Role of MicroRNA-17-92 cluster in the Osteosarcoma Development and Progression

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Dedication

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents, Brajesh and Keerti Varshney whose words of encouragement and push for tenacity ring in my ears. Even though they are thousand miles away their love and support made me feel very much at home.

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Abstract

The acquisition of cancer traits in rare cells is accompanied and caused by a huge variety of driver genetic events. These include chromosomal deletions, translocations, point mutations, and insertions. The acquisition of a metastatic phenotype is currently the biggest problem in cancer treatment. However, genetic driver events that cause metastasis have been elusive and thus treatment to stop metastasis is not available in many cases. It has become recently appreciated that certain cancer traits are conferred by changes in microRNAs (miRNAs). MicroRNAs are a class of small endogenous RNAs that regulate the expression of other genes by binding to and destabilizing mRNAs and/or suppressing their translation. In this work we used a comparative oncogenomics approach to discover miRNAs that cause osteosarcoma progression and investigated their function *in vivo* and *in vitro*. In the first chapter, I review the evidence for a genetic origin of human cancer. Then, I review hallmarks of cancer, with a focus on metastasis and the failure of DNA sequence changes to fully explain metastasic process. Then, I review the role of miRNAs in biology and cancer development. The second chapter focuses on osteosarcoma development, the known roles that miRNAs play in this disease, and the chance that this understanding could lead to better treatments. The third chapter describes my investigation into the role of the miR-17-92 cluster in OS progression and metastasis. Finally, in the fourth chapter, I provide a summary and future perspectives for this work.

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Chapter 1: The Nature of Cancer and microRNAs in Cancer Development

What is cancer?

Cancer is a class of diseases characterized by immortalized cells undergoing uncontrolled division, invading into local tissues, and metastasizing to distant organs. There are six hallmarks of cancer that are acquired during the multistep development of human tumors (Hanahan and Weinberg 2011, Hainaut and Plymoth 2013). They include maintenance of proliferative signaling, evasion of tumor suppressors, avoidance of cell death, persistent replicative immortality, stimulation of angiogenesis, and induction of invasion and metastasis. Underlying these hallmarks is genomic instability, which generates the genetic diversity that advances the accumulation of novel mutations, and inflammation, which fosters an environment amenable to tumor growth. Additionally, two emerging hallmarks of potential generality have been introduced to this list in the past decade: reprogramming of energy metabolism and evading immune destruction.

One of the hallmarks that is still least understood is metastasis. The ability to metastasize is nearly synonymous with tumor malignancy. Serious consideration of cancer metastasis began with an observation from a ploughman (Paget 1889). Paget aspired to understand what organs are suitable for colonization by cells from a primary tumor growing elsewhere in the body. His thoughts were the basis for what he called the “seed and soil hypothesis”, which was fully reviewed by Hart and Fidler in 1980 (Hart and Fidler 1980). His and other work set the stage for understanding this most malignant aspect of cancer, namely metastasis. It was observed that cancer cells are present

throughout the body of affected patients but cannot develop into secondary tumors in most places, and instead are restricted to specific sites prone to metastasis. Today's research heavily focuses on the molecular mechanisms that bring seed (cancer) and soil (tissue environment) together to foster metastasis.

The genetic basis of cancer

In the early 20th century Dr. Theodor Boveri established associations between genetic aberrations he could observe at the level of whole chromosome alterations and cancer formation, leading to his early hypotheses of a genetic basis for cancer (Boverie and Belaiche 1991). Dr. Peyton Rous achieved the next big milestone in understanding the genetic basis of cancer. He identified an avian retrovirus, later named as Rous Sarcoma Virus (RSV), which could cause oncogenic transformation in chicken cells, inducing the reproducible formation of sarcomas (Rous 1910, Rous 1910). This discovery led to the identification of several mammalian retroviruses that could also induce tumors, and eventually the identification of the first retroviral oncogene, *src*, which is encoded in the RSV genome (Stehelin, Fujita et al. 1977). The discovery of a mammalian cell homolog of *src, c-src,* and studies on the activation of the *HRAS* oncogene in human tumors contributed to our early understanding of the genetic basis of human cancer.

Additional evidence for the genetic basis of cancer came from detailed studies of chromosomes in human tumor cells. University of Pennsylvania researchers first reported the presence of abnormal minute chromosomes present in white blood cells from patients with a chronic myeloid leukemia (CML) diagnosis (Nowell and Hungerford 1960). This

was designated the Philadelphia chromosome. Further analysis of the Philadelphia chromosome, using Giemsa staining, led to the realization by Dr. Janet Rowley, that it was produced by a balanced chromosomal translocation, t(9;22). Since then, recurrent chromosomal translocations have been found to characterize many tumors. Examples, include the, t(15; 17) in acute promyelocytic leukemia and t(14;18) in B cell lymphoma. Cloning of the specific breakpoints that cause translocations subsequently led to the realization that these chromosomal translocations produce activated oncogenes. In some cases, these oncogenes are novel in-frame fusions of two open reading frames (e.g. BCR-ABL resulting from the t(9;22) translocation in CML) while in others the translocations bring a new promoter to an open reading frame resulting in ectopic and/or overexpression of proto-oncogenes (e.g. activation of MYC by the t(14;18) in B cell lymphoma).

