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Augmentation of Ras-induced cell transformation : a new role for miR-200a in malignancy.

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AUGMENTATION OF RAS-INDUCED CELL TRANSFORMATION:

A NEW ROLE FOR MIR-200A IN MALIGNANCY

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DEDICATION

To my dear Brothah

who always calls me jolly

even when I’m not.

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Science does not exist in a vacuum, and neither do dissertations.

I would like to wholeheartedly thank my parents for their unending love and support; without them, nothing I've ever done would have been possible.

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And finally I would like to thank Damien. He knows why.

ABSTRACT

AUGMENTATION OF RAS-INDUCED CELL TRANSFORMATION:
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Cancer is a multistep disease that begins with malignant cell transformation and frequently culminates in metastasis and death. MicroRNAs (miRNAs) are small regulatory 21-25-nt RNA molecules and are frequently deregulated in cancer. The majority of miRNAs are estimated to be co-expressed with neighboring miRNAs as clusters. Many miRNA clusters coordinately regulate multiple members of cellular signaling pathways or protein interaction networks. miR-200a is a member of the miR-200 family, which are known to be strong inhibitors of the epithelial to mesenchymal transition. As such, the tumor suppressive role of miR-200a in oncogenesis has been well studied; however, recent studies have found a proliferative role for this miRNA as well as a pro-metastatic role in the later steps of cancer progression. In this study, we employed a biphasic approach to determine miRNA involvement in malignant cell transformation. First, we screened 366 human miRNA minigenes to determine their effects on the four major cancer signaling pathways culminating in AP-1, NF-κB, c-Myc, or p53

transcriptional activity. The second phase of this study was an epithelial cell screening assay to determine the ability of miRNAs to transform epithelial cells. In our miRNA cluster profiling study, we found that miR-200a down-regulates p53 activity. miR-200a was demonstrated to directly target p53, reduce protein levels, and inhibit apoptosis. We also found that miR-200a enhances Ras-mediated transformation of MCF10A cells. Furthermore, miR-200a transforms MCF10A cells and induces tumorigenesis in immunocompromised mice by cooperating with a Ras mutant that activates the RalGEF effector pathway. These results demonstrate a role for miR-200a in malignant transformation and reveal a specific cellular environment in which miR-200a acts as an oncomiR rather than a tumor suppressor by cooperating with oncogene activation in the classical two hit model of cell transformation.

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CHAPTER I

INTRODUCTION: CANCER AND MICRORNAS

Cancer Statistics and Cancer Progression

One in three Americans will be diagnosed with cancer in his or her lifetime. In 2013, an estimated 1.6 million new cases of cancer were diagnosed, and over half a million Americans died of cancer. It is the number two leading cause of death in the United States, accounting for nearly one in every four deaths. The most frequently occurring cancers in men are prostate, lung, and bladder. In women, the most frequently occurring cancers are those arising in the breast, digestive system, and respiratory system. In both men and women combined, lung cancer was responsible for 159,480 deaths in 2013, over a third of all cancer related deaths. The second most deadly cancers in men and women are prostate and breast, respectively. Colon cancer claims the third most lives for both sexes. Despite these grim numbers, cancer death rates are declining in both sexes [1]. In 2009 alone, 152,900 cancer related deaths were avoided, thanks in part to advances in treatment efficacy. Even more important, however, are advances in detecting and diagnosing cancer at early, more treatable stages [2,3].

There are multiple forms of cancer, classified by the cell type from which they arise. The five most common types of cancer—breast, prostate, lung, colon, and

bladder—are examples of carcinomas, or cancers arising from epithelial cells. Carcinomas are the most common form of cancer.

Cancer is comprised of a progressive series of steps, beginning with malignant cell transformation, which leads to primary tumor formation. Vascularization of the tumor occurs to allow survival and growth. Tumor cells become invasive and motile by undergoing an epithelial to mesenchymal transition (EMT). During this process, epithelial cells, once constrained by the necessity of cell:cell and cell:basement membrane contacts for survival, experience changes in gene expression that result in an elongated, mesenchymal phenotype and the ability to migrate and invade local stroma [4]. This mobile, mesenchymal phenotype also allows for subsequent intravasation of invasive cancer cells into the blood stream, allowing them to circulate throughout the body. Circulating cancer cells will then extravasate from the blood stream to colonize new sites on distal tissues. New studies have shown the importance of the reversal of EMT, the mesenchymal to epithelial transition (MET), in metastatic colonization at distal sites [5-7]. By developing a more stable epithelial phenotype, cells that were once mobile and invasive acquire the characteristics necessary for formation of a secondary metastatic tumor. It is rarely the primary tumor that is responsible for cancer mortalities, but rather the late stage metastatic disease that accounts for over 90% of cancer related deaths [8-10]. The study of the very first step of cancer progression—cell transformation—is thus crucial to provide the means for diagnosing and treating cancer before progression to the deadly metastatic stage.

Malignant Cell Transformation

Malignant cell transformation occurs when a normal somatic cell develops the ability to proliferate indefinitely and escape the cell cycle control checkpoints and apoptotic signaling that normally eliminate cells with hyper-activated proliferative signaling pathways [11]. Cells that acquire this unrestrained proliferation phenotype *in vivo* can develop into a tumor. In 2000, Robert Weinberg elegantly described the Hallmarks of Cancer, identifying the six abilities acquired by cancer cells that allow them to form tumors, induce angiogenesis, and metastasize [12]. Four of these six hallmarks pertain specifically to cell transformation: sustaining proliferative signaling, resisting cell death, evading growth suppressors, and enabling replicative immortality. More recently, Weinberg updated these hallmarks to include four new emerging hallmarks of cancer: dysregulating cellular energetics, avoiding immune destruction, genome instability and mutation, and tumor-promoting inflammation. Genome instability and mutation influences the genetic changes that induce malignant cell transformation, and so they will be discussed within the context of the four hallmarks of transformation.

Sustaining Proliferative Signaling

Normal somatic cells receive external and internal signals throughout their normal lifespans that regulate proliferation. During development, mitogenic stimuli signal differentiated cells to proliferate into a specified organ, and then proliferation ceases. Under normal conditions, homeostasis of the cells, and thus the organ itself is maintained via cell:cell signals, cell:basement membrane signals, as well as various paracrine and endocrine signals in the entire organism. While much of the homeostatic cell:cell

signaling under normal conditions is not completely understood, proliferative signals in cancer progression are well studied. The crux of the hallmark of sustaining proliferative signaling relies on the propagation of cell signaling pathways that result in proliferation despite a lack of actual proliferative stimuli.

Activation of mitogenic downstream signaling pathways; a focus on Ras

Unrestrained proliferation can occur through multiple mechanisms; however, a common feature of hyper-proliferative cancer cells is the activation of downstream components of mitogenic signaling pathways. A potent example is the classical oncogene, Ras. Ras is the most frequently mutated gene in pancreatic cancer and its associated preneoplastic lesions; it is known to stimulate cell proliferation and contribute to the induction of pancreatic cancer [13].

Ras activation occurs in response to growth factor signaling. Growth factors bind receptor tyrosine kinases, which become activated and recruit the guanine nucleotide exchange factor, SOS, to the plasma membrane where it facilitates the exchange of Ras-bound GDP to GTP [14-16]. Binding of GTP induces a conformational change in Ras to its active state, allowing it to bind its potent downstream effectors at the plasma membrane where they are then activated. The three main effectors in the Ras signaling pathway are PI3K, RalGEF, and Raf [17-19]. Stimulation of these pathways activates Akt signaling, Jnk/AP-1 transcriptional activity, and the MAP kinase cascade, respectively. Ras-mediated activation of multiple cell signaling pathways is tightly regulated by GTPase activating protein, which stimulates the inherent GTPase activity of

Ras shortly after Ras becomes activated. The resulting hydrolysis of GTP to GDP returns Ras to its inactive form, shutting off the signaling cascade [17,20].

Normal human Ras is a protooncogene, which is a broad class of genes that control growth or proliferation and, if dysregulated, can cause cancer. The most well studied mutation of Ras is a G12V mutation where glycine 12 is mutated to valine [21]. This amino acid change ablates the GTPase activity of Ras, regardless of the presence of GTPase activating protein. Loss of GTPase activity causes constitutive Ras activation, and thus continual stimulation of downstream proliferative signaling pathways, thereby effectuating its conversion from protooncogene to full-fledged oncogene.

In addition to mutations in Ras itself, components of its downstream effector pathways may also be mutated to lead to excessively high levels of proliferative signaling. Amplification of Akt has been documented in pancreatic, colon, breast, endometrial, and ovarian cancers [22]. Akt has a wide variety of targets that it phosphorylates including Mdm2, an inhibitor of p53; Raf, an upstream component of Map kinase signaling; and mTOR, a signaling protein responsible for angiogenesis as well as upregulation of cell cycle progression genes like c-Myc [23-26]. Akt can also phosphorylate targets to inhibit their activity, for example, Caspase-9, an apoptotic effector protein, and p21, an inhibitor of cell cycle progression [27,28]. The crucial element of the oncogenic activity resulting from activating mutations in, or amplification of, genes like Ras and Akt is the lack of dependence on growth factor signaling to induce activity. In other words, the cell continually propagates signals to proliferate despite a lack of normal upstream proliferative stimuli.

Disruption of proliferation-regulating pathways

In addition to over-activation of proliferative signaling, *e.g.* the activating G12V Ras mutation or amplification of Akt described above, loss-of-function of regulatory members of proliferative signaling pathways can lead to sustained proliferative signaling and induction of cancer. These regulatory proteins are generally known as tumor suppressors due to their role in inhibiting cellular processes that, if left unchecked, can induce malignant cell transformation or contribute to cancer progression. The tumor suppressor Phosphatase and Tensin Homolog (Pten) is a potent regulator of the PI3K/Akt activation pathway. PI3K signaling is stimulated when Ras is activated [29]. After being recruited to the plasma membrane, it phosphorylates Phosphatidylinositol 4,5-bisphosphate (PIP_2) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3 serves as a docking site for Phosphoinositide-dependent Kinase 1 (PDK1) which phosphorylates Akt on threonine 308 to serve as its initial activation step [30]. Pten is a phosphatase that dephosphorylates PIP_3 to PIP_2, blocking the recruitment of PDK1 to the plasma membrane and ultimately inhibiting the activation of Akt. Pten mutations and loss of heterozygosity have been found in brain, prostate, breast, ovarian and pancreatic cancers [22]. Loss of this kind of regulatory mechanism also allows for inappropriate proliferative signaling.

Senescence

A natural barrier to malignant cell transformation is oncogene-induced senescence. Senescent cells are characterized by lack of proliferation, lack of response to growth factors, and an enlarged, flattened morphology. Induction of this phenotype can

occur in response to direct DNA damage or in response to oncogene activation [31]. Cells with activated oncogenes stimulate proliferative downstream signaling that induces DNA replication at inappropriately high levels, which triggers a DNA damage response that signals the cell to undergo senescence [32]. When Ras is constitutively activated in cancer, its effector, Raf, initiates the Map kinase signaling cascade. Map kinase signaling is potently mitogenic, and is particularly responsible for inappropriately high levels of DNA replication [33-35]. The prodigious number of replication forks during oncogene-induced hyper-replication induces a DNA damage response through the ATM pathway, which ultimately up-regulates p53 activity [32,36]. The transcriptional target of p53, $p21^{WAF1/CIP1}$, is induced when p53 levels are increased in response to DNA damage, and it inhibits the activity of Cyclin-Dependent Kinases (CDKs) which phosphorylate Retinoblastoma protein (Rb). Phosphorylated Rb sequesters the transcription factor E2F, blocking its ability to promote progression through the cell cycle from G1 to S phase [37]. Other cell cycle arresting pathways converge, and ultimately, if the cell fails to repair the damage that initially induced the DNA damage response, the cell will permanently withdraw from the cell cycle and become senescent [37,38].

Senescence induction as a means of protection from oncogene activation is a first line of defense against allowing malignant cell transformation to occur, but it is also a dynamic process that continues throughout tumor formation [39]. By working to prevent the consecutive steps of cancer progression that lead to metastasis and death, senescence within existing tumors can continue to protect against more severely damaging effects of cancer [8,40].

Resisting Cell Death

Organized cell death, or apoptosis, is the process by which a cell systematically disassembles itself to be digested by surrounding cells and specialized phagocytes. It is characterized by chromatin condensation and membrane blebbing, and is carried out by Caspase proteins. Caspases are thiol proteases that are present in the cell as inactive pro-caspases that are autolytically cleaved to form their active Caspase form in response to apoptotic signaling. Once active, these proteases cleave structural proteins and other important cell components. Apoptosis is necessary during normal development to eliminate extraneous cells. For example, the cells between a developing mammal's phalanges undergo apoptosis to allow for formation of individual digits. Apoptosis is also important for the elimination of cells that pose a threat to the organism, *e.g.* cells with the potential to become cancerous. Cells of this nature are typically cells that have excessive levels of DNA damage or mutations that either activate proto-oncogenes or down-regulate tumor suppressors.

Apoptosis

Apoptosis is exacted through two pathways: an intrinsic or mitochondrial pathway and an extrinsic pathway induced by death ligands binding to cell surface receptors. Both pathways ultimately result in the activation of effector Caspases, which are thiol proteases that cleave after aspartic acid residues. These apoptotic-specific proteases cleave structural proteins, signal transducers, regulators of transcription, repair factors, and many other targets within the cell. The apoptotic cell also prepares itself for phagocytosis by actively flipping phospholipids, specifically phosphatidyl serine, from

the inner to the outer leaflet of the cell membrane, creating a signal for phagocytosis of the disassembled cell by macrophages [41].

Genes upregulated by p53 in response to DNA damage include Puma and Noxa. These proteins are pro-apoptotic BH3 domain-containing Bcl-2 family members that exert their effects by antagonistically binding anti-apoptotic Bcl-2 family members, causing the release of BH123 family members such as Bax, another p53 transcriptional target. Release of Bax allows for its oligomierization and translocation to the mitochondrial outer membrane, where it causes permeabilization of the outer membrane and release of Cytochrome C. Cytochrome C is an important second messenger that binds and activates the adapter protein Apaf-1. Apaf-1 binds procaspase 9 and forms aggregates, allowing for autolytic cleavage to the active form of Caspase 9. Activation of Caspase 9 triggers a Caspase cascade that leads to activation of more Caspase proteins, which can then begin degrading structural proteins to prepare the cell for phagocytosis. This tightly regulated mechanism of cell death is necessary for maintenance of healthy tissue, and loss of this crucial process contributes to cell transformation and the persistence of cells with dangerous mutations.

Cancer cells frequently harbor dysregulated apoptotic machinery. An example is the translocation of anti-apoptotic Bcl-2 to an immunoglobulin locus causing upregulation of Bcl-2 expression, and promotion of cell survival in follicular lymphoma [42]. In addition, p53 is mutated in nearly half of all cancers [43,44], and its loss of function contributes to the survival of cancer cells.

DNA Damage Response

DNA damage is caused by endogenous and exogenous insults to the cell. Endogenous causes of DNA damage include reactive oxygen species and replication fork collapse. Exogenous sources of DNA damage range from radiation *i.e.* UV light, to chemical mutagens, to viruses. DNA damage includes the formation or addition of bulky adducts such as the thymidine dimers caused by excessive UV light and breakage of DNA strands [45]. The most deleterious form of this is the double stranded break (DSB) [46].

When a cell acquires DNA damage, several mechanisms are activated to allow for repair. The first signal relayed is that of the presence of DNA damage such as a DSB. In response to this form of damage, normally supercoiled DNA relaxes [45]. This change in chromatin structure initiates the recruitment of Poly-ADP-ribose Polymerase (PARP) proteins and binding of kinases such as Ataxia-Telangiectasia Mutated (ATM) and DNA-PK to the break site in order to modify histones. Histone modifications include covalent linkage of Poly-ADP-ribose (PAR) chains by PARP proteins and phosphorylation of lysines by ATM and DNA-dependent protein kinase (DNA-PK). The presence of PAR chains stops replication and transcription in the area of the DSB, and phosphorylated histones recruit DNA damage response proteins such as p53 and BRCA1 [47]. BRCA1 is a well-studied tumor suppressor, recognized largely for its mutation in familial breast cancer [48]. The transcription factor and tumor suppressor p53 has long been known as the guardian of the genome. Its functions are well studied and include transcriptional upregulation of proapoptotic genes [49]. p53 is maintained at low basal levels in the cell by its inhibitory protein, Mdm2, which inhibits p53 function by acting as an ubiquitin

ligase to target p53 for proteasomal degradation as well as by binding to and blocking the DNA binding domain of p53, inhibiting its activity as a transcription factor [50]. Upon detection of DNA damage, DNA-PK and ATM bind the DSB and phosphorylate histone H2AX [51]. This DSB-detection signal recruits p53, which is then phosphorylated on its N-terminus at serine 15 by DNA-PK or ATM. Phosphorylation at this site blocks inhibition by Mdm2 and promotes binding to p53 response elements in the promoter regions of proapoptotic genes [41,52,53].

Other signal cascades involve activation of cell cycle check point proteins and DNA repair enzymes to arrest the cell cycle to allow time for appropriate repair of damaged DNA. Repair of DSBs can occur through two pathways: Homologous repair (HR), and Non-homologous end joining (NHEJ). NHEJ frequently introduces errors, and in the case of multiple DSBs, may lead to fusion of nonhomologous chromosomes. Once the cell repairs a DSB, the checkpoint machinery is deactivated, and the cell is once again allowed to progress through the cell cycle [54]. Newly introduced errors that were not corrected become mutations that can have deleterious effects such as deactivation of tumor suppressors like p53 and other pro-apoptotic genes, up-regulation of oncogenes, and other gross changes to the cell's morphology and metabolism.

Oncogene Activation

Propagation of genetic errors such as point mutations, gene amplifications, and chromosomal translocations can lead to disruptions in gene expression and/or gene function. A potent example is the proto-oncogene Myc, which is the most frequently amplified oncogene in human cancers and whose translocation was first discovered as the

driving force in Burkitt Lymphoma [55-57]. Myc is a tightly regulated transcription factor whose target genes include ODC and Gadd45α, both of which are implicated in c-Myc driven proliferation [58]. Low levels of Myc activity are tolerated in the cell and act as a signal to grow and proliferate; however, high levels of Myc activity trigger activity of Arf, a tumor suppressor that induces cell cycle arrest via p53 and can ultimately lead to apoptosis [59,60]. In this way, the strongly oncogenic signaling of Myc is quelled before out of control proliferation occurs. Other proto-oncogenes also induce apoptosis as a safety mechanism if their signaling or activity becomes abnormally high.

Overall, loss of the apoptotic response allows cells to acquire and propagate mutations that would normally induce the DNA damage response pathway and lead to apoptosis. Propagated mutations that activate oncogenes result in unrestrained proliferation or growth, which are normally controlled by feedback mechanisms that detect such inappropriate signaling levels and induce apoptosis. Loss of apoptotic pathway components then results in unimpeded cell survival.

Evading Growth Suppressors

As established earlier, malignant cell transformation depends on loss of tumor suppressors that induce apoptosis, arrest the cell cycle, or regulate proliferative signaling. These include p53, Rb, and Pten, respectively, demonstrating the significant overlap of Weinberg's Hallmarks of Cancer within the malignant cell transformation. Impediments to unrestricted cell survival and proliferation are frequently lost or mutated in cancer, and their mechanisms are well studied; however, more intricate processes of cell growth suppression exist including contact inhibition and differentiation.

Contact Inhibition

Non-transformed cells, when grown in culture, will experience a slowing of proliferation as they reach confluence. This phenomenon was first discovered in the 1960s when cells were first being experimentally cultured in laboratories, and was termed the Hayflick phenomenon. It has recently been studied more extensively, however it is not completely understood, and its relevance *in vivo* is not completely established [31]. What has been deduced is that cells, particularly epithelial cells, experience growth inhibition with respect to adjacent cells. The cell signaling pathways responsible for this are not fully known, but it has been shown under various conditions and with multiple cell types that non-transformed cells will grow into a single-layered sheet of cells *in vitro*, mimicking the single epithelial layer seen *in vivo* such as in the intestinal wall or luminal mammary gland ducts [12]. The physical touching of the cells relays signals, most likely through cell:cell junctions and extracellular structural proteins, that inhibit proliferation. A distinct feature of transformed cells is their ability to proliferate despite the growth inhibitory signals being relayed by their contact with other cells. The result of avoiding these particular growth suppressing signals is a mound of cells that are continuously proliferating, or, *in vivo,* a tumor.

A second form of growth control occurs when cells lose cell:basement membrane contacts. This specific form of apoptosis is called anoikis, and is responsible for the organized cell death of cells that lose such crucial structural contacts [61]. A hallmark of cancer cells is their ability to either escape the constraints of normal tissue structures, or

to degrade and invade the basement membrane. This invasive and migratory ability is coupled to the ability to evade the growth-suppressing signals that would induce anoikis in response to loss of these structural contacts [62].

Enabling Replicative Immortality

Telomeres

Telomeres are protein-DNA complexes that protect the ends of linear chromosomes from being targeted by DNA damage-repair machinery. They consist of a double stranded (DS) stretch of tandem TTAGGG repeats that terminates in a single stranded (SS) G-rich 3' overhang. Telomere associated proteins such as the Shelterin complex coat this SS region to prevent its recognition as DNA damage. Telomeric proteins also aid in the formation of complex structures such as G-quadruplexes and T-loops that cap the end of the chromosome, further disguising the chromosome end from being recognized as DNA damage [63]. Replication of telomeres is a complex and important process. Coordinating the removal of this plethora of proteins with dismantling the complex structures within the telomere is a finely tuned process that serves to maintain the delicate balance between a healthy cell and a prematurely senescing cell. Replication also poses a significant challenge to telomeres because with each round of replication, DNA on the end of the chromosome is lost, due to the inability of the replication machinery to replace RNA primers with DNA on the lagging strand. Aberrant replication can lead to fork pausing and unreplicated telomeres which cause premature senescence and cell death. Loss of telomere-associated proteins can lead to severe physiological defects such as the premature aging seen in Werner syndrome. The

leading strand of the telomere, however, does not experience this shortening due to Okazaki fragmentation. Instead, DNA polymerase replicates off the end of the telomere, leaving behind a blunt end in need of resectioning by an endonuclease to form the classical 3' overhang necessary for proper telomere function. Several reports suggest that this nucleolytic activity is performed by Apollo, a nuclease found both at telomeres and double stranded breaks (DSBs). The function of Apollo requires stimulation by the multifunctional protein, Trf2.

Trf2 is a conserved telomeric protein with a wide repertoire of functions. It is a member of the Shelterin complex that helps disguise the telomere from being recognized as DNA damage, and it functions in the formation and stabilization of the t-loop that buries the single stranded 3' overhang within the double stranded portion of the telomere. Trf2 also recruits Apollo [64], whose endonuclease activity is stimulated by Trf2 both to relieve topological stress during replication [65] and to resection the blunt end of the leading strand telomere into a 3' overhang directly following replication [66,67]. Notably, Apollo's interaction with Trf2 at this significant structure is necessary to prevent major telomere dysfunction [67]. Trf2 has also been recently implicated by various sources in the recognition and repair of DSBs in response to various forms of damaging radiation [68-70]. Trf2 is rapidly phosphorylated in response to DSBs. This phosphorylation has proved necessary for both the transient recruitment of Trf2 to these DSBs and the initiation of DNA repair [70]. Interestingly, in times of telomere crisis, or during the alternative lengthening of telomeres (ALT) pathway, phosphorylated Trf2 has been shown to localize to the telomere [70]. This discovery is not unexpected because many DNA damage-related proteins and telomeric proteins have been found to function

within both contexts [69,71,72]. Apollo is also recruited to DSBs and interacts with ATM [73], a crucial signal transducer in the DNA damage recognition pathway that leads to non-homologous end joining (NHEJ). Trf2 is proposed to hold ATM inactive at telomeres and DSBs. Although controversial, it has been shown that ATM may mediate phosphorylation of Trf2 in response to DNA damage [68,70,74].

The presence of the telomere protects important genomic DNA from this loss, but overall, this shortening of telomeres is part of the aging of the cell and eventually results in naturally occurring senescence. With each round of cell division, a cell must replicate its DNA before separating into daughter cells. The DNA polymerase enzyme responsible for replicating the genome is not able to fully replicate the ends of eukaryotic linear chromosomes, and so with each round of cell division, small amounts of DNA remain un-replicated and are lost. The ends of chromosomes thus are comprised of several kilobases of telomeric G-rich repetitive sequence. Cells are protected from the loss of valuable genomic coding sequence DNA by telomeres because the repetitive telomeric sequences do not contain genes, and telomere shortening during cell division serves as an internal clock that senses when the telomeres have become too short for safe replication and stops the cell from replicating. Because of the "internal clock" function of telomeres, they are thought to play an integral role in the aging process. It is notable that the proteins required for maintenance of the cell's "internal clock" also function within the DNA repair pathway, highlighting the complexity and overlap of Weinberg's defined hallmarks.

An enzyme complex called Telomerase is specifically required for synthesis of telomeric sequence, and in normal cells, this enzyme is not fully expressed. Telomerase

is comprised, minimally, of two subunits: TERT, a reverse transcriptase enzyme, and TER, a large RNA subunit. A portion of TER contains the sequence specific template utilized by TERT for adding nucleotides to uncapped telomeres. This sequence is small, compared to the rest of the RNA strand, which is large, and whose sequence is largely divergent between species. Phylogenetic studies have, however, determined the conserved secondary structure of this RNA subunit, which has been determined to assist in the protein interactions required for Telomerase holoenzyme recruitment to the telomere, as well as further recruitment of other interacting proteins. *In vitro*, these two subunits are all that are required for Telomerase reverse transcriptional activity; however, *in vivo*, other interacting proteins, such as RNA binding proteins and DNA binding proteins involved in recruitment and stabilization of the Telomerase:telomere interaction, are necessary for the enzyme's functionality. Telomerase is, in general, not active or expressed in adult somatic cells; however, certain cell types such as highly proliferative germ line cells, smooth muscle cells, and certain lymphocytes, retain Telomerase functionality. In these cells, Telomerase activity and telomere length are maintained by a phenotypic switch between capped and uncapped states of the telomere. As telomeres are replicated, they become progressively shorter; however, telomere binding proteins such as Apollo, Trf2, and other members of the Shelterin complex vivaciously coat the telomere and help induce the secondary structures that telomeres form, *e.g.* T-loops. The coating action of telomere-associated proteins complements the telomeric DNA secondary structures, forming a protective cap on the chromosome's end. This cap prevents recognition of the DNA ends as DSBs, but also regulates Telomerase activity. Capped chromosomes are less likely to be extended by Telomerase, however, as

replicating cells' telomeres are progressively shortened, they are less likely, and less able to form protective caps, allowing for extension by Telomerase. By switching between two forms—capped and uncapped—telomeres are maintained within upper and lower length limits in Telomerase-positive cells. This regulation is crucial to avoid hyper-extension of telomeres as well as recognition of telomeres as DSBs, both of which are implicated in cancer.

