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Award Number: W81XWH-10-1-0556

TITLE: MicroRNA Gene Regulatory Networks in Peripheral Nerve Sheath Tumors

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REPORT DATE: September 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-09-2012	Annual	15 AUG 2011 - 14 AUG 2012

4. TITLE AND SUBTITLE

MicroRNA Gene Regulatory Networks in Peripheral Nerve Sheath Tumors

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-10-1-0556

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Minnesota
Minneapolis, MN 55455

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In recent years, efforts to investigate the mechanisms of tumor initiation, progression and transformation have led to identification of several driver genes and pathways. We are interested in identifying miRNAs which can regulate those driver genes along with many others to drive tumor transformation. Based on the analysis of expression of miRNAs between MPNST and Schwann cells, miR29 family was found to be down-regulated in MPNSTs compared to normal Schwann cells. Many of the known gene targets of miR29 family were also found up-regulated in these conditions, implicating the loss of miR29 leading to deregulation of target genes. Some of those genes include PTEN, TGF, PDGF, SUZ12, DNMTs. Hence we proposed to knock-out all four miRNAs of miR29 family (miR29a, miR29b1, miR29b2 and miR29c) in mice which are present in two clusters. We have made gene targeting constructs for both miR29 clusters and currently screening for successfully targeted mouse ES cell colonies. We have also identified a novel pseudogene SUZ12P as potential factor contributing to tumor transformation through titrating out miRNAs which are critical for regulating key driver genes of cancer. Further, we have shown the pseudogene SUZ12P is regulated by various miRNAs and currently investigating the effects of SUZ12P on various miRNAs, genes and DNA/chromatin modification pathways. Since SUZ12P is a pseudogene of a critical component of polycomb repressor complex SUZ12, This may have a crucial role in tumor transformation through miRNAs and polycomb mediated gene/miRNA regulation.

15. SUBJECT TERMS

MPNST, microRNAs, miR-29 knockout

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	28	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

MPNST are aggressive sarcomas often associated with NF1 gene inactivating mutations. MPNSTs develop due to malignant transformation of pre-existing benign dermal or plexiform neurofibromas, with the latter having more chances of malignant transformation. Development of MPNSTs from neurofibromas is a complex process. Several studies have found differential expression of genes between benign and malignant tumors suggesting the role of several genes and pathways in transformation. Such comparative studies alone could not attribute a 'cause or effect' relationship of these genes and pathways. So, detailed mechanisms of malignant transformation still remain to be understood.

Based on comparative gene/miRNAs expression studies using tumors of various stages and cell lines of neurofibromas, MPNSTs and Schwann cells we have determined various genes and miRNAs that could be involved in malignant transformation. Since miRNAs can regulate multiple genes simultaneously, deregulation of miRNAs leads to wide spread mis-regulation of multiple genes as in the case of key genes operating high in the order of cellular homeostasis pathways. We have observed miR29 to be significantly down-regulated in MPNSTs compared to neurofibromas. Several studies have implicated the role of miR29 in activating p53 and DNMTs, which are major players in tumorigenesis, progression and transformation. We have also noticed the deregulation of miRNAs such as miR34a and miR214, whose role in MPNSTs has yet to be fully established. Hence, we hypothesize that deregulation of 'miRNAs-gene regulatory networks' play a significant role in malignant transformation of MPNSTs.

Body

Our objectives in this study is to determine the gene/miRNAs signatures of MPNST susceptibility which could serve as predictive biomarkers and functionally characterize their role in malignant transformation of neurofibromas into MPNSTs using both *in vitro* and *in vivo* mouse models which might lead to miRNA based therapeutics. We proposed to carry out this work with specific objectives listed below.

Specific Aims:

Aim # 1: Define profiles of MPNST susceptibility based on microRNA and gene expression signatures in human and mouse tumors. To accomplish this, we will:

1.1. Generate miRNA profiles for benign and malignant PNSTs from human and mouse tumors and for human MPNST cell lines.

1.2. Analyze gene expression profiles (mRNA) for human and mouse MPNSTs and human MPNST cell lines.

1.3. Validate the expression profiles of candidate miRNAs and mRNAs by quantitative PCR.

Aim # 2: Decipher the regulatory pathways mediated by microRNAs that are etiologically significant for malignant transformation to MPNST. To achieve this, we will:

2.1. Identify putative miRNA regulatory networks by in *silico* analysis of miRNA and mRNA expression data. We will analyze regulatory interactions involving miRNAs that are differentially expressed in MPNSTs as observed in our preliminary studies (for example, miR-29, miR-214 and miR-34).

2.2. Perform functional validation of miRNA- mRNA association *in vitro* using human MPNST cell lines and understand the biological consequences of miRNA modulation.

2.3. Develop novel miRNA based diagnostic markers of MPNSTs using tissue microarrays.

Aim # 3: Engineer miR-29 family dysregulation *in vivo* in the setting of benign neurofibroma. To accomplish this, we will:

3.1. Develop a mouse model that shows 'accelerated malignant transformation' phenotype with Schwann cell/Schwann cell precursor specific (Dhh-Cre) disruption of miR-29a/b1 and miR-29b2/c.

3.2. Characterize the phenotypic and genotypic features of these engineered mice.

3.3. Identify predictive biomarkers of malignant transformation using blood plasma obtained from engineered mice that have potential to show accelerated malignant transformation phenotype.

This annual report covers the work carried out from Aug 15th 2011 to Aug 14th 2012. The following tasks were approved to complete during this period.

Completed tasks:

1. Begin *in vitro* validation of functional and biological effect of miRNAs found to be associated with malignant transformation process. Begin functional characterization of top 10 miRNAs that are found to be differentially expressed in MPNSTs and neurofibromas. Begin luciferase reporter assay experiments to validate the miRNA-mRNA pairs. High throughput screening of target libraries with 3'UTRs sequences. Months 15-24.

2. Construct miR-29a/b1 and miR29b2/c targeting vectors and sequence verification of constructs. Begin generation of ES cells using these targeting vectors. Develop miR-29a-b1 and miR-29b2-c knockout mice. Months 15-24

1. Generating knock-out mice of miR29 family:

In mice, miR29 family miRNAs viz., miR29a/b1/b2/c are distributed in two clusters. miR29a & miR29b1 are on chromosome 6 and miR29b2 & miR29c are on chromosome 1. We don't have conclusive evidences to confirm whether these miRNAs are expressed / regulated in clusters or individually. In our experiments, we have analyzed expression levels of all four miRNAs of miR29 family and found that their levels are different. However, it could be due to differential stability of these miRNAs. Although these miRNAs share significant sequence similarities they have the potential to regulate different target genes. However, this does not rule out the possibility of redundancy (at least partially) in their functions. So it is essential to knock out all four miR29 family miRNAs individually and also in combinations to study their role in tumor transformation / growth.

Designing a Gene targeting vector for miR29 family.

Since we did not have any conclusive information regarding the transcription start site and promoters of miR29s, it is very challenging to decide the sequences to be deleted to knockout miR29s specifically without affecting other neighboring transcripts. It is also difficult to ensure normal expression of these miRNAs after introducing the lox-p sites for conditional deletion of alleles. So, we analyzed the genomic locus around the miRNAs, all reported transcripts from that locus, CLIP seq data of Argounaute-1,Argonaute-2 & Argonaute-3. We found several uncharacterized transcripts and noncoding RNAs in this locus. After aligning all the sequences we narrowed down to regions which are free of any transcripts and safe to insert the Lox-P sites.

Knock-out construct of miR29ab1:

miR29a/miR29b1 are located on chromosome 6. However, we do not have any conclusive evidence to confirm the co-expression and/or co-regulation of these two miRNAs. Levels of expression of these two miRNAs are usually not similar although they are located very close to each other on the same chromosome. We have observed several transcripts reported from this locus other than miRNAs and their putative precursors. There was one long transcript (#2 in Figure 1A) which includes miR29a/b1 miRNAs, but there is a second transcript (#1 in Figure1A) upstream to the former and ends almost at the beginning of the transcript #2. Hence it was difficult to predict the transcription start site of these miRNAs/transcripts. Further these multiple transcripts restrict the choice of sites for lox-p insertions. We considered all such factors in designing our gene targeting vectors.

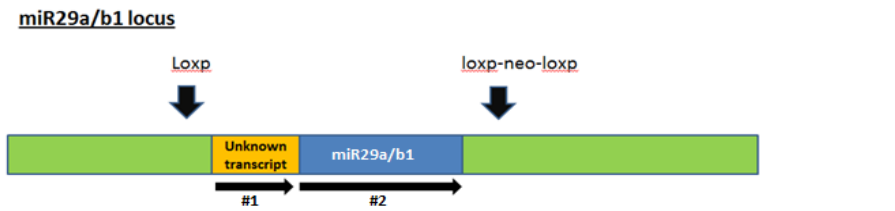

Figure 1A: miR29a/b1 locus with known transcripts indicated in black arrows. The sites for Lox-p insertions are also shown.

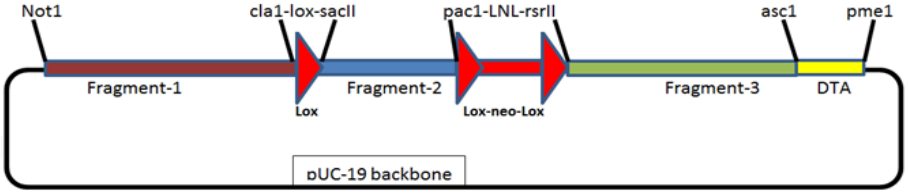

Figure 1B: Map of gene targeting vector designed for miR29a/b1 locus.

In order to construct a gene targeting vector for miR29a/b1, we designed a new vector with pUC19 backbone with a specially designed multiple cloning site consisting of selected restrictions sites. miR29a/b1 locus (of approximately 13kb) was amplified in three fragments. On either side of fragment-2 which consists of miR29a/b1 we inserted lox-p and LoxP-Neo-LoxP cassette (Figure 1B). A schematic map of the gene targeting vector is in Figure 1B. Since the promoter sites/regulatory sequences of miRNAs were not known, we inserted LoxP-Neo-LoxP (LNL) sites downstream of fragment-2.

Gene targeting vector for miR29b2 and miR29c:

miR29b2 and miR29c are located on chromosome 1. Similar to miR29a and b1, we don't have any evidences to confirm whether they are co-expressed and/or co-regulated. Analyzing the transcripts from this locus revealed several overlapping transcripts in both directions, making it very difficult to choose sites for Lox-P sequence insertions

A schematic of the transcripts is shown in Figure 2A**.** miR29b2 and C seems to be a part of a very long non-coding RNA. There are several other smaller transcripts reported from this locus as well in the same orientation (Figure 2A). They could be either degraded products of long noncoding RNAs or independent smaller transcripts. There are two other small transcripts in apposite orientation to miR29b2 and c. considering the possible transcription start sites of such transcripts and promoter sites we could narrow down to 2 very small regions where we can insert lox-p sites (to determine the locus for deletion) as indicated in Figure 2A and B. LoxP-neo-LoxP cassette was inserted after the 2nd fragment to avoid possible leaky transcription/elongation of the unknown transcript and gene expressed in apposite orientation as indicated in the Figure 2A. Lox-p site was inserted upstream of miRNAs, since precise locations of promoters are not known it was the safest approach.