Work in the 1970's and 1980's established that genetic mutations in genes whose normal role was to control and moderate the normal affinity of cells to continuously divide could cause cancer. These genes came to be called tumor suppressor genes (TSGs). It was Alfred Knudson who initially promulgated the idea of "two hits" being necessary to cause sporadic, non-hereditary incidence of cancer and "one hit" to initiate the formation of familial forms of cancer (Knudson 1971). He evaluated 48 cases of retinoblastoma for bilateral or unilateral retinoblastoma tumors and observed that the age versus incidence rates of retinoblastoma suggested at least two rate-limiting steps for sporadic (i.e. non-familial) cases. In bilateral cases (which are often familial), he found that the age versus incidence rates suggested one rate-limiting hit was required for tumor formation, leading to his conclusion that the first hit was likely an inherited mutation

while the second was acquired later in life via a different mechanism. In the sporadic tumors both the hits were somatic with a similar mutation rate. This “two-hit” theory became the basis for what is now our classical model for tumor suppressors. Knudson’s work led to the hypothesis that a “retinoblastoma” gene, later called RB1, must exist. While many scientists had supported the “two hit” model, it was David Comings who articulated a general framework for a role of tumor suppressor genes in different cancer types (Comings 1973). The end of 1980’s established *RB1* and another critical gene *TP53* as two common tumor suppressor genes. Such genetic discoveries led to a new era for drug synthesis that could potentially target the genetic abnormalities in cancer cells.

Molecularly targeted drugs

It was only during the last 10-20 years that we have begun to take advantage of our new understanding of the molecular basis of cancer to develop new therapies. The prototypical example is the development of imatinib (trade name Gleevec) for the treatment of CML. Gleevec is a small molecule tyrosine kinase inhibitor that binds to and interferes with the BCR-ABL kinase domain in tumors carrying that oncogenic fusion protein, suppressing CML cell survival and dramatically enhancing patient survival. The first molecular-targeted monoclonal antibody, trastuzumab (Herceptin), was developed to target HER2 in the 25% of the breast cancer cases where that protein is overexpressed (Konecny, Fritz et al. 2001). In clinical trials, such targeted therapies have proven far more effective than chemotherapy alone for patients who carry the mutated genetic target. Another example is provided by the observation that certain specific mutations such BRCA1/2 (hereditary breast cancer) and MSH2 and MLH1 (hereditary non

polyposis colon cancer) confer sensitivity to specific drugs involved DNA repair (Fishel, Lescoe et al. 1993, Bronner, Baker et al. 1994, Futreal, Liu et al. 1994). The discovery of such cancer specific genetic mutations has led to an era studies into global genetic deregulation and the profiling of various cancer tissue types.

However, there are many complex cancers where such targeted therapies have not shown such efficacy. Gefitinib is such story. Gefitinib inhibits a tyrosine kinase, called the epidermal growth factor receptor (EGFR), that is overexpressed in 40-80% lung cancers but only 10-20% of the patients respond to therapy and those that do, do so only transiently which is followed by the acquisition of resistance (Lynch, Bell et al. 2004, Sequist, Martins et al. 2008).

Metastasis - the beast of them all

The metastatic program encompasses a series of sequential steps: increased cell motility, tissue invasion, intravasation, dissemination through the blood or lymph, extravasation, and finally proliferation at a new site. This incredibly complex and diverse system of alterations makes it one of the most challenging hallmarks to tackle from both the perspective of understating the underlying biology and eventually treating metastatic disease (Eccles and Welch 2007). With the latest revelations about the roles of miRNA in the metastatic program, there are new hopes that this situation could change. Accumulating evidence has pointed to a central regulatory role for miRNAs in the initiation and progression of the vast majority of cancers in which this question has been studied (Nguyen and Massague 2007). Recent reports show that miRNAs may be part of

a very important class of cancer-predisposing genes that could participate in the genetic complexity that give rise to the malignant phenotype (Esquela-Kerscher and Slack 2006, Chivukula and Mendell 2008, Manikandan, Aarthi et al. 2008).

Cancer and microRNAs

microRNAs (miRNAs) are a group of small non-coding RNAs that post-transcriptionally regulate gene expression (Gurtan and Sharp 2013). miRNAs can be located in introns, exons of coding genes, non-coding genes, and intergenic regions. The biogenesis of miRNAs is a complex process. RNA polymerase II (RNA Pol II), or less frequently RNA Pol III, generates a primary miRNA transcript (pri-miRNA), which is then exported to the cytoplasm by exportin-5. There, it is further processed to form precursor miRNAs, called pre-miRNAs (Gurtan and Sharp 2013). RNAse III Dicer and its co-factors then cleave the terminal loop from the pre-miRNA. One of the two strands, generally the 3p which is on the 3' end of the hairloop forms the mature miRNA. The mature miRNA (20-24 nucleotides) then becomes the guide strand that is loaded into a ribonucleotide complex to form miRISC (miRNA induced silencing complex). Depending on the level of complementarity between the guide miRNA and the target mRNA, miRNA can direct either the cleavage of the target with the help of Ago2 or sit on the transcript and induce translational suppression **(Fig. 1)**. miRNA mediated gene regulation is a crucial mediator in every biological system as it can regulate hundreds to thousands of different mRNAs that play essential roles in various metabolic, signaling, or developmental pathways. Therefore, alterations of miRNA expression can result in various human disease conditions, including cancer (Siomi and Siomi 2010).

