COMMENTARY

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MicroRNA-23b regulates cellular architecture and impairs motogenic and invasive phenotypes during cancer progression

Loredana Pellegrino,* Jonathan Krell, Laura Roca-Alonso, Justin Stebbing, and Leandro Castellano

Division of Oncology; Department of Surgery & Cancer, Imperial College; Hammersmith Hospital Campus; Imperial Centre for Translational & Experimental Medicine; London, UK

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*Correspondence to: Loredana Pellegrino; Email: l.pellegrino@imperial.ac.uk

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The cytoskeleton is a dynamic three dimensional structure contained within the cytoplasm of a cell, and is important in cell shape and movement, and in metastatic progression during carcinogenesis. Members of the Rho family of small GTPases, RHO, RAC and cell cycle division 42 (Cdc42) proteins regulate cytoskeletal dynamics, through the control of a panel of genes. We have recently shown that the microRNA (miRNA) miR-23b represents a central effector of cytoskeletal remodelling. It increases cell-cell interactions, modulates focal adhesion and reduces cell motility and invasion by directly regulating several genes involved in these processes.

Introduction

Tumor progression often culminates in the development of metastasis, which usually results in the death of patients with solid malignancies.[1] After establishment of the primary tumor, cancer cells acquire aggressive traits that allow them to detach, migrate and invade, and these events denote the first steps of metastatasis.[2] Changes in the cellular cytoskeleton are central to the acquisition of a metastatic phenotyope. This generally requires the disruption of existing cell-cell contacts, the establishment of cell-matrix adhesions, the degradation or rearrangement of the extracellular matrix, and finally movement of the tumor cell. The actin cytoskeleton constitutes the structural support to cell morphology, polarity, adhesion and migration. Primary regulators of cytoskeletal dynamics are three members of the Rho family of small GTPases, RHO, RAC and cell cycle division 42 (Cdc42).[3] Under physiological conditions they ensure spatial and temporal cytoskeletal organization via the coordination of a plethora of effectors, including transcription factors. Conversely, aberrant activity of Rho GTPases has been significantly implicated in tumor aggressiveness and metastasis[4] Rho GTPases are activated in response to pro-migratory signals through various transmembrane receptors that are usually overexpressed or hyperactivated in cancer, thus leading to sustained Rho GTPase function. This results in dysregulation of the Rho GTPase cytoplasmic effectors and disruption of the actin cytoskeleton, leading to increased migration and invasion. In addition, aberrant activity of Rho GTPases induces changes in transcriptional activity, resulting in the expression of genes that further promote motility and invasion.[5] Accordingly, it is well-known that multigenic reprogramming events are necessary to promote the sustained expression of genes that initiate metastasis and coordinate the seeding of cancer cells to specific distant target organs.[6-8] In addition to the genetic and epigenetic alterations involved in malignant transformation, microRNAs (miRNAs) appear to play an important role in the establishment of the distinctive genetic signatures that characterize metastatic cells, owing to their ability to regulate the expression of multiple genes.[9]

MicroRNAs are a class of small, noncoding RNAs that function as post-transcriptional negative regulators in a wide range of physiological and pathological

processes. In malignant contexts, miRNAs play either oncogenic or tumor suppressor roles and deregulation of miRNA expression has been detected in many different human cancers.[10] Recently, it has been proposed that miRNA activity can promote or inhibit metastatic gene expression programs; this arises from the observation that specific miRNAs can control multigenic regulatory networks that operate at different steps of the metastatic process.[11] This is exemplified by miR-31, a miRNA that is preferentially downregulated during breast cancer progression, and that mediates coordinated repression of at least three pro-metastatic genes, *RhoA*, integrin α5 (*ITGA5*) and radixin (*RDX*) to suppress motility, invasion, resistance to anoikis and colonization of the lungs during breast cancer metastasis.[12]

Our group has recently identified miR-23b as a potent modulator of cytoskeletal dynamics via co-repression of a set of cytoskeleton specific genes, affecting motile and invasive properties necessary for breast cancer cells to metastatize.[13] Here, we attempt to unravel the molecular mechanisms employed by the miR-23b regulatory network in the inhibition of important features of cancer progression and metastasis.