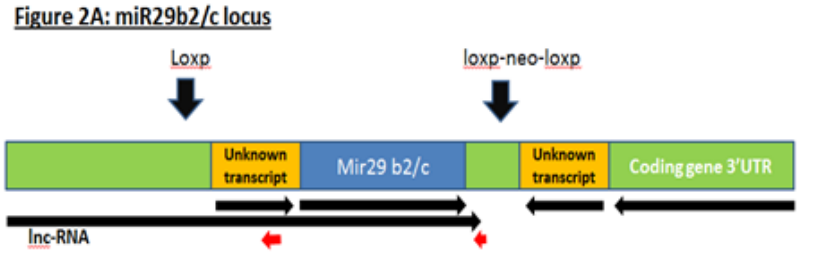

Figure 2A: miR29b2/c locus with known transcripts indicated in black and red arrows. The sites for lox-p insertions are also shown.

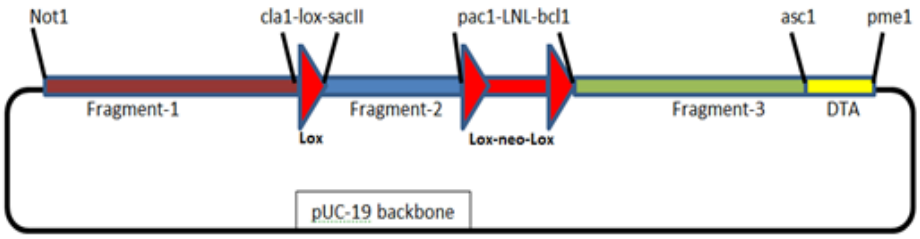

Figure 2B: Map of gene targeting vector designed for miR29b2/c locus.

A schematic map of the designed gene targeting vector is shown in Figure 2B. Both these vectors were designed for conditional deletion of included regions. Even the choices of Lox-p and LoxP-Neo-LoxP cassette were chosen based on the direction of miRNA transcription and the neighboring genes and non-coding RNAs. In our view this is the best possible way to delete the miR29 family miRNAs without/least affecting other neighboring genes and transcripts.

Gene targeting in mouse ES cells:

In order to do gene targeting with both miR29a/b1 and miR29b2/c knock-out vectors, we linearized both gene targeting vectors and transfected them into C57BL/6J ES cells individually. Currently we are screening selected clones for site specific integration of gene targeting vectors.

II. Role of a pseudogene in transformation of MPNST

In order to investigate the mechanisms of transformation of tumors, people have tried to explore the role of genes, miRNAs, CNVs etc. Most of them are studied in human cell lines and animal models where it is possible to establish cause and effect relationships. However there could be many human specific elements in causing this transformation. Here we have identified one such factor which might play a role in transformation of neurofibroma into MPNST.

Careful analysis of NF1 locus which is often deleted/mutated in MPNSTs revealed that there are some hotspots between which micro-deletions are usually observed. Micro-deletions are often seen in SUZ12 gene and a pseudogene of SUZ12 called SUZ12P (Figure 3A). Although SUZ12 and SUZ12P share more than 88% similarity in genomic regions, SUZ12P transcripts are relatively smaller (~700nts) and share very little sequences (of ~500bases) with SUZ12 gene (Figure 3B). This facilitates intra-chromosomal recombination leading to micro-deletion of locus between them. The deleted locus contains many genes, miRNA and pseudogenes including a tumor suppressor *Nf1* [1] and many other genes, miRNA and pseudogenes. SUZ12 is a component of polycomb repressor complex (PRC2) which plays very critical role in cell differentiation and development.

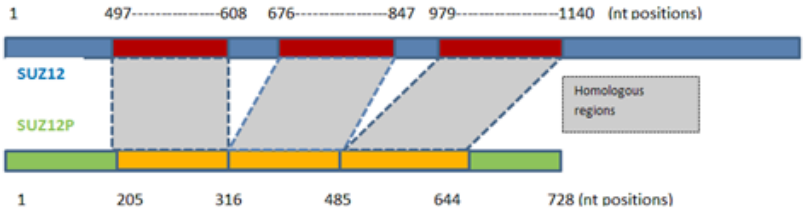

Figure 3B: A schematic representation of SUZ12 and SUZ12p. Homologous regions are highlighted and nucleotide positions are indicated.

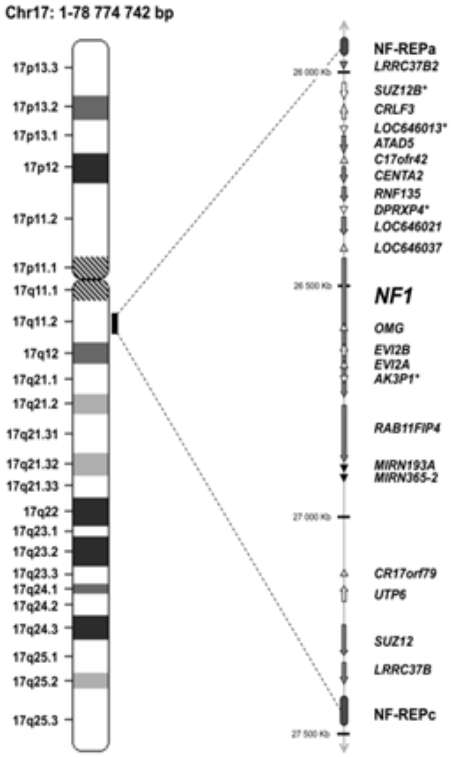

Figure 3A: Schematic diagram showing NF1 locus; * indicates pseudogenes

These polycomb genes are very critical in early developmental stages of an organism and highly conserved across species. They execute their function through *Polycomb Repressor Complex* (PRC1/2/3) which induce methylation of histones (markers of inactive chromatin) leading to suppression of genes. PRC are essential to maintain stemness of a cell and have to be suppressed to undergo differentiation during early development [2]. But in most of the cancers these genes are overexpressed implicating a de-differentiated state of tumor/transforming cells or at least tumor initiating cells [2, 3]. But their mechanisms need to be understood.

Pseudogenes are often found in most of the species and some are specific to humans (e.g: SUZ12P). Depending on the location of the pseudogene they might play a serious role in genomic recombination leading to chromosomal aberrations like deletions, recombination etc. Many such chromosomal aberrations are associated with various cancers. Often pseudogenes are transcribed but the transcripts could be different from the homologous functional gene itself (at least partially). Their expression is spatially and temporally regulated. Therefore it is possible that they play significant role in regulating expression of various genes and noncoding RNAs and might be involved in other pathways including RNAi and Chromatin modifications.

Our preliminary analysis of PAR-Clip data of Ago1 and Ago3 revealed *SUZ12P* transcripts are associated with RNAi machinery [4, 5] implicating their role in RNAi mediated regulation. *Overall, SUZ12P pseudogene and transcripts have the potential to play important role in gene/genome regulation.*

In our efforts to establish a correlation with cancer, we have observed that *SUZ12P* is overexpressed in MPNSTs compared to benign tumors. We observed a similar trend in Schwann cells and MPNST cell lines as well (Figure 4**)**.

We have predicted and observed that several microRNAs can target SUZ12 &/or SUZ12P. Unlike commonly used prediction methods which consider only 3'UTRs of genes, we included coding regions, 5'UTRs and 3'UTRs avoiding any bias. Some of the predicted miRNAs include miR200c (involved in EMT, tumor transformation and metastasis) [6, 7, 8, 9], miR302 (inducing pluripotency) [10, 11] and many other important miRNAs like miR200b, 203, 214, 182, 21, 29, 520, 503 etc., An interesting outcome of our prediction is that miR-503, 182 and 520 may target 5'UTR, coding region and promoter region of SUZ12 respectively, and all of these can target SUZ12P transcripts as well. We have observed that overexpression of miR29 and miR503 leads to suppression of SUZ12P **(Figure 4)**. Hence we think SUZ12P can regulate the levels of SUZ12 by titrating-out/ quenching of miRNAs that can suppress SUZ12 gene. This kind of buffering/titration is a novel mechanism of tuning the expression levels of genes by competing for the same regulatory RNAs. In a broader perspective, SUZ12P when overexpressed in cancer cells can thus release the suppression of many other genes inhibited by miR200b/200c/302/503/182/214 etc., which are known to be involved in EMT, pluripotency and other pathways driving tumor progression and transformation. If confirmed this mechanism would be a first of its kind where a pseudogene SUZ12P regulates polycomb mediated pathways, RNAi mediated pathways & gene/ miRNA/ *lnc*-RNAs expression contributing to tumor transformation.

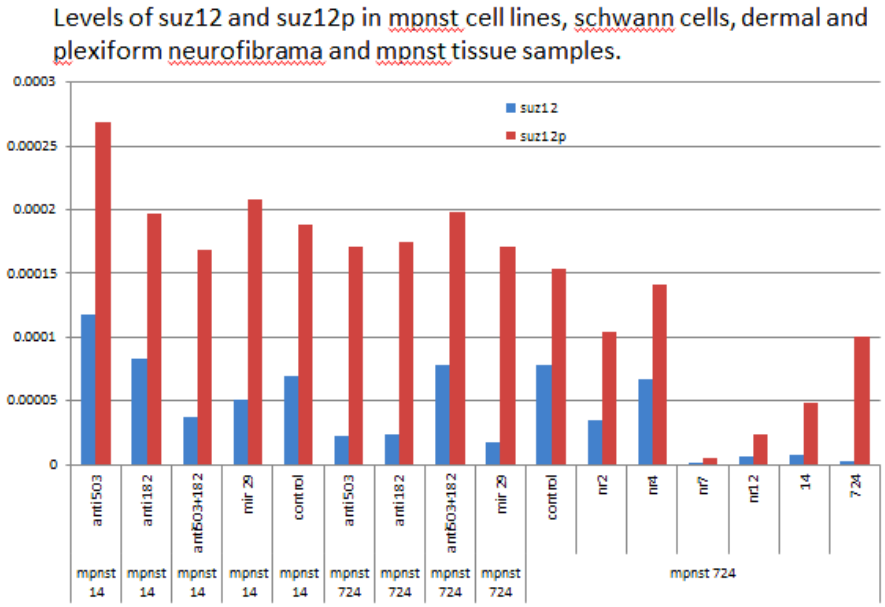

Fig4: figure shows the levels of suz12 and suz12p in schwann cells (nr2), dermal neurofibroma (nr4), plexiform neurofibroma (nr7) and MPNST (nr12) tissues and cells lines mpnst 14 (14), and mpnst 724 (724). Mpnst 14 and 724 cells were transfected with mimics of miR29, and antimirs of mir182 and miR503 individually and in combinations. Levels of suz12 and suz12p are plotted.