Cancer is considered a complex genetic disease that involves long-term accumulation of various mutations in coding as well as non-coding genes. With the increasing awareness of such small RNAs and long non-coding RNAs, it is crucial to understand the role of these regulatory RNAs on various cellular pathways and how their dysregulation can transform a normal cell into a cancerous one. miRNAs can bind and deregulate tumor suppressor genes such as p53 (Hermeking 2012). p53 plays a crucial role in regulating a plethora of cell processes such as cell cycle progression, migration, the epithelial–mesenchymal transition, stemness, metabolism, differentiation, and cell survival (Hermeking 2012). Thus, targeting miRNA levels in cancer patients may be a potential therapeutic avenue to explore.

miRNAs that are altered in various cancers can act as potential biomarkers to help us understand the disease state and progression. There are various reports that support the diagnostic utility of miRNAs, the advantage being that only a small quantity of tissue sample or body fluids is needed to assess miRNA levels. Additionally, miRNA expression profiles can easily distinguish normal tissue from tumor tissue and further differentiate subtypes of cancer (Hoshida, Villanueva et al. 2008). Moreover, assessing miRNAs, is a stable and a less invasive diagnostic tool, and miRNA levels can help predict the likelihood of recurrence and metastasis (Budhu, Ji et al. 2010). Together, miRNAs can function as potential diagnostic and prognostic markers and further aid in therapeutic development (Liu, Yuan et al. 2008, Miyakawa, Ishihara et al. 2009). In this thesis, I have focused on microRNAs and their crucial role in tumorigenesis and metastasis in various types of cancer.

miRNAs in common types of carcinomas

Oral Cancer

Oral cancer arises from different regions of the oral cavity (Sankaranarayanan, Masuyer et al. 1998). Oral cancer is heterogeneous in nature and has a dismal 5 year survival rate of ~50% that has not changed for decades. It is the sixth most common cancer occurring globally and accounts for 30% of all cancers in India (Elango, Gangadharan et al. 2006). Thus, a better understanding of the molecular basis of tumorigenesis is needed to facilitate the development of drugs and strategies that will lead to improved clinical outcomes.

Soga et al. were the first to perform a comprehensive miRNA profiling study of oral cancer (Soga, Yoshiba et al. 2013). Using TaqMan miRNA Array 2.0 they found that 12 miRNAs (miR-31*, miR-31, miR135b, miR-193a-5p, miR-103, miR-224, miR-93, miR-200c, miR-183, miR-203, miR-21 and miR-223) were upregulated more than 4 fold in oral cancer compared to the normal subjects. miR-21, miR-203, miR-31, and miR-31* are functionally validated to play a crucial role in the oncogenicity of various malignancies (Iorio, Ferracin et al. 2005, Schetter, Leung et al. 2008, Saini, Majid et al. 2011, Alder, Taccioli et al. 2012, Chang, Kao et al. 2013) and miR-31 and miR31* have been shown to contribute to oral cancer (Shao, Yu et al. 2012).

Cheng et al. discovered that 23 miRNAs are differentially expressed between six oral cancer cell lines and five lines of normal oral keratinocytes (Lu, Chen et al. 2012). Out of the 23 miRNAs, they found that miR-10b was the most upregulated in cancer cell lines and a xenograft oral cancer mouse model (20 fold) (Lu, Chen et al. 2012). miR-10b

is crucial for cell migration and invasion in many cancers (Sun, Liu et al. 2013, Han, Yan et al. 2014). miR-10b was found in the plasma of cancer patients but was absent in normal subjects, indicating its diagnostic potential. The other miRNAs, including miR-196a, miR-196b, miR-582-5p, miR-15b, miR-301, miR-148b, were also found to be upregulated in oral cancer patients. On the other hand miR-128a, miR-503, and miR-31 were downregulated in patients compared to normal subjects.

In summary, studies have demonstrated that miRNAs hold potential as diagnostic and/or prognostic biomarkers for oral cancer. However, many of these miRNAs have yet to be functionally validated in relation to oral cancer.

Esophageal cancer

Esophageal cancer (EC) is the eighth most common cancer worldwide, with 481,000 new cases (3.8% of the total) estimated in 2008, and the sixth most common cause of death from cancer with 406,000 deaths (5.4% of the total) (2008). Despite being a very commonly diagnosed cancer, the prognosis of EC is poor (He, Yin et al. 2012). Thus it is urgent to find novel diagnostic and prognostic markers to aid in the treatment of the disease. Guo et al identified seven miRNAs (upregulation of miR-25, -424 and -151; downregulation of miR-100, -99a, -29c and -140) that have potential value as biomarkers to distinguish malignant EC from normal tissue (Guo, Chen et al. 2008). Additionally, several studies have shown that some miRNAs are consistently detected in the systemic circulation of patients diagnosed with EC, yet are absent in the circulation of healthy individuals (He, Yin et al. 2012). For instance, miR-21 is overexpressed in various types of EC and is upregulated in the plasma of Esophageal Squamous Cell Carcinoma (ESCC)

patients compared to healthy individuals. Similarly, a panel of serum miRNAs (miR-10a, -22, -100, -148b, -223, -133a and -127-3p) was found upregulated in ESCC, suggesting the group could be used to distinguish stage I/II ESCC patients from the control group (Zhang, Wang et al. 2010).