Overlook of miRNA Biogenesis and Function

Most of mammalian miRNAs derive from intergenic or intronic genomic regions and go through a model of maturation events necessary for their ultimate function.[14,15] Mammalian single or clustered miRNA genes are generally transcribed by polymerase II, and less frequently by Polymerase III, into a primary miRNA transcript (pri-miRNA).[15] Owing to regions of imperfect complementarity, pri-miRNAs typically acquire double-stranded hairpin-like conformations flanked by single-stranded 5' and 3'ends that are typically capped and polyadenylated, respectively. The pri-miRNA hairpin structure is recognized and bound by the RNA-binding protein DGCR8, which allows the first cleavage event of the miRNA maturation process.[16] The binding of DGCR8to the ribonuclease Drosha enables it to process pri-miRNAs at the base of their hairpin region, thus releasing a ~70-nucleotide stem-loop intermediate, known as miRNA precursor (pre-miRNA).[16,17] Notably, a subclass of miRNAs undergo a noncanonical maturation pathway that does not require Drosha/DGCR8, but instead uses cellular splicing machinery for the formation of the pre-miRNA.[18] Completion of the miRNA maturation requires translocation of the pre-miRNA from the nucleus to the cytoplasm that is actively performed by exportin-5.[19] Once in the cytoplasm, the ribonuclease Dicer in complex with its cofactors, the RNA-binding proteins TARBP2 and PACT, cleaves off the loop of the pre-miRNA, yielding a 19–26-nucleotide-long double stranded miRNA-miRNA* duplex.[15,16,20] This miRNA duplex is then loaded onto the RNA-induced silencing complex (RISC), which consists of the Argonaute (Ago) protein family members and other proteins.[15,21] After strand separation, the guide strand or mature miRNA is preferentially retained into the active RISC, whereas the complementary strand (miRNA*) is discarded and degraded. The mature miRNA then guides Ago to selective recognition of a miRNA-specific target mRNA.[22] The specificity of target selection is generally determined by complementary matches between a "seed" sequence (positions 2 to 8 from the 5'end of the mature miRNA) and a miRNA-binding site usually embedded in the 3'untranslated region (3'UTR) of the target mRNA. Upon this interaction, the mature miRNA inhibits the expression of its target mRNA through Ago-mediated translational repression and/or deadenylation and decay of the messenger sequence.[23]

miR-23b Regulates the Actin Cytoskeleton and the Metastatic Process

miR-23b is encoded from a unique primary transcript that also contains miR-27b and miR-24–1. A few studies have implicated miR-23b in cancer progression, metastasis and cytoskeletal remodelling in several tumor types. In human colorectal cancer, miR-23b shows tumor suppressor functions by reducing in vitro migration and in vivo experimental metastatic formation, by inhibiting the expression of a cohort of pro-metastatic genes including uPA, c-MET, PAK2, FZD7, and MAP3K1.[24,25] MiR-23b-mediated repression of uPA has been shown to be critical in the regulation of migration in human cervical cancer cells.[26] In addition, this miRNA functions as an in vivo metastatic suppressor in prostate cancer, coordinating repression of the proto-oncogenes Src kinase and AKT, and inhibiting epithelial to mesenchymal transition (EMT) by reduction of the mesenchymal markers Vimentin and Snail and upregulation of E-cadherin.[27]

In our study, we showed that experimental suppression of miR-23b in breast cancer cell lines modulates cellular architecture and promotes in vivo tumor growth, invasion and lymph-node metastasis.[13] By combining bioinformatic and experimental analyses, we found that miR-23b is a master regulator of cytoskeletal dynamics in breast cancer cell models, with a robust impact on cell-cell interactions, cell-matrix adhesions, cell spreading and protrusion formation. We initially performed gain and loss of function experiments that revealed the ability of miR-23b to modulate in vitro migration and invasion of breast cancer cells.[13] Subsequently, we performed RNA-sequencing (RNA-seq) following miR-23b perturbation in cancer cell lines to investigate how its effect is mediated at the molecular level. This strategy allowed us to identify and validate a subset of cytoskeletal and pro-metastatic genes as direct miR-23b targets, namely the previously documented Pak2 and uPA, Arhgef6, Limk2, Cofilin-2 (Cfl2), and Annexin 2 (Anxa2). Coordinated repression of these targets along with mir-23b-mediated perturbation of the transcriptome state of breast cancer cells enabled our understanding of the molecular mechanism(s) that underpin the cellular phenotypes controlled by miR-23b.

