to those not expressing the integrin (Fig. 3.2D), supporting a relationship between β4 expression and the regulation of miR-29a.

Gene set enrichment analysis of the published Affymetrix GeneChip data (76) was conducted to substantiate the role of miR-29a in the regulation of β4-mediated targets. This analysis examines the population of β4-regulated mRNAs for an over-representation of genes predicted to be targeted by our miRNA of interest. Our analysis revealed a significant enrichment ($p < 0.001$) for miR-29 predicted targets in mRNAs upregulated by β4 (Fig. 3.3). In contrast, no enrichment was detected for miR-93, a miRNA selected as a negative control on the basis that it was expressed at robust levels in all samples (data not shown). As part of this analysis, a list of leading edge genes was generated, consisting of a group of mRNAs that are the important contributors to the detected enrichment. The list of leading edge genes contained 116 mRNAs (Table S3.1),

the top 25 of which are listed in Table 3.1 ranked in order of contribution to the detected enrichment. As anticipated, SPARC appears on this list. Of interest, other genes in this table have also been implicated in the invasive process in breast carcinoma and other cancers, including LOXL2 and MAPRE2 (201-206). COL1A2 appears on this list as well and has been linked to increased cell motility and metastatic disease (207, 208). These observations raise the possibility that miR-29a regulates a pro-invasive pool of target genes, and that SPARC actively cooperates with many of these molecules to promote carcinoma invasion.

β4-mediated repression of miR-29a can promote SPARC-dependent invasion

The findings described above raised the issue of whether miR-29a represses invasion by targeting SPARC. To test the hypothesis that repression of miR-29a is required for invasion, a synthetic chemically-modified miRNA mimic was used to overexpress the mature form of miR-29a in the MDA-MB-435/β4 transfectants. Transfection of the β4 transfectants with the miR-29a mimic decreased invasion 6.6-fold compared to cells transfected with a non-specific negative control mimic (Fig. 3.4A). These findings were extended to SUM-159 cells, an invasive breast carcinoma cell line that endogenously expresses β4 and contains levels of miR-29a similar to those of the β4 transfectants (Figs. 3.2B, 3.2C, and 3.4A). We then pursued the possibility that loss of functional miR-29a is sufficient to induce an invasive phenotype. Mock transfectants, which are poorly invasive and express relatively high levels of miR-29a, were transfected with a miR-29a functional inhibitor. This hairpin inhibitor is an RNA oligonucleotide designed to inhibit the function of the endogenous miRNA. Expression of the inhibitor

diminishes levels of functional miR-29a and, thus, mimics β4-induced miR-29a repression. The results from this experiment demonstrate that inhibition of miR-29a is not sufficient to induce the invasive phenotype of cells in the absence of β4 (Fig. 3.4B), consistent with our observation that overexpression of SPARC in the mock transfectants resulted in no change in invasion (data not shown).

To establish that miR-29a represses SPARC as a function of β4 expression, SPARC expression was examined following manipulation of miR-29a levels in both the MDA-MB-435/β4 and mock transfectants. Transfection of the β4 transfectants with the miR-29a mimic produced a significant decrease in SPARC expression compared to mock transfected cells and cells transfected with a non-specific negative control mimic (Fig. 3.4C). Conversely transfection of the mock transfectants with a miR-29a inhibitor substantially increased SPARC expression compared to mock transfected cells and cells transfected with a non-targeting negative control inhibitor (Fig. 3.4C). Importantly, these data substantiate the invasion assays described above by confirming that the mimic and hairpin inhibitor are functional, since functionality is established by their ability to regulate target gene expression. Furthermore, the protein data from the inhibitor studies provide a control for the invasion assay presented in Figure 3.4B, insuring that the poorly invasive phenotype of the cells transfected with the miR-29a inhibitor is not due to a technical problem with the inhibitor.

To determine whether SPARC is necessary for β4-mediated invasion, β4 transfectants were subjected to an invasion assay following incubation with a functional blocking antibody to SPARC. The ability of these cells to invade Matrigel was decreased

2.5-fold compared to cells receiving no treatment and cells pre-incubated with normal mouse IgG (Fig. 3.4D), establishing a role for this β4 target in mediating invasion downstream of the integrin.

β4 can regulate SPARC independently of miR-29a

Although the β4 transfectants possess some constitutive activity and can mediate β4-function in a ligand independent manner (78, 88), ligation of β4 either by adhesion to laminin or antibody-mediated clustering should in principle further repress miR-29a and upregulate SPARC expression. Interestingly, our data indicate that β4 signaling can upregulate SPARC expression independently of the miRNA. As depicted in Figure 3.5A, ligation of β4 in the β4 transfectants by adhesion to laminin induces SPARC protein expression compared to suspension control. Given that the β4 transfectants retain expression of the β1 integrin subunit (53), antibody-mediated clustering experiments were conducted to substantiate these data and further implicate β4 signaling in the regulation of this effector molecule. Specifically, clustering with an antibody to the α6 subunit of the integrin (mAb 2B7) upregulates SPARC protein compared to cells clustered with an antibody to β1 (mAb AIIB2), confirming that this regulation is specific to integrin α6β4 (Fig. 3.5A).

Our observation that SPARC induction occurs in the absence of further miR-29a repression (data not shown) prompted us to examine the expression of SPARC message under these conditions. SPARC mRNA levels are unchanged in cells clustered with the α6 antibody compared to the β1 control (Fig. 3.5B), suggesting that β4 plays a role in regulating SPARC protein stability or translation. Considering that ligation of this

integrin is known to upregulate mTOR signaling and VEGF translation (95), we treated cells with rapamycin, an inhibitor of TOR cap-dependent translation. As depicted in Figures 3.5A and 3.5B, rapamycin blocked β4-mediated induction of SPARC protein, as well as pS6K and p4E-BP1 signaling intermediates. Our data suggest that while steady-state levels of SPARC can be regulated by miR-29a in this system, rapid changes in SPARC expression occurring in response to β4 ligation arise through a TOR-dependent translational mechanism.

We next assessed the relationship between β4 and SPARC in breast carcinoma cells that express endogenous β4. For this purpose, the SUM-159 cell line was selected because it is an invasive breast carcinoma cell line in which SPARC is robustly expressed (Fig. 3.5D). Interestingly, transient depletion of β4 using siRNA diminished SPARC protein expression but it had no effect on SPARC mRNA levels (Figs. 3.5C and 3.5D). These data support the hypothesis that β4 can regulate SPARC expression. Depletion of β4 expression, however, did not increase miR-29a (data not shown). Based on our observation that β4 can regulate SPARC independently of the miRNA in the MDA-MB-435 system, we examined the possibility that this translational mechanism was also at play in the SUM-159 cells. As depicted in Fig. 3.5D, levels of pS6K and p4E-BP1 signaling intermediates were diminished upon loss of β4. To establish that this pathway is required for maintenance of SPARC expression, SUM-159 parental cells were treated with rapamycin. After six hours, a detectable decrease in SPARC protein levels was observed (Fig. 3.5E), suggesting that β4 regulates SPARC expression in this system through a TOR-dependent translational mechanism.

To assess the role of β4 ligation and signaling in regulating SPARC translation in SUM-159 cells, these cells were plated on laminin in the presence or absence of rapamycin. Work from our laboratory has established that α6β4 is the predominant laminin-binding integrin in these cells (96). Laminin-mediated clustering of β4 induces SPARC expression at the protein level compared to suspension control (Fig. 3.5F), while SPARC mRNA levels remain unaffected (Fig. 3.5G). As anticipated, this induction is abrogated upon treatment with rapamycin (Fig. 3.5F).

Discussion

The major conclusion of this study is that the β4 integrin can regulate the expression of SPARC in breast carcinoma cells. This finding is significant because this integrin is known to facilitate the invasion of carcinoma cells, and its regulation of SPARC adds to our understanding of how β4 can contribute to the invasive process. In addition, our data reveal a novel function for the β4 integrin in repressing the expression of a specific miRNA, miR-29a that can impede invasion. To our knowledge, this is the first report that integrins can regulate the expression of miRNAs. One mechanism by which miR-29a impedes invasion is to target SPARC. This mode of miR-29a regulation by β4 is manifested in cells that express high levels of miR-29a. In other cells that express endogenous β4 and low levels of miR-29a, we provide evidence that β4 expression and signaling can enhance SPARC translation. These findings indicate that β4 has the ability to regulate SPARC expression by distinct mechanisms.

Our data support the notion that SPARC, a secreted extracellular matrix glycoprotein with counter-adhesive properties, functions to promote invasion. This role for SPARC is supported by the findings that SPARC can promote cell motility and invasion in various carcinoma cells, including breast (209-216). Moreover, SPARC expression has been associated with basal-like breast cancers (217). This observation is relevant to our findings because we correlated β4 integrin expression with basal-like breast cancers in a previous study (138), and the cell lines used in the current study exhibit a basal phenotype. Some reports, however, have questioned the role of SPARC in breast cancer invasion and progression (218, 219). SPARC has also been shown to decrease the mitogenic potency of various growth factors including VEGF and platelet-derived growth factor (PDGF) by antagonizing their ability to bind to their cognate receptors (220, 221). In contrast, there is evidence that SPARC can enhance integrin and growth factor receptor-regulated kinases, thereby upregulating key signaling pathways involved in cell motility (215, 222-226), observations that are consistent with our data. This dichotomy of SPARC function may be explained by the hypothesis that SPARC inhibits early stages of tumorigenesis but potentiates later stages of progression, analogous to the TGF-β pathway (227), a growth factor signaling pathway that SPARC has been shown to regulate (226, 228-232).

Our data provide the first indication that β4 has the ability to regulate the expression of specific miRNAs and that such miRNAs can influence β4-mediated migration and invasion. Since the initial reports that the β4 integrin has the ability to promote the migration and invasion of epithelial and carcinoma cells, numerous

mechanisms have been reported to account for this phenomenon. These mechanisms include activation of signaling pathways, especially the PI3K pathway and Rho GTPases, transcription factors (NFAT), and cap-dependent translation of key effector molecules (53, 74, 78, 79, 95, 233, 234). The ability of β4 to regulate the expression of miRNAs adds a new dimension to our understanding of how β4 mediates invasion and other functions. The repression of miR-29a that occurs in response to exogenous expression of β4 is significant in this context because miR-29a represses invasion and targets SPARC. While our data indicate that β4-mediated repression of miR-29a is required for invasion, our observation that functional inhibition of this miRNA did not induce an invasive phenotype in the poorly invasive, mock transfectants suggests that a single miRNA is unlikely to be solely responsible for a cellular process. Although we observed that this regulation of miR-29a by β4 is manifested in specific cell types, especially those that express high levels of miR-29a, the paradigm that miRNAs contribute to the execution of integrin-mediated functions may be widespread.