Recently, miRNAs have been shown to regulate invasion and metastasis, exacerbating the mortality of many cancers (He, Yin et al. 2012). In EC, miR-143, -145 and -133a/b significantly inhibited cell growth and invasion by targeting *FSCN1*, which promotes cell motility and growth (Wu, Xu et al. 2011). Moreover, a significant correlation was found between miR-10b and inhibition of the tumor suppressor gene *KL4,* which resulted in enhanced invasiveness (Tian, Luo et al. 2010). miR-92a was another crucial miRNA that is overexpressed in various tumor tissues and correlates with lymph node metastasis status and TNM staging, that led to suppression of cadherin1 (*CDH1*) expression (Chen, Zhao et al. 2011). Consequently, inhibition of miR-17-92 cluster member miR-19a by antisense oligonucleotides induced apoptosis and inhibited tumor growth *in vivo*, likely by targeting tumor necrosis factor-α (TNF-α) (Liu M 2011). The aforementioned studies suggest that miRNAs play a role in the progression of EC and may be used to determine the stage of carcinogenesis.

Colon cancer

Colon cancer (CC) is one of the leading causes of cancer death worldwide and is the third most common cancer affecting both men and women (Jemal, Siegel et al. 2008). Deregulation of signaling pathways such as WNT, RAS−MAPK, PI3K, TGFβ, p53, and DNA mismatch-repair pathways leads to initiation and progression of CC (2012). Most

studies classify CC on the basis of microsatellite instability (MSI is located primarily in the right colon and is frequently associated with the CpG island methylator phenotype (CIMP and hyper-mutation) and those that are microsatellite stable but chromosomally unstable (Fearon 2011). The Cancer Genome Atlas Project has made leaps in profiling genomic changes in 20 different cancer types and has thus far published results on ovarian cancer, glioblastoma, and colon cancer (2008, Li, Li et al. 2011, 2012). A comprehensive integrative analysis was performed on 224 colorectal tumor and normal tissue pairs, and the tumors were identified on the basis of possible biological differences in colon and rectal tumors. The analysis revealed that a majority (94%) of the non-hypermethylated tumor samples, irrespective of copy number, anatomic origin had a mutation in one or more members of the WNT signaling pathway, predominantly in *APC* (2012). Recently, our group identified differential expression of 39 miRNAs including miR-135b, -96, -182, -1, and -133a depending on mismatch repair status of colon cancer tissues relative to normal colon tissue (Sarver, French et al. 2009). Further, we have examined 52 normal colonic mucosa, 41 adenomas (polyps), 158 adenocarcinoma with proficient DNA mismatch repair (pMMR), and 64 adenocarcinoma with defective mismatch repair (dMMR) that were selected on the basis of sporadic (n=53) and inherited colon cancer (n=11) (Oberg, French et al. 2011). We observed that all sporadic dMMR had inactivated *MLH1* due to promoter hypermethylation (Oberg, French et al. 2011). Based on unsupervised PCA and cluster analysis, we were able to demonstrate that miRNAs could distinguish between normal colon tissue, adenomas, pMMR carcinomas, and dMMR carcinomas (Oberg, French et al. 2011). In a comparison between pMMR and

dMMR tumors, we identified four miRNAs (miR-31, -552, -592 and -224) that had statistically different expression levels (≥2-fold change)(Oberg, French et al. 2011).

We have identified common miRNAs that are perturbed in multiple tumor types, including colon cancer, rhabdomyosarcoma (both ARMS and ERMS types), and synovial sarcoma (Sarver, Li et al. 2010). The analysis revealed that miR-183 was significantly upregulated in colon cancer, ERMS, ARMS, and synovial sarcoma. miR-135b was upregulated in 3 cancer types excluding ERMS. In concurrence with the analysis, we found that miR-183 cluster members (miR-96 and miR-182) were also upregulated in colon cancer. Further, we determined that miR-183 regulates *EGR1*, a tumor suppressor gene that in turn regulates *PTEN*, another important tumor suppressor gene. With the knockdown of miR-183 in CC cell lines, we found a significant deregulation of a miRNA network composed of *miR-183–EGR1–PTEN* (Sarver, Li et al. 2010). Thus, miR-183 is a potential oncogenic miRNA that regulates tumor suppressor genes, such as *EGR1* and *PTEN*. The deregulation of this miRNA regulatory network is conserved in many cancer types.

Hepatocellular carcinoma

Hepatocellular Carcinoma (HCC), one of the most common cancers, results from the deregulation of multiple intracellular and extracellular signaling pathways. miRNAs have been shown to play a major role in such deregulation (Gramantieri, Fornari et al. 2008). For instance, the miR-199a/a* family and miR-1 are downregulated in HCC. These miRNAs post transcriptionally regulate *MET*, which is overexpressed in 40-70% of

HCCs (Datta, Kutay et al. 2008). It has been shown that miR-199a/a* and miR-1 are methylated in HCCs, especially in primary tumors. Additionally, the RAS family presents downstream of RTK receptors that contains multiple complementary sites for binding of let-7 family miRNAs. Overexpression of let-7 in various cancer cells has shown reduced RAS protein levels (Johnson, Grosshans et al. 2005). Thus, let-7 downregulation may play a role in the development and progression of HCC.