We first showed that ectopic expression of miR-23b enhances epithelial characteristics of breast cancer MCF-7 cells, indicating that it may have a role in the inhibition of EMT during breast cancer progression[13]; this notion was supported by previous evidence in prostate and colorectal cancers where miR-23b

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overexpression abolished features of EMT and upregulated the epithelial marker E-Cadherin.[24,27] Furthermore, under conditions of prolonged miR-23b administration we revealed that miR-23b failed to reverse EMT in- mesenchymal-like MDA-MB-231 cells and did not affect E-cadherin expression in either MDA-MB-231 or MCF-7 cells indicating that it specifically acts during EMT by improving the strength of existing junctions. In support of this hypothesis, sustained miR-23b ectopic expression improved the overall architecture of epithelia formed by 2-dimensional cultures of MCF-7 cells, which exhibited more orderly and stable cell-cell junctions compared with control-treated cells. Taken together, these data suggested that miR-23b activity confered enhanced tension between existing E-cadherin-mediated cell-cell junctions rather than inducing their de novo formation. Consistent with this hypothesis, analysis of our RNA-seq data revealed that prolonged overexpression of miR-23b in MCF-7 cells induced transcriptional upregulation of *Nectin1* and *LMO7*, which encode for cell-adhesion molecules that are highly involved in cell-cell junction formation. Nectin1 is a member of the Nectin family of transmembrane receptors that are involved in early establishment of cell-cell contacts, known as tight and adherens junctions.[28] Upon cell-cell interactions, clusters of Nectins on the lateral surface of the cell interact with similar clusters assembled on the membrane of the adjacent cell; simultaneously, on the intracellular side of both cells, Nectins recruit Afadin, an actin-binding protein that connects filamentous actin (F-actin) to the nectin-based adhesive structures. E-cadherin participates in the formation of adherens junctions following Nectin-mediated contacts.[29] Clusters of E-cadherin on the membrane of two adjacent cells connect to each other and are internally stabilized by interaction with F-actin via several actin-binding proteins. The interaction of additional adaptor molecules such as LMO7 with Afadin and E-cadherin-associated actin-binding proteins allows aggregation of Nectin and E-cadherin adhesive structures and strengthens the adhesive capabilities of E-cadherin.[30] Maturation of adherens junctions requires the assembly of additional E-cadherin-F-actin units and the accumulation of RHOA-activated myosin II. The latter generates a contractile force that brings two adjacent cadherin-based clusters together and maintains junctional tension.[31] This suggests that miR-23b may actually induce de novo formation of both tight and adherens junctions through a mechanism that requires Nectin1 upregulation and is independent of E-cadherin expression; in a later stage, miR-23b may stabilize existing and newly formed adherens junctions via upregulation of the adaptor molecule LMO7. Moreover, mir-23b likely contributes to enhance the junctional tension during assembly of the epithelia, as we detected increased myosin light chain II (MLCII) phosphorylation, indicative of myosin II activity, upon miR-23b overexpression in different cell lines, including MCF-7 cells.

In mesenchymal like breast cancer cells, miR-23b activity controls cytoskeletal dynamics to stimulate maturation of focal adhesions and cell spreading on the extracelluar matrix and impairs lamellipodia extension. The physiologic functions of the miR-23b targets identified in our study may elucidate the effects of miR-23b on the motile and invasive abilities of mesenchymal-like MDA-MB-231 cells.