Studies have shown that Telomerase is frequently reactivated in human cancer by various mechanisms. Some reports show that Myc hyper-activation can induce TERT expression, and it is one of the genetic changes found necessary by Weinberg's lab for oncogene cooperation-induced malignant cell transformation of human cells. Another important mechanism of Telomerase reactivation in cancer cells is the re-activation of a telomere maintenance program in order to bypass telomere damage-induced crisis and senescence. Rapidly dividing cells frequently incur DNA damage due to the plethora of replication forks that overwhelm the cell. Even more problematic during replication is the process of dismantling the precarious secondary structures and intricate protein-DNA complexes that cap the ends of the chromosomes. This process can pose problems during normal replication, but during the rapid proliferation seen in cancer, this invariably leads to damaged chromosomes. These telomere damage-induced foci (TIF) trigger a p53 and Rb dependent DNA damage response, but the massive influx of DNA damage typically overwhelms the cell and induces crisis. Crisis is comprised of misguided attempts by the DNA repair machinery to repair the linear ends of chromosomes, having recognized them as DSB. The end result is more damage as telomeres become fused and chromosomes break. This cycle overwhelms the cell and eventually causes senescence. In the absence

of p53 activity or intact DNA damage response pathways, or perhaps even due to the loss of such pathways during crisis, a cell will survive crisis and propagate its gross genetic damage as mutations. A crucial component of this is the re-activation of telomere maintenance machinery. This almost always involves the re-expression of TERT. The mechanisms of Telomerase reactivation are not fully known, but promoter mutations have been found in many cancers, including thyroid, central nervous system and skin cancers, glioblastoma, and over half of bladder cancers [75]. By establishing a telomere maintenance program, the cell is able to avoid normal cellular lifespan limits, *i.e.* achieve immortality. Immortality is one of the first steps in malignant cell transformation, and expressing Telomerase is crucial in this immortalization process. However, primary mouse embryonic fibroblasts MEFs, which express Telomerase, will not grow indefinitely in culture; p53KO MEFs will, indicating that while Telomerase function is involved in malignant cell transformation, it is a component of a complex, dastardly process. It is notable that rodents constitutively express Telomerase, making their cells easier to indefinitely culture *in vitro,* but presenting a major difference when studying malignant cell transformation in rodent models.

Oncogene Cooperation

Weinberg's hallmarks of cancer indicate the multiple processes that occur in cancer, or, with respect to the specific four described above, malignant cell transformation. In molecular biology terms, the requirement of multiple processes to induce cell transformation translates to changes in cell signaling pathways, either by up-regulation of proto-oncogene activity, or loss of either function or expression of tumor suppressors. Although certain proteins, for example Ras, can activate multiple

downstream effector pathways, multiple genetic changes are required for cell transformation.

Historically this was attributed to viral oncogenes that stimulated Telomerase activity, inhibited apoptotic and senescence-inducing machinery, and stimulated proliferation [76-78]. However, Weinberg's landmark paper in 1983 demonstrated the requirement of two oncogenic hits to transform primary fibroblasts, and in so doing, described for the first time the synergistic effect of Ras and Myc signaling to induce cell proliferation without triggering apoptosis or senescence, which occurs when each oncogene is over-expressed individually [79]. Cooperation of Ras and Myc coselects for loss of p53 function. Dysregulation of Ras and c-Myc signaling delineates a classical paradigm of oncogene cooperation, and continued activation of both of these pathways allows cells to bypass senescence, escape apoptosis, and enter into a malignant, hyperproliferative state [79,80].

In 2005, Boehm et al. delineated the nonviral genes necessary for human cell transformation [81]. While Ras and c-Myc transform primary rodent cells, which express Telomerase, human cells maintain more stringent checkpoints and require reactivation of Telomerase, as well as loss of function of tumor suppressors. The specific changes in human cellular pathways originally identified are activation of Ras, Myc, and TERT, along with the loss of function of p53, Retinoblastoma protein, and PTEN [81]. All of these combine to allow replicative immortality, hyperproliferation, and deactivation of cell cycle checkpoints that would not under normal circumstances allow propagation of genetic mutations that drive cell transformation [81-83]. The specific mutations necessary to transform human cells is more complicated than in rodent cells, but the

ultimate result for both is the survival of cells that are no longer governed by pathways that regulate survival and proliferation. Such unrestrained cells are then able to form a tumor.

MicroRNAs

History

MicroRNAs (miRNAs) are an abundant class of regulatory noncoding RNAs that regulate thousands of genes across a vast array of signaling networks and cellular functions [84]. The first miRNAs discovered were lin-4 and let-7 in *C. Elegans* [85,86]. These miRNAs were found to be expressed temporally and play crucial roles in development. The field expanded to the cornucopia of small regulatory RNAs that it is today with over 2500 mature miRNAs identified in humans alone. As research progresses, it has become clear that miRNAs are key regulators in all major cellular processes.

Genomic Location and Biogenesis

miRNAs are present in introns within coding or non-coding transcribed units, or as exons, *i.e.* independent genes. miRNAs may be present as single autonomous miRNA genes; however, over half of human miRNAs are present as miRNA clusters that are co-expressed as polycistronic units [87]. Members of miRNA clusters frequently target similar or related genes, culminating in an increased overall effect on a single pathway or protein complex, rather than broad, unrelated targeting by multiple miRNAs [88-91]. Many miRNA clusters occur because of gene amplifications or insertions, resulting in

multiple copies of identical or similar sequences [92,93]. This in turn results in nearly identical targeting patterns. Similarly, related mRNAs harboring binding sites corresponding to the same miRNA or miRNAs indicates a coordinated mechanism of gene regulation [94].

The majority of miRNAs are processed from introns of mRNA. Experimental evidence shows that processing of miRNAs need not occur only after splicing out of the intron, and that miRNA processing does not affect mature mRNA assembly [95]. Intronic and Exonic miRNAs are transcribed by RNA Polymerase II, and exonic miRNAs are processed similarly to mRNA, in that they are capped with 5'meG and polyadenylated. After miRNAs are transcribed, intra-strand regions of complementarity result in the formation of an imperfect hairpin loop (pri-miRNA). The functional miRNA sequence is present on the arm of this loop [87]. The biogenesis of this primary transcript is what differentiates miRNAs from other small interfering RNA within the RNA-mediated gene silencing field. Rather than cleavage into multiple small regulatory molecules, pri-miRNAs are "cropped" in the nucleus into small hairpin structures called pre-miRNAs. This cropping enzyme is known as the microprocessor, and is comprised of the class II Ribonuclease III enzyme, Drosha, and DGCR8, a double stranded RNA binding protein [96]. Cropping by the microprocessor complex is the initiating event of miRNA processing, as loss of these enzymes leads to accumulation of pri-miRNAs and reduced pre-miRNA levels. Following microprocessor cropping, pre-miRNAs are exported to the cytoplasm via Exportin5/RanGTP [97]. Exportin5 is a nuclear transporter that exports pre-miRNAs in complex with RanGTP. The entire complex is shuttled across the nuclear membrane to the cytoplasm where RanGAP stimulates hydrolysis of

GTP to GDP, and the pre-miRNA is released into the cytoplasm [98]. Once in the cytoplasm, they are cleaved by the class I Ribonuclease III, Dicer, to form imperfect duplexes. The 3' overhang of pri-miRNA is recognized by the PAZ domain of Dicer, and its catalytic RNAse III subunit cleaves the stem loop into a ~22 nt miRNA duplex. In general, the strand with the least stable 3' end base pairing functions as the guide strand while the other strand, often annotated miR*, is degraded. However, both the canonical guide strand as well as the star strand of several miRNAs have been shown to be functional.

Mechanism

To effect gene silencing, the guide strand is loaded into the RNA-induced silencing complex (RISC), which binds target mRNA 3' Untranslated regions (UTRs) in a miRNA seed sequence-directed manner [84]. The RISC is a large enzyme complex whose major functional unit is Argonaute (Ago2). Ago2 is a versatile enzyme involved in RNA-induced silencing. Perfect complementarity between the silencing RNA and the mRNA directs mRNA strand cleavage. This is always the case with siRNA, and occasionally with miRNA-mediated silencing. The mechanism of gene silencing employed by miRNAs, however, revolves around translational repression, in which Ago2 does not cleave target mRNA [84,99]. Indeed, most miRNA targets are not based on perfect base pairing; however, the perfect base pairing dictating mRNA cleavage is not a hard and fast rule. Ago2 has recently been found to associate with P-bodies, and one potential mechanism of gene silencing may involve sequestering of RISC-associated mRNA in P-bodies to down-regulate protein expression [99].

The Argonaute complex is targeted to specific mRNA by base pairing between the miRNA and the 3' UTR of the target mRNA [84]. Nucleotides 2-7 on the 5' end of the miRNA are known as the seed sequence. Complementarity between these bases and target mRNA 3'UTRs results in the specific targeting and regulation of those mRNA. Complementarity is rarely perfect, and binding sites vary in their affinities based on sequence complementarity, as well as the presence of an Adenosine within the mRNA UTR in line with the first nucleotide of the miRNA. Canonical sites include the 7mer-A1, in which there is perfect complementarity between the miRNA seed sequence and the mRNA, as well as an mRNA adenosine at the first nucleotide position; the 7mer-m8 site, in which there is perfect base pairing within the seven nucleotides of the seed sequence, as well as at the 8^{th} nucleotide position, which is not included as part of the canonical seed sequence; and the 8mer site, in which there is perfect complementarity between the seed sequence and the 8^{th} nucleotide, as well as an mRNA adenosine at position 1. Marginal binding sites are 6mers, in which there is complementarity between the miRNA seed sequence and the target mRNA, but no compensatory adenosine at position 1, and no match at the 8^{th} nucleotide. Similarly, other 6mers include complementarity at six contiguous bases that occur only partially within the seed sequence. Targeting is also enhanced by compensatory complementarity that occurs several bases downstream of the seed sequence site. This particularly enhances targeting when seed sequence complementarity contains one or more mismatched bases. Canonical and marginal complementarity sites are summarized in Table 1.

Table 1. Canonical and Marginal miRNA binding sites. Lower strand: miRNA Upper Strand: target mRNA. ORF: Open reading frame, N: complementary nucleotide, NNNNNN: seed sequence, A: position 1 adenosine.

7mer-A1	ORF.....NNNNNNA.....Poly(A) 3′-nnnnnnnnnnnnnnnNNNNNNn-5′
7mer-m8	ORF.....NNNNNNN.....Poly(A) 3′-nnnnnnnnnnnnnnnNNNNNNn-5′
8mer	ORF.....NNNNNNNA.....Poly(A) 3′-nnnnnnnnnnnnnnnNNNNNNn-5′
6mer	ORF.....NNNNNN.....Poly(A) 3′-nnnnnnnnnnnnnnnNNNNNNn-5′ or ORF.....NNNNNN.....Poly(A) 3′-nnnnnnnnnnnnnnnNNNNNNn-5′

MicroRNAs in Cancer

Because of their regulation of such crucial cell signaling pathways, dysregulation of miRNAs contributes to disease, for example cancer [100]. Just as protein coding genes become dysregulated in cancer [82,101], miRNA genes may also become aberrantly over expressed or silenced [100,102]. miRNAs within introns are subject to regulation by changes in the promoter controlling the host gene [95]. Other miRNAs expressed from the genome as independent transcripts may be epigenetically modified at their promoters to affect expression levels [100,103-107]. miRNAs are also subject to gene amplification and deletions [108,109].

By targeting one or more tumor suppressors, miRNAs can function as oncogenic miRNAs (oncomiRs) when aberrantly over-expressed. miR-21 has emerged as a canonical example of an oncomiR. miR-21 is up-regulated in nearly all epithelial cell-derived solid tumors including breast, pancreas, lung, gastric, prostate, colon, head and neck, and esophageal cancers. It is also reported to be up-regulated in hematological malignancies such as leukemia, lymphoma and multiple myeloma. miR-21 is over-expressed in glioblastoma, osteosarcoma, and spermatocytic seminoma. Thus, miR-21 is, as yet, the only gene that is found to be overexpressed in all major classes of human cancers derived from epithelial cells, connective tissues, hematopoietic cells, or nervous cells. Its target genes include regulators of apoptosis, cell cycle progression, growth factor signaling, and proliferation [41]. It has been found to directly target such well known tumor suppressors as Pten and Bcl2. miR-21 overexpression in MCF-7 human breast cancer cells promotes colony formation by directly targeting the tumor suppressor

PDCD4 and suppressing its expression [110]. The role of miR-21 in cancer exemplifies the diverse targeting and dastardly effects of oncomiRs.

In addition to dysregulation of single exonic miRNA genes, entire miRNA clusters may be dysregulated in cancer, for example the miR-17~92 cluster is frequently up-regulated in cancer, specifically, its genomic locus is amplified in diffuse large B-cell lymphoma [108]. It contains several homologous miRNAs that target pro-apoptotic genes such as p21 and Bim [100]. Dysregulation of this coordinated targeting of major cell signaling regulators contributes strongly to the progression of cancer. Overall, the over-expression of oncogenic miRNAs leads to both the targeting and loss of tumor suppressive mechanisms.

In contrast, miRNAs may function as tumor suppressors by targeting oncogenes and regulating their expression. Tumor suppressor miRNAs are often down-regulated in cancer [100,102]. The let-7 family is a well-studied tumor suppressor miRNA family comprised of 12 miRNA family members. These family members have been found to target and regulate expression of the proto-oncogenes Ras and Myc, as well as other oncogenic proteins such as CDK6 and Cyclin D, cementing its role as a tumor suppressor by regulating proliferation and crucial cell cycle promoting enzymes [100]. Expression of the let-7 miRNA family is frequently down-regulated in human lung cancer cases, which also exhibit higher levels of Ras expression [111]. This is in contrast to cancers that express mutated, constitutively active forms of Ras, strongly implicating involvement of let-7 family dysregulation in cancer pathogenesis. Similarly, in 31-64% of medulloblastomas, the most common central nervous system tumor in children, Myc is over-expressed; however, the Myc gene is amplified in only 5-8% of medulloblastoma

cases. The locus containing miR-33b is frequently lost in medulloblastoma, and miR-33b has been shown to directly target Myc and repress its expression in medulloblastoma cell lines [112]. These studies provide evidence for the involvement of miRNAs in regulation of proto-oncogenes, indicating their importance in homeostasis and, in turn, the role their dysregulation plays in human disease.

Figure 1. The miR-200 family

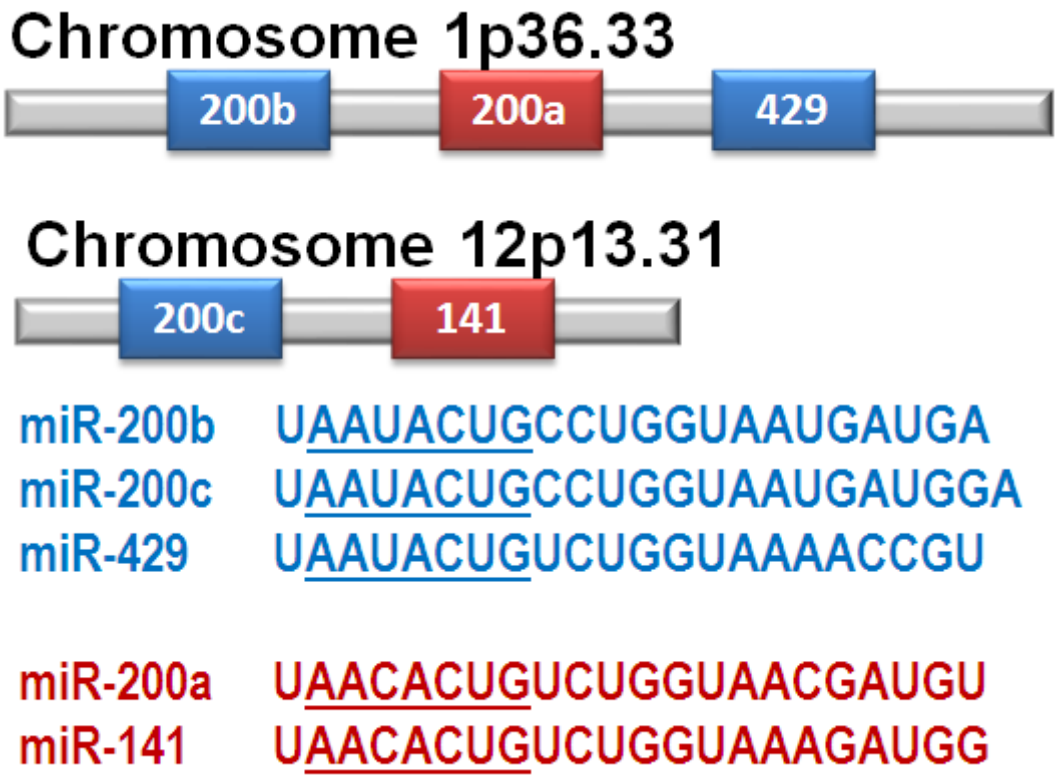

Figure 1. The miR-200 family Upper: Genomic organization of miR-200 family.

Lower: miR-200 family grouped by seed sequence

miR-200a

miR-200a is a member of the miR-200 family. This family is comprised of five members present in the genome as two clusters (Figure 1). The first cluster is located on chromosome 1 at locus 1p36.33 and contains miR-200b, miR-200a, and miR-429. The second cluster is located on chromosome 12 at locus 12p13.31 and contains miR-200c and miR-141. miR-200a and miR-141 contain identical seed-region sequences of AACACUG, and miR-200b, miR-200c, and miR-429 contain identical seed sequences that differ from the other seed sequence by one nucleotide: AAUACUG. Because of these shared seed sequences, miR-200a and miR-141 are predicted by targeting prediction algorithms to regulate the same genes. While the entire family is frequently expressed together and demonstrated to regulate the same targets [113], both clusters do not always have identical functions [114].

Along with its family members, the first and most well studied function of miR-200a is in maintenance of epithelial cell morphology through regulation of the pro-mesenchymal Zeb transcription factors [115,116]. Zeb1 and Zeb2 directly bind the promoter of the epithelial marker E-cadherin to down-regulate its transcription. Likewise, Zeb1/2 directly bind the promoter and stimulate transcription of the mesenchymal marker Vimentin. By regulating these pivotal transcription factors, miR-200a promotes epithelial cell morphology, and inhibits the epithelial-mesenchymal transition (EMT). EMT is the critical initiating step of metastasis and as such, is crucial in the progression from primary tumor to deadly metastasis [117]. As a potent regulator of this process, miR-200a is frequently an inhibitor of metastasis, and thus functions as a tumor suppressor [118,119]. Several studies in cancer cell lines and tumor samples

demonstrate the down-regulation of miR-200a in mesenchymal metastatic cells [105,120,121].

Conversely, emerging studies show cancer cell populations and tumor samples that over-express miR-200a and rely on its over-expression for metastatic colonization, growth, and survival [5,62,122,123]. Many of these studies remain focused on EMT and suppose that maintenance of an epithelial-like morphology by the miR-200 family either accounts for heterogeneity of tumor cells [124], or promotes a reversal of EMT, allowing for metastatic colonization at sites distant from the primary tumor [123]. A study in an isogenic series of breast cancer cell lines with increasing invasiveness and metastatic potential, demonstrated that the cell line that is able to fully invade, metastasize and colonize distant organs from the primary tumor site is the only cell line in the series that expresses the miR-200a family. Targeting of Sec23 by the miR-200 family was demonstrated to affect the cells' secretome and regulate the ability of these cells to invade. The authors concluded that the miR-200a family promotes the ability to colonize distal sites during metastasis, a novel role for the miRNA family often regarded as a tumor suppressor. The authors speculated that promotion of an epithelial phenotype is necessary for establishing metastatic tumors. Another breast cancer cell line study demonstrated that an enhancer specific to the miR-200b~429 cluster, which also includes miR-200a, is responsible for high expression levels of miR-200a in epithelial breast cancer cells [107].

In pancreatic cancer cells, miR-200a has been shown to be hypomethylated and differentially over-expressed, along with its family member miR-200b [105]. Meanwhile, its canonical target, Zeb1, was found to be hypermethylated and silenced,

indicating that the consequences of miR-200a overexpression in this pancreatic cancer model were occurring separately from its role in EMT inhibition via Zeb1 repression. miR-200a and miR-200b were also found at higher levels in the sera of pancreatic cancer patients, highlighting the importance of this miRNA in a clinical disease setting, as well as the potential use of miR-200a and its family members as biomarkers in disease.

A miRNA expression analysis study revealed that miR-200a was expressed at levels higher in epithelial ovarian cancer samples than in benign cysts. Further stratification revealed that miR-200a expression was associated with early stage tumors, and that late stage metastatic tumors expressed significantly lower levels of both miR-200a and E-Cadherin [125]. Overexpression of miR-200a in earlier stages of cancer, rather than the metastatic steps associated with a mesenchymal phenotype, is consistent with both the established role of miR-200a in promoting an epithelial phenotype as well as the newer studies that demonstrate a role for miR-200a in proliferation and growth.

Another novel target of miR-200a is Yap1, a key mediator in the Hippo signaling pathway, and a known regulator of anoikis [62]. miR-200a was found to directly target Yap1 in breast cancer cells, and overexpression of miR-200a resulted in anoikis resistance of human breast cancer cells in animal models. By targeting such a crucial regulator of cell homeostasis, miR-200a was able to enhance metastatic potential of these breast cancer cells.

miR-200a has also been shown to up-regulate PI3K signaling and Akt activity by targeting Fog2, which binds and inhibits the regulatory subunit of PI3K [102]. This study demonstrates a role for miR-200a in promoting cellular growth, unrelated to its role in

maintenance of an epithelial phenotype. miR-200a has a dichotomous role in the promotion and inhibition of different metastatic steps, and has an emerging role in cellular growth and survival.

Hypothesis and Research Strategy

Taken together, it is clear that miRNAs play crucial roles in cancer progression and are frequently dysregulated during all stages of oncogenesis. It is crucial to study the molecular events that occur early in cancer progression in order to enhance detection and diagnostic techniques as well as to aid in cancer prevention. To characterize specific miRNA roles in cancer initiation, the following hypothesis was proposed:

MicroRNAs function as oncogenes in malignant cell transformation.

In this study, a biphasic approach was employed to characterize the role of miRNAs in cancer initiation. We first examined the role of miRNAs grouped according to genomic cluster in four major cancer signaling pathways: AP-1, NF-κB, c-Myc, and p53. Our second step was to screen miRNAs for the ability to transform epithelial cells.

In our first step, we found that miR-200a directly suppresses p53 and inhibits apoptosis. In the second step, we found that miR-200a transforms immortalized rat epithelial RK3E cells, and, when expressed with Ras, miR-200a enhances transformation of immortalized human epithelial MCF10A cells. Further characterization of the mechanism behind miR-200a's ability to transform cells revealed that miR-200a cooperates with the RalGEF effector pathway of Ras to transform MCF10A cells and

induce tumorigenicity. Taken together, these results reveal a new role for miR-200a in malignancy.

CHAPTER II

A SYSTEMATIC SCREEN REVEALS MICRORNA CLUSTERS THAT SIGNIFICANTLY REGULATE FOUR MAJOR SIGNALING PATHWAYS.

Chapter Overview

MicroRNAs (miRNAs) are encoded in the genome as individual miRNA genes or as gene clusters transcribed as polycistronic units. About 50% of all miRNAs are estimated to be co-expressed with neighboring miRNAs. Recent studies have begun to illuminate the importance of the clustering of miRNAs from an evolutionary, as well as a functional standpoint. Many miRNA clusters coordinately regulate multiple members of cellular signaling pathways or protein interaction networks. This cooperative method of targeting could produce effects on an overall process that are much more dramatic than the smaller effects often associated with regulation by an individual miRNA. In this study, we screened 366 human miRNA minigenes to determine their effects on the major signaling pathways culminating in AP-1, NF-κB, c-Myc, or p53 transcriptional activity. By stratifying these data into miRNA clusters, this systematic screen provides experimental evidence for the combined effects of clustered miRNAs on these signaling pathways. We also verify p53 as a direct target of miR-200a. This study is the first to provide a panoramic view of miRNA clusters' effects on cellular pathways.

Introduction

MicroRNAs (miRNAs) are small RNA molecules 20-25 nucleotides in length. Through complementary base pairing, miRNAs bind the 3' UTR of target mRNAs to post-transcriptionally down-regulate gene expression. Originally discovered in *C. elegans*, the first miRNA was found to be a key regulator of development [87,126]; however, subsequent studies have revealed a myriad of roles for miRNAs in virtually all biological processes. Studies highlighting the biological function of miRNAs have emerged alongside studies that reveal the detrimental effects of miRNA dysregulation [127]. Many miRNAs, when lost or over-expressed, become crucial players in the oncogenic process [128,129]. miRNAs may target a wide variety of genes, including those most closely associated with the processes of cancer development, particularly the hallmarks of cancer [41,82]. By inhibiting expression of tumor suppressors, miRNAs may function as oncogenes. Conversely, miRNAs can also exhibit tumor suppressive properties by repressing oncogenes.

miRNAs are transcribed and processed from intronic or intergenic regions, and may be transcribed as individual miRNA or as polycistronic transcripts (clusters) [87,95]. Primary miRNA transcripts (pri-miRNA) are processed into imperfect stem-loop structures called pre-miRNAs by Drosha in the nucleus and then exported into the cytoplasm by Exportin V. These pre-miRNAs are cleaved by Dicer to form mature miRNAs, which are then incorporated into the RNA-induced silencing complex (RISC). Imperfect complementary base-pairing between the miRNA and mRNA directs the RISC to the 3' UTR of target mRNA. This targeting leads to down-regulation of translation of the mRNA, and is often accompanied by a decrease in mRNA levels [87].

Nearly half of all miRNA genes are within 50 kilobases of another miRNA gene [88]. These clusters range from 2 miRNAs, for example miR-200c and miR-141, to as many as 46 miRNAs, as seen in the largest miRNA cluster in primates, Chromosome 19 miRNA Cluster (C19MC) [88,130,131]. miRNAs within clusters frequently contain high sequence homology, particularly within the seed sequence, resulting in identical targets [5,123]. Recent evidence, however, points to clustered miRNAs that target different genes within a specific pathway or protein complex [89,132]. miRNAs are also predicted to target downstream effectors of cellular signaling pathways such as second messengers and transcription factors (TFs) more frequently than upstream ligands and receptors or housekeeping and structural genes [133]. TFs are key players in cell signaling pathways. By responding to a plethora of extra- or intra-cellular stimuli and regulating transcription of the many genes necessary for a cellular response, TFs act as crucial cell signaling hubs. Dysregulation of major TFs is often a key event in oncogenesis [134]. Such TFs include AP-1, NF-κB, c-Myc, and p53 [57,135-137]. Many individual miRNAs target these pathways [138-141], but little data exists regarding the full effect of miRNA clusters. While it is clear that miRNA clusters are frequently predicted to target specific cell signaling pathways, no experimental evidence based on systematic screening has been provided. In this study, we intend to address these deficiencies by analyzing the role of 366 human miRNAs as clusters in these four major signaling pathways using an existing genetic library [142].

Experimental Procedures

miRNA Screen

The method involves a published lentiviral-based miRNA genetic library that contains a large number of human miRNA minigenes [142]. To screen miRNAs that specifically target TFs of interest, we utilized luciferase constructs plus the miRNA library. For instance, pTRF-p53-Luc (Systems Biosciences) contains a firefly *luciferase* gene (*luc*) under the control of a minimal CMV promoter. This promoter is only activated when p53 binds to the p53-specific transcription response elements (TREs), eight tandem repeats of ACATGTCCCAACATGTTGTCG. Similarly, TRE constructs for the other TFs are as follows: pTRF-NF-κB-Luc: four repeats of GGGGACTTTCC; and pTRF-AP1-Luc: four repeats of TCCGGTGACTCAGTCAAGCG. c-Myc activity was measured using an E2F2-Luc reporter vector consisting of the E2F2 promotor with four distinct E-boxes, CACGTG [143]. The parental vector, pSIF[142], substituted for the miRNA construct, serves as a normalization control for miRNA expression. *Rluc* from pRL-TK (Promega) is used to normalize transfection efficiency and total protein synthesis.