Another study showed that miR-221 is upregulated in various cancers, including HCC, suggesting its oncogenic role (Fornari, Gramantieri et al. 2008). Its oncogenic function was further confirmed by its ability to modulate the expression of the cyclin-dependent kinase inhibitor CDKN1B/p27, a key controller of cell cycle progression. More recently, miR-221 has been shown to target another cyclin-dependent kinase inhibitor, CDKN1C/p57 BH3-only protein BMF (le Sage, Nagel et al. 2007, Schmelzle, Mailleux et al. 2007). Through this mechanism, miR-221 can protect cancer cells from anoikis, leading to metastasis.

Also, miR-122 is downregulated in more than 70% of HCCs (Xu, Liu et al. 2010). Studies revealed that miR-122 targets cyclin G1, which is a negative regulator of p53 which activates *MDM2*, thus leading to p53 degradation (Okamoto, Li et al. 2002). As a result, cyclin G1 overexpression can result in cell proliferation (Kampmeier, Behrens et al. 2000). Recently, it was shown that the absence of cyclin G1 was associated with a lower susceptibility to liver tumors, likely due to increased p53 tumor suppressor activity in a mouse model (Jensen, Factor et al. 2003). Therefore, the upregulation of cyclin G1

due to miR-122 downregulation in human HCC may lead to p53 downregulation and promote tumorigenesis. Further, miRNAs can react to the changes in cancer microenvironment more than healthy tissue. For example, interferon treatment is not effective in all hepatic cancer patients, but levels of miR-26 in patients suffering from hepatic cancer can help them stratify for interferon treatment (Ji, Shi et al. 2009).

Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is considered the thirteenth most common cancer related death in the world (Bryant, Mancias et al. 2014). Aberrant miRNA expression patterns have been identified in PDAC. The miRNAs that were found to be upregulated were miR-221, -424, -301, -100, -376a, -125b-1, -21, -16-1, -181a/c, -92-1, -15b, -155, -212, -107, -24, let-7f-1, and let-7d. The significantly downregulated miRNAs include miR-345, -142-3p, and -139 (Szafranska, Davison et al. 2007). Another group did similar miRNA array profiling studies and found that only two miRNAs, miR-217 and miR-196a, could distinguish PDAC from normal pancreas and pancreatitis (Bloomston, Frankel et al. 2007). Additionally, various miRNAs have been identified as agents that promote invasion and metastasis in PDAC (Zhang, Jamaluddin et al. 2011). For instance, EP300, a histone acetyl transferase that plays an important role in cell growth and division, is regulated by a group of miRNAs (miR-194, -200b, -200c, and -429) in PDAC (Mees, Mardin et al. 2010). miR-27a is also upregulated in various PDACs and in other malignancies (Liu, Tang et al. 2009). One of the main targets of miR-27a is *SPRY2*, which plays a

crucial role in inhibiting tumor growth and metastasis through Ras/MAPK pathway inactivation (Ma, Yu et al. 2010).

Further, there are various miRNAs that function as tumor suppressors. For instance, miR-96 is a potential tumor suppressor as it directly targets and inhibits KRAS activation (Yu, Lu et al. 2010). In PDAC, miR-96 is downregulated compared to normal pancreatic tissues. Thus, it is possible that miR-96 holds potential therapeutic use in KRAS-driven PDAC. miR-20a is another crucial miRNA that has potential metastasis-suppressing effects by negative regulation of STAT3, proliferation inhibition, and invasion by PDAC cells *in vivo* as well as *in vitro*. Therefore, controlled expression of miR-20a expression may be useful as a therapeutic treatment for human PDAC in the future (Yan, Wu et al. 2010).

Cervical cancer

With over 528,000 cases occurring every year, cervical cancer is the fourth most commonly occurring cancer in women after breast, colorectal, and lung cancers (Ferlay, Steliarova-Foucher et al. 2013) (Reshmi and Pillai 2008). It is also the fourth leading cause of death in women and one fifth of all new cases are diagnosed in India. Cervical cancer commonly occurs due to chronic infection with human papillomavirus (HPV). HPV infects the basal cells of the cervix and is maintained as an episome (Holowaty, Miller et al. 1999). The oncogenic transformation begins once the viral DNA is incorporated into host DNA. The E6 and E7 genes of the viral DNA inactivate common tumor suppressor genes, including p53 and RB, and induce neoplastic transformation. E6

also inhibits cellular apoptosis, while the E7 protein degrades Rb family proteins necessary for cell cycle progression (Ghittoni, Accardi et al. 2010).