In order to confirm the role of SUZ12 and SUZ12P in tumor transformation, we would like to perform both *gain-of-function* and *loss-of-function* studies of both these gene/pseudogene. Therefore, we have constructed shRNA constructs to knock-down the expression of SUZ12/SUZ12P individually and both together and also plasmids expressing full length transcripts to overexpress them. Our vectors have a luciferase reporter in an independent cassette to facilitate in-vivo tracking of cells in xenograft models. We would like to investigate the effect of loss and gain of SUZ12/SUZ12P on cell proliferation, viability, migration and invasion. We found Transient overexpression/ suppression of SUZ12/SUZ12P in cells showed only mild effects. Therefore, currently we are selecting stably integrated cells of MPNST with all overexpression and shRNA constructs. We are also making lentiviruses out of all these constructs to get better transduction in transient experiments.

In order to confirm whether the role of SUZ12P is through RNA or some unknown translated product, we have designed SUZ12P constructs fused to T7 RNA polymerase promoter sequences to produce RNA by in-vitro transcription. The *in vitro* transcribed RNA was transfected into MPNST cells and cells were harvested to collect RNA and protein. Currently we are analyzing the expression levels of various genes/miRNAs and cellular parameters like viability, invasion, proliferation and migration.

Key research accomplishments:
1. Completed generation of knockout constructs for both miR-29a/b1 and miR-29 b2/c

2. DNA electroporation and screening of positive clones.

3. Identified SUZ12P as a potential regulator of microRNAs and driver of malignant transformation through integrated genomic approaches

Reportable outcomes:
The work is on going, we have published a paper on genes that can function as competing endogenous RNA that can affect the microRNA networks.

Publications
A research and review article was published in this project period. These articles are attached in appendices.

1. Sarver A, Subramanian S. Competitive endogenous RNA Database. *Bioinformation* 2012 8(15): 731-733.

2. Subramanian S and Kartha RV. MicroRNA mediated gene regulations in human sarcomas. *Cell Mol Life Sci 2012* (*in press*)

Conclusions:
We are extensively moving forward with our *in vitro* and knockout studies and expect to have the miR-29 knockout phenotypes analyzed in this upcoming project period.

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Appendices: PDF files of two articles published.

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Volume 8(15)

Competing endogenous RNA database

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Received July 27, 2012; Accepted July 28, 2012; Published August 03, 2012

Abstract:
A given mRNA can be regulated by interactions with miRNAs and in turn the availability of these miRNAs can be regulated by their interactions with alternate mRNAs. The concept of regulation of a given mRNA by alternate mRNA (competing endogenous mRNA) by virtue of interactions with miRNAs through shared miRNA response elements is poised to become a fundamental genetic regulatory mechanism. The molecular basis of the mRNA-mRNA cross talks is via miRNA response elements, which can be predicted based on both molecular interaction and evolutionary conservation. By examining the co-occurrence of miRNA response elements in the mRNAs on a genome-wide basis we predict competing endogenous RNA for specific mRNAs targeted by miRNAs. Comparison of the mRNAs predicted to regulate PTEN with recently published work, indicate that the results presented within the competing endogenous RNA database (*ceRDB*) have biological relevance.

Availability: http://www.oncomir.umn.edu/cefinder/

Key words: ceRNAs, MRE, microRNA response elements, database, competing endogenous RNAs database, ceRDB

Background:
MicroRNAs (miRNAs) play an important role in almost all biological functions **[1].** Transcriptional deregulations in miRNAs have been implicated in disease processes including cancers and developmental disorders **[2]**. It has been well established that a single miRNA can regulate the expression of many mRNAs/ transcripts and an mRNA can be regulated by multiple miRNAs **[1]**. miRNA gene regulation is mediated by a complex set of proteins termed RNA induced silencing complex. The miRNAs are guided to the miRNA response elements (MRE) present in the target mRNAs, which may result in transcript degradation and/or translational inhibition **[3].** Recently it has been established that miRNA activity on the target gene can be influenced by the presence or absence of other competing endogenous (ceRNA) mRNAs that contain shared MREs **[4-7]**. These miRNA activity modulators can act as a sponge, absorbing and releasing miRNA based on the level of the transcript. Several modulators of miRNA activity have been recently characterized **[8].** Salmena et al proposed a hypothesis that these modulators can communicate with each other in a miRNA dependent manner mediated through MREs **[9].** This complex miRNA-mRNA network and interactions opens up a new chapter in miRNA-mediated gene regulation. However, currently there are no publicly available resources that identify and catalog the list of genes that can act as miRNA activity modulators or ceRNAs. Here we developed a comprehensive and easy to use resource named '*competing endogenous RNA database (ceRDB)*' that lists potential MRE containing genes that can act in a sponge like fashion for a given mRNA based on a set of scoring and ranking criteria.

Methodology:
MiRNA-mRNA target interactions were obtained from http://www.targetscan.org Release 5.2 June 2011. The predicted conserved target information file was parsed to obtain 54979 conserved human miRNA-mRNA interactions. To explore the structure of the dataset, the list of interactions was converted into a matrix containing 153 miRNA families on the X-axis and 9448 target mRNAs on the Y-axis. The presence of a predicted conserved miRNA-mRNA interaction is defined by the presence of a '1' at the defined gene row miRNA column corresponding to the interaction. The absence of an interaction is defined by the presence of a '0' at the corresponding interaction. To shuffle the matrix, interactions between each miRNA and mRNA were randomly assigned maintaining the total number of interactions for each mRNA. Both the real matrix and the shuffled matrix were filtered to only show genes with more than 5 miRNA binding sites and these were

clustered using Gene Cluster 3.0 hierarchical clustering of both the X and the Y-axis using Centroid linkage. The resulting clustered matrixes were visualized using Java Treeview. To score potential ceRNA interactions, the 54979 human interactions were loaded into a mySQL database and when the user selects a given mRNA all predicted miRNA targets for the given mRNA are obtained. These miRNA are then used to define all mRNAs that contain binding sites for the set of miRNAs. For each mRNA, an interaction score is then defined by adding up the total number of miRNA binding sites that overlap with the miRNA for a given mRNA. This interaction score is then used to sort the results and the top 50 predicted potential ceRNAs are returned. This process is carried out on the fly using PHP interactions with mySQL in a similar fashion as previously described in our publicly available databases such as sarcoma microRNA expression database (S-MED).

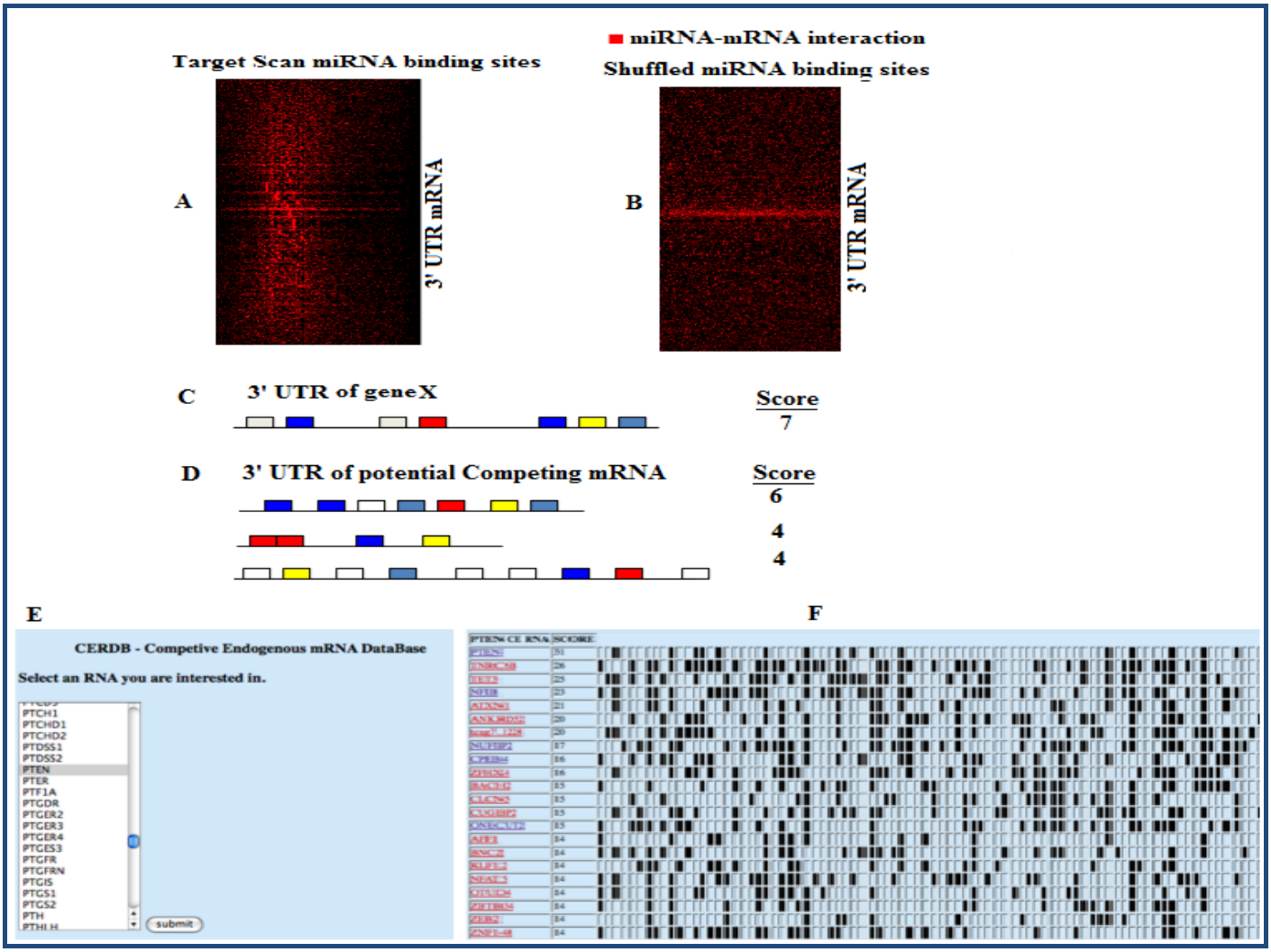

Figure 1: Visualization of co-occurrence in predicted miRNA-mRNA interactions**.** Heat map showing the presence of predicted miRNA-binding sites on the X-axis and the genes that contain the binding sites in the 3'UTR on the Y-axis. Only genes that show more than 5 binding sites are shown for **(A)** predicted interactions and **(B)** predicted interactions after shuffling. **(C)** Predicting competing mRNA via miRNA-mRNA interactions. miRNA binding site predictions in the 3'UTR are shown as colored boxes. The 'Score' is generated by counting the number of conserved predicted interactions. In this hypothetical case shown there are 7 predicted binding elements in the 3'UTR of the gene. **(D)** To predict potential competing RNA for the gene shown in A, binding sites for the predicted miRNA found in A are obtained and summed in all genes. The genes are then sorted by total number of overlapping binding sites and returned to the user. **(E)** Example of competing mRNA predictions from ceRDB for PTEN. The user selects an mRNA of interest from the list of available mRNA. In the case shown here the PTEN tumor suppressor is chosen. **(F)** Starting with the list of miRNA binding elements present in PTEN the tool predicts potential competing RNA and visualizes the extent of overlap between the miRNA binding sites. Only a representative subset of the matrix is shown, the full matrix is available online. Each predicted gene is linked back to the TargetScan database to visualize the position and total numbers of each miRNA element.