Cell Culture Experiments

293T and H1299 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM media supplemented with 10% FBS and antibiotics at 37°C with 5% CO_2. Lipofectamine LTX (Invitrogen) was used for all transfections according to manufacturer's instructions. Luciferase assays were conducted using the Dual-Glo® Luciferase Assay System (Promega) 48 hours post-transfection in 96-well plates.

Relative Luciferse Units (RLU) were normalized to Renilla luciferase expression. The parental vector pSIF was used to normalize plate-to-plate variation. Apoptosis was measured using an ApoTarget™ Annexin-V FITC Apoptosis Kit (Invitrogen, Carlsbad, CA) as described previously [142]. Briefly, transfected cells were washed twice with PBS, resuspended in Annexin-V binding buffer, and then incubated in Annexin-V FITC and Propidium Iodide Buffer in the dark for 15 minutes at room temperature. Stained cells were then analyzed on an LSR II flow cytometer (BD Biosciences) using FL1 (FITC) and FL3 (PI) lines. Cell cycle was analyzed as described [144]. Experimental groups were analyzed in triplicate, and data represent three independent trials.

Western Blot

Total protein was isolated from cells in 6-well plates using M-PER mammalian protein extraction reagent (PIERCE, Rockford, IL). Protein concentration was measured using a BCA kit (PIERCE, Rockford, IL). 30-50 µg of protein were separated on 12% to 15% Bis-Tris polyacrylamide gels (Bio-Rad, Hercules, CA) and then transferred to PVDF membranes (Bio-Rad). Protein membranes were incubated in blocking buffer (1× Tris-buffered saline, pH 7.5, 5% nonfat dried milk, 0.05% Tween 20) for 2 hours at room temperature, followed by anti-p53 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-β-actin antibody (Sigma-Aldrich) overnight at 4 ^{0}C. The membranes were washed with 1× Tris-buffered saline containing 0.05% Tween 20, incubated with horseradish peroxidase-linked goat anti-mouse Ig (Santa Cruz) or goat anti-Rabbit Ig (Cell Signaling) for 1 hour at room temperature, washed, and visualized with the SuperSignal West Dura/ Femto Chemiluminescent Substrate kit (PIERCE).

Statistical Analysis

Boxplots of the luciferase results for all clusters were plotted to show what the observations look like for each end point variable (AP-1, NF-κB, c-Myc, or p53). For each end point, residual plots indicated that the observations with log-transformation are more likely to be normally distributed and have equal variances among different clusters. For each variable, one way analysis of variance (ANOVA) was applied to examine whether the observations at log-scale from different clusters are significantly different from the overall means at log scale. Residual plots indicated that the log-transformed responses are more likely to be normally distributed and have equal variances among different clusters. The Fisher's least significant difference tests were applied to examine which clusters are significantly different from the overall least square mean [145]. Based on the analytic results, we painted the boxplots red for the clusters with significantly high readings (observations), and green for the clusters with significantly low readings. The clusters with a pink diamond are significantly different from the overall mean (Figures 3-6).

Results

miRNA Library Screening

We used an established TF luciferase-based screen to determine miRNAs affecting pathways that regulate TF activity (Figure 2). 293T cells were transfected in triplicate with a plasmid containing a firefly luciferase gene under the control of a

minimal CMV promoter along with a second plasmid containing a member of our miRNA library [142]. Transcription response elements (TRE) corresponding to each TF were placed upstream of the promoter. A third plasmid containing a Renilla luciferase gene driven by the HSV-TK promoter served as a normalization control. Luciferase gene expression was measured with a luminometer to determine which miRNA expression resulted in inhibition or promotion of TF activity. Luciferase expression was normalized to Renilla luciferase to yield Relative Luminescence Units (RLU) for each miRNA before being normalized to the parental vector. This approach has been used to identify individual miRNAs in the p53, NF-κB, and c-MYC pathways [142,144,146]. To analyze the impact of miRNA clusters in reporter activities, mean RLU values for each cluster were calculated and normalized to the mean values of all miRNAs. This allowed us to determine statistical significance of miRNA regulation of specific TFs when miRNA data were stratified into clusters compared to baseline overall miRNA effects. For each TF, clusters with values significantly lower than the overall cluster mean were identified as down-regulators of the specified TFs. Clusters with values significantly higher than the cluster mean were deemed up-regulators of the specified TF.

Figure 2: Schematic of luciferase-based microRNA screen.

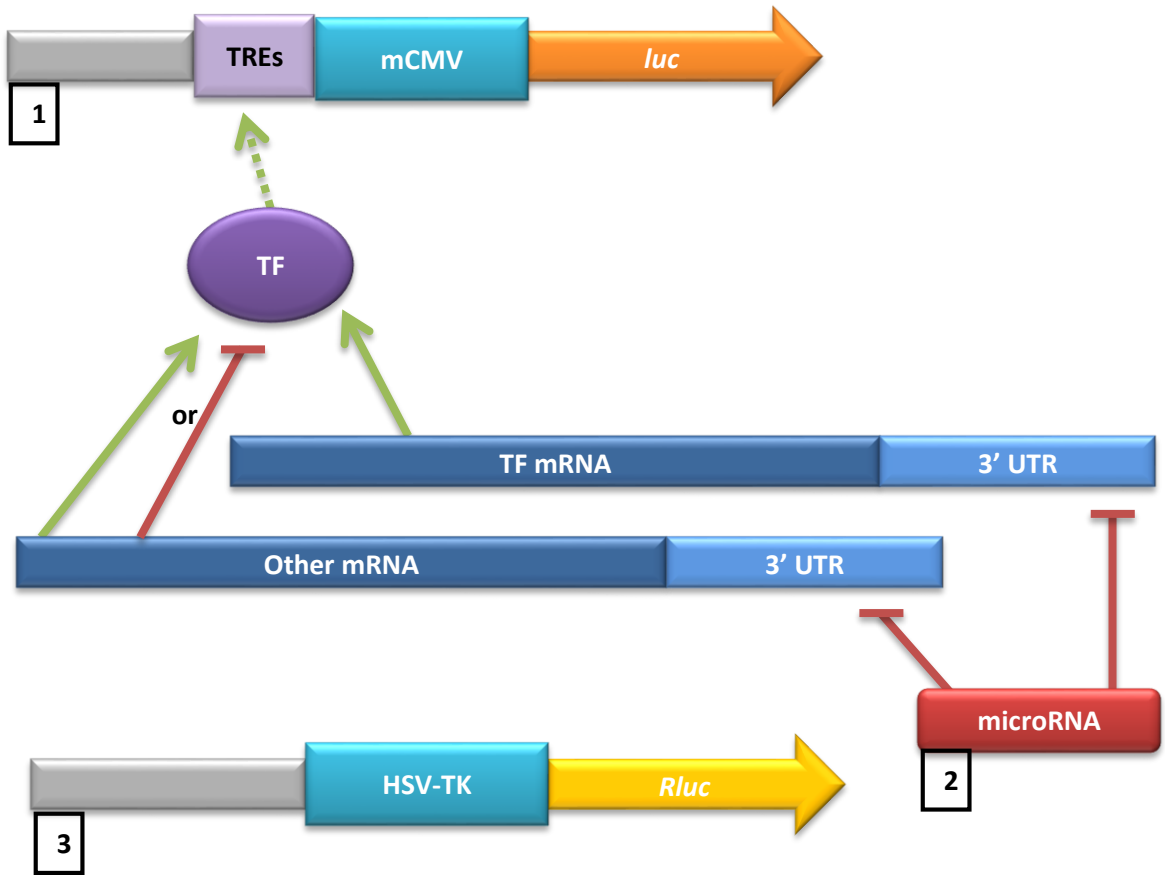

Figure 2: Schematic of luciferase-based microRNA screen. 293T cells were co-transfected with: 1) a vector containing a luciferase gene under control of regulatory elements recognized by AP-1, NF-kB, p53, or c-Myc; 2) a member of our microRNA library, and 3) a Renilla luciferase vector for normalization of luciferase values. Following transfection, cells were analyzed by luciferase assay to measure the effects of miRNA regulation of TF-driven luciferase expression.

TRE: Transcription factor regulatory element, TF: Transcription factor, luc: luciferase, Rluc: Renilla luciferase, UTR: Untranslated region

AP-1

Activating protein 1 (AP-1) is a dimeric TF consisting of Jun, Fos, or Activating TF (ATF). Combinations of these subunits allow for hetero- and homo-dimerization, resulting in differing DNA recognition and functions of AP-1. The TRE used in this screen is predominantly recognized by the cJun-cFos as well as cJun homodimers to a lesser extent [147,148]. AP-1 is activated in response to many signals such as stress, bacterial and viral infections, cytokines, growth factors, and oncogenic stimuli. Post-translational regulation occurs through interactions with other TFs, proteolytic turnover, and phosphorylation [147,149]. Data from the miRNA screen point to five miRNA clusters that yield an overall negative effect on AP-1 directed transcription (Figure 3 and Table 2). These clusters may target genes that are upstream of the pathway directly regulating AP-1 turnover, or genes within signaling cascades that lead to AP-1 activation. Five clusters were found to have an activating effect on AP-1 transcriptional activity. One such noteworthy cluster is 10a~196a. Studies have established a pro-proliferative role for this cluster in multiple cancers including pancreatic cancer and acute myeloid leukemia [150-152]. This role is consistent with our finding that it positively regulates activation of a TF known for its role in promoting proliferation, particularly in the context of cancer [147].

Figure 3. Boxplot showing logarithmic values of AP-1-mediated luciferase expression for microRNAs grouped according to cluster.

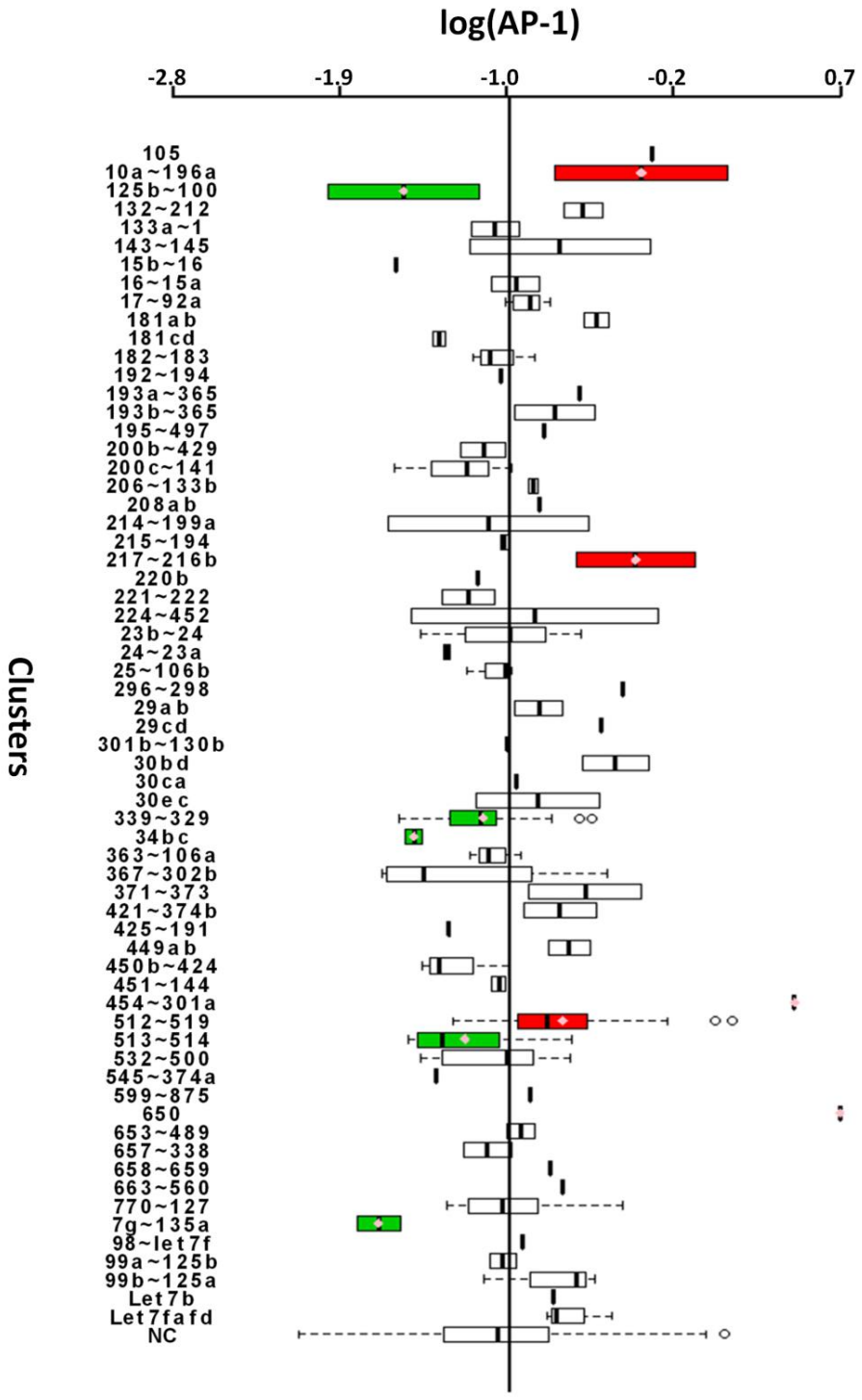

Figure 3. Boxplot showing logarithmic values of AP-1-mediatedluciferase expression for microRNAs grouped according to cluster. Clusters that yielded values significantly different from the overall mean are marked with a pink diamond and annotated in Table 1. MicroRNA clusters that caused significant up-regulation of AP-1-driven luciferase gene expression are highlighted in red. MicroRNA clusters that down-regulated this expression are marked in green. 293T cells were transfected with the indicated miRNA in triplicate. NC: non clustered miRNAs.

NF-κB

NF-κB is a TF that consists of Rel protein dimers that bind κB sites in the promoters of target genes to regulate transcription. The Rel family of proteins consists of five members: p100 and p105 which are proteolytically processed into p50 and p52, respectively, and RelA, RelB, and c-Rel, which do not require proteolytic processing. The TRE in this screen is specifically recognized by the heterodimer made up of p50 and RelA, which is the most abundant form of NF-κB in most cells. This heterodimer is held inactive in the cytoplasm by inhibitors of κB (IκB) [139]. The classical pathway of NF-κB activation is triggered by exposure to bacterial or viral infections and pro-inflammatory cytokines such as TNF-α. These signals go through the Toll-like receptor (TLR) to activate IκB kinases (IKK) which phosphorylate IκB, targeting it for ubiquitin-mediated degradation. NF-κB is released and translocates to the nucleus to promote transcription [139,153]. One of the major functions of NF-κB is inhibition of apoptosis, though its role in cancer development and progression is cell-type dependent. Suppression of NF-κB activation abrogates transcription of critical anti-apoptotic genes such as c-FIIP, cIAP1, cIAP2, and BCL-X_L [153]. This screen revealed seven clusters that negatively regulate NF-κB-mediated transcription (Figure 4 and Table 2). Inhibition of NF-κB signaling implies a potential anti-inflammatory role for these clusters. Five clusters were found to up-regulate NF-κB activity. Among these is cluster 454~301a. miR-301a has recently been implicated as an NF-κB inducer in pancreatic cancer [142]. Cluster 99b~125a was also found to up-regulate NF-κB activity. A recent study found that miR-125a and miR-125b directly target TNFAIP3, a ubiquitin editing enzyme that negatively regulates NF-κB activity by disrupting the activation of IKK [120].

Figure 4. Boxplot showing logarithmic values of NF-κB-mediated luciferase expression for microRNAs grouped according to cluster.

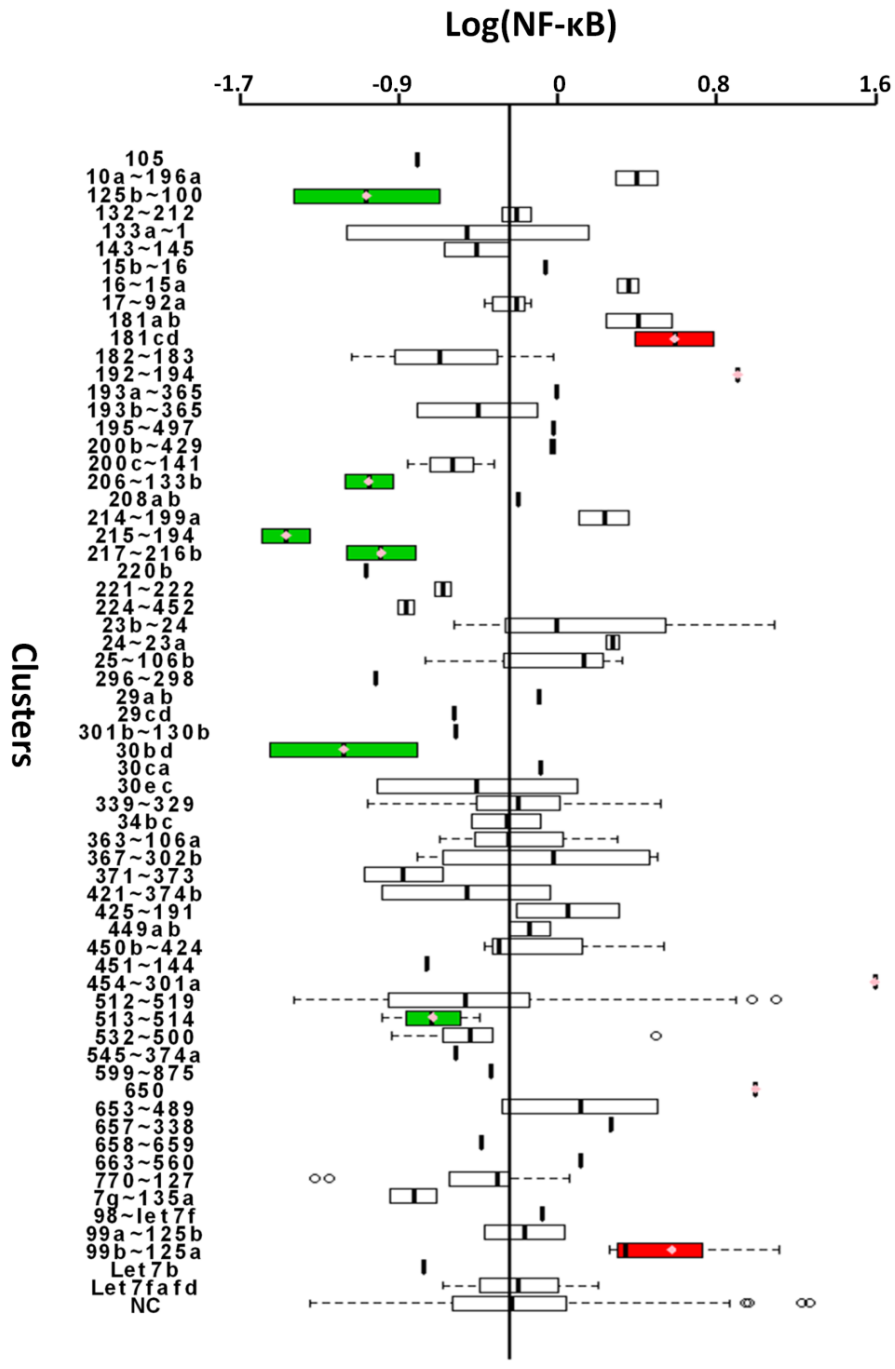

Figure 4. Boxplot showing logarithmic values of NF-κB-mediated luciferase expression for microRNAs grouped according to cluster. Clusters that yielded values significantly different from the overall mean are marked with a pink diamond and annotated in Table 1. MicroRNA clusters that caused significant up-regulation of NF-κB-driven luciferase gene expression are highlighted in red. MicroRNA clusters that down-regulated this expression are marked in green. 293T cells were transfected with the indicated miRNA in triplicate. NC: non clustered miRNAs.

c-Myc

c-Myc is a TF that heterodimerizes with Max to bind E-boxes within the promoters of its target genes [154]. It is a multifunctional protein that regulates a wide variety of cellular processes such as cell cycle progression, growth and metabolism, differentiation, and apoptosis [134]. Because of its function in positively regulating processes that contribute to tumorigenesis, Myc is a proto-oncogene. Aberrant expression of Myc is seen in the majority of cancers, resulting from genomic amplification, or lack of negative regulatory pathways [154]. Our screen returned four miRNA clusters that down-regulate Myc-induced transcription (Figure 5 and Table 2). Notably, Cluster 512~519a negatively regulates Myc-mediated transcriptional activation. Also striking was up-regulation of Myc-mediated transcription by the entire miR-200 family (Clusters 200c~141 and 200b~429). In addition, we have confirmed miR-33b as a bona fide c-Myc regulator [146].

Figure 5. Boxplot showing logarithmic values of c-Myc-mediated luciferase expression for microRNAs grouped according to cluster.

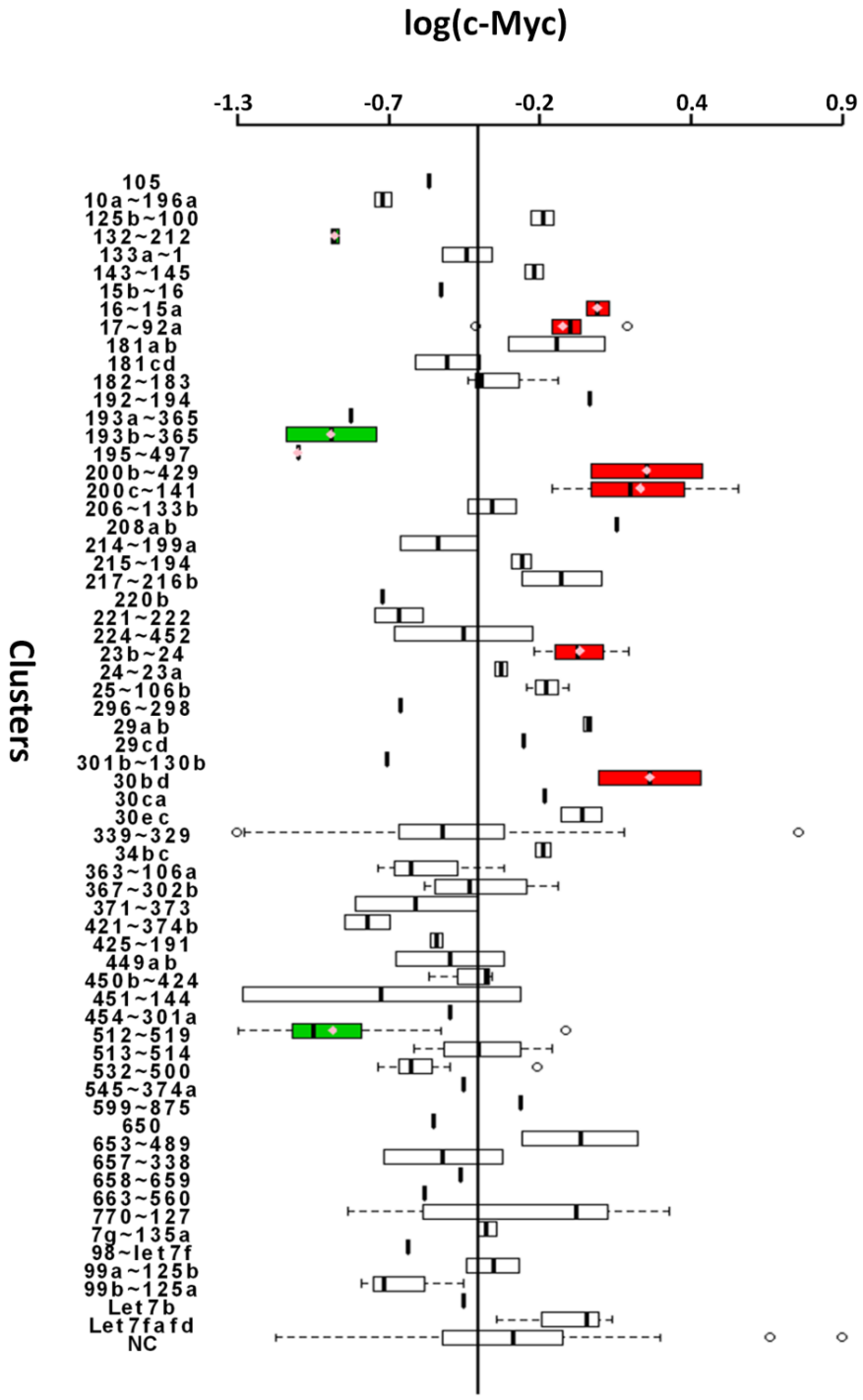

Figure 5. Boxplot showing logarithmic values of c-Myc-mediated luciferase expression for microRNAs grouped according to cluster. Clusters that yielded values significantly different from the overall mean are marked with a pink diamond and annotated in Table 1. MicroRNA clusters that caused significant up-regulation of c-Myc-driven luciferase gene expression are highlighted in red. MicroRNA clusters that down-regulated this expression are marked in green. 293T cells were transfected with the indicated miRNA in triplicate. NC: non clustered miRNAs.

p53

p53 has long been known as the guardian of the genome. Its transactivational functions are well studied and include induction of proapoptotic genes like Puma, Noxa, and Bax as well as cell cycle regulatory proteins such as p21 [49]. p53 is maintained at low basal levels in the cell by its inhibitory protein, Mdm2 [155]. Mdm2 inhibits p53 function by acting as an ubiquitin ligase to target p53 for proteasomal degradation as well as by binding and blocking the DNA binding domain of p53, inhibiting its activity as a TF. Upon detection of DNA damage, oncogene hyperactivation, or other cellular stresses, p53 is phosphorylated on its N-terminus, which blocks inhibition by Mdm2 and promotes its binding to p53 response elements. In our screen, we found 7 miRNA clusters that significantly up-regulate p53-mediated luciferase expression (Figure 6 and Table 2). Among these is Cluster512~519a, also known as C19MC. Comprised of 46 pre-miRNAs, it is the largest miRNA cluster conserved in primates. It is an imprinted gene, and the paternal allele is expressed specifically in the placenta [130,156]. This tissue specificity is noteworthy in the context of its up-regulation of p53 activity. Enhanced apoptosis and increased p53 expression in the placenta during pregnancy are associated with fetal growth restriction, preeclampsia, intrauterine growth restriction, and HELPP syndrome [157,158]. Our screen implicates a role for this miRNA cluster within the tightly regulated process of developmental or pathological apoptosis. Among the 5 clusters that down-regulated p53-mediated luciferase expression is 200b~429, one of two clusters that comprise the miR-200 family (Figure 6 and Table 2). The miR-200 family is largely known as tumor suppressive because of its inhibition of the epithelial-mesenchymal transition (EMT) through direct targeting of Zeb1 and Zeb2 TFs [116,159].

Our data support an oncogenic role for this miR-200 family and we performed ensuing studies to examine the role of miR-200a in the p53 pathway (see below). Cluster 25~106b also significantly down-regulated p53 activity, and we noted that miR-25 has been verified to directly target p53 [144].

Figure 6. Boxplot showing logarithmic values of p53-mediated luciferase expression for microRNAs grouped according to cluster.