Large-scale miRNA microarray analysis has shown that cervical cancer and normal tissue samples have distinct miRNA profiles. Among the differentially expressed miRNAs, miR-21 is highly over-expressed in tumor tissue. miR-21 is a negative regulator of programmed cell death 4 (PDCD4), which is a tumor suppressor with apoptotic function that blocks translation and tumor growth (Yao, Xu et al. 2009). Another oncogenic miRNA highly expressed in cervical cancer is miR-10a, which causes tumor growth, metastasis, and invasion by suppressing CHL1, a tumor suppressor gene (Long, Wu et al. 2012). miR-19a and miR-19b target Cullin-5 (CUL5) (Xu, Wang et al. 2012). The CUL5 gene is a core component of E3 ubiquitin ligase that forms the proteasomal degradation complex (Mehle, Goncalves et al. 2004). miR-20a, another miRNA from the same cluster, positively regulates the oncogene tyrosine kinase, nonreceptor 2 (TNKS2), causing enhanced cellular invasion and metastasis (Kang, Wang et al. 2012).

Many studies have examined tumor suppressor miRNAs that play a crucial role in opposing cervical cancer. For instance, miR-138 expression is lower in cervical cancer cells than in normal tissue. miR-138 is known to suppress telomerase activation and cellular immortalization (Chakrabarti, Banik et al. 2013). In addition, miR-7 was identified as a likely tumor suppressor because it decreased cellular growth and increased cellular apoptosis in cancerous tissue. Similar function was observed by Cui et al. when they overexpressed miR-125b in cervical cells and found that it could inhibit cell growth, induce apoptosis, and decrease tumorigenicity by suppressing the phosphoinositide 3-

kinase catalytic subunit delta (PIK3CD) by targeting the PI3 K/Akt/mTOR signaling pathway (Cui, Li et al. 2012).

It is clear that miRNAs play a critical role in cervical cancer. Many of these miRNAs such as miR-21, miR-27a, miR-34, miR-34a, miR-146a, miR-155, miR-196a, miR-203, and miR-221 are overexpressed and present at high levels in the serum of cancer patients (Gilad, Meiri et al. 2008, Gocze, Gombos et al. 2013). This indicates that miRNAs could likely be used as diagnostic markers for cervical cancer. miRNAs are also believed to have therapeutic potential, as researchers are currently developing drugs to target specific miRNAs (e.g. miR-21) that are overexpressed in cervical cancer (Wang, Xu et al. 2012). In addition, strategies are being developed to deliver miRNAs like miR-143 that are expressed in low levels in cancer and are known to negatively regulate oncogenes (Osaki, Takeshita et al. 2008). These findings highlight the potential medicinal value of miRNAs in cervical cancer and suggest that miRNAs may have a future role in personalized medicine.

Ovarian cancer

New cases of ovarian cancer have been estimated to be 225,000 worldwide in 2008, accounting for around 4% of all cancers diagnosed in women (Ferlay J 2010). The incidence rates vary considerably across the world, with developed countries having rates nearly twice as high as those in less developed countries. Moreover, there is preliminary evidence that screening can improve survival, but the impact of screening on mortality from ovarian cancer is still unclear (Rauh-Hain, Krivak et al. 2011).

One recent study by Zhang et al. used an integrative genomic approach in human epithelial ovarian cancer (Zhang, Volinia et al. 2008). The group compared miRNA expression levels in 18 ovarian cancer cell lines, non-neoplastic cell lines, and normal ovarian epithelial cells. They identified 4 miRNAs (miR-26b, -182, -103 and -26a) that were upregulated and additionally found downregulation of known tumor suppressor miRNAs let-7d and miR-127 (Zhang, Volinia et al. 2008). Overexpression of miR-26b, -182, -103 and -26a was due to the amplification of a chromosomal region. Downregulation of let-7d and miR-127 was due to epigenetic regulation. In addition to this study, there are other miRNA studies that investigate miRNA expression levels in different subtypes of ovarian cancer (Zaman, Maher et al. 2012). For instance, miR-519a was significantly upregulated in serous and clear cell carcinoma compared to the mucinous subtype (Kim, Kim et al. 2010). Overexpression of miR-519a was positively correlated with poor survival outcomes. Similarly, downregulation of miR-153 and miR-485-5p had a positive correlation with advanced clinical stage FIGO (International Federation of Gynecology and Obstetrics) grade 3. miR-519a expression was increased in clinical stages III and IV (advanced clinical stages) as compared to stages I and II (early clinical stages). Additionally, miR-100 has been shown to be downregulated in clear cell ovarian carcinoma cell lines and, when over-expressed, it enhanced sensitivity to rapamycin analog RAD001 (everolimus) by inhibiting the mTOR pathway (Nagaraja AK 2010). Such differential miRNA profiles are helpful in distinguishing different subtypes and are used to improve diagnostics and prognosis of the cancer. For instance, patients

with low let-7a methylation had a poor survival outcome compared to those with high methylation. The miRNA-200 family also plays a crucial role in ovarian cancer, and the miR-200 family cluster that includes miR-200a, -200b, and -429, when downregulated, is correlated with poor survival (Hu X 2009). Yang et al., confirmed that miR-214, -199*, and -200a were associated with high-grade and late stage tumors (Yang, Kong et al. 2008).

Another interesting ovarian cancer study of miRNAs from tumor-derived exosomes found eight miRNAs to have diagnostic potential (miR-21, -141, -200a, -200c/b, -203, -205 and -214) had similar expressions between cellular and exosomal miRNAs, with an absence of exosomal miRNAs in control samples (Taylor and Gercel-Taylor 2008). Additionally, the ratio of high-mobility group AT-hook 2 (*HMGA2*), an early embryonic gene, to let-7 has been identified for prognostic studies (Shell, Park et al. 2007). *HMGA2* is a known target of the let-7 family. Patients with higher *HMGA2*/let-7 ratios exhibited decreased 5-year progression-free survival (<10%) as compared to patients with lower ratios. Therefore, miRNAs play an important role in identifying different subtypes and exosomes present in ovarian cancer compared to benign/ normal ovarian epithelial tissue.