Results:

In order to define the information content present within miRNA-mRNA predicted interactions we clustered a matrix containing miRNA families on the Y-axis with genes on the X-axis. Predicted binding interactions are labeled with a '1' and the lack of an interaction is labeled with a '0' at corresponding

points in the matrix. A heatmap of the clustered images as well as the branch structure indicate that miRNA binding sites coexist within 3′UTR at a much higher rate than would be expected at random. To visually show this we randomized the interaction matrix and clustered the results **(Figure 1A & B).** Within the cell, each miRNA has many mRNA targets and each mRNA has potentially many miRNAs capable of regulation leading to a complex and dynamic regulatory system. One heretofore overlooked consequence of this system is that manipulation of the transcript level of a given mRNA may lead to changes in the concentration of available miRNAs leading to changes in alternate mRNA regulation via miRNA-mRNA interactions. In order to predict these interactions for a given target mRNA we determined all possible miRNA binding to this target mRNA and then found mRNAs (ceRNAs) that contained binding sites to these miRNAs. The potential for competition was ranked for each mRNA by counting the number of overlapping miRNA binding sites shared between the given mRNA and the potential ceRNA **(Figure 1C & D).** Competing endogenous mRNA rankings were generated using the conserved mRNA-miRNA interactions. To access the data, we built a simple to use web interface and have made it available at http://www.oncomir.umn.edu/cefinder/. The user enters an mRNA they are interested in finding potential competing mRNAs that can regulate the gene of interest, and the tool returns potential ceRNA regulators. The list is sorted based on the overlap of the miRNA binding sites in the each of the pairwise relationships **(Figure 1E & F).** Additionally the miRNA interactions present within the 3′UTR of the primary mRNA and all potential regulators are visualized in the final table**.**

Discussion:

In recent years miRNAs have taken center stage in many aspects of post transcriptional gene regulation. The complexity of miRNA-mediated gene regulation is compounded by the presence of multiple mechanisms that modulate either the levels of miRNAs and/or its target gene. Recently, the Pandolfi group proposed a novel concept in which mRNAs can regulate each other via common miRNA response elements **[4, 8]**. Through this cross talk novel mRNA-mRNA interactions have been identified in multiple cancer types. These findings suggest that modulation of miRNA activity by changing the levels of competing endogenous RNA is a key fundamental mechanism of gene regulation that will be applicable for many biological functions. Here we present a general and straightforward tool for identifying competing endogenous RNAs (ceRNAs) for a given gene of interest. Starting with the conserved set of miRNA-mRNA interactions, we observe that there is high degree of co-occurrence of miRNA binding sites within the miRNA-mRNA interaction dataset. This is consistent with the reports of Shalgi et al **[10].** We then use the co-occurrence of miRNA binding sites to predict and rank potential ceRNAs for all mRNAs. Our predictions are experimentally validated for PTEN and likely very relevant for a large number of additional genes **[5].** Several recent articles have described ceRNAs that are capable of regulating *PTEN* via competing reactions **[4, 5].** In these cases, loss of a competing mRNA releases miRNAs for interaction with the tumor suppressor *PTEN* leading to decreased *PTEN* expression. Our database predicts many of the biologically validated interactions previously reported and uses a very straightforward algorithm in identifying these competing endogenous RNAs. Our search for ceRNAs for many established tumor suppressors in our database revealed some interesting observations. For example, genes such as *ONECUT2*, *NFIB* and *TNRC6B* appeared in many of the ceRNAs gene lists, these genes contains long 3′UTRs of up to 14kb in length and are predicted to contain many MREs that can potentially act as a sponge for multiple miRNAs. We are tempted to speculate that these ceRNAs with long 3′UTR can act as a 'master' MRE containing gene whose regulation may be affected in multiple disease conditions. Recently, TNRC6B was predicted to function as a ceRNA for *PTEN* and the downregulation of *TNRC6B* reduced the expression of *PTEN* **[5].**

In conclusion, we have developed the ceRDB resource to in the future accommodate multiple species such as model organisms and other types of sequences such as long non-coding RNAs and pseudogenes that can potentially also function as ceRNAs. We believe that the concept of competing endogenous RNA is likely to become a canonical central theme of gene regulation and having the ceRDB resource will significantly enhance our understanding of this fundamental gene regulatory mechanism.

Conflict of Interest:

We declare no conflict of interests

Author contributions:

AS and SS developed the idea. AS wrote the code and implemented. AS and SS wrote the manuscript.

Funding:

We acknowledge the funding from Department of Defense grant number # W81XWH-10-1-0556.

Acknowledgement:

We profusely thank Drs. Clifford Steer, Reena Kartha, Praveensingh Hajeri and Venugopal Thayanithy for their helpful discussions. We also wish to acknowledge the Minnesota Supercomputing Institute for providing access to computational resources.

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Edited by P Kangueane

Citation: Sarver & Subramanian, Bioinformation 8(15): 731-733 (2012)

Cell. Mol. Life Sci.
DOI 10.1007/s00018-012-1127-x

Cellular and Molecular Life Sciences

MULTI-AUTHOR REVIEW

MicroRNA-mediated gene regulations in human sarcomas

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Received: 8 August 2012 / Revised: 9 August 2012 / Accepted: 9 August 2012

Abstract Sarcomas are a heterogeneous group of tumors with mesenchymal origins. Sarcomas are broadly classified into bone and soft tissue sarcomas with over 50 subtypes. Despite recent advances in sarcoma classification and treatment strategies, the prognosis of some aggressive sarcoma types remains poor due to treatment infectiveness and development of drug resistance. A better understanding of sarcoma pathobiology will significantly increase the potential for the development of therapeutics and treatment strategies. Recently, expressions of microRNAs (miRNA), a class of small non-coding RNAs, have been found to be deregulated in many sarcomas and are implicated in sarcoma pathobiology. Comprehensive understanding of gene regulatory networks mediated by miRNAs in each sarcoma type and the conservation of some shared/conserved miRNA-gene networks could be potentially investigated in the prevention, diagnosis, prognosis and as multi-modal treatment options in these cancers. In this review, we will discuss the current knowledge of miRNA–gene regulatory networks in various sarcoma types and give a perspective of the complex multilayer miRNA-mediated gene regulation in sarcomas.

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Keywords Sarcoma · MicroRNA · Soft tissue sarcoma · Bone sarcoma · Markers · Expression · Gene networks · Osteosarcoma · Rhabdomyosarcoma · MPNSTs · GIST · Synovial sarcoma · Liposarcoma

Introduction

Sarcomas are mesenchymal tumors that accounts for about 1 % of all malignant tumor types in humans [1]. Approximately 15 % of all malignant tumors in children are pediatric sarcomas. Sarcomas can occur anywhere in the body, but the majority occur in the extremities and can be broadly classified into bone and soft tissue sarcoma with over 50 subtypes [2, 3]. Sarcoma nomenclature is generally based on the cell and/or tissue type, as in osteosarcoma (OS), angiosarcoma, rhabdomyosarcoma (RMS) and liposarcoma. Even within a specific sarcoma type, tumors are highly heterogeneous and the histopathological and clinical features are not always distinct, causing a diagnostic challenge. Several sarcoma types, however, are characterized by chromosomal translocations resulting in tumor-specific fusion transcripts [4, 5]. The presence or absence of such fusion transcripts are widely used as diagnostic markers for certain subtypes [6]. In addition, DNA copy number changes are used in sarcoma diagnosis [7]. Despite these markers, a significant number of sarcomas are characterized by complex karyotypes and remain unclassified [6]. The risk of metastasis and the prognosis also depends on the sarcoma type and grade [2]. Generally, the standard-of-care for high-grade tumors includes chemotherapy and/or radiation. Despite advances in treatment strategies, the 5-year survival rate remains static for certain sarcoma types [8]. This bleak prognosis could be attributed to the relative ineffectiveness of the treatment as well as the complex

Published online: 25 August 2012

tumor biology leading to drug resistance [9]. A better understanding of sarcoma biology will significantly increase the potential for identification and development of novel therapeutic targets and treatment strategies.

Significant efforts have been made to generate gene expression profiles of various sarcoma types [10–14]. These profiles have enhanced our understanding of the biology and classification of various sarcoma types [15]. In recent years, microRNA (miRNA) expression patterns of several sarcoma types have been determined [16, 17]. MicroRNA signature-based classification and subgrouping of sarcomas were largely based on lines of differentiation [16]. It is well established that miRNAs play a significant role in posttranscriptional gene regulation [18]. Substantial expression data have been generated using sarcoma tumor tissues and cell lines, allowing us to integrate gene and miRNA expression patterns and perform complex correlative and functional studies [19] to better decipher the sarcoma biology. In this review, we will discuss our current understanding of miRNA-mediated gene regulatory networks in various sarcoma types and give our perspective on the complex layers of gene regulation mediated by miRNAs in these cancers.

MicroRNA biogenesis and function

MicroRNAs play a fundamental but significant role in most aspects of cellular biology [20–25]. miRNA biogenesis pathways and the mechanisms of regulatory functions are described elsewhere in detail [25–28]. Here, we briefly outline the biogenesis of miRNA. miRNAs are evolutionarily conserved small non-coding RNAs that post-transcriptionally regulate gene expression. In humans, miRNAs are present in exons, introns or intergenic regions. miRNAs are initially transcribed as primary miRNAs (pri-miRNAs), either as monocistronic (single) or polycistronic (multiple miRNAs) transcripts [29]. These pri-miRNAs are processed by an RNAse III enzyme, Drosha, to become precursor miRNA (pre-miRNA), a stem loop structure of about 70 nucleotides. Pre-miRNAs are exported to cytoplasm by exportin 5 and subsequently processed by Dicer to form mature miRNAs, 18–24 nucleotides in length. The mature miRNA duplex is then bound to a miRNA-induced silencing complex (miRISC), where the passenger or star strand is selectively degraded, retaining the active strand. The '*seed*' sequence in the active strand directs the miRISC to complementary sites in the mRNA transcripts (typically in the 3′UTRs) and negatively regulates gene expression [30, 31]. Protein translation regulation or mRNA degradation of the target is determined based on the miRNA complementarity with the target sequences [31, 32]. The biogenesis of miRNAs is graphically illustrated in Fig. 1.

miRNAs have emerged as major gene regulators and are implicated in many disease processes including cancers [25]. More than 60 % of human transcripts are directly or indirectly under the control of miRNAs. miRNAs associated with cancer are collectively known as oncomiRs [33]. A miRNA can function either as an oncogene or a tumor suppressor based on the cell types and the target genes it regulates [34, 35]. The role of miRNA can be attributed to the established hallmarks of cancer. For instance, over 40 miRNAs are implicated in cellular apoptosis [36] and some may have overlapping roles in other cancer mechanisms such as cell angiogenesis, invasion and metastasis [37–39].