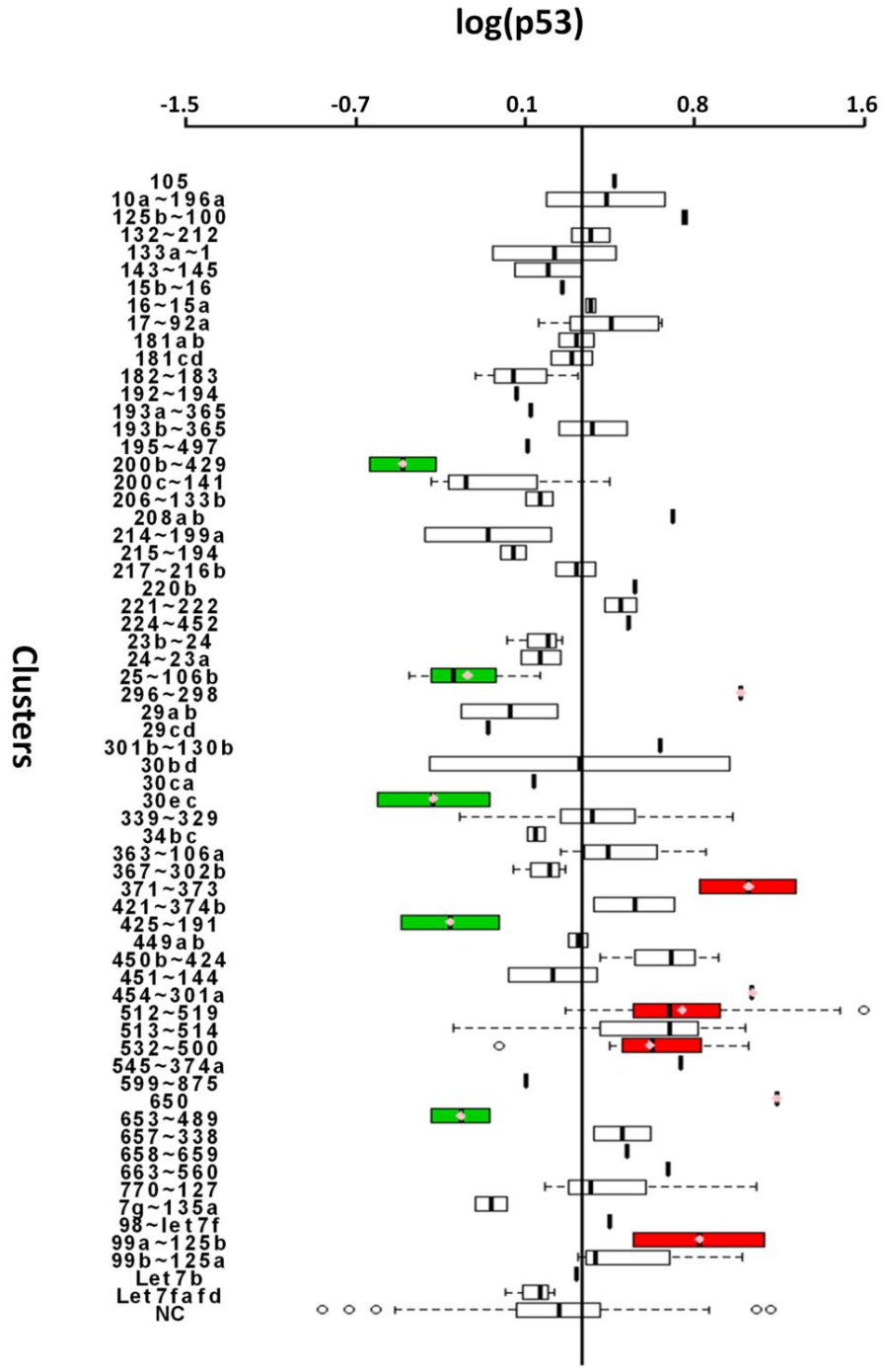

Figure 6. Boxplot showing logarithmic values of p53-mediated luciferase expression for microRNAs grouped according to cluster. Clusters that yielded values significantly different from the overall mean are marked with a pink diamond and annotated in Table 1. MicroRNA clusters that caused significant up-regulation of p53-driven luciferase gene expression are highlighted in red. MicroRNA clusters that down-regulated this expression are marked in green. 293T cells were transfected with the indicated miRNA in triplicate. NC: non clustered miRNAs.

Table 2. Top microRNA clusters that significantly modulate reporter expression.

	Inhibiting Clusters	Difference from mean	p-value	Activating Clusters	Difference from mean	p-value
AP-1	Let7g~135a	-0.78068	5.14E-03	512~519a	0.183747	1.78E-02
	125b~100	-0.65192	1.92E-02	217~216b	0.567326	4.14E-02
	34bc	-0.59466	3.26E-02	10a~196a	0.596606	3.20E-02
	513~514	-0.3289	3.05E-02	454~301a	1.399706	3.86E-04
	339~329	-0.23763	3.22E-03	650	1.636268	3.57E-05
NF-κB	215~194	-1.17405	1.90E-04	99b~125a	0.822977	2.81E-03
	30bd	-0.87412	9.13E-03	181cd	0.839874	1.22E-02
	125b~100	-0.75574	2.40E-02	192~194	1.167321	1.33E-02
	206~133b	-0.74421	2.62E-02	650	1.262463	7.49E-03
	217~216b	-0.68132	4.17E-03	454~301a	1.876999	7.89E-05
	513~514	-0.41319	2.39E-02			
c-Myc	195~497	-0.66248	3.15E-02	17~92a	0.305999	1.74E-02
	193b~365	-0.54136	1.35E-02	23b~24	0.368437	4.00E-02
	512~519a	-0.52607	8.16E-17	16~15a	0.432454	4.80E-02
	132~212	-0.52607	1.63E-02	200c~141	0.589838	1.08E-03
				200b~429	0.312486	5.25E-03
p53	200b~429	-0.83273	6.44E-04	532~500	0.295012	2.61E-02
	30ec	-0.69189	4.46E-03	512~519a	0.444204	1.78E-10
	425~191	-0.61609	1.12E-02	99a~125b	0.522585	3.12E-02
	653~489	-0.56796	1.93E-02	296~298	0.712016	3.71E-02
	25~106b	-0.53544	7.25E-03	371~373	0.747797	2.14E-03
				454~301a	0.766234	2.49E-02
				650	0.877633	1.03E-02

miR-200a

The miR-200 family is comprised of two clusters (Figure 1). Cluster 200b~429 is located on chromosome 1 and contains miR-200a, miR-200b, and miR-429. Cluster 200c~141 is located on chromosome 12 and contains miR-200c and miR-141. The most thoroughly studied function of the miR-200 family is inhibition of EMT. EMT is characterized by cellular acquisition of mesenchymal morphology and phenotypes and is largely associated with tumor metastasis. In particular, the TFs Zeb1 and Zeb2 are responsible for repressing transcription of E-cadherin and other epithelial markers to promote EMT [115,160]. The miR-200 family directly targets the 3' UTRs of Zeb1 and Zeb2 to inhibit their expression, and Zeb1 and Zeb2, on the other hand, bind the promoters of both miR-200 family clusters to reciprocally inhibit their transcription [124]. This miRNA family inhibits proliferation as well as EMT through its targeting of Zeb1 and Zeb2 [161]. Recently, however, new tumor-suppressor targets of the miR-200 family have been discovered, suggesting this miRNA family may have a pro-proliferative function [5,102,162]. In addition, a recent study has investigated the miR-200 family's promotion of an epithelial morphology in the context of a mesenchymal-epithelial transition, thus promoting metastatic colonization, and providing further evidence for an oncogenic role for this miRNA family [123].

Our screen revealed a p53-suppressing role for cluster 200b~429, which contains miR-200a, miR-200b, and miR-429. TargetScan predicts a miR-200a binding site in the 3' UTR of p53 (Figure 7A). This predicted target is conserved between humans and chimpanzees. To determine direct targeting of p53 by miR-200a, a luciferase assay was performed using constructs with a wild type 3' p53 UTR (WT) or a 3' UTR with a

mutated miR-200a binding site (Mut) downstream of a luciferase reporter gene. Luciferase assay was performed to measure differential reporter expression resulting from this binding site mutation in p53-null H1299 cells (Figure 7B). Compared to empty vector control, miR-200a caused a significant reduction in WT construct luciferase expression. This reduction of expression was not seen in cells with the mutant 3' UTR. This suggests that miR-200a directly targets the 3' UTR of the human p53 gene. Western blot was performed to determine the effects of miR-200a on p53 protein levels. H1299 cells were transfected with miR-200a or its empty vector control, and either p53 coding sequence with a wild type 3' UTR (WT) or that with a mutated miR-200a binding site in its 3' UTR (Mut). Compared to the control, miR-200a caused a significant down-regulation of p53 protein levels in cells with a WT 3' UTR, but not those with a Mut 3' UTR (Figure 8A). These results show that direct targeting of the p53 3' UTR by miR-200a down-regulates p53 at the protein level. To determine the functional significance of p53 suppression by miR-200a, we analyzed apoptosis and cell cycle in response to miR-200a over-expression in H1299 cells containing a p53 expression cassette with either WT or Mut 3' UTR. We found that re-expression of p53 in H1299 cells led to significant cell apoptosis and cell cycle arrest at the G1 phase (G1 arrest) even in the absence of DNA damage (Figure 8B and 8C), in agreement with previous reports [163-165]. miR-200a significantly decreased apoptosis in H1299 cells with the WT p53 construct (Figure 8B). Apoptosis was unaffected in cells containing the Mut p53 construct. In addition, G1 arrest was also inhibited by miR-200a compared to the vector control (50.7% *versus* 60.1, $P \leq 0.05$) only when the exogenous p53 had a WT 3' UTR (Figure 8C). Taken together, these results provide a new mechanism of oncogenic action for miR-200a. By directly

targeting the 3’ UTR of p53, miR-200a down-regulates p53 protein expression, resulting in a significant reduction in apoptosis and G1 arrest.

Figure 7. miR-200a directly targets the human p53 gene.

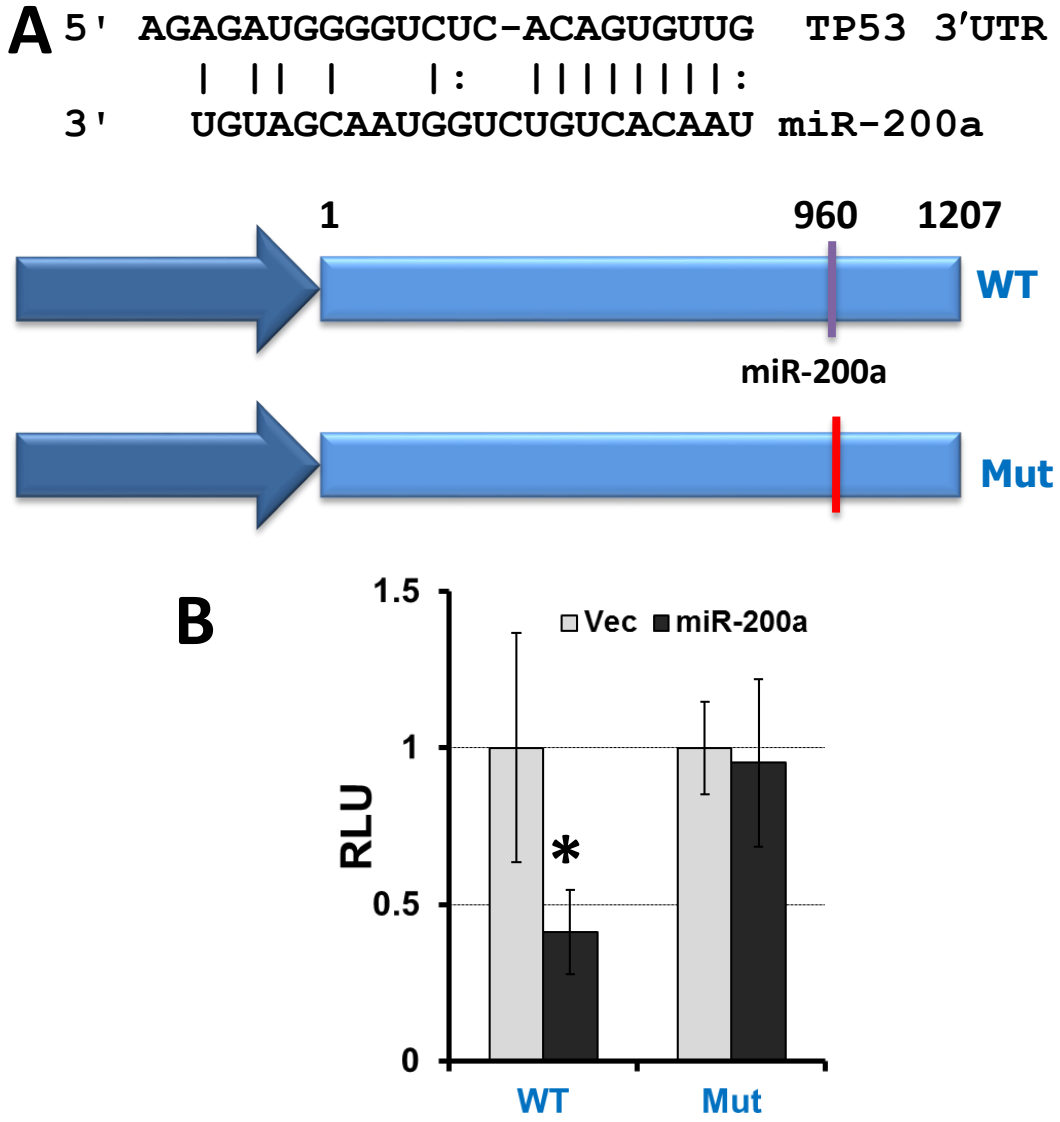

Figure 7. miR-200a directly targets the human p53 gene. (A) Schematic representation of miR-200a: p53 3'UTR. Top: seed sequence base paring between miR-200a and the 3'UTR of p53 mRNA. Bottom: p53 constructs with the wild type miR-200a binding site (WT) or a mutated miR-200 binding site (Mut) in the 3'UTR. (B) A reporter assay to determine whether the p53 3'UTR is targeted by miR-200a. Y axis denotes relative luminescent units (luc/Rluc) in H1299 cells expressing WT or Mut p53 3'UTR constructs and miR-200a.

Figure 8. miR-200a downregulates p53 protein expression and inhibits apoptosis.

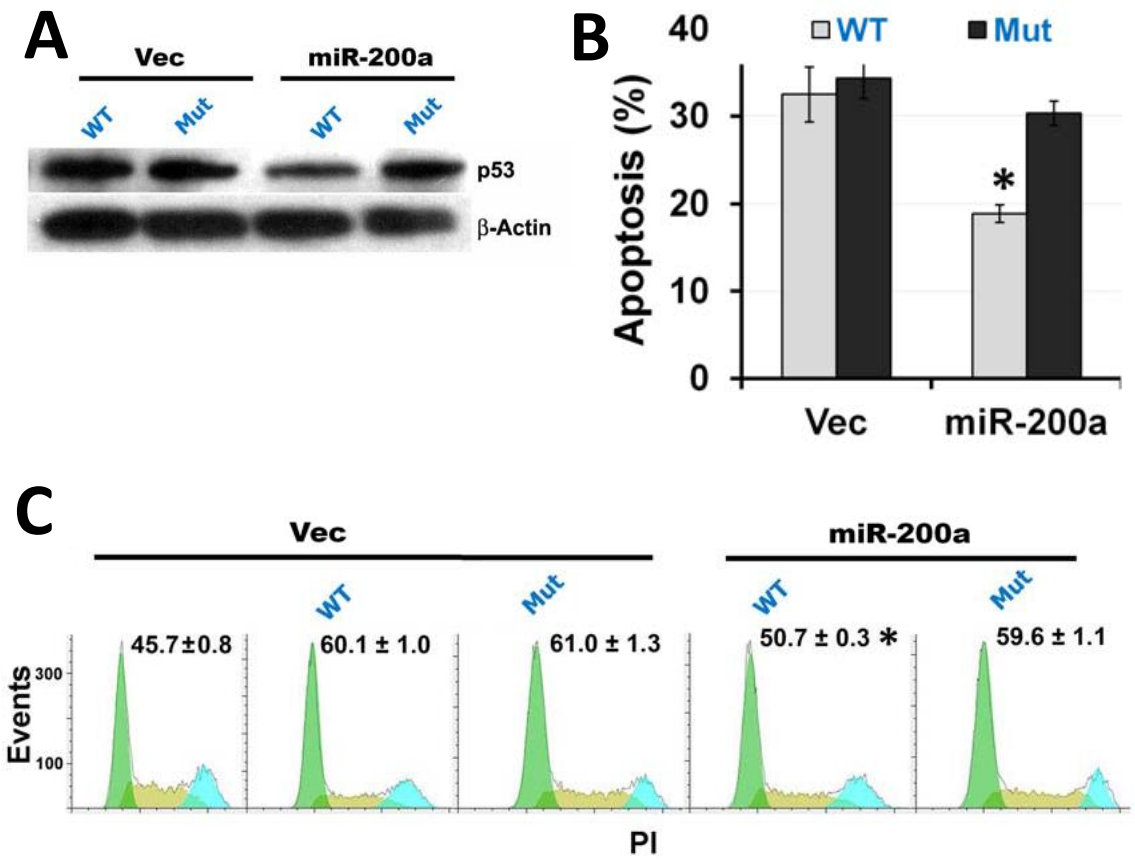

Figure 8. miR-200a downregulates p53 protein expression and inhibits apoptosis.

(A) Western blotting analyses of H1299 cell extracts. H1299 cells were transfected with miR-200a and WT or Mut p53 3'UTR constructs. (B) Apoptosis assay of H1299 cells transfected as in C. E. Cell cycle analysis of H1299 cells transfected as in (C) The Y axis denotes events (the number of cells) and the X axis denotes the emitted fluorescent light of the DNA dye (PI), that is, DNA content. Values like indicate the percentages of cells in the G1 phase with standard error of the mean. *P≤0.05 with n=3.

Discussion

Most miRNA studies revolve around finding novel targets of single miRNAs, yet half of all miRNAs are co-expressed as clusters [88]. Most of the miRNAs within clusters are likely to be transcribed as a whole unit, so these coexpressed miRNAs shall be investigated together for their biological and pathological function. By stratifying our screen of miRNAs that target TF signaling pathways into miRNA clusters, we were able to collect data that describes the effects of an entire miRNA cluster on a signaling pathway culminating in regulation of a major TF. Several mechanisms exist behind multiple coexpressed miRNAs regulating a wide variety of targets, thus the *modus operandi* of miRNA clusters is not fully understood. Individual miRNAs are predicted to, and have been found to target a wide array of genes and affect multiple cellular functions [41,166]. Based solely on this, a miRNA cluster could potentially target any and all cell signaling pathways. However, bioinformatics, as well as an increasing number of molecular biology approaches have parsed out a much more ordered pattern of target suppression by miRNA clusters [89-91,133]. miRNA clusters are predicted to target interacting members of protein complexes [89], multiple proteins within a single pathway or biological process [90,133], or multiple clustered miRNAs may simultaneously target and strongly repress a single key regulator of a pathway [167]. In this way, rather than the small scale fine tuning of hundreds of targets [87], a cluster would provide a large combinatorial impact on an entire biological process or pathway. In miRNA clusters comprised of closely related family members, for example both clusters of the miR-200 family or many members of C19MC, similar or same seed sequences provide a clear mechanism for multiple cluster members to target identical sets

of genes [123]. This combinatorial system of multiple clustered miRNAs regulating an entire system does not preclude the presence of a single major effector miRNA within a cluster regulating a specific pathway [90]. Xu and Wong propose this mechanism for cluster mmu-miR-183-96-182, which is predicted to control 12 signaling pathways. miR-96 is predicted to target the majority of the genes within these pathways, indicating it as the major effector miRNA of this cluster [90]. Cluster 17~92a, is a well-studied oncogenic cluster whose most oncogenic member, miR-19, has been experimentally validated as the most active player in the oncogenic process [168]. The 25~106b cluster, an ortholog of 17~92, significantly down-regulated p53 reporter activity. We have verified that miR-25 directly targets the p53 gene [144]. It is noted that each miRNA in the 25~106b cluster is upregulated in multiple myeloma, a cancer with little p53 mutation [169]. miR-25 is the most significantly upregulated miRNA in multiple myeloma, and its expression is inversely correlated with p53 mRNA levels, suggesting that miR-21 upregulation could be responsible for p53 inactivation in cancers without p53 mutation [144]. How other members of the 25~106b cluster upregulate p53 transactivational activities, however, remains elusive. Similarly, we have verified that miR-301a up-regulates NF-κB by inhibiting Nkrf [142], yet the role of miR-454 (the other member of the 301a~454 cluster) in the NF-κB pathway needs further investigation.

We experimentally pursued the down-regulation of p53 activity by cluster 200b~429 and demonstrated the direct targeting of p53 by miR-200a. miR-200a and its orthologs, miR-200b, miR-200c, and miR-141 were first found tumor suppressors as they inhibit EMT through targeting Zeb1 and Zeb2 [115,116]. Recently, studies have begun investigating the role of miR-200a in the reverse process, mesenchymal-epithelial

transition, which enhances the metastatic potential of cancer cells [123]. Down-regulation of p53 and subsequently apoptosis and G1 arrest by miR-200a illuminates a novel function for this miRNA. This, coupled with emerging studies that show evidence for an oncogenic function for miR-200a and its family members [5,102,162], provides a strong foundation for the oncogenic potential of miR-200a.

While our screen provides new, preliminary experimental data regarding the effects of miRNA clusters on TF pathways, there are several limitations that must be considered. First, this screen was performed with a single cell line (293T), which does not account for any bias that may arise from tissue or cell-type specific targeting. Second, our screen may return false negatives or positives because other cellular changes may compromise the luciferase reading. For example, miR-34c is a tumor suppressor, identified as such by its direct targeting and repression of c-Myc [168]. However, cluster 34bc was not found in this screen to down-regulate c-Myc activity. Finally, single transient transfections of miRNA-containing plasmids do not replicate endogenous miRNA levels, which may be subject to further regulation when the entire cluster is expressed. This may bias the screen toward miRNAs that are expressed at low endogenous levels. These limitations can be mitigated by further experimental validation using multiple cell lines or performing miRNA inhibition experiments [144,146].

To summarize, this study provides a panoramic view of miRNA clusters' effects on AP-1, NF-κB, c-Myc, and p53 signaling pathways and will serve as a base for thoroughly interrogating the contribution of miRNAs in these pathways.

CHAPTER III

THE ROLE OF MIR-200A IN MAMMALIAN EPITHELIAL CELL TRANSFORMATION

Chapter Overview

Cancer is a multistep disease that begins with malignant cell transformation and frequently culminates in metastasis and death. MicroRNAs (miRNAs) are small regulatory 21-25-nt RNA molecules and are frequently dysregulated in cancer. miR-200a is a member of the miR-200 family, which are known to be strong inhibitors of the epithelial to mesenchymal transition. As such, the tumor suppressive role of miR-200a in oncogenesis has been well studied; however, recent studies have found a proliferative role for this miRNA as well as a pro-metastatic role in the later steps of cancer progression. Little is known about the role of this miRNA in the early stages of cancer, namely, malignant cell transformation. Here we show that miR-200a cooperates with Ras to enhance malignant transformation of immortalized mammary epithelial cells. Furthermore, miR-200a induces cell transformation and tumorigenesis in immunocompromised mice by cooperating with a Ras mutant that activates only the RalGEF effector pathway, but not Ras mutants activating PI3K or Raf effector pathways. This transformative ability is in accordance with miR-200a targeting Fog2 and p53 to activate Akt and directly repress p53 protein levels, respectively. These results

demonstrate a role for miR-200a in malignant cell transformation and provide a specific cellular context where miR-200a acts as an oncomiR rather than a tumor suppressor by cooperating with an oncogene in the classical two-hit model of malignant cell transformation.

Introduction

Cancer is an often fatal disease that requires multiple steps to progress from a normal state to full phenotypic disease [1,170]. Cancer mortality, while comprising one quarter of all deaths in the United States, is declining, due largely to improvements in screening and detection. Diagnosis of early stage cancer is strongly associated with better survival [1-3,171]. Thus it is crucial to understand the molecular events that occur early in this progressive disease.

Malignant cell transformation is the initiating step of cancer progression [172]. During this process, a cell must bypass senescence and avoid apoptosis, allowing for uncontrolled proliferation, which leads to formation of a primary tumor [12]. The hyper-proliferative, anti-apoptotic phenotypes that arise during malignant cell transformation are conferred by genetic mutations and abnormalities that upregulate proto-oncogene activity and ablate tumor suppressor gene function [173]. The first oncogene/tumor suppressor combinations associated with cell transformation were investigated by in an attempt to delineate endogenous, non-viral proto-oncogenes [79,81]. The classical model of cell transformation identified the cooperation between the Ras and Myc oncogenes in selecting for a dominant-negative p53 tumor suppressor mutation and transforming primary rodent cells [81]. Numerous transforming oncogenes and tumor suppressor

mutations have been identified since these landmark studies [82], demonstrating the complexity of cancer initiation.

Recently, noncoding RNAs, *e.g.* microRNAs (miRNAs), have garnered interest as potent mediators of malignant cell transformation and cancer progression [100,106,129]. miRNAs are a regulatory class of small RNAs that bind the 3' UTR of target mRNA to post-transcriptionally repress gene expression [84]. miRNAs are frequently dysregulated in cancer through altered epigenetic modifications, deletions, translocations, and amplifications [100,103]. The subsequent changes in expression patterns and/or function result in differential repression of target genes. By repressing expression of oncogenes or tumor suppressors, a miRNA may function as a tumor suppressor or oncogene, respectively [129].

Among miRNAs dysregulated in cancer, miR-200a has emerged as a key mediator of the oncogenic process, though its overall role during cancer progression is not clear. Gene expression profiling reports that miR-200a is frequently down-regulated in cancer, including melanoma, breast, and nasopharyngeal cancers [118,119,121]. Its most well studied function is the suppression of Zeb1/2 transcription factors to inhibit the epithelial to mesenchymal transition (EMT) and promote a more stable, epithelial phenotype [115,124]. EMT is a crucial early step in the progression of transformed primary tumor cells into invasive metastatic cells that invade local stroma, travel through vasculature, and colonize distant sites in the body [4,174]. These metastases are responsible for the gross majority of cancer related deaths [171]. By blocking EMT and inhibiting metastasis, miR-200a functions as a tumor suppressor [116,124].

However, miR-200a has also been found to promote oncogenesis. miR-200a is overexpressed in several cancers including endometrial, pancreatic, and ovarian cancers [105,175,176]. Our studies demonstrate a specific role for miR-200a in early cancer progression that may apply to such clinical cases. Recent studies show that mesenchymal to epithelial transition (MET), the reversal of EMT, is a later step in metastasis that allows motile, invasive cells to revert back to a stable epithelial phenotype more conducive to forming new metastatic tumors at distant sites [177]. By inducing an epithelial phenotype in this context, miR-200a promotes metastasis [5,123,178]. In addition to its involvement in EMT/MET, a limited number of studies have observed a hyper-proliferative role for miR-200a including stimulation of PI3K signaling in hepatocellular carcinoma cells and up-regulation of miR-200a in a rat model of hepatocellular carcinoma [102,179]. My recent study demonstrates the anti-apoptotic function of miR-200a due to its direct targeting of *p53* [53] (Chapter 2). In this study, we determined the effect of miR-200a overexpression on transformation of rodent cells and immortalized human MCF10a cells and characterized the underlying mechanism of the ability of miR-200a to cooperate with Ras to transform MCF10a cells.