During the migration process, a cell needs to acquire a polarized morphology, by extending membrane protrusions at the leading edge, such as lamellipodia and filopodia, that are stabilized by the formation of new focal adhesions (FAs). FAs are adhesive multiprotein complexes that connect the actin cytoskeleton to elements of the extracellular matrix.[32] Ectopic expression of a miR-23b mimic induced a significant increase of the FA area in MDA-MB-231 cells spreading on collagen I matrixes. Larger FA sites are caused by an excessive maturation of newly formed FAs, a process that requires local activity of myosin II.[32] At FA sites, myosin II activity is tightly regulated by the p21/RAC/Cdc42-activated kinases (PAKs). PAKs control the activation state of myosin II either by directly phosphorylating MLCII or by inhibiting the activity of MLCK, which results in reduced myosin II phosphorylation. PAK2, one of our identified miR-23b gene targets, has been shown to be crucial in limiting the size of FAs. Indeed, PAK2 depletion in breast cancer cells resulted in larger FA sizes and increased MLCII phosphorylation.[33] Accordingly, we observed that overexpression of miR-23b in MDA-MB-231 cells led to repression of PAK2 and enhanced phosphorylation of MLCII,. Conversely, inhibition of miR-23b activity using an in house sponge construct led to an increase of PAK2 levels which may explain the effect of miR-23b in inducing an increase in FA areas observed in these cells. Fully developed FAs promote cell spreading on the extracellular matrix and are associated with slower migration rates;[34,35] therefore a miR-23b-promoted increase of FA size may contribute to transient cell spreading and to the reduction in migratory phenotypes that we observed in miR-23b-overexpressing MDA-MB-231 cells.[13]

Localization of PAKs at FA sites is mediated by their direct interaction with αPIX (PAK-interacting exchange factor α), also known as COOL2 (Cloned out of library 2) or ARHGEF6 (RAC/Cdc42 guanine nucleotide exchange factor 6), strikingly, an additional miR-23b target validated in our study. ARHGEF6 is a well-described activator of RAC and Cdc42, which in turn activates PAKs.[36] ARHGEF6-mediated localization of PAKs at FA sites induces PAK activation through local stimulation of RAC and Cdc42. In this respect, along with repression of PAK2 expression, miR-23b may enhance FA maturation by limiting local PAK2 activity through downregulation of ARHGEF6.

It has been shown that ARHGEF6 overexpression dramatically promotes the formation of lamellipodia and filipodia protrusions, likely as a result of ARHGEF6-mediated activation of RAC and Cdc42.[37] Indeed, once activated, RAC and Cdc42 stimulate actin polymerization in order to support an extension of membrane protrusions, via a plethora of cytoplasmic effectors. Among them, activation of the PAK1/LIMK/cofilin pathway by both RAC and Cdc42 is crucial for actin polymerization dynamics and appropriate formation of lamellipodia at the leading edge.[38] RAC/Cdc42-activated PAK1 catalyzes phosphorylation and activation of LIMK which in turn

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phosphorylates cofilin, resulting in its inhibition. Cofilin is an actin-binding protein responsible of F-actin severing; yet, it promotes lamellipodia assembly by inducing F-actin turnover that produces free barbed ends for the polymerization of new actin filaments.[39] Nevertheless, uncontrolled cofilin activity leads to accelerated F-actin turnover resulting in widening of the lamellipodium. PAK1-induced inactivation of cofilin ensures correct formation of lamellipodia by limiting their extent of expansion, that are under cofilin control.[40] We found that, miR-23b-overexpressing MDA-MB-231 cells exhibited significant impaired formation of lamellipodia protrusions at their leading edge and in contrast MDA-MB-231 stably expressing a sponge construct able to inhibit miR-23b activity showed larger lamellipodia. It is reasonable to infer that by coordinated repression of ARHGEF6, LIMK2 and CFL2, miR-23b disrupts crucial steps of the molecular pathway that supervises correct extension of lamellipodia. Notably, miR-23b-induced corruption of lamellipodia formation likely encompasses the suppressive effect of miR-23b on MDA-MB-231 cell migration as lamellipodia formation is crucial for cells to migrate. This effect can be also explained by the fact that downregulation of several miR-23b cytoskeletal targets, such as LIMK2, CFL2, and PAK2 inhibit migration and invasion of these cells.[13,41,42]