The half-life of specific miRNAs could be a significant factor in their potential repression by integrin signaling. Given that the reported half-life of miR-29a is greater than 12 hours (200), a detectable decrease in miR-29a following the transient signaling events induced by integrin ligation would require degradation of the pre-existing miRNA. This assumption is supported by our result that antibody-mediated clustering of β4 on MDA-MB-435 cells for times up to four hours had no significant effect on miR-29a expression. We surmise from these data that exogenous expression of β4 in β4-deficient cells results in a long-term and sustained repression of miR-29a expression. This

possibility is supported tangentially by our finding that the expression pattern of β4 in breast carcinoma cell lines correlates inversely with miR-29a expression, and previous reports that β4-mediated signaling and function can occur independently of its ligation (78, 88). It is also worth noting in this regard our analysis of published microarray data that revealed a significant enrichment in miR-29a predicted targets in mRNAs upregulated by expression of β4. This finding suggests that a miRNA can broadly affect gene expression downstream of an integrin and corroborate the importance of miR-29a in the regulation of genes whose expression is mediated by β4.

We also provide evidence that SPARC can be regulated at the level of protein translation by β4, particularly in cells that express endogenous β4 and low levels of miR-29a. Ligation of β4 by adhesion to laminin or antibody-mediated clustering upregulates SPARC protein expression in both MDA-MB-435/β4 transfectants as well as SUM-159 cells. This finding is consistent with a previous report demonstrating that β4 can facilitate the cap-dependent translation of VEGF in breast carcinoma cells (95). In principle, this mode of regulation would enable SPARC expression to be altered rapidly in response to microenvironmental cues that impact β4. Moreover, the β4-mediated regulation of SPARC by miRNA repression and cap-dependent translation mechanisms need not be mutually exclusive.

Materials and Methods

Cell Lines, Antibodies, and Reagents: MDA-MB-435 and MDA-MB-231 cells were obtained from the Lombardi Cancer Center (Georgetown University, Washington, DC).

SUM-159 and SUM1315 cells were obtained from Dr. Stephen Ethier (Wayne State University School of Medicine, Detroit, MI). MDA-MB-435 and MDA-MB-231 cell lines were maintained in low glucose DMEM medium (Gibco, Carlsbad, CA) supplemented with 10 mM HEPES, 5% fetal bovine serum, and 1% streptomycin and penicillin. SUM-159 were maintained in Ham's F12 medium (Gibco) supplemented with 5% fetal bovine serum, insulin (5 µg/ml), hydrocortisone (1 µg/ml), and 1% streptomycin and penicillin. SUM1315 cells were maintained in Ham's F12 medium (Gibco) supplemented with 5% fetal bovine serum, insulin (5 µg/ml), EGF (10 ng/ml), and 1% streptomycin and penicillin. All cell lines were grown at 37°C in an incubator supplied with 5% CO_2. MDA-MB-435 mock transfectants (6D2 and 6D7 sublcones), β4 transfectants (3A7 and 5B3 subclones), and β4ΔCYT transfectants (5D5) were generated and characterized as previously described (53). Antibodies to SPARC (Haematological Technologies, Essex Junction, VT), pS6K (Cell Signaling, Beverly, MA), p4E-BP (Cell Signaling), tubulin (Sigma, St. Louis, MO), and actin (Sigma) were used for immunoblotting. The same SPARC antibody was used as a functional blocking antibody for invasion assays. The 505 antibody to β4, used for immunoblotting, and the 2B7 antibody to α6, used for clustering, were produced by our laboratory as previously described (86, 182). The AIIB2 antibody to β1 (Development Studies Hybridoma Bank, University of Iowa, Iowa City, IA) was used for clustering experiments. For inhibitor experiments, rapamycin (Sigma) was used at a concentration of 50 nM.

Immunoblotting: Cells were solubilized on ice for 10 min in Triton X-100 lysis buffer (Boston Bioproducts, Ashland, MA) 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) (Lysis Buffer A). Nuclei were removed by centrifugation at 16,100 × *g* for 10 min. Culture media was concentrated 8-fold using Ultra-4 Centrifugal Filter Units with a 10 kDa cutoff by spinning at 340 × *g* for 25 min (Millipore, Indianapolis, IN). Concentrations of total cell lysate and culture media were assayed by Bradford method. Lysates (50 µg) and concentrated culture media (25 ug) were separated by electrophoresis through 10% SDS-PAGE and transferred to 0.2 µm nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat milk in Tris-buffered saline/Tween 20 for 1 h and blotted with the antibodies to SPARC (1:10,000), pS6K (1:500), p4E-BP (1:1000), β4 (1:4,000), actin (1:5,000), or tubulin (1:10,000) overnight at 4°C. Proteins were detected by enhanced chemiluminescence (Pierce, Rockford, IL) after incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies.

miRNA and RNA Isolation and Detection: Total RNA was isolated using the miRVana RNA Isolation Kit according to manufacturer protocol (Ambion). qPCR detection of mature miRNAs was performed using TaqMan miRNA Reverse Transcription Kit and TaqMan human Microarray Assays for miR-29a (Applied Biosystems, Austin, TX) according to manufacturer protocol. U6 small nuclear RNA was used as an internal control. qPCR detection of SPARC mRNA was performed using Superscript II reverse transcriptase (Invitrogen) and Power SYBR Green (Applied Biosystems) according to manufacturer protocol. GAPDH was used as an internal control. miRNA and SPARC expression levels were quantified using the ABI Prism 7900HT Sequence detection

system (Applied Biosystems). Primers to SPARC (5'-AGCACCCCATTGACGGGTA-3' and 5'-GGTCACAGGTCTCGAAAAAGC-3') and GAPDH (5'-ATCATCCCTGCCTCTACTGG-3' and 5'-GTCAGGTCCACCACTGACAC-3') were used for analysis.

Gene Set Enrichment Analysis: For miRNA target enrichment analysis, mRNA expression data generated by Chen et. al. (76) were downloaded from the NCBI Gene Expression Omnibus (GEO), series number GSE11466. Affymetrix CEL files were processed with the robust multi-chip average (RMA) algorithm (186) using BRB-ArrayTools. TargetScanHuman Release 5.1 (235, 236) was used to predict conserved mRNA targets. Using total context score, the top 500 targets for miR-29 or miR-93 were compiled into gene set lists. miR-93 targets were used as a negative control gene set because miR-93 is highly abundant, yet it did not change expression in the β4 versus mock miRNA array analysis. Log base 2 mRNA data was loaded into the Broad Institute's Gene Set Enrichment Analysis (GSEA) software v2.06 (163, 187). 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.

Oligonucleotide Transfection: miRIDIAN- microRNA Mimics are synthetic chemically modified mature miRNAs (Dharmacon, Lafayette, CO). MDA-MB-435 β4 transfectants were transfected with 20 nM hsa-miR-29a mimic or a miRNA mimic negative control at 50% confluency using DharmaFECT 4 Transfection Reagent (Dharmacon). At 72 h

post-transfection, cells were plated for invasion assays or harvested for total cell lysate. A miRIDIAN microRNA Hairpin Inhibitor to mature miR-29a was used for loss-of-function analyses along with a hairpin inhibitor negative control (Dharmacon). MDA-MB-435 mock transfectants were transfected with 20 nM miR-29a inhibitor or negative control inhibitor as described above. At 72 h post-transfection, cells were harvested for protein or total RNA as described above.

Invasion Assays: The upper surfaces of the transwells were coated with 0.5 μg Matrigel (BD Biosciences, Bedford, MA) and allowed to dry overnight at room temperature. Cells were harvested at 80% confluency by trypsinization and resuspended low glucose DMEM containing 0.25% heat-inactivated fatty acid-free bovine serum albumin. The coated surfaces of the transwells were blocked with media containing bovine serum albumin for 60 min at 37°C. For SPARC blocking antibody experiments, cells were incubated with 16 ug/ml of SPARC antibody (Haematological Technologies) or normal mouse IgG for 30 min at room temperature with intermittent agitation. 10^5 cells in a total volume of 100 μl were loaded into the upper chamber and NIH-3T3 conditioned media was added to the lower chamber. Assays proceeded for 4 h at 37°C. At the completion of the assays, the upper chamber was swabbed to remove residual cells and fixed with methanol. Cells on the lower surface of the membrane were mounted in 4′,6-diamidino-2-phenylindole mounting media (Vector Laboratories, Burlingame, CA), and the number of cells was determined for five independent fields in triplicate with a 10X objective and fluorescence.

siRNA Experiments: SUM-159 cells were transfected with 20 nM On-TARGETplus SMARTpool siRNA targeting β4 (Dharmacon) at 50% confluency using DharmaFECT 4 transfection reagent (Dharmacon). A non-targeting siRNA pool (Dharmacon) was used as a control for these experiments. At 72 h post-transfection, cells were harvested for protein or total RNA as described above.

Integrin Clustering: MDA-MB-435/β4 and SUM-159 cells were serum starved overnight in DMEM containing 0.1% BSA and F12 containing 0.1% BSA, respectively. Cells were trypsinized, washed, and the resuspended at a concentration of 10^6 cells/ml. For laminin experiments, cells were plated on laminin (100 μg/10 cm plate) or maintained in suspension. For antibody-mediated clustering experiments, cell suspensions were incubated for 30 minutes with integrin-specific antibodies (2 μg/ml) in DMEM containing 0.1% BSA. The cells were washed and added to plates that had been coated overnight with anti-mouse or anti-rat IgG (33 μg/6 cm plate). For both laminin and antibody-mediated clustering experiments, cells were treated with 50 nM Rapamycin or DMSO for 10 minutes prior to plating cells on coated plates. After incubation at 37°C for 45 minutes, the cells were washed twice with PBS and lysed for protein in a 20 mM Tris buffer, pH 7.4, containing 10% glycerol, 136 mM NaCl, 10% NP-40, 1 mM sodium orthovanadate (Na_3VO_4), 10 mM sodium fluoride (NaF), 2 mM phenylmethanesulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Roche) (Lysis Buffer B) or for total RNA as described above.

Rapamycin Experiments: SUM-159 parental cells were treated with 50 nM Rapamycin or DMSO in serum-containing medium for four or six hours. Cells were lysed using Lysis Buffer B and samples were prepared for analysis as described above.

Statistical Analysis: Data are presented as the mean ± S.E. The Student's t test was used to assess the significance of independent experiments. The criterion $p < 0.05$ was used to determine statistical significance.