Experimental Procedures

Cell Culture

Rat kidney epithelial RK3E cells and human embryonic kidney 293T cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM media supplemented with 10% FBS and antibiotics at 37°C with 5% CO_2. Human mammary epithelial MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse

serum, 20 ng/mL EGF (Invitrogen), 0.5 mg/mL Hydrocortisone (Sigma), 100 ng/mL Cholera Toxin (Sigma), 10 ug/mL Insulin (Humulin), and antibiotics at 37°C with 5% CO_2.

miRNA Screen

RK3E cells were transfected using Lipofectamine LTX/Plus reagent according to manufacturer instructions with individual miRNAs from our laboratory's miRNA library comprised of 366 human miRNA minigenes in the lentiviral PSIF vector [180]. After 48 hrs, wells were visually inspected for three dimensional foci formation.

Transfection and Viral Transduction

Lipofectamine LTX-plus (Invitrogen) was used for all transfections according to manufacturer's instructions. For lentivirus production, 293T cells were transiently co-transfected, 24hrs post plating in 6-well plates, with 2µg of pSIF vector, or miRNA, 1.4µg of pVGV-S and 0.7µg of pFIV-34N packing and expression vectors, respectively. Lentivirus-containing supernatant was collected after 48 hours, centrifuged to remove cellular debris, and supplemented with 8 µg/mL Polybrene (American Bioanalytical) before transducing target MCF10A or RK3E cells. For retrovirus production, 3 µg of the retroviral vectors containing constitutively active RasG12V mutant (Addgene plasmid 1768), c-Myc (Myc construct from Addgene plasmid 16011 cloned into plasmid 12269), p53dd (Addgene plasmid 9058), or RasG12V effector pathway mutants (Addgene plasmids 12274, 12275, 12276) [18] were transfected into Phoenix-Ampho cells, and virus collection and transduction were performed as for Lentivirus. G418 (200 µg/mL), Puromycin (1 µg/mL), or Hygromycin (8 µg/mL) were used to select for positively

transduced cells. Cells were transduced three timesmiR-200a expression levels, as measured by Taq-Man QRT-PCR expression assay (Invitrogen), ranged from a 5 to 15 fold increase above vector control (Data not shown).

Acini Formation in Matrigel

Matrigel (Corning) was used to coat the well bottoms of a 12-well chamber slide (Ibidi). 5,000 positively drug-selected exponentially growing MCF10A cells suspended in 2% Matrigel were layered over top of the first Matrigel layer and allowed to grow 5 or 14 days. Acini were fixed with ethanol while still in Matrigel to prevent disruption of morphology, blocked with goat serum and incubated first with anti-E Cadherin or Cleaved caspase-3 antibodies (Cell Signaling) overnight at 4° and then Alexa488-coupled goat anti-rabbit secondary antibodies for 2 hrs room temperature. Slides were visualized by confocal microscopy.

Colony formation assay

Six well plates were coated with a bottom layer of 0.5% noble agar (Sigma-Aldrich) and 2000 RK3E or MCF10A cells were suspended in a top layer of 0.2% noble agar in triplicate. The cells were maintained at 37˚C in a humidified 5% CO_2 atmosphere for two weeks. Fresh media was added at regular intervals to prevent the plates from drying out. Resulting colonies were stained with 0.05% Crystal Violet and destained with water. Colonies were counted and imaged using a dissection microscope coupled to a digital microscope imager (Celestron). The experiment was performed three times, once for each level.

Cell Cycle Analysis

For synchronization, MCF10A cells were progressively deprived of serum and growth factors over 24 hours, then stimulated with complete media for 18 hours. Cells were collected by trypsinization and washed twice in 1X PBS. Cells were fixed in 1ml of ice-cold 70% ethanol at 4˚C overnight. Cells were then washed twice with 1X PBS and stained with a solution of 50 µg/ml Propidium Iodide, 100 µg/ml Ribonuclease A, and 0.2% Triton X-100 diluted in PBS for 30 mins at 4˚C. Flow cytometry was performed with a FACScan Flow Cytometer. A minimum of 10,000 cells per sample were collected and the FACS files were analyzed using FlowJo software (Tree Star Inc.) for cell cycle analysis [28].

Cell Migration

Transwell (Boyden) chambers (Invitrogen, Carlsbad, CA), with a pore size of 8µm, were placed in triplicate into 12-well plates. Complete MCF10A media, which served as a chemoattractant, was added to wells beneath the Transwell chamber. D283 cells (1×10^4) in low serum media were added to the Transwell chamber and the plates were then incubated at 37˚C in a humidified 5% CO_2 atmosphere for 24 hrs. The cells were fixed with methanol for 10 mins and stained with 0.4% crystal violet for 2 hrs. Non-migrated cells on the upper side of the filter were removed with a cotton swab, and the filter was mounted on microscope glass slides. Slides were imaged using a dissection microscope coupled to a digital microscope imager (Celestron).

Western Blot

Total protein was isolated from cells in 6-well plates using RIPA (Cell Signaling). Protein concentration was measured using a BCA kit (Pierce). 30–50 µg of protein were separated on 12% to 15% Bis-Tris polyacrylamide gels (Bio-Rad) and then transferred to PVDF membranes (Bio-Rad). Protein membranes were incubated in blocking buffer (1× Tris-buffered saline, pH 7.5, 5% nonfat dried milk, 0.05% Tween 20) for 2 hours at room temperature, followed by phospho-p53, p53, PTEN, phospho-Akt, Akt, phospho-Erk, or Erk antibody (Cell Signaling), Fog2 antibody (Santa Cruz Biotechnology) or β-actin antibody (Sigma-Aldrich) overnight at 4°C. The membranes were washed with 1× Tris-buffered saline containing 0.05% Tween 20, incubated with horseradish peroxidase-linked goat anti-mouse Ig (Santa Cruz) or goat anti-Rabbit Ig (Cell Signaling) for 1 hour at room temperature, washed, and visualized with the SuperSignal West Dura Chemiluminescent Substrate kit (PIERCE).

Mice

Athymic male nude (*Foxn1^{nu}/Foxn1^{nu}*) mice (5 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Louisville's AAALAC-accredited animal facility. All animal studies were conducted in accordance with National Institutes of Health animal use guidelines, and a protocol approved by the University of Louisville's Institutional Animal Care and Use Committee. Exponentially growing cells were harvested and injected subcutaneously ($5.0x10^5$ cells/animal) into nude mice (six per group). Each mouse was injected in each flank with MCF10A cells stably over-expressing a Ras effector mutant alone in one flank, and

MCF10A cells stably over-expressing the same Ras effector mutant in combination with miR-200a in the other flank. Side of injection was randomized. Tumor size was monitored once per week for 10 weeks before sacrificing. Tumors were harvested and immediately formalin fixed. Tumors were embedded in paraffin, and tissue sections were stained with hematoxylin and eosin.

Statistical Analysis

Colony formation data was analyzed by multivariate analysis of variance (MANOVA) with T tests post hoc to look for individual effects with standard errors corrected for multiple comparisons. Cell proliferation data were analyzed by linear regression modeling with T tests post hoc to determine individual effects using standard errors corrected for multiple comparisons. Cell Cycle Distribution was analyzed by generalized linear models comparing G1 to combined S/G2 phases with binomial response variables and parameters estimated by maximum likelihood. Log odds ratios were further analyzed by T tests post hoc to evaluate individual effects using standard errors corrected for multiple comparisons.

Table 3. Statistical Analyses of colony formation in soft agar: miR-200a, oncogenes. MCF10A cells stably overexpressing miR-200a alone or in combination with the

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MCF10A COLONY FORMATION ASSAY - miR-200a + Oncogenes

Coefficients: (1 not defined because of singularities)
                  Estimat Error t value Pr(>|t|)
(Intercept)      -1.023e-14  5.821e+00   0.000    1.000
miR200a           3.333e-01  8.233e+00   0.040    0.968
ras               6.400e+01  8.233e+00   7.774 3.68e-07 ***
cmyc              1.367e+01  8.233e+00   1.660    0.114
p53dd             2.300e+01  8.233e+00   2.794    0.012 *
ras:cmyc         -6.667e+01  1.164e+01  -5.726 1.98e-05 ***
miR200a:ras       1.447e+02  1.164e+01  12.425 2.87e-10 ***
miR200a:cmyc     -1.300e+01  1.164e+01  -1.117    0.279
miR200a:p53dd    -5.667e+00  1.164e+01  -0.487    0.632
```

Group 1	Group 2	colonies	t value	p value	
vector	miR-200a	3.33E-01	4.05E-02	4.84E-01	
vector	Ras	6.40E+01	7.77E+00	2.69E-07	***
vector	cMyc	1.37E+01	1.66E+00	5.76E-02	
vector	p53dd	2.30E+01	2.79E+00	6.24E-03	**
vector	Ras + cMyc	1.10E+01	1.89E+00	3.80E-02	*
vector	miR-200a + Ras	1.45E+02	1.24E+01	2.93E-10	***
vector	miR-200a + cMyc	-1.30E+01	-1.12E+00	8.60E-01	
vector	miR-200a + p53dd	-5.67E+00	-4.87E-01	6.84E-01	
miR-200a	miR-200a + Ras	2.09E+02	1.79E+01	8.86E-13	***
Ras	miR-200a + Ras	1.45E+02	1.25E+01	2.82E-10	***
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05					

Table 4 Statistical Analyses of colony formation in soft agar: miR-200a, Ras mutants. MCF10A cells stably overexpressing miR-200a alone or in combination with the indicated Ras effector mutants.

```
Colony formation - miR-200a + Ras Effector Mutants

Coefficients: (1 not defined because of singularities)
                 Estimate Std. Error t value Pr(>|t|)
(Intercept)     -1.641e-14  7.551e+00   0.000   1.0000
miR200a          2.333e+00  1.068e+01   0.218   0.8295
rase37g          5.667e+00  1.068e+01   0.531   0.6022
rast35s          2.333e+00  1.068e+01   0.218   0.8295
raswt            1.160e+02  1.068e+01  10.862 2.47e-09 ***
rasy40c          1.800e+01  1.068e+01   1.685   0.1092
miR200a:rase37g  4.333e+01  1.510e+01   2.869   0.0102 *
miR200a:rast35s  9.333e+00  1.510e+01   0.618   0.5443
miR200a:raswt           NA         NA      NA       NA
miR200a:rasy40c  2.333e+00  1.510e+01   0.154   0.8789
```

Group 1	Group 2	colonies	t value	p value	
vector	miR-200a	2.33E+00	2.18E-01	0.415	
vector	E37G	5.67E+00	5.31E-01	0.301	
vector	T35S	2.33E+00	2.18E-01	0.415	
vector	Ras	1.16E+02	1.09E+01	0.000	***
vector	Y40C	1.80E+01	1.69E+00	0.055	
vector	miR-200a + E37G	4.33E+01	2.87E+00	0.005	**
vector	miR-200a + T35S	9.33E+00	6.18E-01	0.272	
vector	miR-200a + Y40C	2.33E+00	1.55E-01	0.440	
miR-200a + E37G	miR-200a	4.90E+01	3.24E+00	0.002	**
miR-200a + E37G	E37G	4.57E+01	3.02E+00	0.004	**
miR-200a + T35S	miR-200a	1.17E+01	7.73E-01	0.225	
miR-200a + T35S	T35S	1.17E+01	7.73E-01	0.225	
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05					

Table 5. Statistical Analysis of Cell Proliferation Data. MCF10A cells stably overexpressing miR-200a alone or in combination with Ras.

```
Cell Proliferation Curve

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)        0.116063   0.020842   5.569 1.54e-06 ***
time               0.070912   0.006865  10.330 3.19e-13 ***
time:miR200a       0.034543   0.008140   4.244 0.000115 ***
time:ras           0.065368   0.008140   8.031 4.29e-10 ***
time:miR200a:ras -0.056111   0.011511  -4.874 1.53e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.08805 on 43 degrees of freedom
Multiple R-squared:  0.9309,    Adjusted R-squared:  0.9245
F-statistic: 144.9 on 4 and 43 DF,  p-value: < 2.2e-16
```

Group 1	Group 2	Slope	t value	p value	
vector	miR-200a	0.105455	3.052862	0.001939	***
vector	Ras	0.13628	16.74201	0	***
vector	miR-200a + Ras	0.014801	1.285814	0.102695	***
miR-200a + Ras	miR-200a	0.009257	0.267985	0.394996	
miR-200a + Ras	Ras	-0.02157	-1.87369	0.96611	
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05					

Table 6. Statistical Analyses of Cell Cycle Data. MCF10A cells stably overexpressing miR-200a alone or in combination with the indicated oncogenes.