Breast Cancer

Breast cancer is the most common cancer in women worldwide, affecting approximately 1.5 million women. It is also the principle cause of death from cancer among women (Jemal, Bray et al. 2011). Breast cancer is a heterogeneous type of cancer and is generally classified on the basis of the presence or absence of estrogen receptor, human epidermal growth factor (*HER2*) expression, and by using gene expression based classifier or the integrative classification based on genomic and transcriptomic data (Perou, Sorlie et al. 2000). A recent study performed a miRNA expression pattern analysis in a cohort of breast tumors (n=1302) with various degrees of heterogeneity as well as adjacent normal breast tissues (n=116) along with a panel of breast cancer cell lines (n=28) and matched genomic, mRNA, or long-term survival data(Dvinge, Git et al. 2013). The miRNA array demonstrated a global decrease in miRNA in tumors compared to adjacent normal tissues. Additionally, 133 miRNAs were identified in minimal common regions with recurrent copy number alterations in 5% of samples (Dvinge, Git et al. 2013). For instance, they observed gain of miR-17-92 oncogenic family and loss of tumor suppressor miR-31. Interestingly, only 49 out of 227 miRNAs exhibit high correlation with the host mRNA, which includes ER+ marker miR-342. Other miRNAs, such as miR-10a and miR-505, are subtype specific and are co-transcribed with their host genes: *HOXB3* and *HOXB4* genes in ER+ or *ATP11C* in ER- samples (Luqmani, Al Azmi et al. 2009). Additionally, lymphocytic infiltration correlated with 5 miRNAs (miR-150, -155, -146a, -142-3p and -142-5p) that are known to have a pronounced effect on immune regulation (Faraoni, Antonetti et al. 2009, Andreopoulos and Anastassiou 2012, Xu, Lu et al. 2012). In contrast to clinical covariates, mRNA variation and

molecular signature were highly correlated to miRNA expression (Greither, Wurl et al. 2012). For instance, there was a high correlation between regulatory components of signaling and development, extracellular matrix, cell adhesion and morphogenesis, and known tumor-suppressive miRNA families such as miR-143/145 (Peng, Guo et al. 2011), miR-199/214 (Li, Han et al. 2012) and miR-127 (Guo, Hu et al. 2012). Additionally, they observed a correlation between ER-dependent miR-342 and the oncogenic miR-17-92 polycistron with estrogen and progesterone levels, reflecting its activity in breast cancer (Castellano, Giamas et al. 2009). This study was the first detailed systems-level analysis of miRNA expression architecture in a large number of human breast tumors that analyzed miRNA levels and integrated them with matched mRNA expression and DNA copy number.

Prostate cancer

Prostate cancer is considered the second most common cause of death in men over 40 years of age. The major challenge of prostate cancer is the development and acquisition of castrate resistant prostate cancer phenotype that leads to skeletal metastasis, which turns an otherwise treatable form of cancer into an incurable disease (Peng, Guo et al. 2011). Therefore, miRNAs become an attractive area of research to find answers to such aggressive forms of prostate cancer.

Tumor suppressor miRNAs in prostate cancer include miR-15a, -16, -143, -145, -200, and -488 (Hassan, Ahmad et al. 2012). miR-15a and -16 are located at 13q14.3, and the deletions at this region have been found in various cancers including chronic lymphocytic leukemia (CLL), multiple myeloma, mantle cell lymphoma, and prostate

cancer (Aqeilan, Calin et al. 2010). It has been reported that miR-15a and -16 can target oncogenes such as *BCL2*, *CCD1*, *WNT3A*, which promote survival, proliferation, and invasion of prostate cancer (Aqeilan, Calin et al. 2010).

miR-143 plays a crucial role in controlling EMT and is downregulated in prostate cancer (Clape, Fritz et al. 2009). Xu et al. identified that miR-143 regulates *KRAS*, pERK1/2, and cyclin D1, all of which play central roles in cell proliferation, migration, and chemosensitivity in prostate cancer (Xu, Niu et al. 2011). Overexpression of miR-143 in prostate cancer cells significantly decreased proliferation and migration, and enhanced sensitivity to docetaxel by affecting the EGFR/RAS/MAPK pathway. Additionally, they found that the expression levels of miR-143 and -145 were downregulated considerably in metastasis samples (Xu, Niu et al. 2011). Another key miRNA that controls the EMT process in prostate cancer is miR-200 (Kong, Li et al. 2009). It targets zinc-finger E-box binding homeobox 1 (*ZEB1*), *ZEB2,* and *SNAIL2* expression leading to acquisition of mesenchymal phenotype (Kong, Li et al. 2009, Ahmad, Aboukameel et al. 2011). Interestingly, miR-488 has a binding site at the 3'UTR of the androgen receptor (AR) gene and over-expression of miR-488 reduces expression of AR in both Androgen-dependent (LNCaP) and androgen-independent (C4-2B) prostate cancer cells (Sikand, Slaibi et al. 2011).