Table 3.1. Top 25 leading edge genes

Gene Symbol	Gene Title	Rank in Gene List[1]	Rank Metric Score[2]	Running ES[3]
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	17	1.607	0.020
SHROOM2	shroom family member 2	70	1.054	0.030
HDAC4	histone deacetylase 4	90	0.960	0.041
TRIM9	tripartite motif-containing 9	92	0.952	0.053
MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	104	0.910	0.065
LOXL2	lysyl oxidase-like 2	113	0.873	0.075
FAM3C	family with sequence similarity 3, member C	121	0.859	0.086
DCP2	DCP2 decapping enzyme homolog (S. cerevisiae)	134	0.829	0.096
TUBB2A	tubulin, beta 2A	149	0.798	0.106
HMGN3	high mobility group nucleosomal binding domain 3	152	0.796	0.116
COL1A2	collagen, type I, alpha 2	153	0.795	0.126
KCTD3	potassium channel tetramerisation domain containing 3	158	0.786	0.137
MAPRE2	microtubule-associated protein, RP/EB family, member 2	169	0.769	0.146
GMFB	glia maturation factor, beta	193	0.733	0.154
COL5A2	collagen, type V, alpha 2	196	0.727	0.163
FRAT2	frequently rearranged in advanced T-cell lymphomas 2	226	0.690	0.170
MLF1	myeloid leukemia factor 1	266	0.657	0.176
CNOT8	CCR4-NOT transcription complex, subunit 8	270	0.654	0.184
ZFP36L1	zinc finger protein 36, C3H type-like 1	279	0.644	0.192
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	280	0.640	0.200
LAMC1	laminin, gamma 1 (formerly LAMB2)	283	0.638	0.209
PURA	purine-rich element binding protein A	295	0.623	0.216
RERE	arginine-glutamic acid dipeptide (RE) repeats	306	0.617	0.223
GAS7	growth arrest-specific 7	315	0.607	0.231
PPIC	peptidylprolyl isomerase C (cyclophilin C)	316	0.606	0.239

[1] Rank in Gene List refers to position of gene in list of mRNAs ranked in order of greatest change in expression

[2] Rank Metric Score is the score used to position the genes in the ranked list

[3] Running Enrichment Score (ES) reflects the degree to which the gene is overrepresented in the top of the ranked list of genes

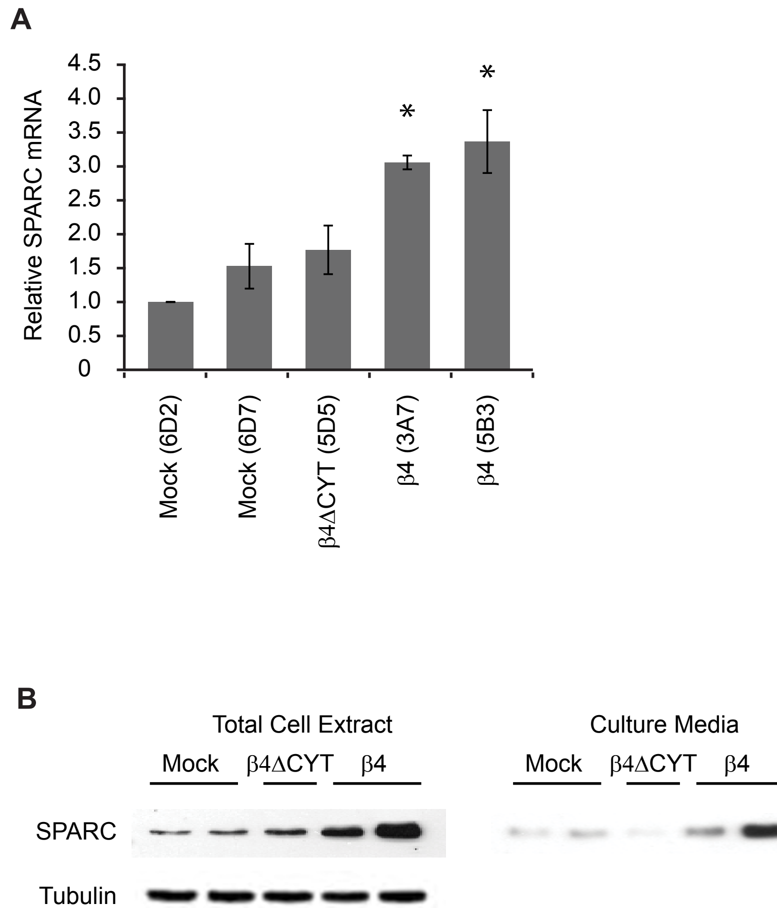
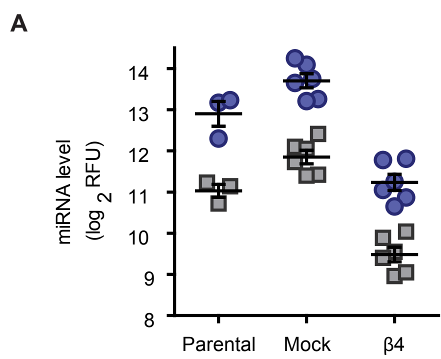
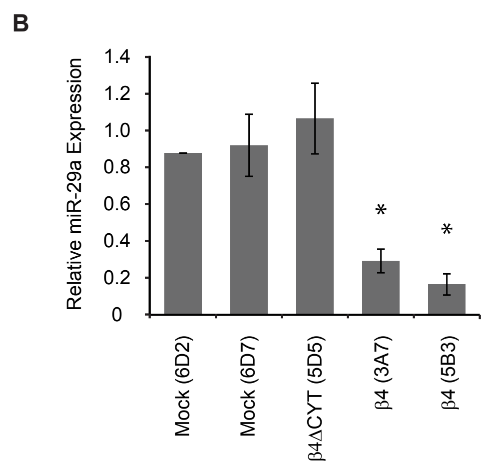


Figure 3.1. β4 integrin regulates expression of SPARC. A, Relative expression of SPARC mRNA by qPCR in mock, β4ΔCYT, and β4 transfectants, *, $p < 0.04$. **B**, Expression of SPARC in total cell extract (50 µg) and culture medium (25 µg) across MDA-MB-435 subclones.

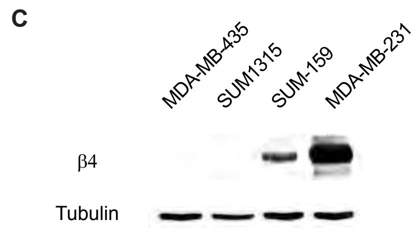
A



B



C



D

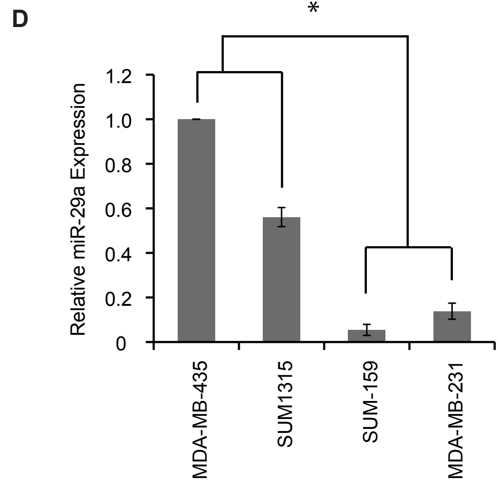


Figure 3.2. β4 expression inversely correlates with miR-29a expression. A, miR-29a and miR-29b expression from qNPA microarray performed in triplicate on the MDA-MB-435 parental cell line, two subclones of the MDA-MB-435 mock transfectants (6D2 and 6D7), and two subclones of the MDA-MB-435 β4 transfectants (3A7 and 5B3). **B**, Relative expression of miR-29a in two subclones of the mock transfectants, one subclone of the β4ΔCYT transfectants, and two subclones of the β4 transfectants based on qPCR, *, $p < 0.001$ when compared to average expression in mock transfectants. **C**, Expression of β4 in total cell extract (50 μg) in MDA-MB-435, SUM1315, SUM-159, and MDA-MB-231 breast carcinoma cell lines *, $p < 0.004$. **D**, Relative expression of miR-29a in MDA-MB-435, SUM1315, SUM-159, and MDA-MB-231 breast carcinoma cell lines. Data represent means ± S.E. from three independent experiments.

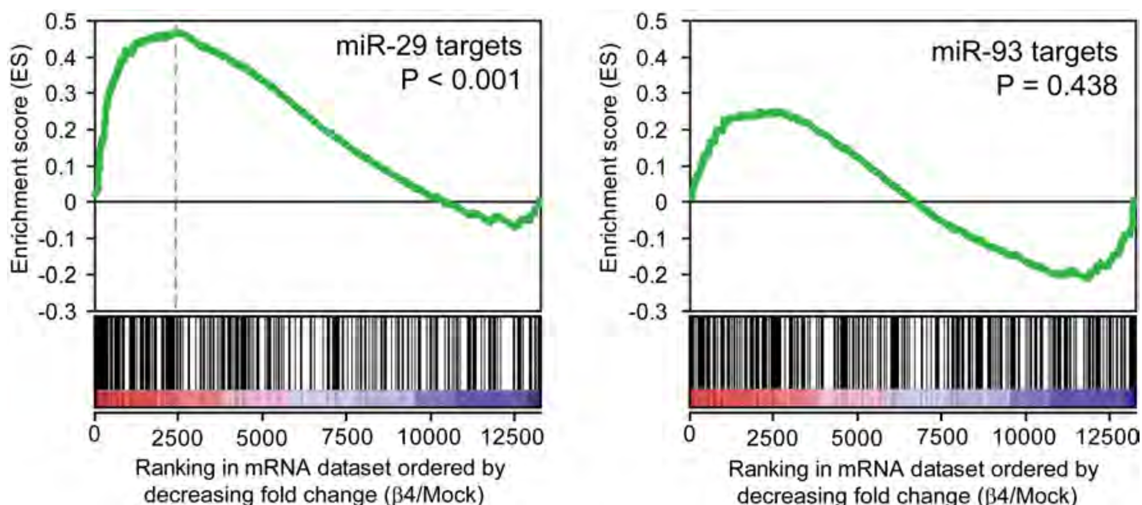


Figure 3.3. Enrichment of miR-29 predicted targets in β4-regulated mRNAs. GeneChip derived mRNA levels were ranked from the most upregulated in β4 transfectants to the most downregulated (x-axis, 1 to 12,300, respectively). Red shading indicates mRNA is upregulated in β4 transfectants, while blue shading indicates mRNA is downregulated. Each vertical black line represents a miRNA target predicted by TargetScan. The left-to-right position of each black line indicates the relative position of the predicted target within the rank ordered mRNA list. Left panel, the miR-29 predicted target gene set is enriched among mRNAs upregulated in the β4 transfectants, as illustrated by the increasing number of black lines on the left side and the positive running enrichment score (ES) marked by the green line ($p < 0.001$). The leading edge subset, the 116 miR-29 targets that contribute the most to the ES, are found to the left of the gray dotted line. Right panel, miR-93 predicted targets, used as a negative control gene set, did not show a significant enrichment ($p = 0.438$).