```
CELL CYCLE DATA

Coefficients: (1 not defined because of singularities)
                 Estimate Std. Error z value Pr(>|z|)
(Intercept)       0.98074    0.01762   55.66   <2e-16 ***
miR200a          -1.20118    0.02405  -49.94   <2e-16 ***
ras              -1.03059    0.02229  -46.23   <2e-16 ***
cmyc             -1.49792    0.02153  -69.56   <2e-16 ***
p53dd            -1.44509    0.02354  -61.40   <2e-16 ***
ras:cmyc          1.81266    0.02994   60.53   <2e-16 ***
miR200a:ras       1.29906    0.03249   39.99   <2e-16 ***
miR200a:cmyc      1.14627    0.03140   36.51   <2e-16 ***
miR200a:p53dd     0.79548    0.03300   24.10   <2e-16 ***
```

Group 1	Group 2	log odds estimate	Fold Change	z value	p value
vector	miR-200a	-0.22044	0.802165767	-9.1659	0.00003
vector	Ras	-0.04985	0.95137212	-2.23643	0.01266
vector	cMyc	-0.51718	0.596199462	-24.0214	0.00003
vector	p53dd	-0.46435	0.628543526	-19.726	0.00003
vector	Ras + cMyc	2.7934	16.33646948	93.29993	0.99997
vector	miR-200a + Ras	2.2798	9.774725269	70.16928	0.99997
vector	miR-200a + cMyc	2.12701	8.389743937	67.73917	0.99997
vector	miR-200a + p53dd	1.77622	5.907483872	53.82485	0.99997
miR-200a + Ras	miR-200a	0.26847	1.307961738	8.263158	0.99997
miR-200a + Ras	Ras	0.09788	1.102830438	3.012619	0.9987

Results

miR-200a transforms immortalized rat epithelial cells

The RK3E cell line is an E1A-immortalized rat kidney epithelial cell line, whose defining characteristic is monolayer growth under normal conditions, and foci formation under transforming conditions such as Ras activation or Myc overexpression [181-183]. In order to determine the role of miRNAs in cell transformation, RK3E cells were transiently transfected with individual miRNAs from our library of 366 miRNA genes [180] and visually screened for foci formation (Figure 9A). A construct expressing c-Myc was used as a positive control [154]. Negative vector control-expressing cells formed no foci. Of the 366 miRNAs screened miR-141 and let-7e formed about the same number of foci as c-MYC, and miR-200a formed more foci than c-Myc. These three miRNAs were selected for an ensuing stringent cell transformation experiments. We stably infected RK3E cells with lentivirus made from each of these three miRNAs or vector control and assayed them for anchorage-independent growth in soft agar. All three miRNAs induced colony formation in soft agar (Figure 9B).

In order to determine tumorigenicity of these miRNAs, RK3E cells stably expressing each of these miRNAs were subcutaneously injected into nu/nu mice. Cells expressing miR-141 or miR-200a formed orthotopic subcutaneous tumors (Figure 10), but let-7e-expressing cells did not produce tumors (data not shown). miR-200a and miR-141 are family members with identical seed sequences, thus miR-200a alone was analyzed further.

To determine potential mechanisms underlying this cell transformation, we analyzed the expression of miR-200a target genes [102,184-186]. Consistent with the literature, Western blot analyses showed that in RK3E cells expressing miR-200a, the epithelial marker, E-cadherin, was up-regulated, and the mesenchymal marker, Vimentin, was down-regulated compared to vector control (Figure 11A). Furthermore, expression of the negative regulators of the PI3K/Akt pathway, Fog2 and Pten, was down-regulated, concomitant with an increase in Akt phosphorylation (Figure 11B).

We next determined whether miR-200a acts as a driving force in cell transformation. We employed the classical two-hit model of primary rodent cell transformation by combining miR-200a with Ras, c-Myc, or p53dd in mouse embryonic fibroblasts (MEFs). miR-200a overexpression in MEFs induces an initial increase in proliferation; however, miR-200a expression alone, or in combination with either Ras or c-MYC was unable to transform MEFs (data not shown). p53dd is known to immortalize MEFs, however miR-200a was unable to cooperate with this tumor suppressor mutation to transform these cells. Ras and Myc coexpression was able to transform primary MEFs. Taken together, these results suggest that other genetic pathways active in RK3E cells, but not in primary MEFs, are involved in miR-200a-mediated cell transformation and that miR-200a is not a strong transforming component of the classical two-hit murine model.

Figure 9. miR-200a transforms RK3E cells.

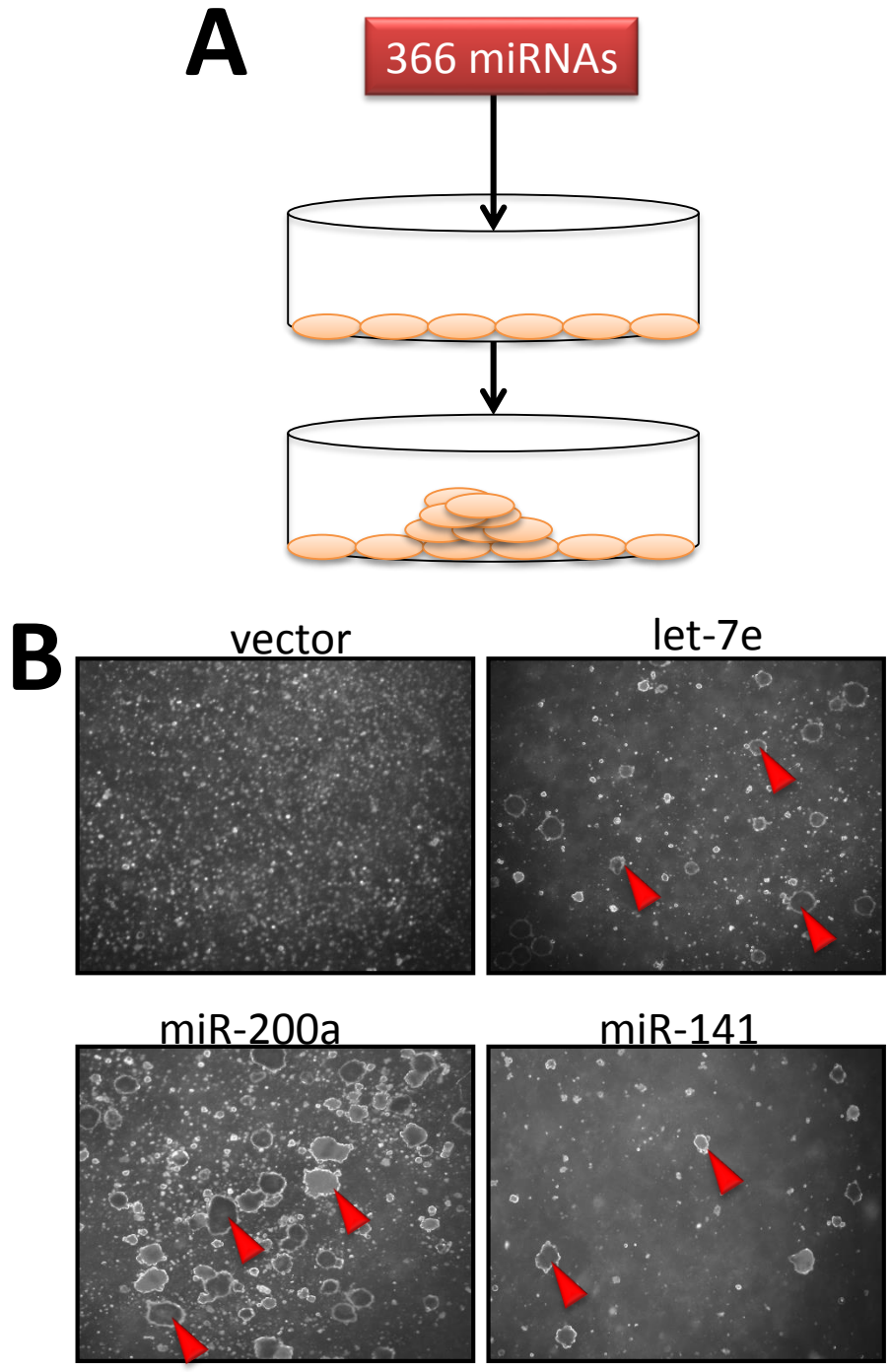


Figure 9. miR-200a transforms RK3E cells. (A) Schematic of foci formation induced by transient transfection of individual miRNA from our library of 366 miRNA minigenes. (B) Phase contrast microscopy of 3-dimensional colonies formed in soft agar by RK3E cells stably overexpressing the indicated miRNAs. Red arrowheads indicate colonies.

Figure 10. miR-200a induces tumorigenesis of RK3E cells in immunocompromised mice

Figure 10. miR-200a induces tumorigenesis of RK3E cells in immunocompromised mice. Subcutaneous tumor formation of RK3E cells overexpressing the indicated miRNAs

Figure 11. miR-200a regulates EMT and stimulates Akt activity.

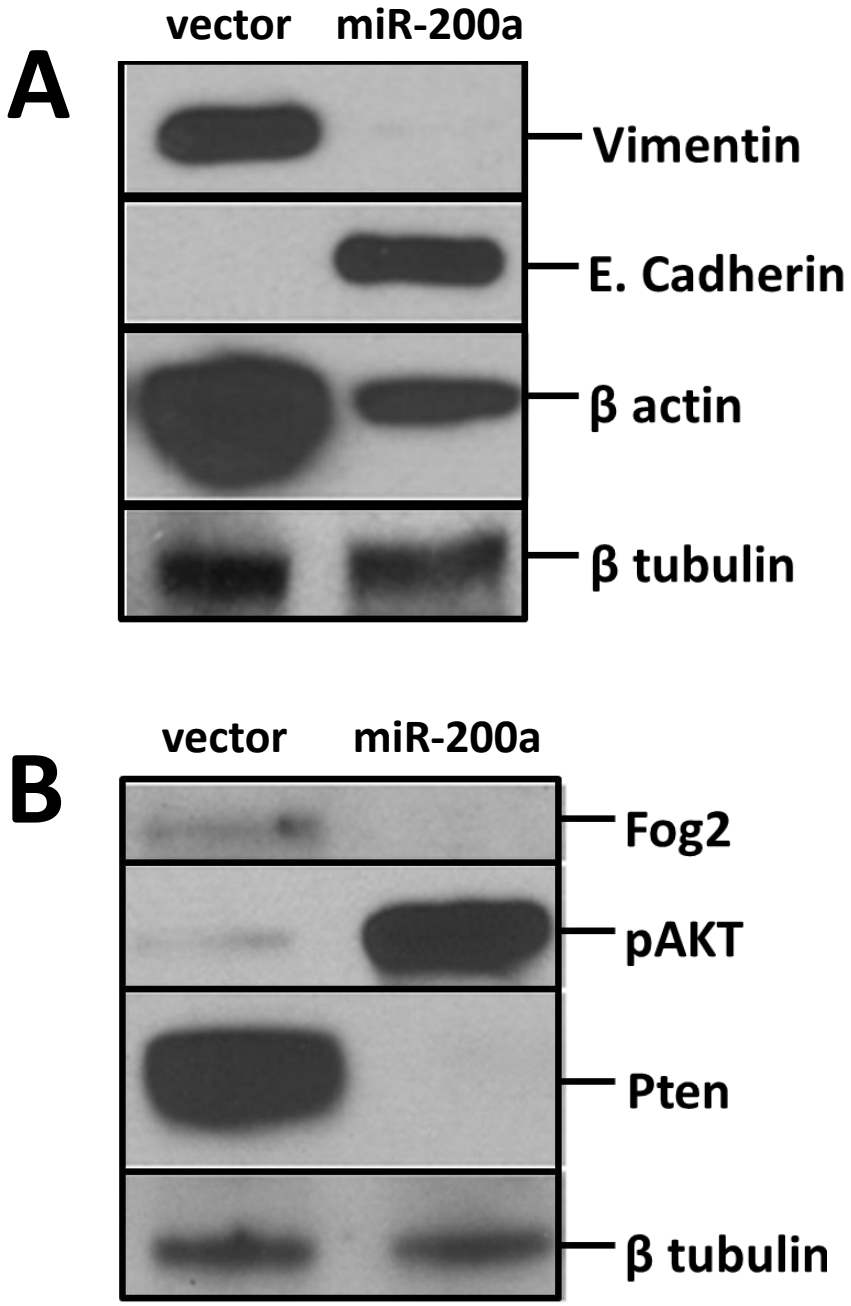


Figure 11. miR-200a regulates EMT and stimulates Akt activity.

(A) Western blot for the mesenchymal marker Vimentin and the epithelial marker, E. Cadherin in RK3E cells lenti-virally infected with miR-200a or empty vector control. (B) Western blot for members of the Akt activation pathway in RK3E cells.

miR-200a augments Ras transformation of immortalized human mammary epithelial cells.

In order to determine the role of the proliferative effects of miR-200a in human cells, we chose the immortalized yet untransformed mammary epithelial cell line, MCF10A, from a patient with fibrocystic disease [187]. This cell line is readily transformed by overexpression of Ras or Myc, and normal morphology in three-dimensional cell culture is disrupted by loss of p53 signaling [188-190]. Consistent with our efforts to determine oncogenic cooperativity, we stably infected this cell line with miR-200a alone, or in combination with Ras, c-MYC, or p53dd. The Ras construct harbors a G12V mutation, rendering it constitutively active. p53dd is a truncated form of the carboxy terminus of the p53 protein that binds wild type p53, resulting in a dominant negative repression of p53 function [191]. To test for transformation, cells were assayed for anchorage-independent growth in soft agar (Figure 12, 13). miR-200a alone did not transform MCF10A cells. When miR-200a was expressed in combination with c-Myc or p53dd, colony formation was not significantly increased compared with that of either oncogene alone. However, colony formation increased more than three times in cells expressing miR-200a in combination with Ras than that in cells expressing Ras alone (Figure 13). Coexpression of Ras and c-Myc induced colony formation, but surprisingly, their combined effects were not synergistic, that is, their combined effect did not show an increase greater than the addition of both oncogene's individual effects. MF10A cells expressing miR-200a at 5-fold, 10-fold, and 15-fold increases above vector control were transduced with empty vector or Ras, and assayed for colony formation. There was no difference in the number of colonies formed in response to varying levels of miR-200a

expression (Data not shown). Cells with a 10-fold increase in miR-200a expression were used for subsequent experiments.

miR-200a synergizes with Ras to inhibit apoptosis.

We next analyzed these cells for changes in cell cycle progression (Figure 14, 15). Compared to vector control, all experimental groups caused a decrease in G1 arrest. Consistent with its effects on colony formation, Ras and Myc coexpression had the smallest effect, decreasing G1 arrest from 70.93% to 55.52%. Compared to p53dd alone, miR-200a in combination with p53dd caused the greatest increase in cell cycle progression, decreasing G1 arrest from 38.48% to 28.88%. Surprisingly, G1 arrest was unchanged between cells expressing Ras alone, and miR-200a in combination with Ras. Furthermore, compared to miR-200a alone, G1 arrest in cells expressing miR-200a combination with Ras increased from 43.83% to 50.15%. Compared to cMyc alone, miR-200a with cMyc did not change G1 arrest levels. This indicates that induction of cell cycle progression is not responsible for the combinatorial effect between Ras and miR-200a on cell transformation. We next generated a cell proliferation curve in order to compare growth rates of cells expressing miR-200a alone and cells expressing miR-200a in combination with Ras (Figure 16). miR-200a alone and in combination with Ras significantly increased proliferation compared to vector control; however, miR-200a combined with Ras did not significantly increase proliferation compared to miR-200a alone. Ras alone showed the greatest increase in proliferating cells. This indicates that stimulation of proliferation is not responsible for the interactive effect between Ras and miR-200a, and that Ras is unable to enhance the proliferative function of miR-200a.

We next examined these cells for changes in migratory ability, a cancer hallmark, and a phenotype frequently regulated by miR-200a and its family members. miR-200a decreased trans-well cell migration of MCF10A cells compared to vector control (Figure 17). Ras alone greatly increased cell migration above vector control. Cells expressing Ras in combination with miR-200a also showed decreased migration compared to Ras alone; however, these cells formed dense, three-dimensional colonies before and after migrating. Inhibition of cell migration is consistent with the literature and miR-200a's established function in regulation of EMT [124,186].

When seeded in Matrigel extracellular matrix, MCF10A cells grow to form polarized acinar structures with a distinct hollow lumen, which resembles normal breast tissue development [190]. To gain insight into the potential effects of the cooperation of miR-200a with Ras on cell growth and morphology in the context of acinar structure formation, we seeded cells in Matrigel, and analyzed three dimensional acinus formation by confocal microscopy (Figure 18). Acini were examined for changes in three characteristics: morphology, E-Cadherin expression, and cleaved Caspase-3 expression. Acini were stained for E-Cadherin to facilitate inspection of morphological and structural changes, as well as changes in E-Cadherin expression and subcellular localization (Figure 19). Vector control cells formed regular spherical structures with hollow lumen. Cells expressing miR-200a alone formed regular spherical structures, with and without regular lumen clearance. Cells expressing Ras alone formed very irregular and lobular three dimensional structures with loosely packed cells and partial lumen clearance. Cells expressing miR-200a in combination with Ras formed densely packed, slightly irregular spherical structures with no lumen clearance. Notably, in acini expressing Ras alone, or

Ras with miR-200a, E-cadherin was localized diffusely in the cytoplasm, rather than at the plasma membrane. This is consistent with studies showing disruption of normal adherens junctions in transformed cells, and sequestering of E-Cadherin away from the plasma membrane in the cytoplasm [192,193]. In order to determine whether the cause of the loss of lumen clearance in response to miR-200a expression was loss of apoptosis, we examined levels of cleaved Caspase-3 in matrigel-seeded cells allowed to grow as acini for 3 days (Figure 18). Acini containing vector or miR-200a expressed high levels of cleaved Caspase-3 and showed normal lumen clearance. This is in contrast to the loss of lumen clearance in acini expressing miR-200a after seven days (data not shown). Acini expressing Ras alone formed lobular structures at 3 days, and expressed lower levels of cleaved Caspase-3. Acini structures expressing Ras in combination with miR-200a showed tightly packed structures with no lumen clearance, and little to no cleaved Caspase-3. Taken together, these results indicate that miR-200a alone decreases lumen clearance by increasing proliferation without decreasing apoptosis (Figures 16 and 19); however, miR-200a synergizes with Ras to inhibit apoptosis as evidenced by the dramatic reduction of cleaved Caspase-3 (Figure 19).

Figure 12. miR-200a augments Ras-induced malignant cellular transformation.

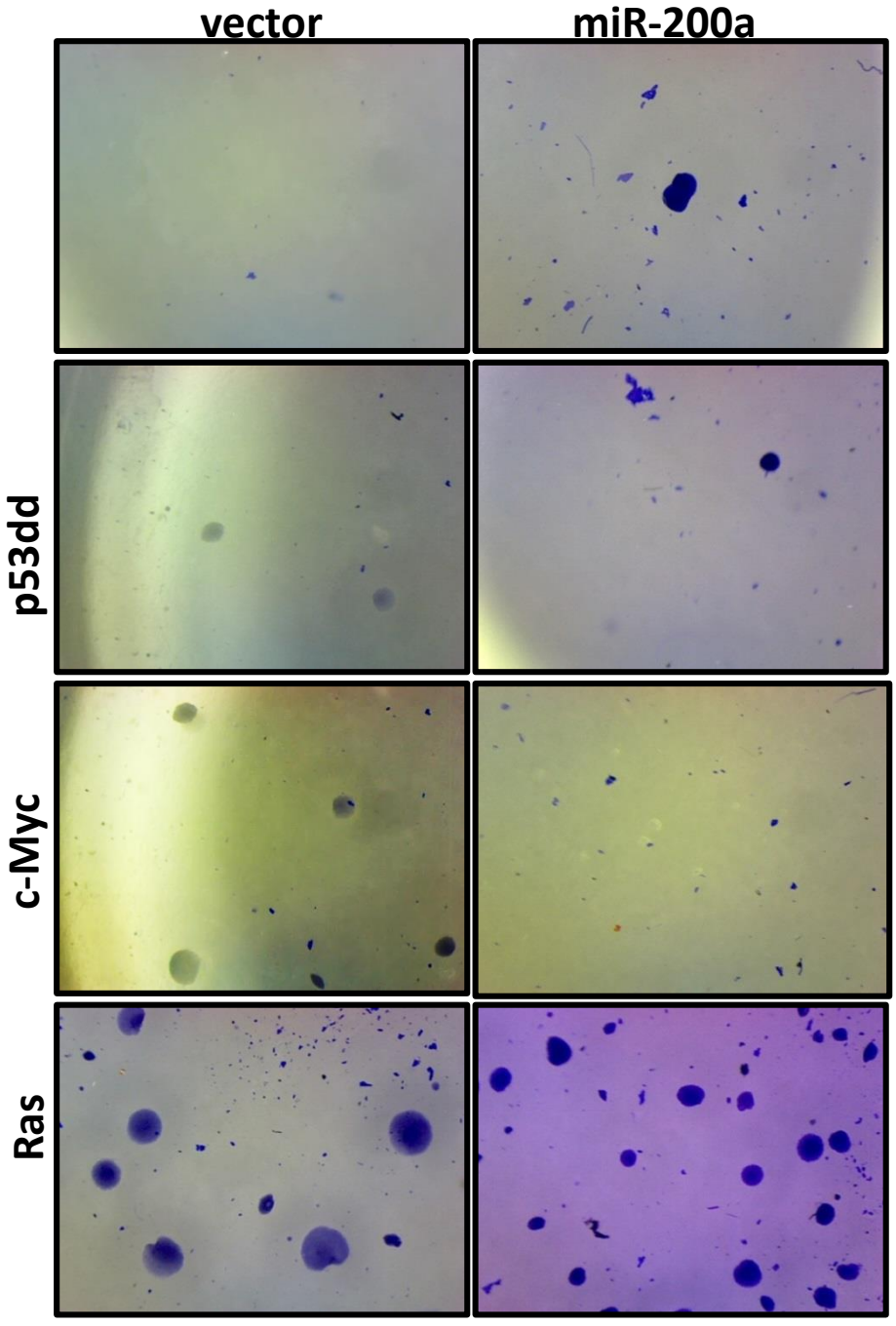


Figure 12. miR-200a augments Ras-induced malignant cellular transformation.

Soft agar colony formation: bright field microscopy of MCF10A cells stably infected with the indicated oncogenes alone (left panels) or in combination with miR-200a (right panels).

Figure 13. miR-200a augments Ras-induced malignant cellular transformation-quantification.

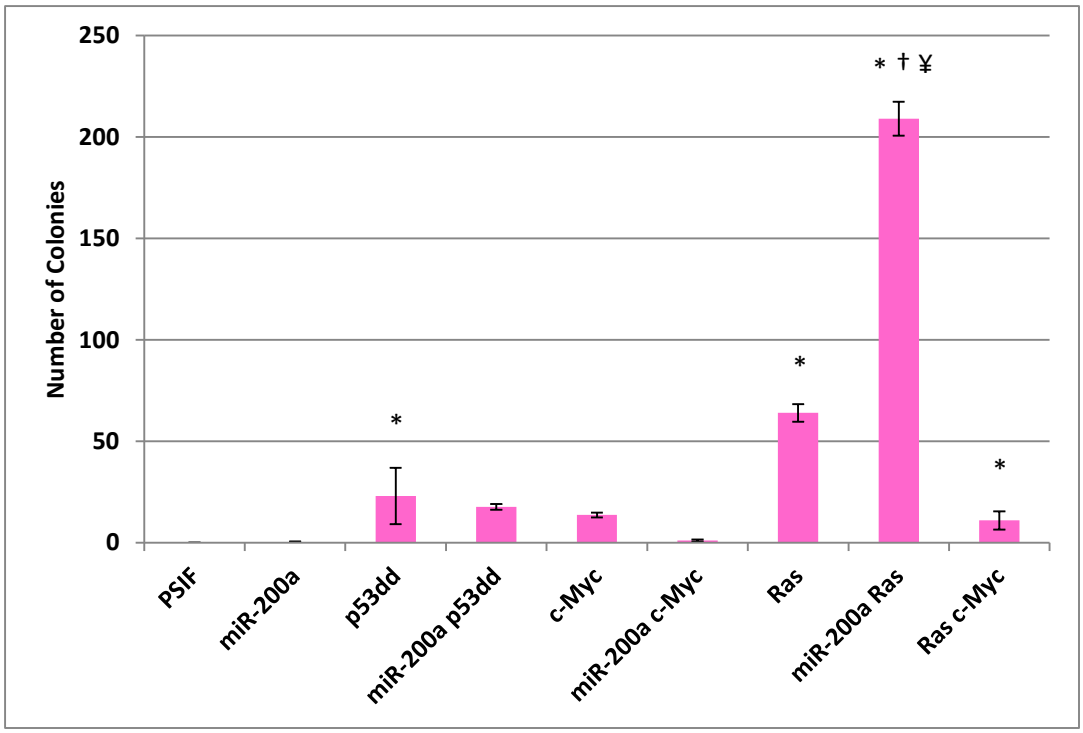


Figure 13. miR-200a augments Ras-induced malignant cellular transformation. Quantification of soft agar colony formation in Figure 12; 2000 MCF10A cells stably infected with miR-200a and the indicated oncogenes were plated in triplicate. The data represents means of three independent experiments +/- standard error.

* $p \leq 0.05$ compared to vector control, † $p \leq 0.05$ compared to miR-200a,

¥ $p \leq 0.05$ compared to Ras.

Figure 14. miR-200a increases cell cycle progression, but not in combination with oncogenes.

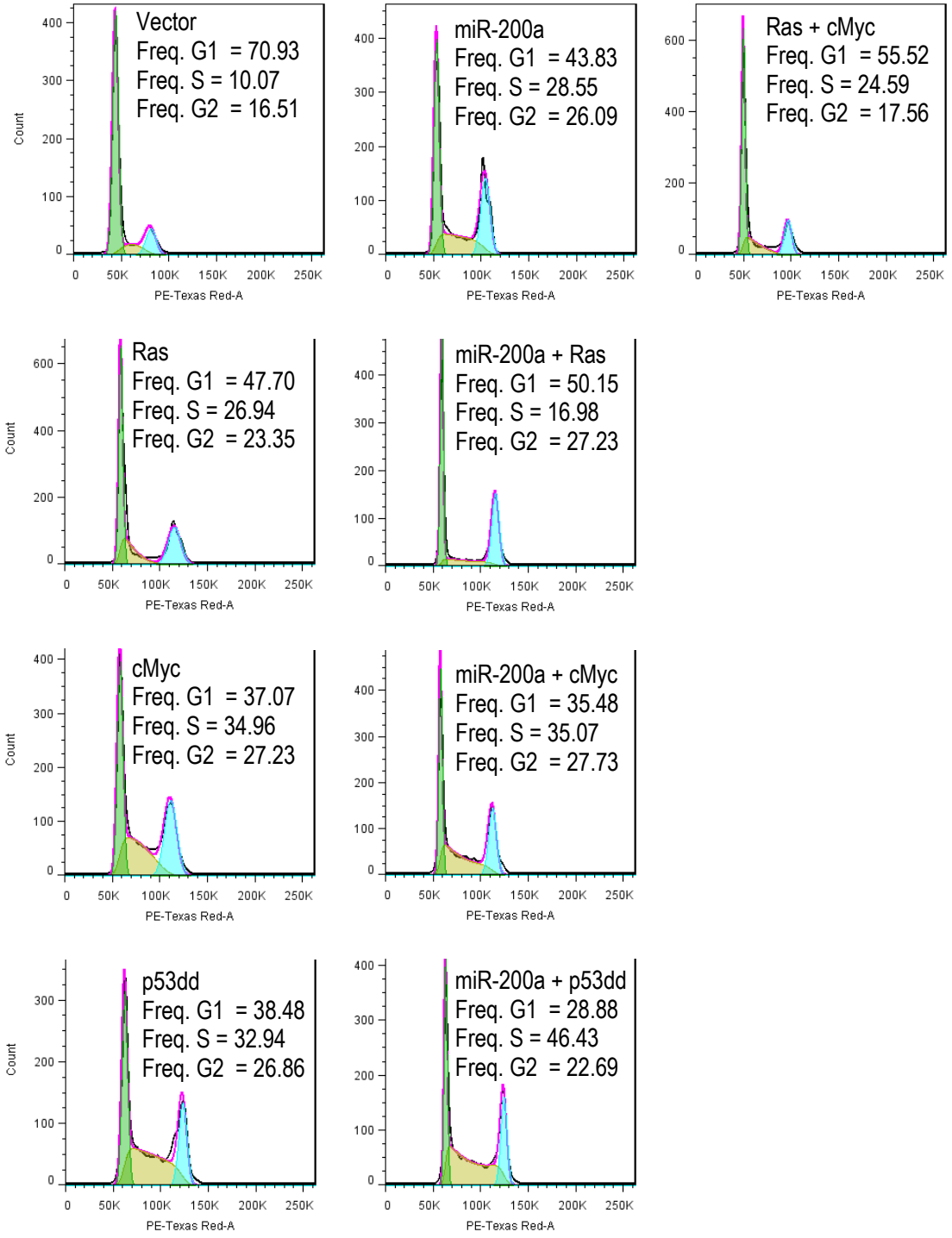


Figure 14. miR-200a increases cell cycle progression, but not in combination with oncogenes. Representative histograms of cell cycle distributions of MCF10A cells infected with the indicated oncogenes alone or in combination with miR-200a.

Figure 15. miR-200a increases cell cycle progression alone, not synergistically with oncogenes.

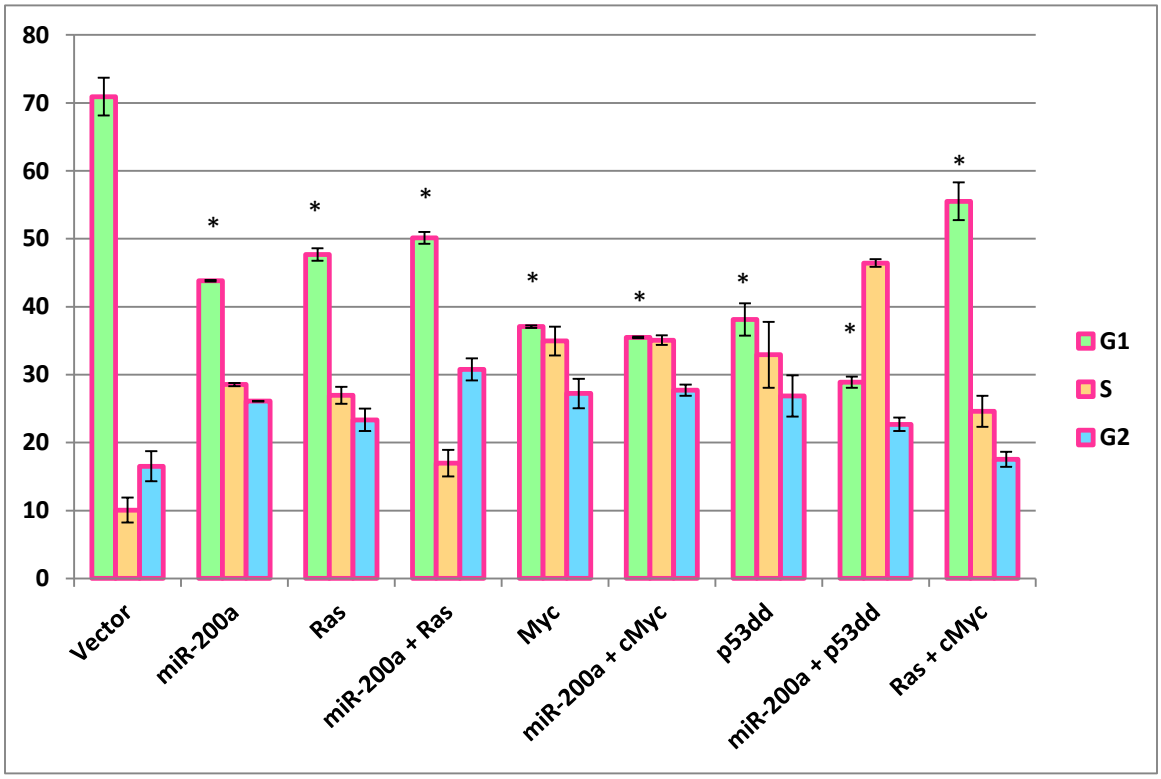


Figure 15. miR-200a increases cell cycle progression alone, not synergistically with Ras. Quantification of cell cycle distribution in Figure 6; MCF10A cells infected with the indicated oncogenes alone or in combination with miR-200a.

* $p \leq 0.05$ compared to vector control, † $p \leq 0.05$ compared to miR-200a,

¥ $p \leq 0.05$ compared to Ras. P-values calculated for cell cycle progression out of G1 phase.

Figure 16. miR-200a mediated increase in proliferation is not enhanced by Ras.

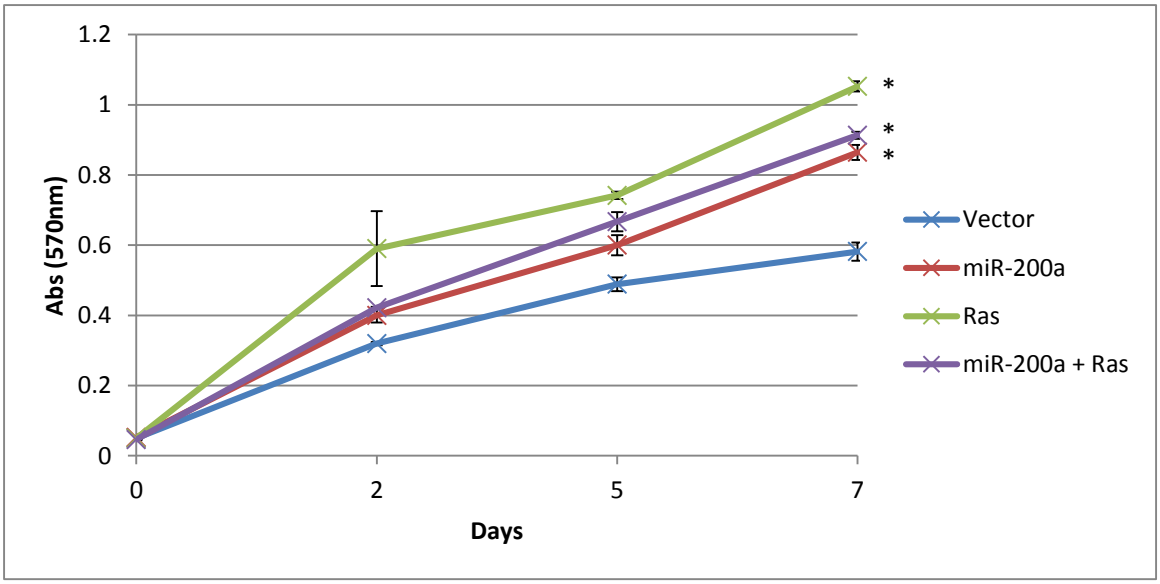


Figure 16. miR-200a mediated increase in proliferation is not enhanced by Ras.

Cell proliferation curve of MCF10A cells infected with miR-200a, Ras, or miR-200a in combination with Ras. Cell proliferation assayed by MTT assay. Cells were plated in triplicate; data are means of three independent experiments +/- standard error.

* $p \leq 0.05$ compared to vector control, † $p \leq 0.05$ compared to miR-200a,

¥ $p \leq 0.05$ compared to Ras.

Figure 17. miR-200a inhibits cell migration.

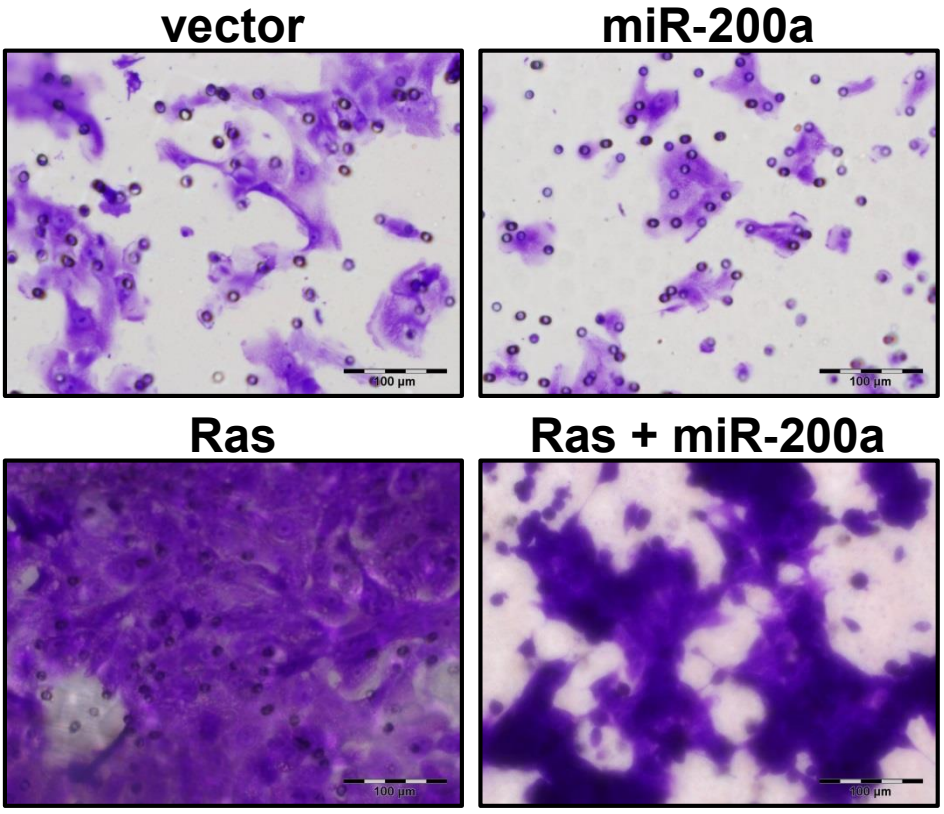


Figure 17. miR-200a inhibits cell migration. Transwell cell migration of MCF10A cells infected with miR-200a, Ras, or miR-200a in combination with Ras and stained with crystal violet.

Figure 18. MCF10A cells form hollow acini in Matrigel 3 dimensional culture.

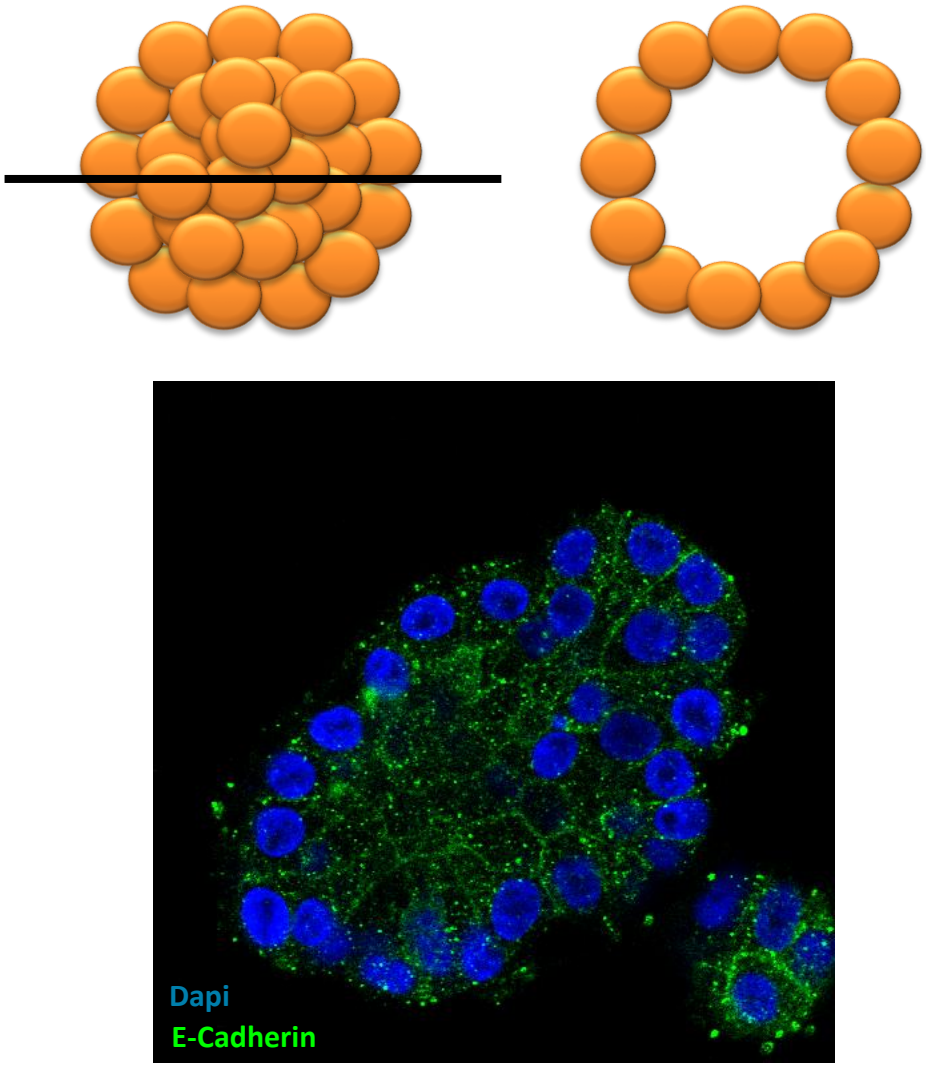


Figure 18. MCF10A cells form hollow acini in Matrigel 3 dimensional culture.

Upper left: Schematic of three dimensional acinus formed by MCF10A cells in Matrigel. Black line indicates equatorial confocal plane of focus. Upper right: Schematic of hollow lumen visualized by confocal microscopy focused at center line depicted in left diagram. Lower: representative image of acinar structures formed after 14 days in Matrigel by MCF10A stably infected with vector control.