We also reported that overexpression of miR-23b greatly reduced invasion of MDA-MB-231 cells in three-dimensional collagen I matrixes; whereas inhibition of miR-23b activity strongly enhanced the invasive properties of these cells. Along with the role of miR-23b cytoskeletal targets in affecting breast cancer cell invasion, miR-23b seems to be closely implicated in the regulation of invasive processes, owing to its ability to directly target uPA and Annexin 2, two main components on the invasive machinery. Indeed, uPA and Annexin 2 play crucial roles in the extracellular cascade that converts plasminogen into plasmin, a serine protease largely implicated in invasion, neo-angiogenesis and metastasis. uPA directly catalyzes proteolytic cleavage of plasminogen, resulting in release of the active form plasmin.[43] Regulation of uPA by miR-23b has already been described in different tumor types[24,25] and we indicated that miR-23b is able to repress uPA expression in breast cancer. Moreover, it has been shown that inhibition of uPA activity abolishes breast cancer invasion and metastasis in vivo,[44] which is consistent with our data showing that miR-23b suppression promotes invasion and metastasis in animal models. On the other hand, we identified and validated Annexin 2 as a direct miR-23b target in breast cancer. Annexin 2 is a phospholipid-binding protein that promotes plasminogen activation and degradation of the extracellular matrix (ECM).[45] Annexin 2 localizes to the plasma membrane of invasive cells, where it is frequently found in heterotetramers known as AIIt. AIIt functions as a cell surface receptor for various secreted proteases, including tPA, Cathepsin B and plasminogen itself.[45] Docked on AIIt, both tPA and Cathepsin B mediate plasmin activation by direct proteolysis of plasminogen and by activating uPA, respectively.[45,46] By interacting with different components of the ECM, AIIt provides a structural linkage between the cellular proteolytic system and the ECM, thus promoting localized matrix degradation, and subsequently cell migration and invasion.[45,46] Overexpression of Annexin 2 is found in many tumors, including highly aggressive breast cancers where it correlates with increased invasiveness, neo-angiogenesis and metastasis; moreover, silencing of Annexin 2 reduced migration of MDA-MB-231 cells.[46,47] These observations suggest that miR-23b may further inhibit the metastatic potential of breast cancer cells through repression of Annexin 2.

By considering the overall role of miR-23b in breast cancer, we extrapolated a complex regulatory network that miR-23b imposes to impede acquisition of aggressive traits. This network employs mechanisms of post-transcriptional repression, transcriptomic changes and intracellular dynamics affecting multiple genes that differentially promote or inhibit breast cancer progression.

Notably, we found that miR-23b expression is inversely correlated with breast cancer metastasis. miR-23b is selectively downregulated in breast cancer cell lines that metastasized to different distal organs compared with cells derived from mammary primary tumors after inoculation in the mammary fat pads of nude mice. Accordingly, we found that, in a large cohort of paired primary-metastatic patient samples, miR-23b expression was significantly reduced in lymph-node metastasis compared with their matched primary tumors. In addition, we showed that AP-1 is a transcriptional suppressor of miR-23b expression in breast cancer and that breast cancer cells stimulated with EGF expressed lower levels of mature miR-23b. EGF is an extracellular ligand that specifically activates EGFR, a member of the HER2 family of receptor tyrosine kinases. Activated EGFR dimerizes with its preferred partner HER2 and triggers multiple signaling cascades leading to metastasis.[48] AP-1 is a transcription factor activated downstream of EGFR/HER2 signaling that controls a motogenic and invasive program that confers aggressive behaviors to tumor cells.[6,48] These data suggest that expression of miR-23b in breast cancer may be regulated by EGFR/HER2-activated signaling cascades involving AP-1 activity. Accordingly, miR-23b has been recently found to be downregulated together with miR-27a and miR-27b in HER2-transformed mammary epithelial cells.[49] Since overexpression of HER2 is detected in ~20% of breast cancers and correlates with increased metastatic potential of these tumors, we propose that a reduction in miR-23b expression and the subsequent disruption of its regulatory network are likely to be crucial events in the initiation of cancer progression and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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