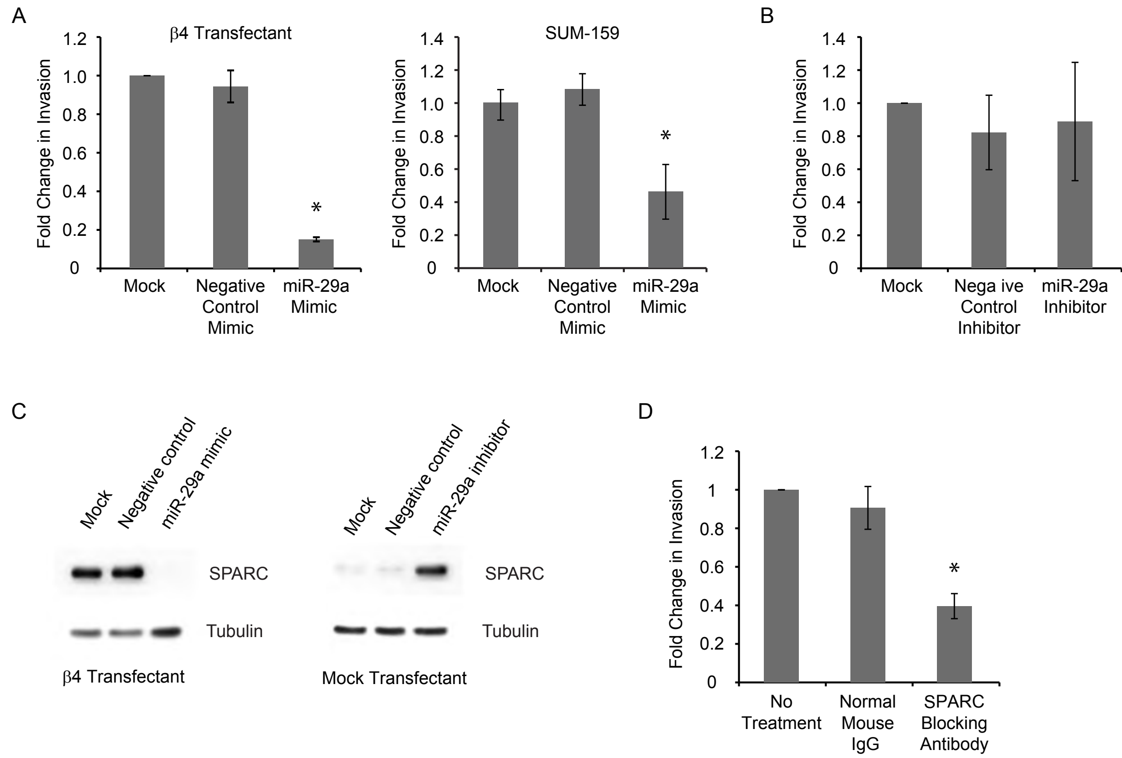
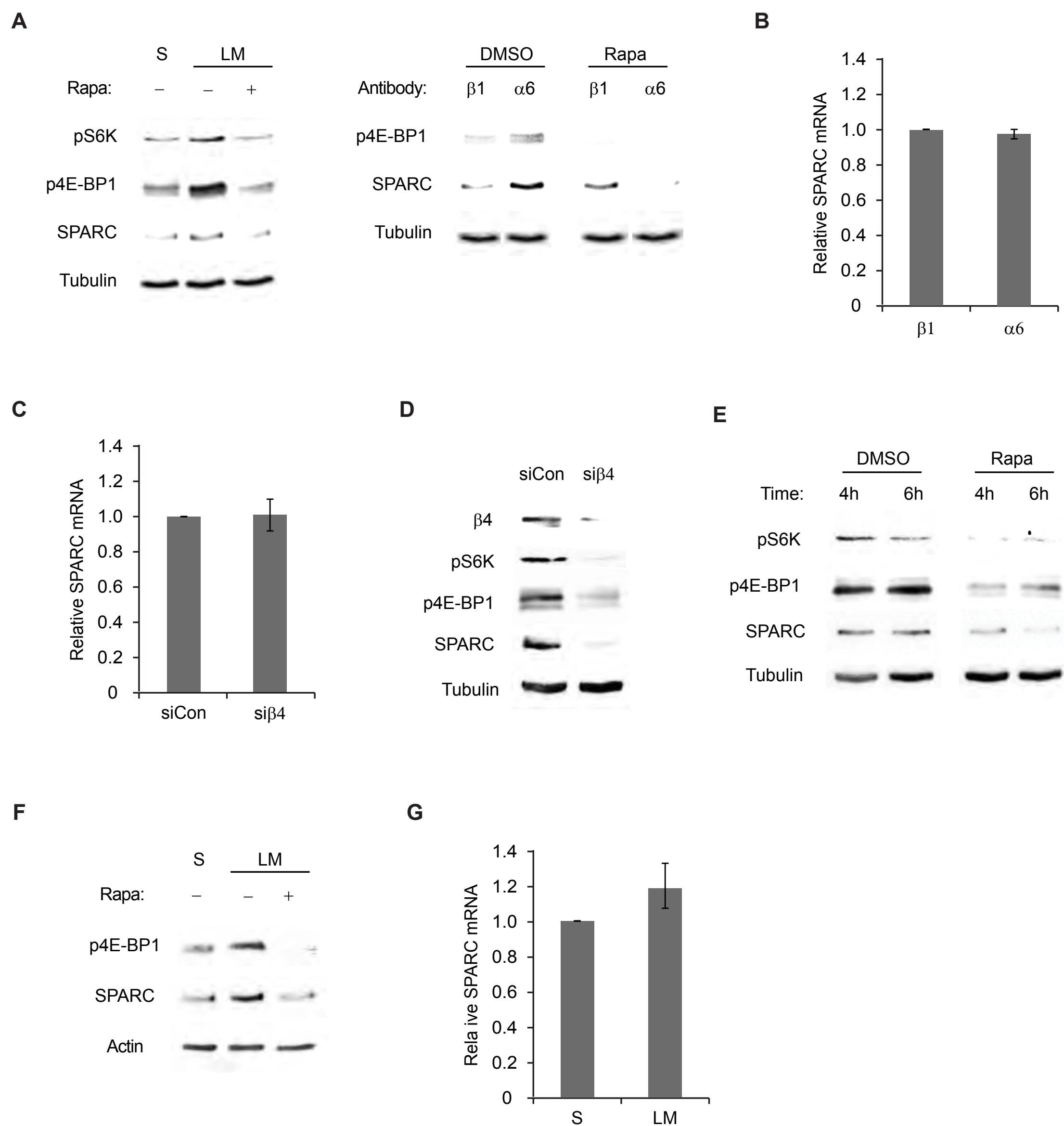

A
β4 Transfectant
Fold Change in Invasion
Mock
Negative Control Mimic
miR-29a Mimic
SUM-159
B
Nega ive Control Inhibitor
miR-29a Inhibitor
C
Negative control
miR-29a mimic
miR-29a inhibitor
SPARC
Tubulin
β4 Transfectant
Mock Transfectant
D
No Treatment
Normal Mouse IgG
SPARC Blocking Antibody

Figure 3.4. β4mediated repression of miR-29a can promote SPARC-dependent invasion. **A**, β4 transfectants and SUM-159 cells were subjected to Matrigel invasion assays following transfection with a miR-29a mimic, *, $p < 0.02$. **B**, Mock transfectants were subjected to Matrigel invasion assays following transfection with a miR-29a hairpin inhibitor. Data for invasion assays represent means ± S.E. from a representative experiment. **C**, Expression of SPARC in total cell lysate (50 μg) following expression of miR-29a mimic in β4 transfectants 72 hours post-transfection or expression of miR-29a hairpin inhibitor in mock transfectants 72 hours post-transfection. **D**, β4 transfectants were subjected to Matrigel invasion assays following 30 minute pre-incubation with normal mouse IgG or a SPARC function blocking antibody, *, $p < 0.001$. Data for invasion assay represents means ± S.E. from three independent experiments.


A
S
LM
Rapa:
–
–
+
pS6K
p4E-BP1
SPARC
Tubulin
DMSO
Rapa
Antibody:
β1
α6
β1
α6
p4E-BP1
SPARC
Tubulin
B
Relative SPARC mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
β1
α6
C
Relative SPARC mRNA
siCon
siβ4
D
siCon
siβ4
β4
pS6K
p4E-BP1
SPARC
Tubulin
E
DMSO
Rapa
Time:
4h
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6h
pS6K
p4E-BP1
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Rapa:
–
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+
p4E-BP1
SPARC
Actin
G
Rela ive SPARC mRNA
S
LM

Figure 3.5. β4 can regulate SPARC independently of miR-29a. A, MDA-MB-435/β4 cells were plated on laminin (LM) or maintained in suspension (S) for 45 minutes (left panel); MDA-MB-435/β4 cells were incubated with integrin-specific primary antibodies prior to plating on secondary antibody-coated plates for 45 minutes (right panel). Rapamycin (50 nM) or DMSO was added 10 minutes prior to plating. Expression of SPARC and signaling intermediates in total cell extract (50 μg) was examined. **B**, Relative expression of SPARC message levels by qPCR in MDA-MB-435/β4 cells clustered with integrin-specific antibodies. **C**, Relative expression of SPARC message levels by qPCR following transient knockdown of β4 at 72 hours post-transfection in SUM-159 cells. **D**, Expression of SPARC and signaling intermediates in total cell extract (50 μg) following transient knockdown of β4 at 72 hours post-transfection in SUM-159 cells. **E**, Expression of SPARC and signaling intermediates in total cell extract (50 μg) following treatment with 50 nM rapamycin. **F**, SUM-159 cells were plated on laminin (LM) or maintained in suspension (S) for 45 minutes. Rapamycin (50 nM) or DMSO was added 10 minutes prior to plating. Expression of SPARC and signaling intermediates in total cell extract (50 μg) was examined. **G**, Relative expression of SPARC message levels by qPCR in SUM-159 cells clustered on laminin (LM) or maintained in suspension.