The functional role of miRNAs has been extensively studied in many cancer types, especially carcinomas and hematopoietic malignancies [35]. In addition, these non-coding RNAs are also implicated in regulating human embryonic stem cells and cancer stem cells [40, 41]. Due to the rarity and modest availability of quality tumor tissues, there are only limited studies of miRNAs in sarcomas. Further, lack of sufficient sarcoma models has also dampened the miRNA studies in sarcomas. Despite these obstacles, a number of studies have been reported on various sarcoma types and on the mechanisms through which these miRNAs might contribute to sarcoma biology. For instance, work by Riggi et al. [42] provides insights into the mechanisms whereby a single oncogene can reprogram primary cells to display a cancer stem cell phenotype in Ewing's sarcoma. They reported that fusion protein EWS/Fli1 and miR-145 form a mutually regulating feedback loop and identified SOX2 as their common target.

miRNA expression patterns in human sarcomas

Expression profiling of large numbers of miRNAs is a powerful technique widely used to understand gene regulatory networks in cancer. It has been reported that, compared to mRNA profiling, miRNA profiling can accurately classify human cancers [43]. Thus, miRNA expression patterns may be more closely linked to tumor differentiation. Our group conducted the first study to report global miRNA profiling in sarcomas [16]. This study included 27 sarcomas, representing 7 sarcoma types. Profiling was performed using microarray technology and/or small RNA cloning and sequencing. The different tumor types showed distinct miRNA expression profiles, as demonstrated by an unsupervised hierarchical clustering. Unique miRNA expression signatures were identified in each tumor class. Remarkably, the miRNA expression patterns were able to successfully distinguish two of the sarcomas that had been misdiagnosed. We confirmed this by reevaluating the tumors using histopathologic and molecular analyses. Further, using a cloning approach, we

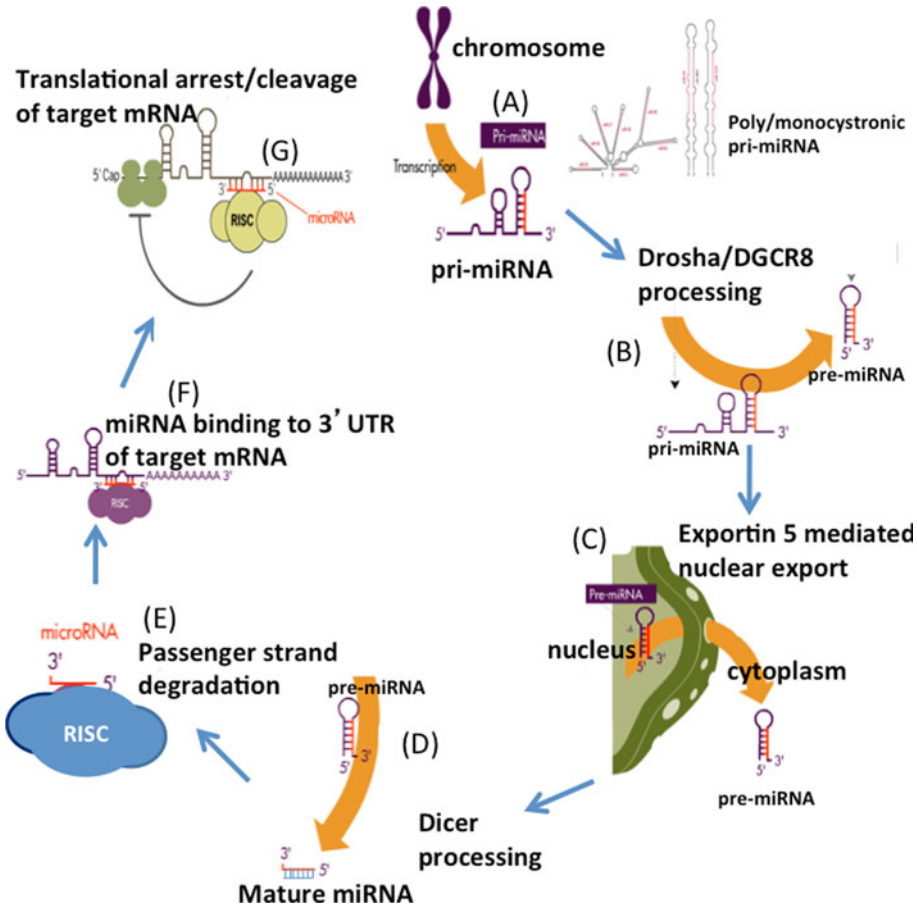

Fig. 1 Schematic representation of microRNA biogenesis. The figure is partly adapted from Subramanian and Steer [36]

identified 14 novel miRNAs in the sarcomas examined. Our data showed that different histological types of sarcomas have distinct miRNA expression patterns, reflecting the apparent lineage and differentiation status of the tumors. The identification of unique miRNA signatures in each tumor type may indicate their role in tumorigenesis and may aid in diagnosis and/or prognosis of soft tissue sarcomas. Recently, Greither et al. [44] reported the expression of hypoxia-regulated miRNA, miR-210, to be significantly associated with the prognosis and the age of tumor onset in a gender-specific manner in soft-tissue sarcoma patients. This evidence on the correlation of expression of a single miRNA with prognosis in sarcoma sheds light on the importance of these small RNA molecules in sarcoma biology.

Recently, our group developed a web-accessible Sarcoma miRNA Expression Database (S-MED), which is a repository that describes the patterns of miRNA expression in 22 human sarcoma subtypes [17]. S-MED provides both basic and advanced data search options for exploration of the data in heat map and text/numerical formats. The database also provides statistical details such as fold changes and *p* values for differentially expressed miRNAs in each sarcoma type and corresponding normal tissue. This comprehensive database is available through the URL link http://www.oncomir.umn.edu/. Together, these high-throughput analyses reveal unique miRNA expression signatures for different sarcoma subtypes, indicating that miRNAs can regulate specific tissue lineages during tumor differentiation. As the cell of origin for many sarcomas remains uncertain, a key difficulty is in determining the normal tissue to which sarcomas expression can be compared. To circumvent this issue, some researchers concentrate mainly on differences in miRNA expression between different sarcoma types. Our database will aid investigators in comparing their sarcoma or miRNA of interest against other tumor subtypes or miRNAs. Further functional characterization of candidate miRNAs has identified the networks of several miRNAs and their target genes that can serve as potential diagnostic/prognostic biomarkers and as therapeutic targets. For the purpose of clarity, the studies are presented for each subtype, although for some extremely rare forms of sarcomas, no reports are yet available.

Bone sarcomas

Bone sarcomas are relatively rare and account for only 0.2 % of all neoplasms. Several subtypes of bone sarcomas

such as OS, Ewing's sarcoma and chondrosarcoma are identified based on the unique histology, cell of origin, clinical features and site distribution. The following are the major histological subtypes of bone sarcoma and their miRNA expression patterns and functions.

Osteosarcoma

Osteosarcoma is the most common sarcoma and the primary malignant bone tumor with an incidence of 4–5 cases per million. It arises from the metaphysis of the long bones of adolescents and young adults. Two studies have shown significant downregulation of miRNAs at the chr.14q32 locus in OS compared to normal bone tissues [45, 46]. Interestingly, neither group observed DNA copy number changes in this locus, suggesting that additional epigenetic mechanisms have contributed to this downregulation. In addition, using bioinformatic predictions, we identified a subset of 14q32 miRNAs (miR-382, miR-369-3p, miR-544 and miR-134) that could potentially target *cMYC* transcript [45]. We also functionally characterized this regulatory network. Restoring the expression of these four 14q32 miRNAs decreased cMYC levels and synergistically induced apoptosis in Saos2 cells. Further exogenous expression of 14q32 miRNAs in Saos2 cells significantly downregulated miR-17-92, a transcriptional target of *cMYC*. The pro-apoptotic effect of 14q32 miRNAs in Saos2 cells was rescued either by overexpression of *cMYC* cDNA without the 3′UTR or with miR-17-92 cluster. Together, our data support a model where the deregulation of a network involving 14q32 miRNAs, cMYC and miR-17-92 miRNAs could contribute to OS pathogenesis [45]. Maire et al. [46] also identified miRNAs that are predicted to target genes involved in diverse intracellular signaling pathways, including Notch, RAS/p21, MAPK, Wnt, and the Jun/FOS pathways. Further, they developed a comprehensive molecular genetic map by integrating the miRNA profiles with previously published array comparative genomic hybridization DNA imbalance and mRNA gene expression profiles from a set of partially overlapping OS tumor samples. Among the different miRNAs examined, an miR-382 and cMYC regulatory circuit was observed to be deregulated in multiple studies and has been functionally validated [45–47]. miRNA-gene network deregulation in OS is illustrated in Fig. 2.

Several other groups have compared miRNA expression in OS tissues, cell lines, and normal osteoblast cell lines. They have identified dysregulated miRNAs and their potential mRNA targets in OS [47–52]. For instance, Braun et al. [53] reported that p53-responsive miRNAs, miR-192 and miR-215 are capable of inducing cell cycle arrest in U2OS cells carrying wild-type p53. Recently, Yan et al. [54] demonstrated that over-expression of p53 transcriptional target, miR-34a, could inhibit tumor growth and metastasis of OS probably through downregulating cMet. Functional studies have enabled identification of miRNAs which have the potential to prevent disease progression. Creighton et al. [55] uncovered a p53-associated role for miR-31 in inhibiting proliferation of OS cell lines. miR-31 was demonstrated as being able to inhibit multiple steps in the metastatic development of breast cancer by downregulating mRNA targets such as intergrin A5, radixin and RhoA [55]. These results suggest that in vivo delivery of miR-31 may have the potential to prevent pulmonary metastasis in OS patients [56]. miRNAs can also be potential biomarkers and therapeutic targets in OS. Duan et al. [47] have reported that restoring miR-199a-3p function may provide therapeutic benefits in OS by decreasing mTOR and signal transducer and activator of transcription 3 (STAT3) expression. miR-125b is yet another miRNA that is frequently deregulated in OS samples and human OS cell lines. It suppresses proliferation and migration of OS cells through regulation of STAT3 [57]. Similarly, suppression of miR-21 which is overexpressed in OS, decreased invasion and migration in MG-63 OS cell lines [58]. miR-21 targets RECK which suppresses invasion of OS cells by decreasing the activity of MMPs [59]. Also, miR-183 plays a role in suppressing migration and invasion