Figure 19. miR-200a cooperates with Ras to inhibit apoptosis.

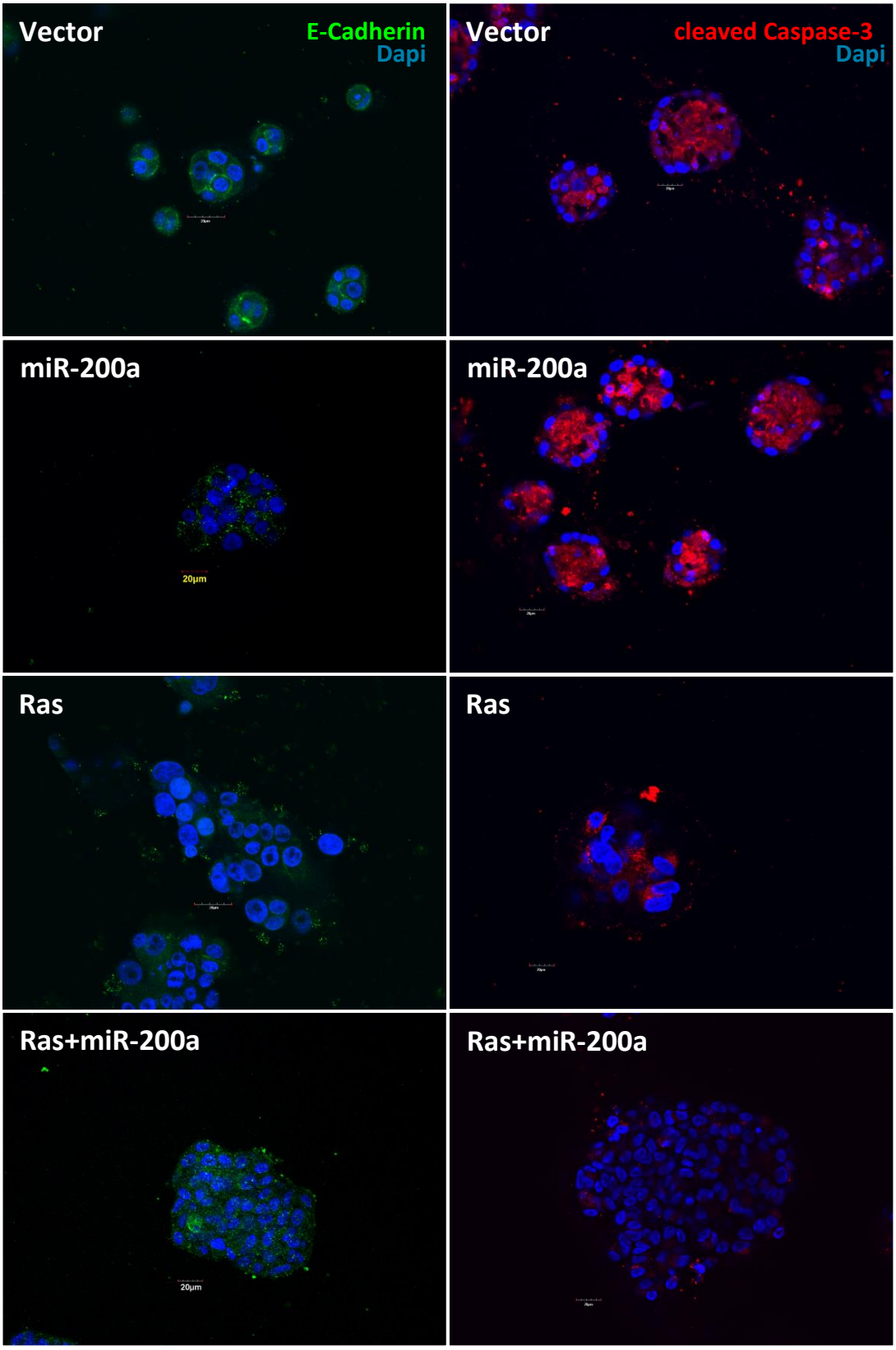


Figure 19. miR-200a cooperates with Ras to inhibit apoptosis. Three dimensional acinar formation after 5 days in Matrigel; MCF10A cells infected as indicated, stained for E-Cadherin (green, left panels) and cleaved Caspase-3 (red, right panels).

miR-200a cooperates with the RalGEF Ras effector pathway

Ras activity results in the activation of a wide array of downstream signaling pathways; however, the three main effectors that are activated by Ras are Raf, PI3K, and RalGEF [29]. We made use of three Ras mutants to determine the mechanism behind miR-200a cooperating with Ras to increase MCF10A cell transformation. The T35S point mutation allows for activation of only the Raf-Erk effector pathway; the Y40C point mutation allows for activation of only the PI3K effector pathway, and the E37G point mutation allows for activation of only the RalGEF effector pathway (Figure 20). We expressed miR-200a along with each of these individual effector pathway mutations to determine which pathway miR-200a specifically cooperates with and assayed them for anchorage-independent growth in soft agar (Figure 21, 22). Compared to T35S alone, miR-200a in combination with T35S caused a small, but insignificant increase in colony formation. miR-200a in combination with Y40C did not cause an increase in colony formation compared to Y40C alone. miR-200a caused a significant ten-fold increase in cells expressing both E37G and miR-200a compared to cells expressing E37G alone. Notably, un-mutated Ras formed more colonies than any of the effector mutants alone or combined with miR-200a, indicating that while miR-200a synergizes with the E37G mutant to enhance cell transformation, these two hits do not completely recapitulate the full effect of Ras.

We next analyzed MCF10A cells expressing Ras effector mutants alone or in combination with miR-200a for changes in cell cycle progression (Figure 23). T35S significantly decreased G1 phase arrest, but when miR-200a was added, the effect was ablated. Y40C alone, or in combination with miR-200a did not show any change in cell

cycle progression, consistent with the lack of effect on colony formation. E37G alone induced a modest but significant increase in G1 phase arrest. However, E37G in combination with miR-200a significantly decreased G1 phase arrest, compared to both vector control and E37G alone, indicating that miR-200a induction of cell cycle progression contributes to its cooperation with RalGEF signaling in cell transformation.

We next analyzed the three dimensional growth in Matrigel of MCF10A cells expressing E37G or T35S Ras effector mutants alone and in combination with miR-200a to determine changes in structure and apoptosis (Figure 24). Cells expressing E37G Ras mutant alone formed regular, round acini with hollow lumen, but had lower levels of cleaved Caspase-3 than the vector control (Figure 19,24). When miR-200a was added, cells expressing E37G Ras mutant formed large, irregularly shaped structures with tightly packed cells and diffuse E-Cadherin staining; punctate cleaved Caspase-3 was visible throughout the structures, along with apoptotic cells; however, no lumen clearance was observed, similar to Ras alone. Cells expressing T35S Ras mutant alone formed regular spherical acini with strong plasma membrane localization of E-Cadherin; cleaved Caspase-3 staining showed uncompromised apoptosis levels in these acinar structures, however, no lumen clearance was observed, possibly due to slower growth. Cells expressing T35S in combination with miR-200a formed irregular acinar structures, most without lumen clearance; structures that did exhibit lumen clearance were part of larger, lobular structures, indicating irregular growth despite lumen clearance; plasma membrane localization of E-Cadherin was retained, and structures showed low levels of cleaved Caspase-3 staining.

These results indicate that miR-200a cooperates with E37G mutant to disrupt acinar growth; however, the change in acinar structure is likely due to increased proliferation rather than a change in apoptosis compared to E37G alone. These effects were not seen when miR-200a was added to T35S, demonstrating that miR-200a does not cooperate with or enhance the effects of Raf signaling on acinar morphology. Taken together, this strongly implicates physiological significance for the interaction between miR-200a and E37G.

Figure 20. Ras effector pathways.

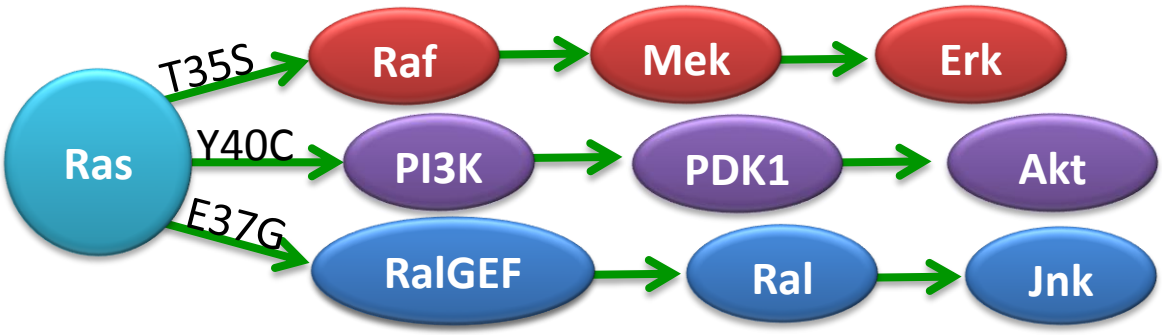


Figure 20. Ras effector pathways. Schematic of the 3 main effector pathways of Ras. Indicated on the leftmost arrows are the activating point mutations that allow for activation of only that pathway.

Figure 21. miR-200a cooperates with the RalGEF pathway to increase cell transformation.

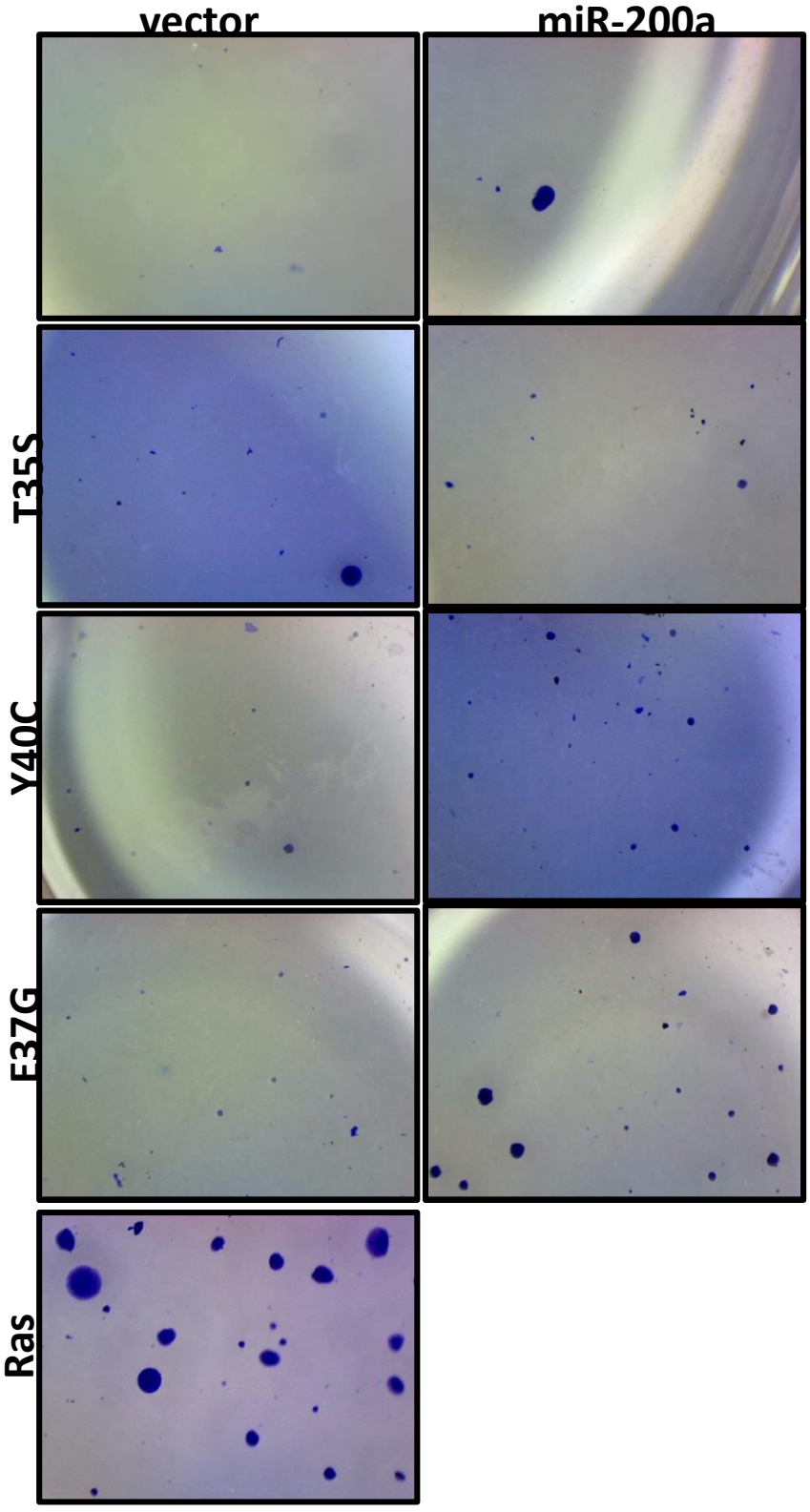


Figure 21. miR-200a cooperates with the RalGEF pathway to increase cell transformation. Soft agar colony formation assay of MCF10A cells stably infected with the indicated Ras effector mutants alone (left panels) or in combination with miR-200a (right panels) compared to constitutively activated Ras (bottom left panel).

Figure 22. miR-200a cooperates with RalGEF pathway activation.

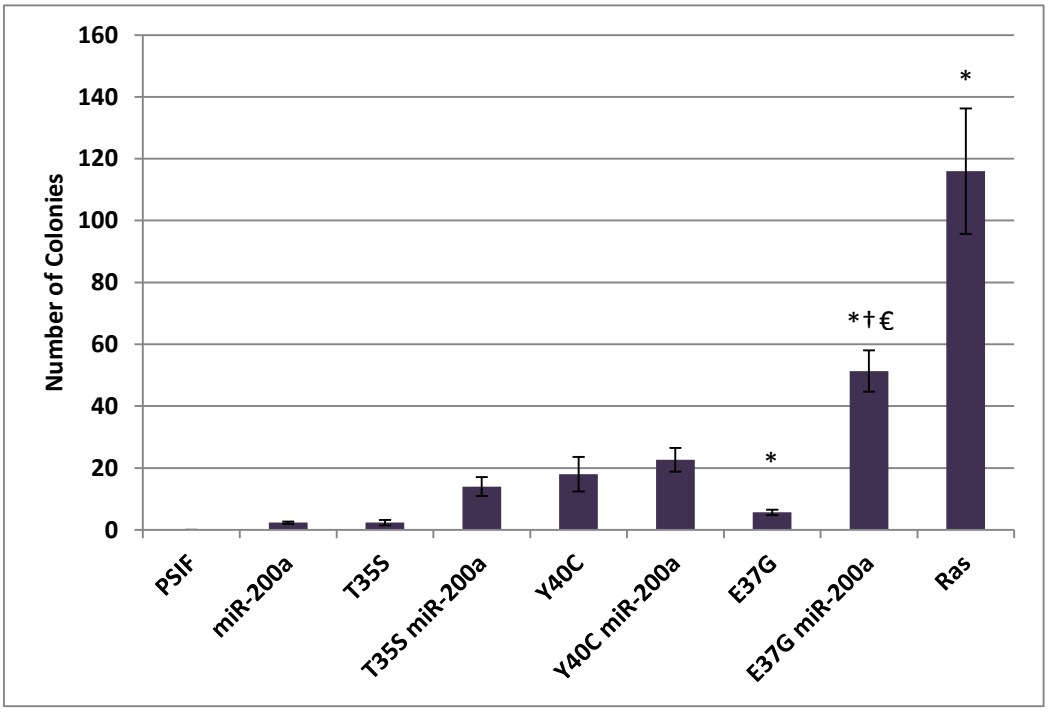


Figure 22. miR-200a cooperates with RalGEF pathway activation. Quantification of soft agar colony formation in Figure 13; MCF10A cells stably infected with miR-200a alone or in combination with the indicated Ras effector pathway mutants. Cells were plated in triplicate. Data represent means of three independent experiments +/- standard error.

* $p \leq 0.05$ compared to vector control, † $p \leq 0.05$ compared to miR-200a,

€ $p \leq 0.05$ compared to E37G.

Figure 23. miR-200a cooperates with the RalGEF pathway to induce cell cycle progression.

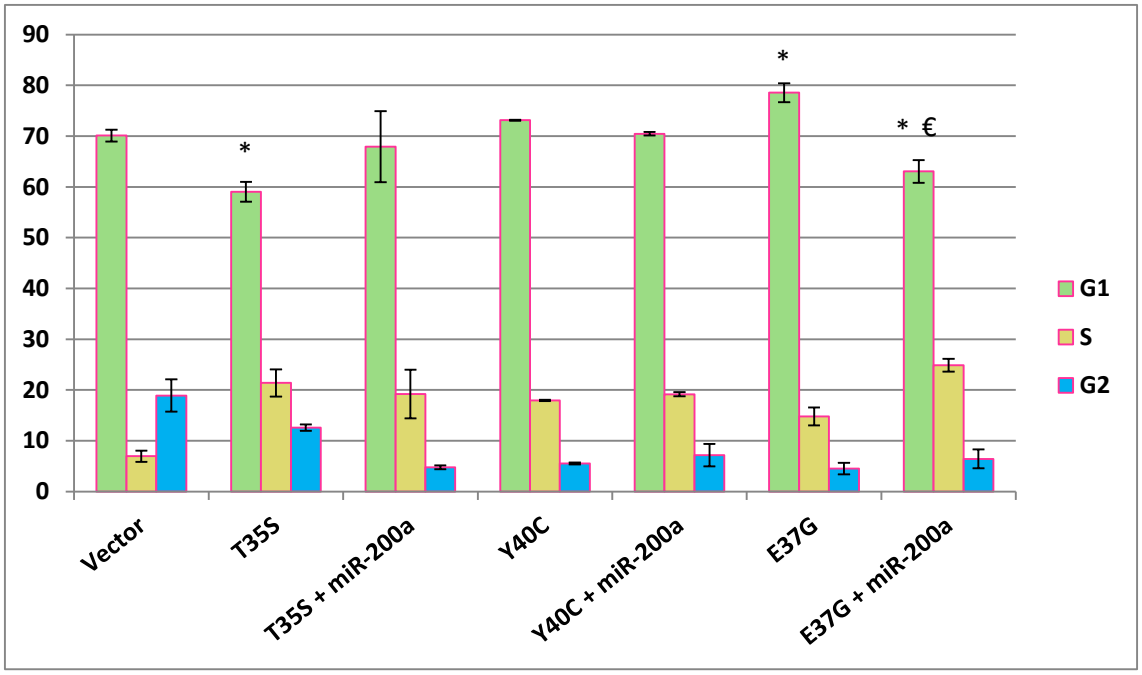


Figure 23. miR-200a cooperates with the RalGEF pathway to induce cell cycle progression. Cell cycle distribution analyzed by flow cytometry; MCF10A cells infected with the indicated oncogenes alone or in combination with miR-200a.

* $p \leq 0.05$ compared to vector control, € compared to E37G. P-values calculated for cell cycle progression out of G1 phase.

Figure 24. miR-200a cooperates with RalGEF signaling to disrupt acinar growth.

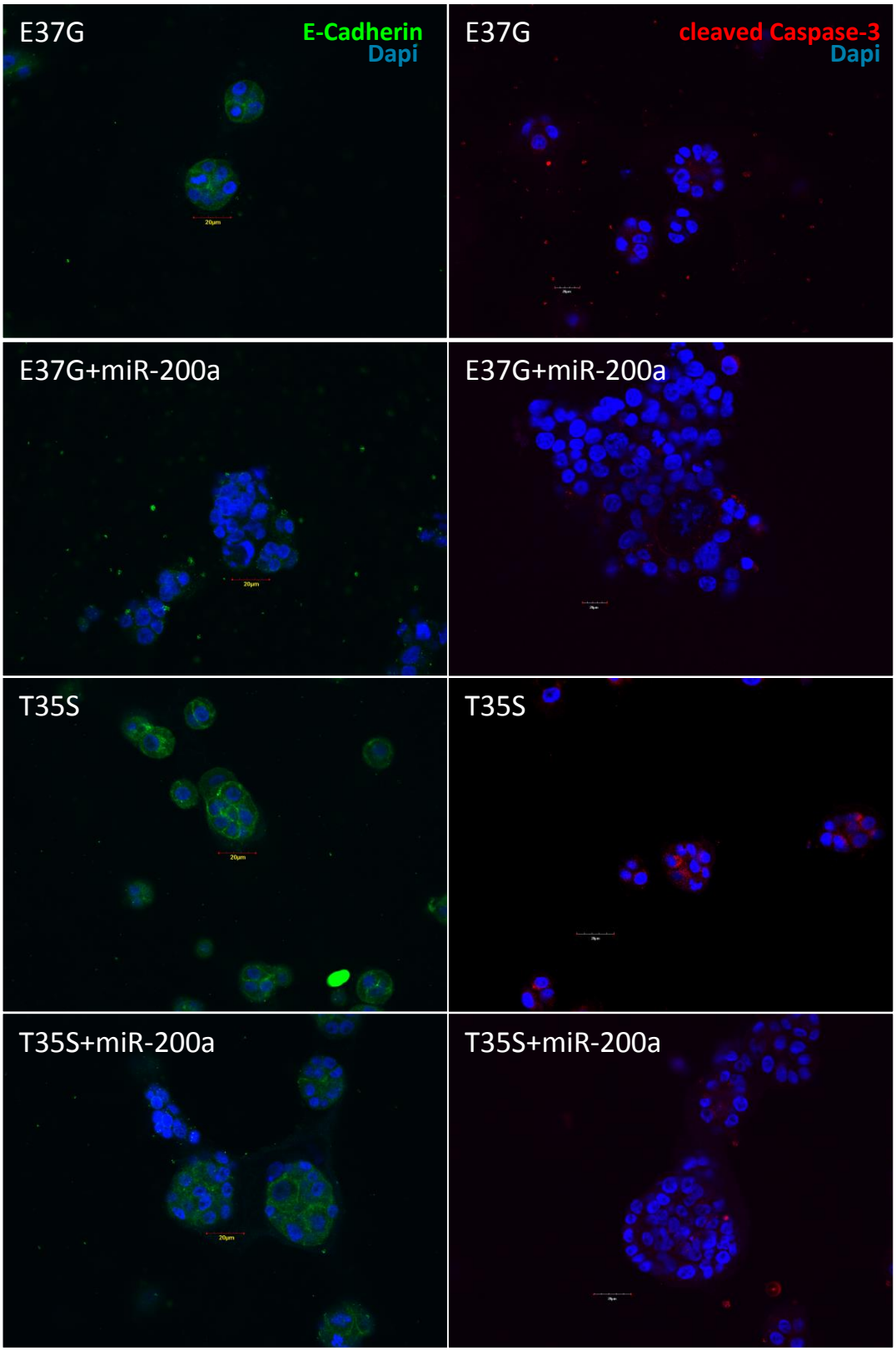


Figure 24. miR-200a cooperates with RalGEF signaling to inhibit apoptosis and induce proliferation. Three dimensional acinar formation after five days in Matrigel; MCF10A cells infected as indicated, stained for E-Cadherin (green, left panels) and cleaved Caspase-3 (red, right panels).

miR-200a stimulates Akt and suppresses p53.

To further elucidate the mechanism of miR-200a in malignant cell transformation, we analyzed MCF10A cells either transiently (Figure 25A) or stably (Figure 25B) over-expressing miR-200a for known targets as well as potential downstream effectors. Similar to RK3E cells, Fog2 expression is decreased in MCF10A cells expressing miR-200a, accompanied by an increase in Akt phosphorylation. However, the miR-200a target Pten is not down-regulated in MCF10A cells. We also analyzed p53 levels in unchallenged and Doxorubicin-challenged MCF10A cells to determine the effect of miR-200a in the apoptotic response. In unchallenged cells, basal levels of p53 were unchanged between vector control and miR-200a expressing cells. In response to Doxorubicin, p53 protein expression and phosphorylation increased in vector control cells, but not in cells expressing miR-200a. Because of the involvement of miR-200a in the Akt pathway and its cooperation with the RalGEF pathway, we also analyzed protein expression and phosphorylation levels of Erk, the third Ras effector pathway. In cells transiently expressing miR-200a, Erk expression and phosphorylation levels decreased compared to vector control; however, in cells stably expressing miR-200a, expression and phosphorylation levels of Erk were unchanged, indicating that miR-200a does not affect the Raf effector pathway of Ras.

Figure 25. miR-200a stimulates Akt signaling and inhibits p53.

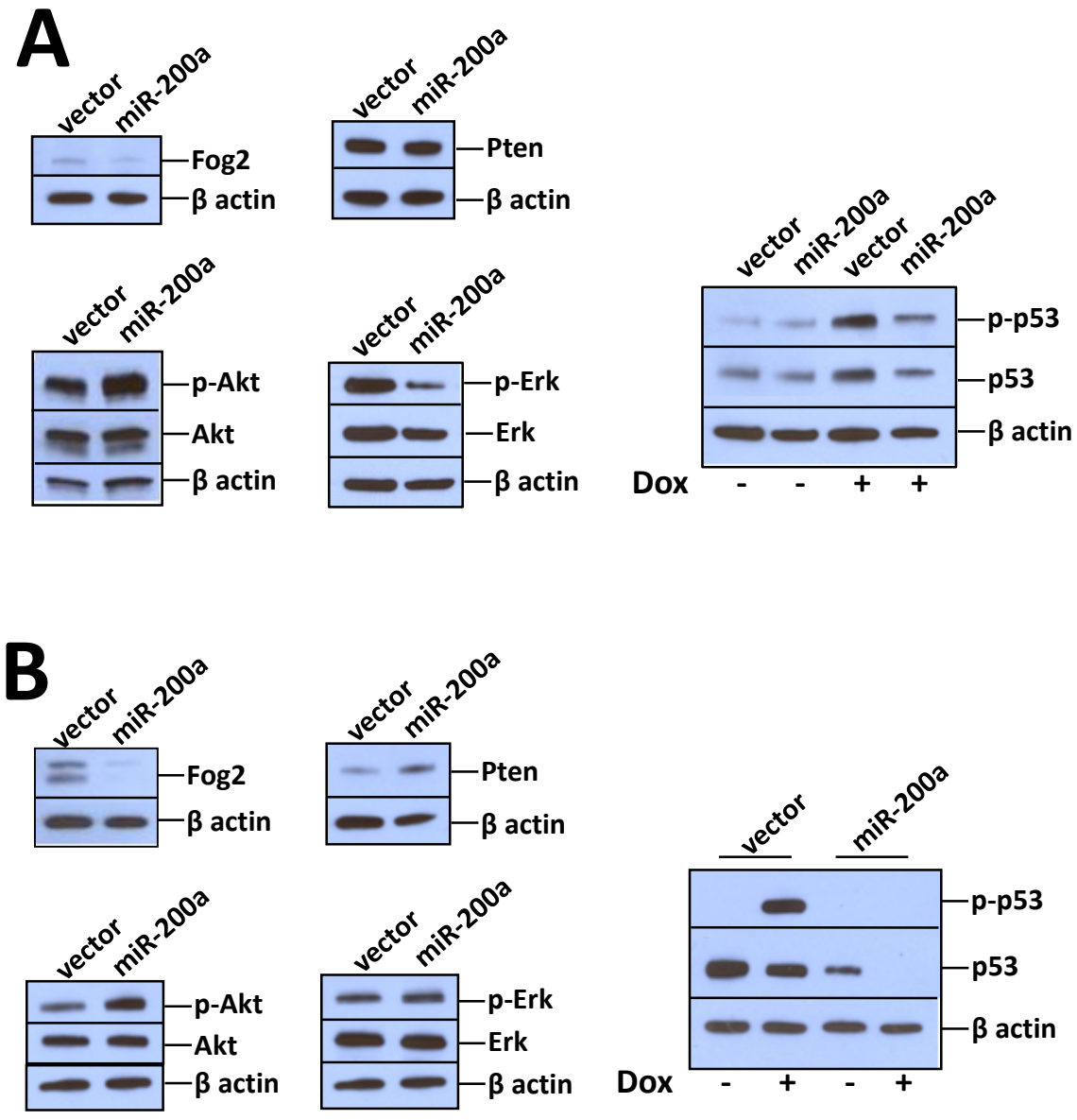
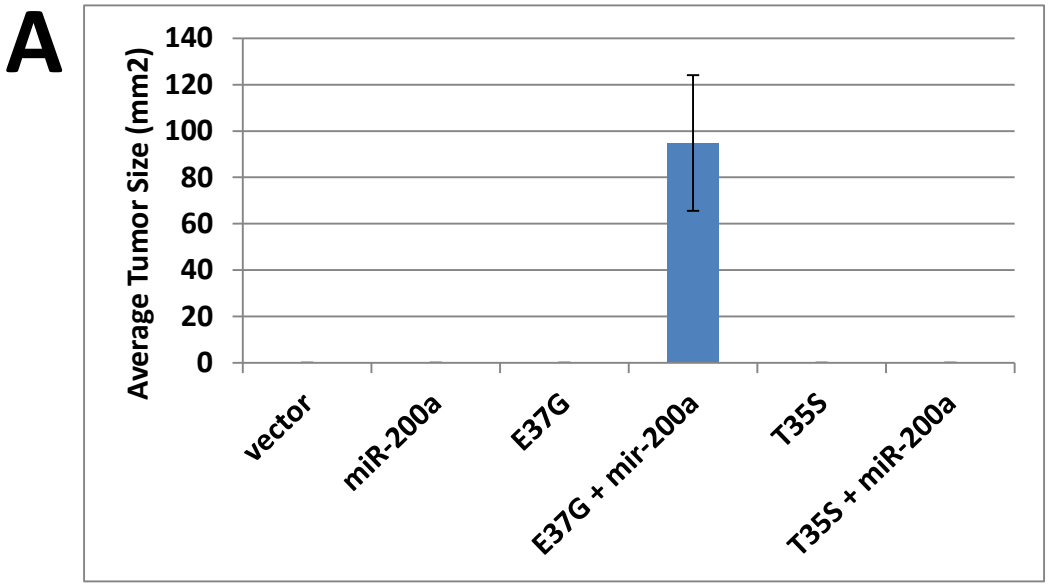


Figure 25. miR-200a stimulates Akt signaling and inhibits p53. (A) Western Blot of MCF10A cells transiently transfected with miR-200a. (B) Western Blot of MCF10A cells stably infected with miR-200a.

miR-200a synergizes with the RalGEF signaling pathway to induce tumorigenesis.

We subcutaneously injected immunocompromised nude mice with MCF10A cells expressing miR-200a in combination with the T35S mutant, or with the E37G mutant and histologically analyzed resulting tumors [194]. MFC10A cells expressing miR-200a in combination with the E37G mutant were the only cells that formed subcutaneous tumors (Figure 26). Transformed MCF10A cells recapitulate human proliferative mammary disease in subcutaneous tumors in nude mice [188]; thus we stained tumor sections with H&E and histologically examined them for abnormal tissue structures (Figure 27). All tumors were vascularized, regardless of size (Figure 27A), and duct formation was either absent (Figure 27C) or severely abnormal (Figure 27B). Defined structures of dense connective tissue were present with intermittent sections of loose connective tissue that infrequently contained small structures resembling ducts, but without regular epithelial lining (Figure 27B). The highly irregular tissue structure and loss of normal duct formation resembles the histology seen in invasive carcinoma of human mammary proliferative disease. The severity of tissue architecture disruption indicates the strong potential of the interaction between miR-200a and the RalGEF pathway to contribute to cancer initiation and tumorigenesis.

Figure 26. miR-200a cooperates with the RalGEF pathway to induce tumorigenesis in immunocompromised mice.



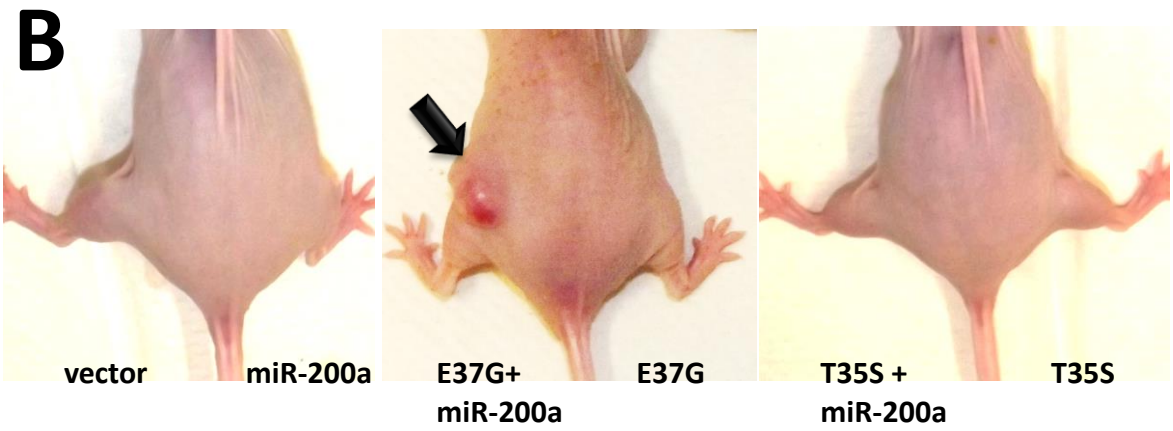


Figure 26. miR-200a cooperates with the RalGEF pathway to induce tumorigenesis in immunocompromised mice. (A) Average tumor size after 10 weeks in nude mice subcutaneously injected with the indicated Ras effector pathway mutants alone or in combination with miR-200a. (B) Representative images of tumor formation. Each mouse was injected in each flank with MCF10A cells stably over-expressing a Ras effector mutant alone in one flank, and MCF10A cells stably over-expressing the same Ras effector mutant in combination with miR-200a in the other flank. N=6 for each group.

Figure 27. miR-200a cooperates with the RalGEF pathway to induce tumor pathology resembling invasive human proliferative breast disease.

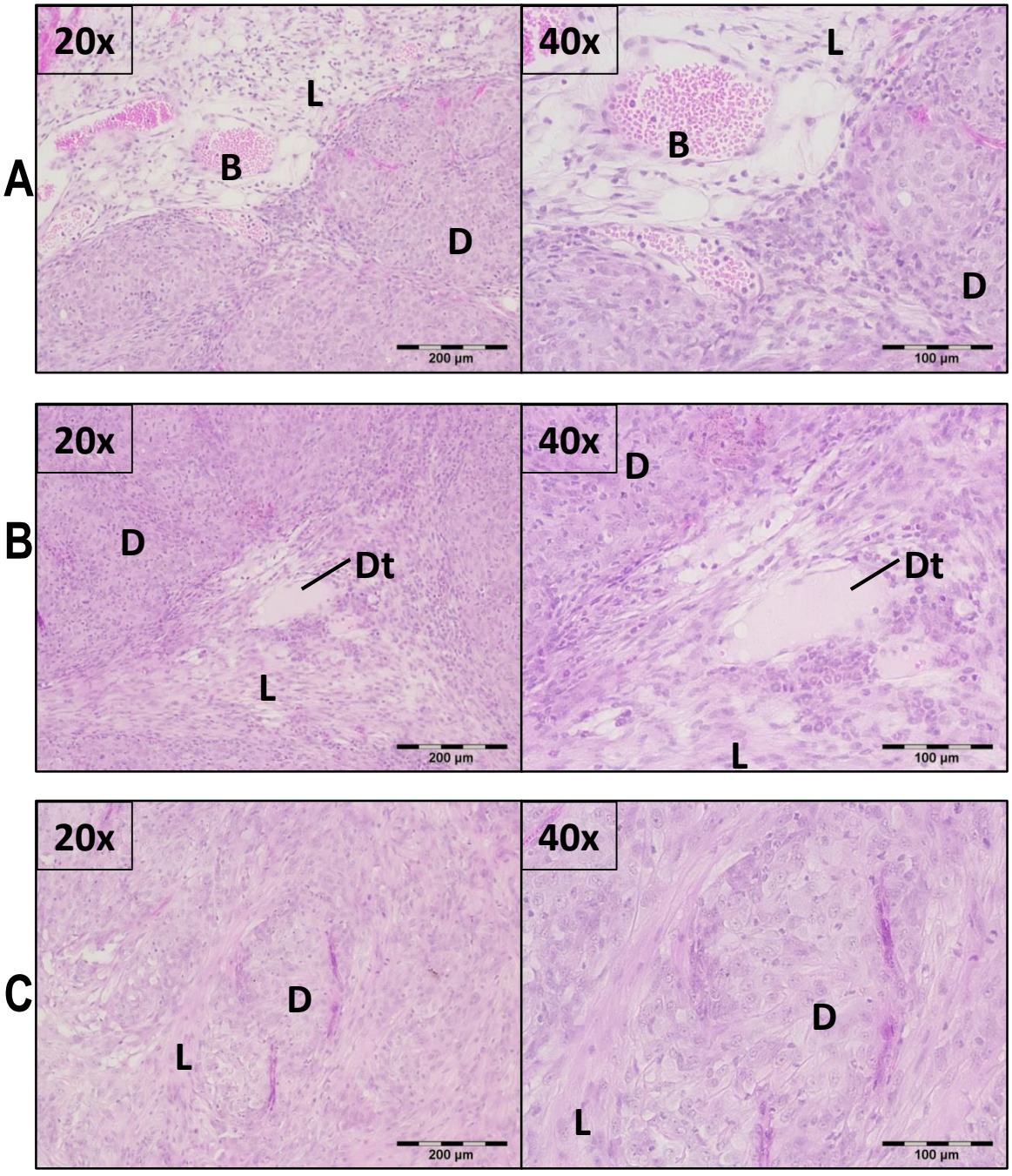


Figure 27. miR-200a cooperates with the RalGEF pathway to induce tumor pathology resembling invasive human proliferative breast disease.

A, B, C. Representative H&E staining of tumors formed by E37G+miR-200a MCF10A cells. Tissue structures resembling human mammary histology are labeled as follows: B: blood vessel, L: Loose connective Tissue, D: Dense connective tissue, Dt: Duct.

Discussion

miR-200a and EMT

miR-200a was originally studied as a tumor suppressor and inhibitor of migration and metastasis because of its inhibition of EMT. We verified that this new oncogenic role of miR-200a occurs alongside upregulation of epithelial markers. Consistent with this, cells overexpressing miR-200a alone or in combination with Ras showed decreased migration compared to vector control or Ras alone, respectively. In nude mice, miR-200a synergizes with the RalGEF pathway to form subcutaneous tumors with histology that resembles invasive proliferative breast disease. This is especially notable because miR-200a has been reported to be a crucial player in the maintenance of epithelial cell polarity, particularly in the mammary gland [195]. The disruption of tissue structure by the combination of miR-200a over-expression with RalGEF signaling highlights the significance of this interactive effect.

miR-200a, Fog2 and Proliferation

Only recently has the oncogenic role of miR-200a in proliferation been studied. It directly targets Fog2 and positively regulates cell growth [102]. Following this, more evidence has revealed the important role of the miR-200 family and Fog2 in regulating PI3K activity in the context of insulin signaling, indicating the significance of the miR-200a/Fog2/PI3K axis in human health [196-199]. We have demonstrated that miR-200a transforms immortalized rat RK3E kidney epithelial cells and augments Ras transformation of immortalized human MCF10A cells. This is consistent with a study by Zhao et al. demonstrating that activated PI3K complements Ras activation in

transformation of human mammary epithelial cells (HMECs) [200]. Our study shows that miR-200a overexpression decreases Fog2 expression and increases Akt phosphorylation as Fog2 is a negative regulator of PI3K activity. Notably, PI3K/Akt signaling is one of the three main Ras-effector pathways, and we show that miR-200a augments Ras transformation of MCF10A cells. More specifically, miR-200a complements the RalGEF/Jnk pathway to enhance colony formation in soft agar.

miR-200a alone significantly increased cell proliferation and cell cycle progression. Yet miR-200a in combination with Ras did not significantly increase cell proliferation or cell cycle progression out of G1 above that seen in cells overexpressing miR-200a alone. Indeed, miR-200a in combination with Ras had a negative interaction effect on cell proliferation compared to Ras alone. This indicates that stimulation of the pro-proliferative PI3K/Akt pathway is not responsible for the synergistic effect between Ras and miR-200a, indicating that Ras activates PI3K/Akt signaling to high levels that can not be further enhanced by miR-200a suppression of Fog2. Conversely, miR-200a in combination with RalGEF signaling causes a significant decrease in G1 cell cycle arrest compared to RalGEF alone, indicating that miR-200a induction of cell cycle progression, rather than inhibition of apoptosis, is important for cooperation with RalGEF signaling.

It is crucial to note that the phenotypic response to miR-200a overexpression in the context of Ras signaling may be cell- or tissue-type specific. miR-200a has a well-studied dichotomous role in cancer, and both its overexpression and down-regulation have been associated with increased tumorgenesis [105,119]. These studies demonstrating miR-200a's contradictory functions emphasize the potential for tissue-specific signaling and pathway interactions.

miR-200a, p53, and Inhibition of Apoptosis

Supporting the synergy between Ras and miR-200a are the changes in acinar structure and intra-luminal apoptosis. miR-200a alone did not decrease cleaved Caspase-3 levels in the lumen of acini. Although lumen formation was curtailed when Ras was expressed alone, cleaved Caspase-3 staining was not ablated, indicating that an increase in proliferation, rather than loss of apoptosis was responsible for the lack of lumen clearance in MCF10A-Ras acini. However, when Ras and miR-200a were expressed together, cleaved Caspase-3 levels decreased dramatically, indicating that miR-200a synergizes with Ras to elicit inhibition of apoptosis. Loss of apoptosis is mechanistically supported by western blot analyses showing down-regulation of p53 protein and phosphorylation levels by miR-200a in MCF10A cells. miR-200a is known to directly target p53 and inhibit apoptosis [53], and expression of p53dd in MCF10A cells has been shown to inhibit apoptosis and cause an EMT-driven loss of normal acini formation including normal structure and lumen clearance [190]. However, miR-200a is a strong inhibitor of EMT, indicating that miR-200a disruption of p53 leading to reduced apoptosis is the dominant force in disrupting normal lumen clearance, not EMT.