Table S3.1. miR-29a leading edge genes

Gene Symbol	Gene Title	Rank in Gene List[1]	Rank Metric Score[2]	Running ES[3]
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	17	1.607	0.020
SHROOM2	shroom family member 2	70	1.054	0.030
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MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	104	0.910	0.065
LOXL2	lysyl oxidase-like 2	113	0.873	0.075
FAM3C	family with sequence similarity 3, member C	121	0.859	0.086
DCP2	DCP2 decapping enzyme homolog (S. cerevisiae)	134	0.829	0.096
TUBB2A	tubulin, beta 2A	149	0.798	0.106
HMGN3	high mobility group nucleosomal binding domain 3	152	0.796	0.116
COL1A2	collagen, type I, alpha 2	153	0.795	0.126
KCTD3	potassium channel tetramerisation domain containing 3	158	0.786	0.137
MAPRE2	microtubule-associated protein, RP/EB family, member 2	169	0.769	0.146
GMFB	glia maturation factor, beta	193	0.733	0.154
COL5A2	collagen, type V, alpha 2	196	0.727	0.163
FRAT2	frequently rearranged in advanced T-cell lymphomas 2	226	0.690	0.170
MLF1	myeloid leukemia factor 1	266	0.657	0.176
CNOT8	CCR4-NOT transcription complex, subunit 8	270	0.654	0.184
ZFP36L1	zinc finger protein 36, C3H type-like 1	279	0.644	0.192
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	280	0.640	0.200
LAMC1	laminin, gamma 1 (formerly LAMB2)	283	0.638	0.209
PURA	purine-rich element binding protein A	295	0.623	0.216
RERE	arginine-glutamic acid dipeptide (RE) repeats	306	0.617	0.223
GAS7	growth arrest-specific 7	315	0.607	0.231
PPIC	peptidylprolyl isomerase C (cyclophilin C)	316	0.606	0.239
SS18L1	synovial sarcoma translocation gene on chromosome 18-like 1	331	0.593	0.246
PER3	period homolog 3 (Drosophila)	337	0.587	0.253
PTP4A1	protein tyrosine phosphatase type IVA, member 1	339	0.587	0.261
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	344	0.584	0.268

TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	351	0.582	0.275
CREB5	cAMP responsive element binding protein 5	373	0.563	0.281
ATXN1	ataxin 1	399	0.548	0.286
CYCS	cytochrome c, somatic	406	0.543	0.293
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	412	0.540	0.300
PDHX	pyruvate dehydrogenase complex, component X	435	0.527	0.305
FEM1B	fem-1 homolog b (C. elegans)	457	0.516	0.310
MAPRE1	microtubule-associated protein, RP/EB family, member 1	468	0.513	0.316
CALM3	calmodulin 3 (phosphorylase kinase, delta)	520	0.484	0.319
HISPPD1	Histidine acid phosphatase domain containing 1	545	0.474	0.323
BLMH	bleomycin hydrolase	548	0.474	0.329
INSIG1	insulin induced gene 1	563	0.465	0.334
JARID1B	jumonji, AT rich interactive domain 1B	592	0.453	0.338
RHOBTB1	Rho-related BTB domain containing 1	597	0.452	0.344
RNF138	ring finger protein 138	633	0.438	0.347
EIF4E2	eukaryotic translation initiation factor 4E member 2	646	0.434	0.351
TRIB2	tribbles homolog 2 (Drosophila)	660	0.429	0.356
PMP22	peripheral myelin protein 22	666	0.427	0.361
DAAM2	dishevelled associated activator of morphogenesis 2	702	0.417	0.364
JARID2	jumonji, AT rich interactive domain 2	726	0.408	0.368
AIM1	absent in melanoma 1	738	0.405	0.372
OTUD4	OTU domain containing 4	741	0.404	0.377
ZHX3	zinc fingers and homeoboxes 3	760	0.397	0.381
RARB	retinoic acid receptor, beta	766	0.395	0.386
PCGF3	polycomb group ring finger 3	779	0.392	0.390
MLXIP	MLX interacting protein	814	0.382	0.393
REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	871	0.368	0.393
SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	873	0.368	0.398
IFI30	interferon, gamma-inducible protein 30	881	0.367	0.402
DDEF2	development and differentiation enhancing factor 2	889	0.364	0.407
BAT2D1	BAT2 domain containing 1	908	0.360	0.410
SLC36A1	solute carrier family 36 (proton/amino acid symporter), member 1	909	0.359	0.415
DICER1	Dicer1, Dcr-1 homolog (Drosophila)	937	0.353	0.417

DYNLT1	dynein, light chain, Tctex-type 1	1089	0.319	0.410
LYPLA1	lysophospholipase I	1091	0.318	0.414
PGAP1	-	1123	0.314	0.416
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	1141	0.310	0.419
NUDT11	nudix (nucleoside diphosphate linked moiety X)-type motif 11	1146	0.309	0.422
BRWD1	bromodomain and WD repeat domain containing 1	1165	0.305	0.425
KIF26B	kinesin family member 26B	1171	0.303	0.429
SNX4	sorting nexin 4	1204	0.298	0.430
TRIM37	tripartite motif-containing 37	1216	0.295	0.433
PLXNA1	plexin A1	1234	0.291	0.436
SNRK	SNF related kinase	1248	0.288	0.438
CDK6	cyclin-dependent kinase 6	1254	0.287	0.442
KCTD5	potassium channel tetramerisation domain containing 5	1323	0.277	0.440
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	1398	0.262	0.438
TDG	thymine-DNA glycosylase	1406	0.261	0.441
KIAA0355	KIAA0355	1436	0.257	0.442
GSTA4	glutathione S-transferase A4	1462	0.253	0.443
PKNOX2	PBX/knotted 1 homeobox 2	1483	0.249	0.445
VANGL1	vang-like 1 (van gogh, Drosophila)	1503	0.246	0.447
ZNF282	zinc finger protein 282	1519	0.244	0.449
ISL1	ISL1 transcription factor, LIM/homeodomain, (islet-1)	1563	0.238	0.449
EPS15	epidermal growth factor receptor pathway substrate 15	1618	0.230	0.448
STX16	syntaxin 16	1625	0.229	0.450
CAV2	caveolin 2	1657	0.226	0.451
MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	1681	0.222	0.452
COL4A5	collagen, type IV, alpha 5 (Alport syndrome)	1695	0.221	0.454
ABHD4	abhydrolase domain containing 4	1759	0.213	0.452
CCNJ	cyclin J	1776	0.211	0.453
SIP1	survival of motor neuron protein interacting protein 1	1812	0.207	0.453
ISG20L2	interferon stimulated exonuclease gene 20kDa-like 2	1846	0.205	0.454
LRP6	low density lipoprotein receptor-related protein 6	1850	0.204	0.456
RLF	rearranged L-myc fusion	1870	0.202	0.457
NKTR	natural killer-tumor recognition sequence	1871	0.202	0.460

CPS1	carbamoyl-phosphate synthetase 1, mitochondrial	1899	0.200	0.460
NASP	nuclear autoantigenic sperm protein (histone-binding)	2039	0.186	0.452
POLE3	polymerase (DNA directed), epsilon 3 (p17 subunit)	2047	0.184	0.454
KIAA0644	-	2050	0.184	0.456
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	2084	0.180	0.456
CHFR	checkpoint with forkhead and ring finger domains	2089	0.180	0.458
DBT	dihydrolipoamide branched chain transacylase E2	2096	0.179	0.460
PARG	poly (ADP-ribose) glycohydrolase	2106	0.178	0.462
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	2179	0.171	0.459
CCNT2	cyclin T2	2211	0.168	0.458
ELF2	E74-like factor 2 (ets domain transcription factor)	2213	0.168	0.461
BCORL1	BCL6 co-repressor-like 1	2243	0.166	0.460
COL2A1	:ollagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital	2256	0.165	0.462
PLP1	proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)	2289	0.162	0.461
PPP1R3D	protein phosphatase 1, regulatory subunit 3D	2290	0.162	0.464
MEST	mesoderm specific transcript homolog (mouse)	2322	0.158	0.463
JARID1A	jumonji, AT rich interactive domain 1A	2346	0.156	0.464
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila)	2364	0.155	0.464
DNM3	dynamin 3	2378	0.154	0.465
UPK1B	uroplakin 1B	2412	0.151	0.465
SOX12	SRY (sex determining region Y)-box 12	2415	0.151	0.467

[1] Rank in Gene List refers to position of gene in list of ß4-regulated mRNAs ranked in order of greatest change in expression
[2] Rank Metric Score is the score used to position the genes in the ranked list
[3] Running Enrichment Score (ES) reflects the degree to which the gene is overrepresented in the top of the ranked list of genes

CHAPTER IV

GENERAL DISCUSSION

The integrin β4 was first identified as a tumor associated antigen whose expression correlated with metastatic disease (26). This early observation was ostensibly at odds with its role as a mechanical device that maintained epithelial integrity. Research over the past two decades has transformed our understanding of this integrin and characterized β4 as a dynamic cell surface receptor that mediates cytoskeletal organization and signal transduction, contributing to both physiological and pathological processes. Its ability to potentiate carcinoma invasion is of particular importance and occurs in part through the regulation of downstream effector molecules.

miRNA Expression Patterns

The work presented in this dissertation documents the first example that integrin expression correlates with specific miRNA patterns. Moreover, integrin β4 status *in vitro* and *in vivo* is associated with decreased expression of distinct miRNA families in breast cancer, namely miR-25/32/92abc/363/363-3p/367 and miR-99ab/100. Further analysis identified overlapping predicted targets of these two miRNA families within a population of genes known to be downregulated by β4 based on published Affymetrix array data (76). An overrepresentation of targets involved in cell migration was detected within this pool of genes, revealing unrecognized β4 targets potentially involved in promoting carcinoma progression. Another miRNA, miR-29a, is significantly downregulated in

response to *de novo* expression of β4 in a breast carcinoma cell line. Further study revealed that expression of this miRNA is inversely correlated with β4 status in several breast carcinoma cell lines. β4-mediated repression of the miRNA is required for invasion, strengthening the link between miRNA expression patterns and cell motility downstream of β4 in the context of breast cancer. Finally, gene set enrichment analysis detected an enrichment in predicted targets of several miRNA families identified by our screen, including miR-92ab and miR-29abc, within β4-regulated genes, substantiating the physiological significance of our data.