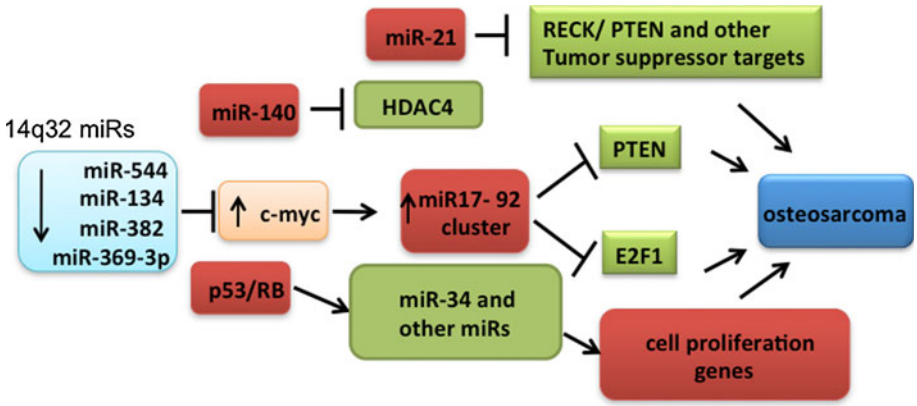

Fig. 2 Deregulation of miRNA-mediated gene networks in osteosarcoma

in OS cells by targeting Ezrin [60]. Further, the expression levels of miR-183 significantly correlate with lung metastasis as well as with local recurrence of OS. A recent study by Jones et al. [49] confirmed the tumor suppressive role of miR-16 and the pro-metastatic role of miR-27a by performing in vitro and in vivo functional validation in OS cell lines.

miRNAs can also act as biomarkers of chemotherapeutic responses in sarcomas. miR-140 is the first reported miRNA candidate associated with drug sensitivity in OS tumor xenografts treated with the chemotherapeutic agents Doxorubicin, cisplatin and ifosfamide (IFO)[61]. In this study, Song et al. [62] revealed consistent high expression of miR-140, which caused chemoresistance to methotrexate (MTX) and 5-Fluorouracil (5-FU) and suppressed cell proliferation in both U2OS and MG-63 OS cells. Further, miR-140 negatively regulates histone deacetylase 4 (HDAC4) which interacts with p21, resulting in 5-FU resistance. The same group also identified miR-215 as playing a significant role in inducing chemoresistance to MTX in U2OS cells. In another study, Gougelet et al. [63] examined miRNA expression profiles in 27 OS paraffin-embedded samples to determine the relevance of miRNA expression on chemoresistance to IFO. Supervised hierarchical clustering identified five candidate miRNAs (miR-92a, miR-99b, miR-132, miR-193a-5p, and miR-422a) that showed a statistically significant ability to discriminate between good and poor responders to IFO. The targets of these miRNAs detected by the in silico approach were involved in cell cycle regulation, invasion, and bone resorption through MAP kinase, TGF-β and Wnt pathways. Recently, Jones et al. [49] reported higher expression of miR-451 and miR-15b in pre-treatment biopsy OS samples that correlated with subsequent positive responses to chemotherapy. This indicates the ability of miRNAs to stratify patients, suggesting that personalized chemotherapy regimens may be viable in the near future. A recent review extensively covers miRNA studies reported in OS [56].

Ewing's sarcoma

Ewing's sarcoma is an aggressive pediatric malignancy that usually begins growing in a bone. It occurs primarily in children and young adults, often appearing during the teen years. It is primarily driven by a fusion of EWS/Ets oncoproteins, which are gain-of-function transcriptional regulators. Two studies analyzed global miRNA profile changes after stable silencing of EWS/Fli1 fusion protein [64, 65]. Mckinsey et al. [65] reported strong repression of a group of miRNAs by EWS/Fli1. Interestingly, these miRNAs have predicted targets in the insulin-like growth factor (IGF) signaling pathway, a pivotal driver of Ewing sarcoma oncogenesis. In the second study, Ban et al. [64] identified miR-145 as the top EWS/Fli1-repressed miRNA. miR-145 was expressed at low levels in primary Ewing's sarcoma tumor samples as compared to mesenchymal progenitor cells. Surprisingly, miR-145 was found to target the EWS/Fli1 transcript directly, functioning in a mutually repressive feedback loop that regulated expression of this fusion protein and miRNA [42, 65]. This regulatory network represents an important component of the EWS-FLI1-mediated Ewing's sarcomagenesis that may open a new avenue to future miRNA-mediated therapy of this devastating malignant disease. Yet another target of this fusion oncoprotein is let-7a [66]. In this study, miRNA arrays were used to compare the global miRNA expression profile of human mesenchymal stem cells and Ewing's sarcoma cell lines, and showed that Ewing's sarcoma displays a distinct miRNA signature that includes induction of the oncogenic miR-17-92 cluster and repression of the tumor suppressor let-7 family. In particular, the authors identified deregulation of let-7a and its target HMGA2 expression to be key events in the development of Ewing's sarcoma.

miRNAs have also been investigated as potential biomarkers of survival in Ewing's sarcoma [67]. In this study, miR-34a appeared to be associated with either event-free or overall survival and emerged as a significant predictor of outcomes. Patients with the highest expression of miR-34a did not experience adverse events in 5 years. The high miR-34a expression could be detected in paraffin-embedded tissues by in situ hybridization, thus contributing to an easy routine evaluation of this miRNA. Further, restoration of miR-34a activity may be useful to decrease malignancy and increase tumor sensitivity to current drugs, doxorubicin and vincristine, thus suggesting its role as a response to therapy marker. In a recent study, an integrated analysis of miRNA and gene copy numbers was performed in xenografts of Ewing's sarcoma [68]. Twenty differentially expressed miRNAs were pinpointed in regions carrying altered copy numbers, attributing changes in miRNA expression to copy number changes.

Chondrosarcoma

Chondrosarcoma is a cancer composed of cells derived from transformed cells that produce cartilage. Peripheral chondrosarcoma is a malignant transformation of multiple osteochondroma (MO), which is a rare skeletal disease characterized by the formation of multiple benign cartilage-capped bone tumors. The most serious complication of this pathology is malignant transformation into chondrosarcoma, which is estimated to occur in 1–5 % of patients. Specific miRNAs have been reported to be involved in chondrogenesis and inflammatory cartilage diseases [69, 70]. Recently, Salvatore et al. [71] reported a

miRNA profiling study in osteochondroma using 19 patient samples compared to normal cartilage growth plate controls. They observed an expression signature comprising of eight miRNAs that were able to further distinguish healthy growth plate controls from MO patients, which can regulate genes involved in MAPK and insulin signaling pathway, Wnt signaling, heparin sulfate and glycan structure and biosynthesis. This is in line with gene expression analyses previously performed in osteochondroma and chondrosarcoma samples showing modulation of signal transduction pathways like TGF-β/Wnt and IHH/PTHLH during osteochondroma formation and malignant progression. Other gene members of IGF family (e.g., IGF2 and IGFBP5) were also identified as differentially expressed between osteochondromas and human growth plates. The overlap between miRNA target predictions by Salvatore et al. [72] and signaling cascades previously related to MO pathogenesis support the potential involvement of the detected miRNAs in MO pathogenesis and malignant transformation.

Chordoma

Chordoma is a rare, slow-growing neoplasm that typically arises from bone in the skull base and anywhere along the spine. A subtype called chondroid chordoma shows histological features of chordoma and chondrosarcoma. Recently, miRNA expression was analyzed in chordoma-derived cell lines and chordoma tissue using miRNA microarray technology with unsupervised hierarchical clustering analysis [73]. In this study, the authors identified miR-1 and miR-206 as being expressed at a significantly lower level or absent in chordoma cells compared to normal cells. Reintroduction of miR-1 inhibited the growth of chordoma cells, with suppression of MET and HDAC4. MET is part of a receptor tyrosine kinase family of oncogenes overexpressed in many human cancers including sarcomas, particularly in chordoma (94.4 %), chondrosarcoma (54.2 %), and OS (23.3 %) [74]. Importantly, a recent study suggested that the MET oncoprotein plays a major role in the metastatic process in chordoma [75].

Soft tissue sarcomas

Cancers that primarily affect connective tissues such as muscle (smooth and skeletal), fat and blood vessels are generally grouped as soft tissue sarcomas. The majority of sarcoma subtypes are classified as soft tissue sarcomas. These malignant mesenchymal tumors account for about 1 % of all human malignant cancers. Soft tissue sarcomas such as gastrointestinal stromal tumor, synovial sarcoma (SS), RMS, leiomyosarcoma (LMS), liposarcoma, fibrosarcoma, and angiosarcoma are discussed in this section.

Gastrointestinal stromal tumor

Gastrointestinal stromal tumor (GIST) is one of the most common mesenchymal tumors of the gastrointestinal tract (1–3 % of all gastrointestinal malignancies). It is typically defined as a tumor with activating mutations in the *c-kit* gene or *PDGFRA* gene. We have profiled miRNA expression of GIST samples and observed that *miR-221* and *miR-222,* which have been shown to target the 3′UTR region of *KIT* in experimental systems [76], were expressed at a lower level. Since 80 % of GIST harbors an activating mutation in *KIT* [77], lower expression of *miR-221* and *miR-222* may allow for increased translation of KIT and further enhance its oncogenic influence on the cell. This is consistent with a recent finding using 54 paraffin-embedded GIST tissues, where the authors observed significant repression of miR-221 and miR-222 in KIT-positive GISTs, compared to normal tissue, which was completely reversed in KIT-negative GISTs [78]. The authors suggest that miR-221/222 can be considered as a tool for future therapeutic strategies for GISTs, especially for tumors with secondary resistance to tyrosine kinase inhibitors. Another therapeutic target that has been investigated recently is miR-494 [79]. Using functional studies, Kim et al. [79] have established miR-494 to be a negative regulator of KIT in GISTs and have shown that overexpression of miR-494 in GISTs may be a promising approach to GIST treatment. Previously, we have shown that miRNA expression patterns can distinguish and identify a misdiagnosed GIST case [16].

Another interesting observation with regard to miRNA regulation in GISTs is the significant downregulation of 44 miRNAs clustered in a genetically imprinted region at 14q32.31 [80]. In this study involving 12 GISTs, the authors observed significantly lower expression of two candidate 14q32 miRNAs in GISTs with 14q loss, and also in GISTs with tumor progression, indicating a correlation between miRNA expression and tumor progression. This was further validated in another study using 20 frozen GISTs. In this study, Choi et al. [81] observed 6 GISTs without 14q loss form a separate cluster distinct from remaining 14 samples. In the 14 GISTs with 14q loss, 5 small bowel GISTs formed a separate cluster, whereas the 9 remaining GISTs divided into two groups according to frequent chromosomal losses and tumor risk. Moreover, overexpression of miR-196a was associated with high-risk grade GISTs showing metastasis and poor survival. The expression of miR-196a was also correlated with expression of HOXC and the lncRNA, HOTAIR [82]. Thus, these

studies demonstrate the efficiency of miRNAs to classify GISTs to various subtypes and tumor grade.

Rhabdomyosarcoma

Rhabdomyosarcoma, a malignant, skeletal muscle-derived tumor is one of the most common soft-tissue sarcomas of childhood. It accounts for 6–8 % of all pediatric tumors and includes two main histological subtypes, embryonal (ERMS) and alveolar (ARMS) RMS [3, 83]. While ERMS has better prognosis (5-year overall survival >70 %), ARMS is more aggressive with poor outcomes [84]. ARMS is often associated with chromosomal translocations, the resulting oncogenic fusion protein (for, e.g., PAX3-FKHR) have greater prognostic values as they characterize a distinctly aggressive subgroup that is frequently unresponsive to conventional therapies and has a high rate of recurrence. Since RMS is widely thought to originate from myogenic precursors, the bulk of studies addressing the role of miRNAs in RMS have focused on myomiRs, i.e., the miR-1/miR-133/miR-206 family. These are miRNAs that specifically control cell fate determination of myogenic precursors and muscle tissue homeostasis.