miR-200a Cooperates with RalGEF

The Raf-Erk effector pathway is considered largely responsible for Ras-mediated transformation of murine cells [201,202]; however, differences exist between the transformation of human cells and rodent cells. The RalGEF effector pathway alone is able to recapitulate approximately 60% of Ras-induced transformation of human embryonic kidney cells, but was not able to induce tumorigenesis *in vivo* [203].

Consistent with this, our study shows that expression of the E37G mutant (activating the RalGEF effector pathway) alone in MCF10A cells was not able to induce tumorigenesis. However, we show that the combination of miR-200a over-expression and RalGEF pathway activation is enough to induce malignant cell transformation and subcutaneous tumor formation *in vivo*. This effect is not seen when miR-200a overexpression is combined with either mutants activating the PI3K pathway (Y40C mutant) or the Raf/Erk pathway (T35S mutant). miR-200a overexpression alone was insufficient to induce colony formation or tumorigenesis, indicating that the combination of Akt activation and loss of p53 is not enough to effect transformation. By cooperating with the RalGEF pathway to transform cells, miR-200a demonstrates that loss of p53 syngergizes with gain of Akt and RalGEF signaling to transform MCF10A cells. This is consistent with a study showing transformation of human embryonic kidney cells by the cooperation of PI3K and RalGEF signaling with loss of p53 in a setting of Rb loss and telomerase gain of function [18]. Rangarajan et al. also reaffirm the importance of RalGEF signaling in human cell transformation compared to mouse cell transformation relying more heavily on the Raf effector pathway [18]. Overall, this shows that suppression of p53 and stimulation of Akt by miR-200a is enough to transform immortalized Rat cells, but not immortalized human cells; however, the addition of RalGEF signaling enables miR-200a to transform immortalized human MCF10A cells and induce tumorigenesis.

In summary, our results show that miR-200a enhances Ras-mediated transformation of human cells. This is the first study that delineates miR-200a's function in malignant cell transformation. We also show that miR-200a synergizes with the RalGEF-activating E37G Ras effector mutant to transform MCF10A cells and induce

tumorigenesis *in vivo*, providing mechanistic insight into the mechanism of action of this dichotomous miRNA. By determining that cooperation with RalGEF is necessary for miR-200a-mediated cell transformation, we have illuminated a new, specific role for miR-200a in malignancy. Furthermore, we anticipate that future studies examining the concomitant genetic changes occurring during malignant transformation will thoroughly reveal the role of miR-200a in cancer initiation and bespeak its prognostic potential.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Cancer is a deadly disease that claims the lives of over half a million people every year in the United States. Cancer is a progressive disease that begins with malignant cell transformation and culminates in metastasis. It is critical to understand cancer initiation to aid in cancer prevention, and to develop methods to detect and diagnose early stages of cancer before deadly metastasis develops.

In this study, we employed a biphasic approach to determine miRNA involvement in malignant cell transformation. The first step involved profiling our library of 366 miRNAs to determine the role of miRNA clusters in four major cancer signaling pathways. In this screen, we were able to provide a panoramic view of the effects of miRNAs on AP-1, NF-κB, p53, and c-Myc signaling. In our miRNA cluster profiling study, we found that 200b~429, which includes miR-200a, down-regulates p53 activity. miR-200a was demonstrated to directly target p53, reduce protein levels, and inhibit apoptosis. These results provide a strong foundation for the study of miR-200a as an oncomiR. miRNA clusters frequently function via cooperative targeting that involves cluster members suppressing different effectors of the same signaling pathway, or multiple subunits of a protein complex in order to produce a larger regulatory effect.

Given miR-200a's role in p53 suppression, it is likely that the entire 200b~429 cluster, as well as its family cluster 200c~141, plays a role in regulating apoptosis.

The second phase of this study was an epithelial cell screening assay to determine the ability of miRNAs to transform epithelial cells. The immortalized RK3E cell line in particular was employed because of its growth as a monolayer that becomes three dimensional foci formation upon transformation with an oncogene. From this assay, we found that miR-200a transforms RK3E cells. We further characterized the transformative potential of miR-200a in the untransformed, immortalized human MCF10A cell line. We found that miR-200a enhances Ras-mediated transformation of this cell line. These results are consistent with a study that shows that knockdown of the high endogenous miR-200a levels in MCF7 breast cancer cells reduces soft agar colony formation [62]. Mechanistic studies showed that miR-200a suppresses p53 protein levels and phosphorylation, as well as Fog2 protein levels. Fog2 is a direct target of miR-200a and a negative regulator of PI3K activity. miR-200a overexpression increased proliferation and cell cycle progression, but when Ras was added, we did not see a combinatorial effect. This indicates that the anti-apoptotic signaling mediated by miR-200a suppression of p53 is responsible for enhancing Ras-induced transformation. This enhancement of Ras-mediated cell transformation implicates a potential role for miR-200a in Ras-driven malignancies, for example pancreatic cancer. Further characterization of the mechasnism of action of miR-200a showed that miR-200a cooperates with the RalGEF effector pathway of Ras to induce soft agar colony formation and tumorigenesis in immunocompromised mice. Inability of miR-200a to transform immortalized human MCF10A cells indicates that miR-200a stimulation of Akt activity and suppression of

p53 is not enough to transform MCF10A cells; however, the addition of RalGEF signaling in this setting induces transformation. These results demonstrate a role for miR-200a in malignant cell transformation and provide a specific cellular context where miR-200a acts as an oncomiR rather than a tumor suppressor.

In summary, this work reveals a new role for miR-200a in malignancy. We show that miR-200a transforms immortalized rat RK3E cells, and when over-expressed with Ras, it transforms immortalized human MCF10A cells. Taken together, these results indicate that miR-200a is pro-oncogenic. We also show that miR-200a transforms MCF10A cells by cooperating with RalGEF pathway activation, but not activation of the PI3K/Akt or Raf/Erk pathways, which are the other two main effectors of Ras. This transformative ability underscores the importance of miR-200a in the Akt and p53 pathways. Our results highlight the importance of the specific cellular environment when characterizing the function of miR-200a. In particular, it is crucial to evaluate miR-200a expression within the setting of concomitant genetic changes occurring during malignant transformation.

Future Directions

miR-200 family

This study focuses on the transforming ability of a single miRNA, miR-200a, which exists both as a member of a miRNA cluster, as well as a member of a five-membered miRNA family. Future studies focusing on the individual effects of miR-200 family members will provide insight into the function of this family, and studies in which the miR-200 family is expressed at disease-relevant levels or ratios will provide an

understanding of the dysregulation of this entire family, as well as its individual members, within the context of cell transformation.

Noncoding RNAs

The field of noncoding RNAs is rapidly expanding to encompass lncRNAs, piRNAs, ceRNAs…the list continues *ad nauseam.* These novel regulators provide insight into the control of miRNA-based gene silencing. For example, ceRNAs, or competing endogenous RNAs, are highly abundant mRNAs that act as miRNA sponges, titrating potent miRNAs out of the pool of gene regulators, thereby unsilencing miRNA targets while the ceRNAs themselves experience insignificant downregulation by these targeted miRNAs. The presence of ceRNAs provides another level of cell context in which miR-200a is functioning.

Global gene expression effects

MicroRNA studies initially began as a race to discover as many new direct miRNA targets as possible without follow-up experiments to determine physiological relevance. As studies have progressed, it has become clear that miRNA effects are likely not due to targeting one single gene, as miRNAs have the potential to 1) target multiple genes at once and 2) be regulated by their own targeting mechanism *e.g.* by ceRNAs. Thus it is crucial to view the "one target per miRNA" perspective with caution, and future studies should take a more global approach to profile changes in gene expression in response to changes in miRNA levels. This study identifies p53 and Fog2 as key targets of miR-200a in the context of cell transformation; however, it is highly probable that miR-200a targets a plethora of genes to regulate multiple signaling pathways. This

view is further complicated by the need to characterize the combined effects of the entire miR-200 family.

Clinical Relevance

Because of the dichotomous role of miR-200a in cancer initiation, progression, and metastasis, it has the potential to serve as a context-specific biomarker for staging cancer diagnoses or providing prognostic information. For example, early stage carcinomas overexpressing miR-200a alone may be less aggressive than tumors overexpressing miR-200a in the setting of constitutive Ras activation, or increased RalGEF signaling.

In particular, changes in expression—both elevations and decreases—of miR-200a are frequently implicated in breast cancer, providing an array of evidence for the contradictory role of mir-200a in cancer. Future studies focused on miR-200a expression in breast cancer tumor samples stratified into cohorts based on prognosis, survival, and estrogen and progesterone receptor status will provide significant insight into the specific contexts in which miR-200a inhibits or contributes to cancer initiation and progression. Understanding the prognostic potential of this miRNA, and by extension the entire miR-200 family, would provide clinicians with the tools to better diagnose and treat cancer based on the personalized molecular profiles of patients.

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Publications

Original Research

1. **Becker LE**, Lu Z, Chen W, Xiong W, Kong M, et al. (2012) A systematic screen reveals MicroRNA clusters that significantly regulate four major signaling pathways. PLoS One 7: e48474.
2. Takwi A, Li Y, **Becker Buscaglia LE**, Zhang J, Choudhury S, et al. (2012) A Statin-regulated MicroRNA Represses Human c-Myc Expression and Function. EMBO Mol Med In Press.
3. Ma X, Kumar M, Choudhury SN, **Becker Buscaglia LE**, Barker JR, et al. (2011) Loss of the miR-21 allele elevates the expression of its target genes and reduces tumorigenesis. Proc Natl Acad Sci U S A 108: 10144-10149.

Reviews

1. **Becker Buscaglia LE**, Li Y (2011) Apoptosis and the target genes of microRNA-21. Chin J Cancer 30: 371-380.
2. Ma X, **Becker Buscaglia LE**, Barker JR, Li Y (2011) MicroRNAs in NF-kappaB signaling. J Mol Cell Biol 3: 159-166.
3. Deng Q, **Becker LE**, Ma X, Zhong X, Young K, et al. (2014) The dichotomy of p53 regulation by noncoding RNAs. J Mol Cell Biol.

Awards and Grants

- 1st place Poster Presentation Award – Biochemistry Department Biennial Retreat-2013
- NIEHS T32 Training grant-2013
- Condict Moore Student Poster Award - James Graham Brown Cancer Center Retreat-2012
- 1st place Poster Presentation Award - Biochemistry Department Biennial Retreat-2011

Presentations

- Research Conference Seminar – Dept of Biochemistry and Molecular Biology – April 2013
  The role of miR-200a in malignant cellular transformation
- Seminar – Dept of Biochemistry and Molecular Biology – November 2011
  The miR-200 family's effects on the Epithelial-Mesenchymal Transition in Cancer
- Seminar – Dept of Biochemistry and Molecular Biology – November 2010
  Protecting Telomeres from Replicative Damage
- Poster Presentation – Research!Louisville – September 2013
  MicroRNA-200a contributes to malignant cell transformation by activating Akt, inhibiting p53, and cooperating with Ras effector pathways.
- Poster Presentation – Dept of Biochemistry and Molecular Biology Biennial Retreat – August 2013
  MicroRNA-200a effects cellular transformation by dysregulating Akt and p53 signaling and cooperating with Ras effector pathways.
- Poster Presentation – Keystone Symposium: RNA silencing – March 2013

MicroRNA-200a Exhibits Transformative Potential Through Dysregulation of Akt and p53 Signaling Pathways.

- Poster Presentation – James Graham Brown Cancer Center Retreat – October 2012
  Transformative Potential of miR-200a: dysregulation of Akt and p53.
- Poster Presentation – Research!Louisville – September 2011
  miR-200a inhibits p53 expression and function.
- Poster Presentation – Dept of Biochemistry and Molecular Biology Biennial Retreat – August 2011
  Transformative Properties of miR-200a

Expertise

Research

- Molecular Biology and Biochemistry laboratory techniques including Western Blot, ELISA, Co-IP, mouse injection, immunofluorescence, immunohistochemistry, tissue culture, transfection, lentiviral transduction, flow cytometry, confocal microscopy, RNA and DNA purification, PCR, QRT-PCR, cloning.
- Computer-based data analysis including Excel, Vector-NTI, FloJo, Photoshop, ImageJ and the statistical analysis program R

Communication

- Highly developed writing skills through preparation of manuscripts, grant proposals, and posters.
- Presentations including departmental seminars and international conference poster presentations.
- Interpersonal communication including time spent as student body president recruiting and interviewing prospective students, and as a member of the graduate education committee working with professors to review education policies and departmental procedures and organize departmental social events.

Teaching

- Teaching Assistant - Methods Course, UofL 2010
    - Prepared reagents for students' experiments
    - Supervised students' lab techniques and assisted with troubleshooting protocols and analyzing data

- Teaching Assistant - Chemistry Department, NAU August 2005-July 2007
    - Prepared and gave lectures on execution of and principles behind introductory chemistry techniques, data analysis, and lab report writing.
    - Prepared reagents
    - Supervised students' lab techniques