An unexpected finding uncovered by our study is the observation that loss of β4 in cells that endogenously express the integrin decreases the expression of miR-29a, results that are seemingly at odds with the aforementioned data. Specifically, qNPA microarray analyses from SUM-159 breast carcinoma cells demonstrate a decrease in the expression of miR-29a upon transient depletion of β4, a finding confirmed by qPCR (data not shown). Interestingly, family member miR-29b is also repressed. Likewise, examination of the MCF10CA1a qNPA microarray identified a decrease in miR-29b and miR-29c in response to loss of β4 (Table 2-S1).

Members of the miR-29 family of miRNAs are transcribed from two bicistronic loci, the miR-29b-1/a cluster on chromosome 7 and the miR-29b-2/c cluster on chromosome 1. Therefore, miR-29a is generated exclusively from the miR-29b-1/a cluster, while miR-29c is generated exclusively from the miR-29b-2/c cluster. Mature miR-29b, on the other hand, can be derived from transcription at either loci. The array data, thus, illustrate a scenario involving β4-dependent maintenance of miR-29b

expression through regulation at distinct transcriptional loci. As miRNA family members share seed regions and therefore largely overlapping pools of putative targets, one hypothesis extending from our finding is that two highly aggressive breast carcinoma cell lines have evolved distinct mechanisms to repress a population of genes through sustained expression of miR-29 family members.

Regarding transcriptional regulation of these family members, efforts early in the course of our study were invested in exploring the role of β4 in the regulation of the miR-29b-1/a cluster by β4. Our initial observation that expression of the integrin in MDA-MB-435 cells repressed both miR-29a and miR-29b prompted us to consider potential downstream effectors of β4 that might be involved in transcriptional repression. An obvious candidate was NFAT, a transcription factor with an established role in promoting β4-mediated invasion in this system (74). More recently, it has been shown to regulate the transcription of β4 targets including autotaxin/ENPP2 and S100A4/metastasin (75-77).

The NFAT family of transcription factors is comprised of five known members. With the exception of NFAT5, all are responsive to fluctuations in intracellular Ca^{++} concentration. NFAT is activated upon deposphorylation by the upstream calcium-responsive phosphatase calcineurin. This event permits translocation of NFAT to the nucleus where it interacts with other transcriptional partners to regulate gene expression (237). NFAT family members may be transcriptional activators or repressors depending upon the genetic context. For example, NFAT1 has been shown to repress the transcription of cyclin-depdendent kinase 4 (CDK4) and cyclin A2 (238, 239), both of

which serve critical functions in cell-cycle regulation and cell proliferation, while upregulating genes involved in immune function, such as interleukin-2 (IL-2) (240).

Based on the ability of β4 to upregulate NFAT family members and to promote an invasive phenotype, we examined the hypothesis that this factor serves as a transcriptional repressor of miR-29a. Interestingly, a report published around this time revealed that NFATc3 promotes the transcription of miR-23a in a model of cardiac hypertrophy (241), demonstrating that members of this transcriptional family can regulate the expression of miRNAs. Analysis using Genomatix MatInspector software identified four putative binding sites for NFAT1 in the 3Kb region upstream of the miR-29b-1/a transcription start site (242). Nonetheless, chromatin immunoprecipitation (ChIP) experiments in the MDA-MB-435/β4 transfectants failed to demonstrate that NFAT1 could bind any of these sites. Moreover, treatment of these cells with pharmalogical agents FK506 and cyclosporine A, inhibitors of calcineurin function, did not induce expression at this locus as assessed by expression of mature miR-29a and miR-29b.

During the course of our study, a report was published characterizing transcriptional regulation at the miR-29b-1/a locus. Mott *et al.* demonstrated that c-myc, hedgehog, and NFκB can bind the promoter of the miR-29b-1/a cluster and induce transcriptional suppression as established by elecrophoretic mobility shift assays, signaling inhibition, and a luciferase reporter construct (243).

In retrospect, our finding that NFAT did not repress transcription at the miR-29b-1/a locus downstream of β4 in the MDA-MB-435 system was not surprising given the fact that β4 may not actually function to repress transcription of this cluster based on the

observation that loss of the integrin decreased the expression of miR-29 family members in two other breast carcinoma cell lines. While expression of β4 clearly appears to impact these miRNAs, the role of the integrin in this process appears complicated and context dependent. To delve deeper into the issue, we employed the miR-29b-1/a promoter luciferase reporter construct developed by Mott *et al*. mentioned above to determine whether β4 repression of transcription at this site could account for reduced levels of mature miR-29a observed in MDA-MB-435/β4 transfectants. The results were again surprising. While expression of both miR-29a and miR-29b is downregulated in the β4 transfectants compared to mock transfectants based on the qNPA microarray and qPCR, increased transcriptional activity was detected at the miR-29b-1/a promoter in the β4 transfectants compared to the mock transfectants (data not shown). Moreover, these data complement observations that ligation of β4 by antibody-mediated clustering modestly increased mature miR-29a levels in the β4 transfectants on a few occasions (data not shown).

Due to this unexpected result and concerns regarding the artificial nature of these luciferase experiments, we considered the possibility that our data were not accurately reflecting activity at the endogenous promoter. Reports have linked cancer to inappropriate methylation of CpG islands in miRNA promoters (126, 244). Along these lines, evidence in the literature indicates the presence of one CpG-enriched site containing five CpG nucleotide pairs in the promoter of this cluster, and that expression of these miRNAs can be linked to epigenetic modifications at this island (245). Based on these observations, we could postulate that β4 induces changes in the methylation profile

at this CpG site to induce transcriptional silencing in the MDA-MB-435 system. While β4 itself undergoes complex epigenetic modifications during the EMT (246), the integrin has never been shown to regulate such modifications in downstream targets nor induce the activity of enzymes involved in this process. Bisulfite sequencing of this site in mock and β4 transfectants would prove informative in this context.

While the luciferase data and ligation data corroborate the results from the array analysis and qPCR in the SUM-159 and MCF10CA1a cells, the disconcordance between promoter activity and mature miR-29a and miR-29b levels in the MDA-MB-435 system suggests some post-transcriptional phenomenon unique to these cells. One possibility is that the exogenous β4 integrated into the genome in a position that somehow affected miR-29a biogenesis. This hypothesis is unlikely given the fact that the integrin undergoes random integration into the genome, and two independent subclones both demonstrate repressed levels of miR-29a. Though unlikely, it is possible that some selective advantage was conferred to subclones in which the integrin integrated in a particular position. In this situation, perhaps integration might interrupt processing machinery genes, for example, involved in miR-29a biogenesis. This is also unlikely given the fact that our analysis of the published Affymetrix array data showed no change in the several genes critical for miRNA maturation (data not shown). It is further unlikely given the fact that the same machinery processes nearly all miRNAs, and the effect of β4 expression on miRNA patterns is not global enough to claim widespread defects in miRNA biogenesis.

Another hypothesis that could account for this incongruity is that the MDA-MB-435 system is unique in its ability to impact miRNA stability. Perhaps β4 generally upregulates expression of these miRNAs but in this system also promotes their decay or interferes with their biogenesis. The most well studied example of miRNA decay involves regulation by LIN-28. Interestingly, high levels of pri-let-7 transcript are detected in embryonic stem cells (ESCs) and other progenitor cells despite low levels of the mature miRNA (247). This discrepancy results from a processing defect in which LIN-28 binds to the hairpin region of the primary miRNA and impedes cleavage by Drosha (248). Interaction of LIN-28 with pre-let-7 can also prevent Dicer-mediated cleavage. Specifically, LIN-28 recruits TUT4, a terminal poly(U) polymerase, and induces 3'-terminal polyuridylation of the precursor, which blocks processing by Dicer (249-253). The uridylated precursor is then targeted for degradation by an unknown RNase (251).

Though this phenomenon has only been reported in let-7 family members (248, 251), it is feasible that a mechanism affecting miR-29a stability could account for low levels of the mature miRNA. Decay of miR-29b, for example, has already been described in cycling HeLa cells, wherein the miRNA is subject to nuclear localization and rapid degradation resulting from a unique trafficking motif on its 3' end (200). Of particular interest, members of the let-7 family of miRNAs were determined by our analyses to be downregulated in response to β4 expression (Table 2.2). Furthermore, let-7 is thought to function as a tumor suppressor, targeting oncogenes such as myc (254). This factor has been linked to the transcriptional repression of the miR-29b-1/a cluster

(243), raising the possibility that β4 indirectly regulates miR-29a through a mechanism involving let-7. Regardless of the mechanism by which low mature miR-29a levels are generated in the MDA-MB-435/β4 transfectants, analysis of primary and presursor levels of the miRNA would be enlightening. For example, if expression profiles of pri-miR-29a across β4 and mock transfectants paralleled the luciferase promoter activity, but levels of pre-miR-29a were equivalent to mature levels, we would conclude that processing of the primary miRNA by the Drosha microprocessor did not proceed as expected. The effect of clustering on transcriptional activity at the miR-29b-1/a locus as assessed by the luciferase reporter construct could also potentially clarify these discrepant data.

Another possibility is that cells null for β4 express unusually high levels of miR-29 family members, and that introduction of the integrin into the system represses its expression. Over time, other mechanisms evolve to maintain suppression, and the integrin assumes a positive role in their regulation rather than a negative one. This hypothesis is consistent with our observation that miR-29a correlates with β4 status in a collection of breast carcinoma cell lines (Fig. 3.2D). This hypothesis that β4 can buffer miR-29 levels is intriguing, though information pertaining to absolute levels of the miRNAs would be useful in testing this idea. Deep sequencing could provide more specific information in this direction. It would further be of interest to determine whether a correlation exists between the amount of functional integrin in a cell and resulting levels of miR-29 family members. Additionally, SUM1315 cells, which do not endogenously express the integrin, could be used to generate β4 transfectants. If this hypothesis were correct, cells expressing β4 should have lower levels of miR-29 than

SUM1315 parental cells. Unfortunately, developing cell lines that express functional β4 is not a trivial task, because the integrin must be appropriately trafficked to the membrane and expressed at physiological levels to recapitulate behavior observed in cells that endogenously express it. One flaw with the buffering hypothesis is the fact that most epithelial cells and many breast carcinomas express β4, so repression of miR-29a in response to the integrin may not be biologically relevant with respect to β4-mediated cell motility.

The hypothesis that β4 actually sustains expression of miR-29 family members challenges our interpretation of their role in carcinoma invasion. Clearly, our data and others have established a role for repression of miR-29a in the invasive process both *in vitro* and *in vivo* (255, 256). Furthermore, the level of miR-29a in β4 positive tumors is significantly reduced compared to β4 negative tumors in the qNPA microarray (Table S2.3), and it is very well established that β4 promotes invasion. One possibility is that miR-29a undergoes differential regulation by β4 to enhance cell motility depending upon the context. For example, repression of miR-29 might permit enhanced expression of genes like matrix metalloproteinases (MMPs) involved in promoting invasion in some settings, while increased miR-29 might block genes that mediate cell adhesion in focal contacts. Thus, miR-29 promotes an invasive phenotype but accomplishes this end through selective regulation of different genes pools. Clearly many molecules have dual biological functions; integrins are a prime example, as they mediate stable adhesions but also facilitate cell motility and cytoskeletal dynamics.