In our initial study, we showed the distinction between ERMS and ARMS using miRNA profiling [16]. Similarly, Gougelet et al. [85] also demonstrated miRNA expression profiling as a useful strategy to discriminate RMS subtypes. Several studies have examined the potential of myomiRs as therapeutic targets in RMS [86]. The myomiRs are largely downregulated in RMS and their functional contribution has been confirmed by gain-of-function studies in which overexpression of myomiRs inhibited RMS cell proliferation [87–90]. Overexpression of miR-1 and miR-133a in an ERMS cell line revealed a tumor suppressor-like role for these myogenic miRNAs [87]. Similarly, re-expression of miR-206 in RMS cells promoted myogenic differentiation and blocked tumor growth in xenografted mice [89]. Further, it has been shown that miR-1 and miR-206 directly regulate MET proto-oncogene, the Met tyrosine-kinase receptor, which is overexpressed in RMS [88, 89].

We have also observed relative downregulation of myomiRs, miR-1 and miR-133 in RMS compared to normal skeletal muscle [16]. Further, in ARMS, miR-335 was found to be overexpressed. This is interesting, since miR-335 resides in the intron 2 of MEST (also known as PEG1). MEST has been indicated to play a role in muscle differentiation [91], and its mRNA expression is high in ARMS (Baer et al. 2004 [92]). MEST is a downstream target of PAX3, the gene involved in the PAX3–FKHR fusion [93]. It thus appears that the PAX3–FKHR fusion may influence the transcription of miR-335 that has several predicted myogenic targets including *CHFR* and *HAND1*.

In addition to myomiRs, non-muscle-specific miRNAs have also been implicated in RMS. miR-29 has been shown to be downregulated in RMS compared to normal muscle tissues [16, 94]. miR-29 is a tumor suppressor in RMS, the overexpression of which leads to cell cycle arrest and differentiation of RMS cell lines. Wang et al. [94] characterized the miRNA regulatory circuit *NFkB-YY1-miR-29*, and indicated that the disruption of this circuit can lead to RMS tumorigenesis. NF-kB activation can lead to overexpression of YY1, which in turn interacts with EZH2, causing sustained downregulation of miR-29b/miR-29c leading to repression of myogenesis. Consistent with this, tumor tissues from RMS patients showed upregulation of YY1 and EZH2. miR-26a is yet another negative regulator of EZH2, the expression of which is downregulated in RMS [95]. We have observed high levels of miR-183 in RMS cell lines and primary tumors and have shown an oncogenic role for this miRNA using knockdown studies [34]. This study also highlights the conserved miRNA gene regulatory networks in several sarcoma types, including RMS and SS. Elevated levels of miR-183 in these tumors significantly reduce the transcripts and protein levels of EGR1, a tumor suppressor and a transcription factor. Since EGR1 is downregulated, its transcriptional target of PTEN is also significantly affected. Blocking the activity of miR-183 using antimiRs elevated the levels of both EGR1 and PTEN. Thus, a single miRNA miR-183 directly and indirectly regulates two tumor suppressors, EGR1 and PTEN [34].

Recently, we examined the regulatory networks underlying miR-1, miR-206 and miR-29 downregulation in RMS [90]. Our data indicate that deregulation of these miRNAs stabilizes the expression of PAX3 and cyclin D2 in both (ERMS and ARMS) RMS types. Ectopic expression of miR-1 and miR-206 shows significant downregulation of PAX3 protein expression only in ERMS, and not in ARMS cell lines. In ARMS, PAX3 forms a fusion transcript with FKHR, and the resultant loss of PAX3 3′UTR in the fusion transcript indicates an oncogenic mechanism to evade miRNA-mediated regulation of PAX3. In addition, miR-29 also targets E2F7, another cell cycle regulator. Overexpression of miR-29 downregulates the expression of these cell cycle genes and induces partial G1 arrest leading to decreased cell proliferation. Taken together, our data suggest that the RMS state is stabilized by the deregulation of multiple miRNAs and their target genes, supporting a tumor suppressor role for these miRNAs [90]. Similarly, miR-29 has been implicated in epigenetic regulation in other cancers. miR-29b can directly target HDAC4 during osteoblast differentiation [96], and the miR-29 family can potentially target DNA methyl transferases in lung cancer, thereby causing global downregulation of DNA methylation [97]. These are potential areas that can be investigated

in RMS biology. Some of the experimentally validated miRNA target gene interactions in RMS are given in Fig. 3.

The clinical relevance of these miRNAs has been highlighted in several studies. Amplification of 13q31-32 chromosomal region, which includes C13orf25 gene harboring miR-17-92 cluster, was observed in a fraction of ARMS patients [98]. Further, in a recent study, it was shown that five of six miRNAs in miR-17-92 cluster were overexpressed in PAX7-FKHR positive ARMS samples with 13q31 amplification [99]. The high levels of these five miRNAs correlated with worse outcome in 13q31-amplified cases indicating a prognostic value for these miRNAs. In a study involving a large cohort of RMS samples, Missiaglia et al. [100] reported lower levels of miR-206 in RMS compared to normal skeletal muscle, the lower miR-206 levels correlating with increased metastasis and poor survival at diagnosis in advanced-stage disease. Moreover, this lower expression was associated with activation of NFκB, ERK and JNK signaling. In an effort to identify non-invasive biomarkers for RMS, Miyachi et al. [101] investigated the levels of muscle-specific miRNAs in human serum samples. miR-206 levels were found to be higher in sera of RMS patients compared to healthy donors or pediatric patients with other tumors. Although miR-206 is a tumor suppressor and downregulated in RMS, it is unclear why miR-206 levels were elevated in serum. These studies indicate the potential of miR-206 expression as a diagnostic and prognostic marker. miRNAs can also regulate drug responsiveness. In RMS cell lines, the downregulation of miR-485-3p can cause NF-YB-dependent decrease in Topoisomerase II (Top2) [102]. NF-YB can bind Top2 promoter and inhibit its transcription, thereby reducing the effect of Top2 inhibitors. Overexpression of miR-485-3p reduces NF-YB and restores Top2 expression, thereby reversing drug resistance. Thus, miRNAs can be potential biomarkers for response to therapy.

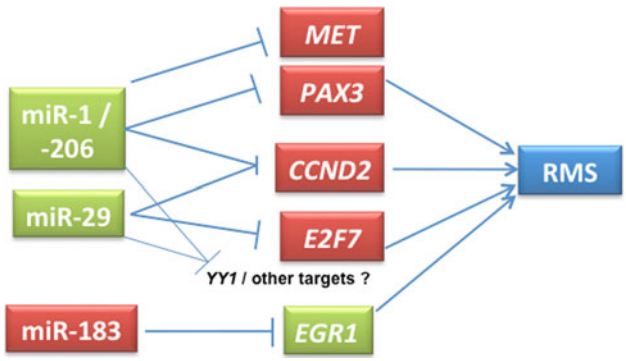

Fig. 3 Representative functionally validated miRNA-target genes in RMS. Partly adapted from Li et al. [90]

Synovial sarcoma

Synovial sarcoma, representing about 8 % of all soft tissue sarcoma, occur near the joints of the arm, neck or leg, and is most common in adolescents and young adults. We performed the first study to examine miRNA expression in this sarcoma [16]. We observed miR-143 to be expressed at very low levels in SS relative to GIST and LMS, as demonstrated by microarray, cloning and northern analyses. miR-143 has been proven to target ERK5 (also known as MAPK7), which is known to promote cell growth and proliferation in response to tyrosine kinase signaling [103]. *SSX1*, a common 3′-fusion partner gene resulting from a t(X; 18) in SS, is predicted to be another target for miR-143. Recently, our group has identified miR-183 to have a potential oncogenic role in SS [34]. miR-183 targets the tumor suppressor, EGR1. In SS, the SS18-SSX fusion protein represses *EGR1* expression through direct association with the *EGR1* promoter. Further, miR-183 knockdown in SS cell lines revealed deregulation of a miRNA network composed of the *miR-183–EGR1–PTEN* pathway. Global miRNA expression profiles in this translocation-associated sarcoma have been examined by Hisaoka et al. [104]. In this study, 35 miRNAs were observed to be differentially expressed in SS in comparison to other tissue types. There were 21 significantly upregulated miRNAs, including some miRNAs, such as let-7e, miR-99b, and miR-125a-3p, clustered within the same chromosomal loci. Further, using antimiR inhibitors against let-7e and miR-99b, the authors demonstrated suppression in cell proliferation and modulation in expression of their targets, HMGA2 and SMARCA5, suggesting potential oncogenic role for these proteins. The unique miRNA expression patterns observed in this study warrant further investigation in order to develop a better understanding of the oncogenic mechanisms and future therapeutic strategies for SS.

Leiomyosarcoma

Leiomyosarcoma is a malignant cancer of uterine smooth muscle. The benign form of this tumor is leiomyoma. In our initial characterization of sarcoma, we included six LMS samples [16]. Significance analysis of microarray comparison of LMS and normal smooth muscle samples identified significant overexpression of miR-1, miR-133a and miR-133b in LMS. This is in contrast to what is observed in RMS. These miRNAs play a major role in myogenesis and myoblast proliferation [105]. Our analysis also indicated LMS to be molecularly heterogeneous [16]. Recently, miRNAs were shown to play a pivotal role in the control of smooth muscle differentiation. Danielson et al. [106] demonstrated the remarkable ability of miRNA patterns to subclassify different tumors of the smooth muscle

lineage. They showed differential expression of >70 miRNAs, including members of the oncogenic miR-17-92 cluster, in LMS compared to normal myometrium. However, the role of these miRNAs was not functionally characterized in this study. Recently, Shi et al. [107] examined the functional correlation between let-7 and its target HMGA2. Overexpression of HMGA2 is common in uterine leiomyomas. This group observed that overexpression of HMGA2 and let-7-mediated HMGA2 repressions are relevant molecular alterations in LMS. Further, using in vitro assays, they demonstrated that disruption of the control of HMGA2 and let-7 pairs promotes LMS cell growth.

miRNAs have also been investigated as ideal biomarkers for diagnosing malignant phenotypes in LMS. For instance, in a recent study, Nuovo and Schmittgen [108] utilized upregulation of miR-221 expression as an accurate way to differentiate LMS from benign metastasizing leiomyoma. They observed that metastasizing leiomyomas are most likely benign lesions and not the malignant LMS. Moreover, miRNAs have also been investigated as a marker of response for hormonal therapy. Pan et al. [108] examined the expression, regulation and function of miR-21 in leiomyomas. They observed miR-21 to be aberrantly expressed and hormonally regulated in these benign tumors. Further, the authors propose a feedback regulatory interaction between miR-21, TGF-β and ovarian steroids, necessary to balance their different functions in a cell- and tissue-dependent context, influencing events such as inflammatory response, cell growth regulation and tissue turnover, leading either to cellular transformation and tumorigenesis or tissue fibrosis. These studies indicate the ability of miRNAs to modulate differentiation and act as biomarkers, thus rendering them attractive therapeutic agents against poorly differentiated sarcomas.