The exact nature of the biological contexts in which miR-29 family members could be differentially regulated by β4 remains to be seen. Perhaps integrin conformation contributes to this process. It has been established that the MDA-MB-435 system can function in a ligand-independent manner, as discussed in Chapter I. One possibility extending from these observations is that the integrin is in an active conformation with an open headpiece at the cell surface or is constitutively active due to a mutation that prevents association of the transmembrane legs of the α and β subunits. Such events could promote signaling cascades not observed in cells in which the integrin is endogenously expressed and unbound by ligand. Crystallography to ascertain the conformation of the integrin at the cell surface in the β4 transfectants, however, is obviously not a useful pursuit.

Perhaps an easier, though less structurally informative, approach to determining whether β4 is constitutively active in the β4 transfectants would be to examine basal levels of signaling intermediates in pathways known to be activated by the integrin. Notably, levels of pAkt and p4E-BP1 are elevated in the β4 transfectants compared to mock transfectants in the absence of ligand (95). This raises the question as to whether activation of signaling pathways occurs in response to transfection as a sort of stress response, since it has been established that p85, the catalytic subunit of PI3K, can play a role in activating JNK (c-Jun N-terminal kinase) stress pathways (257). However, levels of signaling intermediates in cells that have been depleted of endogenously expressed β4 are also diminished (255). These observations refute the hypothesis that constitutive activity of the integrin is unique to the β4 transfectants, thus, accounting for the

biological context in which miR-29a is differentially regulated. These data do, however, strongly suggest that secretion of endogenous ligand plays a role in β4 signaling across systems, perhaps accounting in part for the phenomenon of ligand-independent signaling and function to be discussed below. Nonetheless, it is likely that autocrine laminin does not entirely justify the ligand-independent function as evidenced by the fact that a truncated β4 containing only the transmembrane and cytoplasmic domains still confers signaling potential (69, 88).

What likely matters more in the regulation of miR-29 family members is the larger biological context. As described in the second chapter of this dissertation, β4 expression correlates with differential expression of many miRNAs and miRNA families. The idea that β4 can regulate networks of miRNAs, which in turn regulate pools of target genes is likely a more physiologically accurate depiction. These networks are important for fine-tuning gene expression and coordinating specific cellular functions, thus examination of a single miRNA might not prove very practical or informative.

Along these lines, targeting of large gene pools by miR-29a is likely more biologically accurate than the idea that miR-29a regulates a single downstream target, such as SPARC, to promote invasion. Inspection of putative targets of this miRNA reveals a number of potentially interesting genes. Among these, three laminin chain isoforms are predicted targets of miR-29a: α2, γ1, and γ2. In fact, a report exploring the role of family member miR-29c in nasopharyngeal carcinoma confirmed that γ1 is a bonafide target of the miRNA (258). Laminins are heterotrimeric glycoproteins composed of a single α, β, and γ chain that assemble in a cross-like configuration (259).

Nomenclature for this family of proteins derives from the isoform of each chain, e.g. laminin-332 represents α3, β3, and γ2 (260). The γ1 and γ2 chains are intriguing targets based on their links to carcinoma progression. Specifically, overexpression of laminin-511 correlates with the aggressive phenotype of invasive breast cancers (261, 262). Overexpression of laminin-332 is associated with poor prognosis in a variety of cancers, though the role of this isoform in breast cancer is less clear. While downregulation of laminin-332 has been reported (263), a recent article identified elevated levels of the isoform, in particular the γ2 chain, in the interface zone of invasive ductal carcinoma (264).

The hypothesis that β4 could regulate secretion of its own ligand through a miRNA-dependent mechanism is an intriguing idea, particularly given the report that the integrin functions in a laminin-332 autocrine loop to promote survival of anchorage-independent breast carcinoma cells in a three-dimensional environment (98). Neoplastic cells are believed to secrete their own matrix proteins (265, 266), conferring a selective advantage in the metastatic environment. This phenomenon could revise our understanding of the ligand-independent function attributed to β4 in tumor cells, which might in part represent an autocrine loop involving secretion of endogenous laminin, subsequent ligand-binding, and integrin activation. Such a pathway could provide a novel mechanism for β4-mediated carcinoma invasion. Interestingly, a report from the literature indicates that laminin-332, the preferred ligand for β4, is not expressed in MDA-MB-435 cells (267). In concordance with this study, our examination of the γ2 chain of laminin in both β4 and mock transfectants revealed nearly undetectable levels of

the protein. Furthermore, inhibition of miR-29 in the mock transfectants using a functional inhibitor failed to induce γ2 expression (data not shown). Perhaps this is not surprising since these cells do not express β4 endogenously and might not have, therefore, evolved mechanisms to signal through the integrin in an autocrine manner. These cells do express α6β1, though, another laminin-binding integrin. Nonetheless, we examined a cell line that endogenously expresses the integrin, SUM-159 cells. Again, manipulation of miR-29a in these cells had no effect on γ2 protein expression. These observations do not negate the possibility β4 can potentiate carcinoma invasion through miR-29a-dependent regulation of laminin, as the γ1 chain present in laminin-111 and laminin-511 might also function as a target, nor to they exclude the potential for β4 to execute this autocrine loop through regulation of another miRNA.

Another potentially interesting target of miR-29a is p85, the regulatory subunit of the PI3K. Perhaps elevated levels of phosphorylated Akt observed in the β4 transfectants are attributable to increased overall expression of the upstream lipid kinase. A recent study reported that miR-29a targets p85, thereby inducing p53-dependent apoptosis in breast carcinoma cells (Park et al. 2009). As before, no detectable difference in p85 protein expression was appreciable between β4 and mock transfectants, and manipulation of miR-29a did not induce a change in p85 expression. Furthermore, a point mutation in the *p53* gene in these cells renders the tumor suppressor incapable of inducing apoptosis (93, 268). A recent report does, however, suggest that β4 confers a proliferative advantage mediated by signaling through mitogen-activated protein kinase (MAPK) and Mnk (269). Retrospectively, our observations that protein levels of both γ2 and p85 were

unchanged in the β4 transfectants compared to control is not surprising given the fact that our analysis of the published Affymetrix array failed to identify a change in transcript levels of these two genes, meaning that miR-29a-mediated regulation of their expression in this setting unlikely due to the fact that most miRNAs induce changes in mRNA levels that parallel changes in protein levels (125).

SPARC

Another major conclusion of this study is that β4 integrin expression and ligation can regulate the expression of SPARC in breast carcinoma cells, a phenomenon that further enhances our understanding of how β4 contributes to chemoinvasion. Our data reveal distinct mechanisms by which β4 promotes SPARC expression. Specifically, in cells lacking expression of the integrin, introduction of β4 decreases miR-29a levels while concomitantly increasing the expression of SPARC. Ligation of the integrin in this system can further induce SPARC expression through a TOR-dependent translational mechanism. Likewise, in cells that express β4 and, thus, low levels of the miRNA, ligation of the integrin also enhances SPARC translation. Furthermore, SPARC is required for the invasive phenotype downstream of integrin β4. Our observation that distinct mechanisms have evolved in cells to regulate SPARC expression downstream of β4 suggests that this event is biologically significant.

While these data suggest that translational and miR-29a-mediated mechanisms occur independently to regulate SPARC expression, they do not exclude the possibility that these two phenomena may be functionally linked. For example, miR-29a may

directly regulate SPARC by binding to its 3'UTR to silence expression, as well as target translational machinery involved in biosynthesis of the protein. Interestingly, miR-100, one of two miRNAs downregulated across all three of our arrays in response to β4 expression, has been shown to target mTor, a kinase involved in promoting cap-dependent translation (270, 271). Assessing the ability of β4 to induce SPARC expression upon ligation by laminin or antibody-mediated clustering in cells overexpressing miR-29a could help to explore this possibility.

It is possible that β4 mediates SPARC expression through additional mechanisms. Early studies into the mechanism of β4-regulation of this protein included examination of other signaling pathways regulated by the integrin. Specifically, we tested the effect of PD98059 on SPARC expression in the MDA-MB-435/β4 transfectants. PD98059 is a potent inhibitor of MEK, a kinase upstream of ERK 1/2 in the Ras/MAPK pathway. Preliminary evidence suggests that expression of SPARC decreases upon treatment with the MEK inhibitor (data not shown). Interestingly, a recent study reported that ribosomal S6 kinases RSK1 and RSK2 are effectors of the Ras/MAPK signaling pathway and induce an invasive phenotype in breast carcinoma cells in part through transcription factor FRA1 (272). Earlier reports also link FRA1, a Fos homologue, to the migratory phenotype of breast carcinoma cells (273-275). Such observations are particularly intriguing in light of the fact that c-Jun/FRA1 heterodimers can bind to the SPARC promoter *in vitro* (276), raising the possibility that β4 can signal through the Ras/MAPK/RSK pathway to induce FRA1-dependent transcription of SPARC.

The possibility that β4 promotes SPARC transcription does not exclude miRNA-

mediated or translational mechanisms. In fact, computational analyses have recently confirmed the presence of type II circuits in mammalian cells, networks in which transcription of a miRNA and its target gene are oppositely regulated by upstream events, thereby reinforcing expression of the target gene (277). This is consistent with the idea that miRNAs generally produce subtle effects on target gene expression (108), and that SPARC expression in the MDA-MB-435/β4 transfectants was dramatically increased (Fig. 3.1B). Furthermore, a transcriptional phenomenon might be unique to the MDA-MB-435 system, as depletion of β4 in the SUM-159s downregulated SPARC protein without affecting mRNA levels.

As an aside, the use of signaling inhibitors to identify pathways through which β4 represses mature miR-29a expression in this system was initially appealing. While concerns regarding the half-life of the miRNA made interpretation of clustering experiments unfeasible, concerns about the stability of miR-29a are no longer at play in this context due to the fact that inhibition of signaling pathways would in theory increase expression of miR-29a. Nevertheless, results were inconsistent across the board, and we were ultimately unsuccessful at derepressing expression of the miRNA through inhibition of any known pathways downstream of integrin β4.