Liposarcoma

Liposarcoma is a malignant tumor that arises in fat cells in deep soft tissue. It is the most common mesenchymal cancer, with a mortality rate of 60 % among patients with this disease.

Urgas et al. [109] investigated the alterations in miRNA expression associated with liposarcomagenesis. They compared miRNA expression between normal adipose tissue, well-differentiated liposarcoma, and dedifferentiated liposarcoma using microarrays and deep sequencing studies. This analysis identified over 40 miRNAs that were dysregulated in dedifferentiated liposarcomas including upregulation of miR-21 and miR-26a and downregulation of miRNAs that are highly abundant in adipose tissue (miR-143 and miR-145). Further functional characterization of miR-143 demonstrated its tumor suppressor role in liposarcoma, potentially by decreasing expression of BCL2, topoisomerase 2A, protein regulator of cytokinesis 1 (PRC1), and polo-like kinase 1 (PLK1). In another study, Zhang et al. [110] identified important functions for miR-155 and β-catenin signaling in progression of liposarcoma. miR-155 was the most overexpressed miRNA in this analysis, and functional investigations assigned its role in the growth of dedifferentiated liposarcoma cell lines. Casein kinase 1α (CK1α) was identified as a direct target of miR-155 control which enhanced β-catenin signaling and cyclin D1 expression, promoting tumor cell growth. In a recent study, Taylor et al. [111] examined genetic alterations contributing to liposarcomagenesis by sequencing the genome, exome, transcriptome, and cytosine methylome of a primary and recurrent dedifferentiated liposarcoma from treatment-naive patients. These studies established a role for small RNAs in liposarcomagenesis, typified by methylation-induced silencing of miRNA-193b in dedifferentiated liposarcoma, but not in their well-differentiated counterpart. Patterns of miRNA expression in both dedifferentiated and well-differentiated liposarcomas are given in S-MED [17].

Malignant peripheral nerve sheath tumor

Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive soft tissue tumors that occur, either sporadically or in patients with neurofibromatosis type 1. These tumors have a 5-year survival rate of about 40 % [3]. Gene expression analysis performed by our group has identified an expression signature indicating p53 inactivation in the majority of MPNSTs [112]. Subsequently, we performed miRNA profiling in benign and malignant PNSTs. This analysis indicated a relative downregulation of miR-34a in most MPNSTs compared to neurofibromas. Using functional studies, we demonstrated that exogenous expression of p53 or miR-34a promote apoptotic cell death in MPNSTs. In addition, miR-214 was highly upregulated in MPNSTs compared to neurofibromas. miR-214 is a direct transcriptional target of TWIST1 [113], a regulator of metastasis [114]. It is to be noted that TWIST1 is highly expressed in MPNSTs. miR-214 targets PTEN, hence the *TWIST1-miR-214-PTEN* pathway could be potentially explored to decipher MPNST pathogenesis. Collectively, our findings suggest that deregulation of miRNAs has a potential role in the malignant transformation process in PNSTs [112]. This was further confirmed by a series of studies examining the role of several candidate miRNAs by other groups. For instance, Chai et al. [115] reported upregulation of miR-10b in primary Schwann cells isolated from neurofibromas and in MPNST tumors and cell lines. Using functional studies in multiple cell lines, the authors demonstrated that the inhibition of miR-10b reduced cell

proliferation, migration and invasion by affecting NF1 expression and RAS signaling. In another study, miR-21 was shown to play an important role in MPNST tumorigenesis and progression through its target, PDCD4 [116]. Subsets of MPNSTs are characterized by the presence of a 1.4-Mb microdeletion. Pasmant et al. [117] identified the presence of two miRNAs, miR-193A and miR-365-2, in this microdeletion. However, expression analysis of these miRNAs did not show any significant difference between human dermal and plexiform neurofibromas and MPNSTs.

Schwannomas are benign tumors characterized by mutations in the NF2 gene. Saydam et al. [118] reported miRNA profiling of schwannomas and characterized the tumor suppressor function of miR-7, which was one of the most downregulated miRNAs in these tumors. In addition to the known oncogene targets, epidermal growth factor receptor (EGFR) and p21-activated kinase 1 (Pak1) for miR-7, this group identified associated cdc42 kinase 1(Ack1) oncogene as a direct target. These findings suggest miR-7 as a potential therapeutic molecule for schwannoma treatment, and prompt clinical evaluation of drugs that can inhibit the EGFR, Pak1, and Ack1 signaling pathways to treat this tumor type. miR-21 was also observed to be consistently overexpressed in vestibular schwannomas. This was correlated with decreased levels of tumor suppressor, PTEN, a known molecular target of miR-21 [119]. Both gene and miRNA profiles of schwannomas were also compared with those of neurofibroma and MPNSTs. It can be noted that neurofibroma and schwannoma clustered separately compared to MPNSTs [112]. A schematic representation of miRNA deregulations in the malignant transformation of neurofibroma to MPNST is given is Fig. 4.

Fibrosarcoma

Fibrosarcoma (fibroblastic sarcoma) is a malignant tumor derived from fibrous connective tissue and is characterized by the presence of immature proliferating fibroblasts or undifferentiated anaplastic spindle cells. Not many studies have examined miRNA expression in this rare sarcoma. Recently, two studies have characterized miRNA expression and function in human fibrosarcoma cell line HT1080. Liu and Wilson [120] have demonstrated that miR-520c and miR-373 increases the expression of MMP9 by directly targeting the 3′UTRs of mRNAs of mTOR and SIRT1 and suppressing their translation. This results in activation of the Ras/Raf/MEK/Erk signaling pathway and NF-κB finally increasing MMP9 levels and activity, thereby enhancing cell migration and cell growth in these cells. In the second study, Weng et al. [121] showed that miR-409-3p inhibits tumor growth, vascularization and metastasis through downregulating angiogenin expression in HT1080 cells. Angiogenin is an angiogenic and tumorigenic factor that is elevated in various types of cancers. This was further confirmed by the inverse correlation of the expression of miR-409-3p with angiogenin in mouse xenograft tissues and in human fibrosarcoma samples.

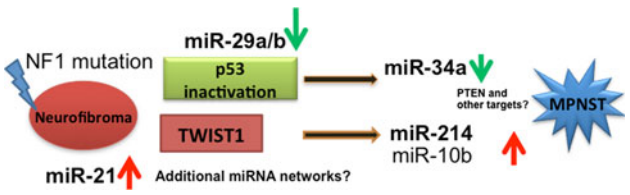

Fig. 4 Potential miRNA gene networks in the malignant transformation of neurofibroma to MPNSTs

Angiosarcoma and other sarcoma types

Our group has experimentally validated differentially expressed miRNAs in angiosarcoma. Using S-MED, we identified miR-515-3p, 515-5p, 517a, 517c, 518b, 519a, and 522 as upregulated specifically in this tumor type when compared to the other sarcomas, and this could be exploited to aid angiosarcoma diagnosis [17]. miRNA expression patterns of many sarcoma types have been characterized; however, the role of miRNAs in several sarcoma types is not yet understood. For instance, the role of miRNAs in sarcomas such as hemangiosarcoma and pleomorphic undifferentiated sarcoma have not yet been reported.

Conclusion

Although diagnosis and treatment of sarcoma pose many challenges, there are several opportunities for investigating sarcoma biology. For instance, by small RNA sequencing of various sarcomas and corresponding normal types, we have identified 14 novel human miRNAs [16]. Thus, the potential for discovering novel RNA transcripts, including small and long non-coding (lncRNAs), is high in sarcomas, since sarcoma genomics is not extensively studied. Further, these novel RNA transcripts may also play a role in other tissues and cancer types. This is relevant, especially in the characterization of conserved gene regulatory networks in sarcomas and other epithelial and hematopoietic malignancies. Common oncogenes and tumor suppressors are deregulated in most cancer types. Both p53 and cMYC can induce the expression of multiple miRNAs and can be regulated by sets of miRNAs that are conserved and found to be deregulated in multiple cancers [24, 122]. For instance, activation of miR-17-92 mediated by cMYC is a characteristic feature in OS and several other carcinomas [24, 45]. Similarly, deregulation of miR-29 is noticed in

both sarcomas (RMS and MPNSTs) [90, 112] and other malignancies [123, 124]. miR-29 family members activate p53 [123], and their loss of expression can limit p53 levels when required in the cell. Subsequently, loss or inactivation of p53 expression leads to downregulation of miR-34, a transcriptional target of p53 [122]. Thus, understanding the factors that modulate cMYC and/or p53 expression in sarcomas can also provide valuable insights in the pathobiology of other malignancies.

miRNA-gene deregulations are conserved in certain types of sarcomas and other malignancies; however, investigating miRNA biology in sarcomas can be challenging. First, a single miRNA can regulate many genes, and, depending on the cell type and target availability, a miRNA can function either as an oncogene or tumor suppressor. Thus, roles of miRNAs cannot be generalized across multiple cancers or cell types. Second, modulation of miRNAs can also have off-target effects. Third, deregulation in miRNA expression leads to '*noise*' in the cellular gene expression, and these noise levels vary in different cancers. Thus, based on miRNA deregulations, the resultant noise in tumor cells and in surrounding stromal cells, outcomes in terms of tumor initiation, progression, invasion and metastases can vary extensively and contribute to the heterogeneity within a specific sarcoma subtype.

miRNAs are known to have hundreds of targets, and many of them may function as passengers in the tumorigenic process [19]. Driver genes are key players in tumorigenesis, and, depending on their function, they can be both oncogenic and tumor suppressors. Investigating the interactions between miRNAs and driver genes (*driver of driver* genes) will significantly enhance our understanding of the sarcoma pathobiology. These miRNA–gene networks can also work cooperatively with the competing endogenous RNAs (ceRNAs) [125] and regulate/be regulated by a plethora of gene networks that are maintained in an intricate balance for normal cellular functioning. Understanding these multilayered gene regulatory mechanisms may pose a major challenge in understanding cancer biology such as drug resistance and tumor recurrence.

Focusing on key '*driver of driver* genes' may reduce the miRNA-gene network complexities to a certain extent. The future research directions for the roles of miRNAs in sarcoma may include (1) development of body fluid (serum/plasma) based miRNA biomarkers, (2) miRNA-based targeting of genes in a cancer signaling pathway using miRNA mimics and or antimiRs/sponges instead of targeting single gene/protein product, (3) investigation of potential ceRNAs as alternative drug targets for cancer genes that are currently undruggable by regular therapeutic approaches, and (4) modulating miRNA(s) expression levels may be explored as differentiation, intercellular communication and stem cell-based cancer therapies.

Acknowledgments Due to space restrictions, we could not cite many significant contributions made by numerous other investigators in this important and rapidly progressing field. We thank Ms. Jennie W. Knoot for assisting with manuscript editing. This work is supported by grants from Minnesota Medical Foundation, Academic Health Center, University of Minnesota and The Karen Wykoff Sarcoma Foundation and the Department of Defense (W81XwH10-1-0556).

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