An intriguing, though somewhat unrelated, conclusion drawn from work on RSK as a downstream effector of Ras/MAPK involves the fact that integrin β4 is one of the pro-invasive genes whose expression is upregulated in response to signaling through this pathway. As ligation of β4 can induce Ras/MAPK signaling, these observations raise the possibility that such an event could positively feedback to increase β4 transcription and

expression, enhancing the biological potency of this integrin during carcinoma invasion. Although reports indicate that β4 induces activation of the Ras/MAPK pathway to promote carcinoma invasion (81), signal transduction through this pathway appears to be most significant in the context of anchorage-independent growth within the MDA-MB-435 system (68).

An extension of this hypothesis is that SPARC might also participate in a positive feedback loop and serve to modulate β4 expression and function. SPARC is involved in a myriad of cellular processes, including the ability to regulate growth factor and integrin signaling pathways. This phenomenon has been studied largely in the context of angiogenesis, where SPARC has been shown to bind VEGF-A and drive VEGF-A/VEGFR2-mediated signaling and angiogenesis in a model of choroidal neovascularization (220, 278, 279). SPARC is also known to directly bind PDGF family members to modify cell behavior (221, 280) and to indirectly regulate fibroblast growth factor 2 (FGF2)-induced signaling (281). The role of SPARC in the regulation of TGF-β1 signaling is also well established, as SPARC can either induce or antagonize signaling through this pathway depending upon the cellular milieu (226).

Perhaps most interesting in the context of this dissertation is the ability of SPARC to affect integrin expression and signaling. Interestingly, SPARC has been shown to bind β1, an integrin subunit that can pair with α6 as a laminin receptor, to induce signaling through integrin-linked kinase (ILK) in lens epithelial cells (224). The ability of SPARC to induce ILK has also been extended to a glioma model, in which loss of SPARC impedes tumor cell survival and invasion associated with decreased ILK and FAK

activity (215). SPARC-mediated regulation of ILK has also been shown to regulate extracellular matrix remodeling (222). Furthermore, SPARC can induce integrin-mediated migration in both prostate carcinoma and dental pulp cells through interaction with αV integrin family members (282, 283). Other data, to the contrary, suggest that SPARC can antagonize the expression of α6 (284) and αV (285) integrins in different settings. These observations coupled to the fact that SPARC can promote signaling through PI3K (213, 286) raise the possibility that interaction of SPARC with cell surface receptors or even β4 could induce expression or signaling events downstream of this integrin.

Along the lines of feedback loops, SPARC could potentially function to impact the expression of miR-29 family members. While miRNAs are classically thought to participate in unidirectional gene regulation, accumulating evidence reveals that feedback loops involving miRNA targets function to regulate the expression of select miRNAs through complex regulatory networks (287). While SPARC has not been linked to signaling pathways implicated in miR-29b-1/a transcriptional repression, namely c-myc, hedgehog, and NFκB, additional investigation may establish a connection between SPARC and these transcriptional effectors. Alternatively, further characterization of the miR-29b-1/a promoter may identify other factors that participate in the regulation of miR-29 family members downstream of SPARC-mediated signaling events.

Further evidence that β4 can regulate SPARC through various mechanisms stems from the MDA-MB-435 TrkB system generated previously by our laboratory. TrkB is a cell surface receptor involved in neuronal guidance and is analogous in many ways to

integrins (288). Interestingly, brain-derived neurotrophic factor (BDNF), the ligand for TrkB, shares similarities with laminin (289). A chimeric receptor was generated by fusing the TrkB extracellular domain to the cytoplasmic and intracellular domains of β4. A truncated TrkB construct consisting of the extracellular and cytoplasmic domains of this neuronal receptor was generated as a control. The results of functional analyses using this TrkB system revealed that the cytoplasmic domain of β4 contains intrinsic signaling potential but is not sufficient to transduce all signals that have been attributed to the full-length integrin (69). Specifically, upregulation of the SFK signaling pathway occurs in response to dimerization of the chimeric receptor, while induction of PI3K and Ras/MAPK signaling pathways are not detectable.

We chose to examine SPARC expression in the TrkB system. Surprisingly, our analysis revealed that TrkB β4 infectants express higher levels of SPARC in both the total cell lysate and the culture media compared to cells expressing the truncated TrkB construct (data not shown), suggesting that neither the extracellular domain nor ligation of the integrin by laminin are required for β4-regulation of SPARC. Moreover, due to the fact that Akt phosphorlyation is not induced by dimerization of the chimeric receptor, it seems unlikely that the changes in SPARC expression could be attributable to a TOR-dependent translational mechanism, as Akt is a kinase upstream of 4E-BP1 in the mTOR signaling pathway. Instead, members of the Src family of kinases might contribute to SPARC regulation. Notably, treatment of MDA-MB-435/β4 transfectants with PP2, an inhibitor of SFKs, failed to consistently decrease SPARC expression; however, these

cells are inherently different than the TrkB β4 system and may employ different mechanisms to regulate SPARC.

Palmitoylation of β4 is critical for trafficking of the intergrin to tetraspanin-enriched microdomains on the cell surface. This process, in turn, permits cell spreading and signaling through p130 Crk-associated substrate (p130CAS) (63), an integrin adapter protein involved in cytoskeletal remodeling, focal adhesion turnover, and cell migration (290). This signaling intermediate is often phosphorylated by members of the Src family of kinases and FAK (290). Along these lines, it would be interesting to determine whether β4 palmitoylation and localization in tetraspanin-enriched microdomains promotes SPARC expression, as data from the TrkB/β4 system suggests a role for SFKs in β4-mediated regulation of this protein.

Integrin β4 has been shown to cooperate with growth factor receptors to promote activation of signaling cascades and carcinoma invasion. These findings prompted us to consider the possibility that β 4regulation of SPARC, and in turn invasion, occurs through collaboration with another cell surface receptor. Work previously published by our laboratory investigated the ability of specific factors to cooperate with β4 to promote the chemotaxis of MDA-MB-435/β4 transfectants. Of the growth factors examined, including EGF, basic fibroblast growth factor, HGF, insulin-like growth factor type I, TGF-α and -β, PDGF (AA and BB), somatostatin, thrombin, and lysophosphatidic acid (LPA), only LPA induced chemotaxis at levels comparable to NIH-3T3 conditioned media (78). Furthermore, this factor induced the formation of large ruffling lamellae, a phenomenon specific to the β4 transfectants. Findings from this study and others suggest

that LPA binds heterotrimeric Gi proteins on the cell surface of the MDA-MB-435 cells to mediate these effects (78, 291). Based on the data reported in this study, we tested whether LPA could induce SPARC expression in these cells. Despite observing the appropriate morphological changes in response to LPA treatment, we did not detect an increase in SPARC expression (data not shown), raising the likely possibility that β4 functions independently to regulate this downstream effector.

A remaining question generated from our work involves the mechanism by which SPARC promotes invasion. One likely possibility, as explored above, involves its ability to mediate integrin dynamics and expression. Moreover, SPARC is known to induce signaling pathways important for invasion, such as PI3K. Whether such signal transduction occurs in concert with integrin function remains to be seen. Another possibility is that SPARC is involved in focal adhesion turnover. Early studies examining the ability of SPARC to impact interactions between cells and their environment established that addition of this counter-adhesive protein to bovine aortic endothelial cells decreases the formation of focal adhesions and promotes redistribution of actin to peripheral regions of the cell (292). Given these findings, as well as the fact that SPARC can induce the activity of FAK, the primary signaling protein involved in focal adhesion turnover, it is possible that SPARC functions downstream of β4 in this capacity to promote cell motility. Regulation of MMPs is another likely path through which SPARC promotes invasion. Members of this family of proteolytic proteins are considered to be the primary regulators of matrix proteolysis and turnover, and their role in promoting the invasive behavior of tumor cells is well established (293). SPARC is

known to induce activity of MMPs through a direct interaction with α helices of its E-F hand region (294). In breast cancer, SPARC is known to upregulate MMP-2 activation (210), giving rise to the possibility that β4-induced expression of SPARC functions to activate MMPs involved in promoting invasion.

Concluding Remarks

The work presented in this dissertation enhances our understanding of integrin function and regulation of downstream effector molecules. Specifically, our data define a novel role for β4 in promoting cell motility in breast cancer. Expression of this integrin correlates with distinct miRNA patterns, potentially important for driving cell behavior downstream of the integrin. Additional work is necessary to dissect the mechanism by which β4 induces differential expression among members of this regulatory class of small RNAs. Whether such effects arise through transcriptional changes, modifications in biogenesis, or influences on stability remains to be seen. Furthermore, characterizing more fully the contribution of miRNAs to the invasive process will be of value, particularly through identification and confirmation of downstream targets aside from SPARC. While this study has focused on the ability of differentially expressed miRNAs to potentiate an invasive phenotype in carcinoma cells, future work will investigate the ability of such miRNAs to participate in other functions mediated by β4 integrin, including hemidesmosome organization, anchorage-independent growth, and cell survival.

While the material presented in this dissertation explores the role of β4 integrin in the context of carcinoma progression, specifically in the process of invasion, it has been well established that the integrin functions under normal conditions to maintain homeostasis. For example, in the breast, β4 is expressed in contractile myoepithelial cells that separate the secretory luminal cells of mammary alveoli from the underlying basement membrane (295), whereas in the skin, the integrin is expressed in basal keratinocytes at epidermis-dermis interface and maintains epithelial integrity (40, 41). Studies exploring the mechanisms that mediate wound closure in keratinocytes have established an important role for β4 in this phenomenon, one that necessitates effective cell migration and parallels the process of tumor cell invasion (38). The idea that SPARC or specific miRNAs might play a role in reepithelialization or maintaining general homeostasis is intriguing. Monitoring changes in the expression of these effector molecules in keratinocytes during a scratch assay, which functions as an *in vitro* method of monitoring cell migration, could shed light on this question. Furthermore, the ability of SPARC or specific miRNAs to modulate wound closure in this setting could be assessed following manipulation of their expression levels.

From a clinical standpoint, the idea of miRNAs as targets in cancer therapeutics has gained increasing attention. The recognition that aberrant miRNA expression contributes to human disease has prompted an interest in the development of strategies aimed at correcting the inappropriate deficiency or accumulation of specific miRNAs. In most cancers, miRNA expression is globally downregulated. Targeted therapies will, therefore, likely focus on reintroduction of select miRNAs and augment current

chemotherapeutic regimes. Delivery of these agents could be accomplished through infection of cancer cells using viral vectors that encode short hairpin RNAs; these hairpins would be subject to processing by endogenous miRNA biogenesis machinery upon transcription (296). Advances in this field of research may ultimately hinder cancer progression and improve prognosis and outcome in patients with aggressive disease.

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