In contrast to tumor suppressor miRNAs, there are oncogenic miRNAs that have been found to be upregulated in prostate cancer (Pang, Young et al. 2010). For instance, miR-221 and miR-222 are both considered oncogenic as they were found to be associated with the development and metastasis of prostate cancer (Pang, Young et al. 2010). These

miRNAs act by binding to p27Kip1, resulting in its suppression and ultimately tumor growth. It is notable that miR-221 is higher in more invasive prostate cancer cells, e.g., LNCaP-AI cells compared to LNCaP (less invasive). This suggests that miR-221 promotes invasion of prostate cancer cells (Zheng, Yinghao et al. 2012). Another important miRNA that is upregulated in various cancers (glioma, breast cancer, colorectal cancer, stomach/gastric cancer, hepatocellular carcinoma, pancreas cancer, lung cancer, cholangiocarcinoma, leukemic cancer, and prostate cancer) is miR-21. Major targets of miR-21 that play a role in carcinogenesis include, *TPM1*, *PDCD4*, and *MARCKS* (Li, Li et al. 2009). Additionally, studies have also shown that transfection of mature miR-125b causes prostate cancer cell growth that targeted the 3'UTR of *BAK1* (a pro-apoptotic member of the BCL-2 gene family that is involved in initiating apoptosis) transcripts leading to cell proliferation (Lee, Kim et al. 2005). These studies suggest that the role of miRNAs could be explored to understand the stratification of prostate cancer.

miRNAs in hematopoietic cancers

Lymphoma and leukemia are the most commonly found blood cancers with multiple origins. Lymphoma is a type of blood cancer that occurs due to increased proliferation of B and T lymphocytes, whereas leukemia is a condition caused by an increased number of immature white blood cells. These diseases accounted for nearly 9.5 percent of the deaths from cancer in 2010, based on the total of 569,490 cancer deaths (Vardiman, Thiele et al. 2009).

There are two commonly found acute types of leukemia, acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL). AML consists of cytogenic abnormalities (50%) and chromosomal abnormalities that remain undetectable (Yuan, Kasar et al. 2012). A recent study found similar chromosomal alterations in both these leukemia types however, expression difference in 27 miRNAs especially; miR-146a distinguished these types when comparing AML patient samples with ALL patient samples(Mi, Lu et al. 2007). Additionally, the study found that miR-146a expression was inversely correlated to survival in both AML and ALL patients (Wang, Li et al. 2010). However, this study was not focused on understanding the miRNA abnormalities in these types of cancer but only miRNA expression profiles.

In a microarray profile study of 122 AML samples that consisted of 60 untreated cases and 50 relapsed or refractory cases with CD34+ normal cells, they observed downregulation of several miRNAs (miR-126, -130a, -93, -125a, and -146). Additional correlation studies of cytogenic abnormalities with observed miRNA expressions patterns in AML elicited that there are 14 downregulated and 8 upregulated miRNAs, which were associated with 11q23 translocation versus other AML types (Garzon, Volinia et al. 2008). For instance, the overexpression of miR-199a and miR-191 was identified in AML with trisomy 8 and was associated with poor outcome. This was the first study to identify distinct miRNA profiles between AML patients and normal controls, and the subsets of miRNAs related to cytogenetic groups and disease outcome (Garzon, Volinia et al. 2008). Also, in remission AML patients, levels of miR15a/16 were upregulated in contrast to relapse patients.

Around the same time, a study showed that nucleophosmin (*NPM1*) mutations are the most common molecular abnormalities in AML and are associated with upregulation of miR-10a, -10b, -196a and -196b, located within the homeobox genes (*HOX*) (Jongen-Lavrencic, Sun et al. 2008). Also, miR-21 was upregulated in AML samples when compared to normal CD34+ cells, thus strengthening the case for the importance of miR-21 in AML (Jongen-Lavrencic, Sun et al. 2008).

Chronic lymphocytic leukemia (CLL), another common leukemia is characterized by the accumulation of malignant B cells in peripheral lymphoid organs, bone marrow, and peripheral blood (Hallek, Cheson et al. 2008). CLL cells have genomic instability, chromosomal alterations (11q23 deletions: *ATM*; miR-34b/c cluster; trisomy 12 (increased *MDM2*), 17p deletion (TP53), 13q14 deletions (miR-15a/16-1)), and other genetic abnormalities (Dohner, Stilgenbauer et al. 2000). Several miRNAs such as miR-15a/16-1, -34 cluster, -155, -29, and -181b have been implicated in the pathogenesis of CLL (Dohner, Stilgenbauer et al. 2000). miR-155, -150, and -21 expression is shown to be increased in B-CLL cells compared to normal B cells (Fulci, Chiaretti et al. 2007). Around 50-60% of CLL patients exhibit deletion of the 13q14 region that encodes miR-15a/16-1 (Calin and Croce 2006). The downregulation of miR15a/16-1 is crucial as these target key cell cycle regulation and antiapoptotic proteins such as cyclin D1 and BCL2 (Cimmino, Calin et al. 2005). Interestingly, spontaneous models of CLL (NZB mice) also exhibit 50% reduction of miR-15a/16-1 (Raveche, Salerno et al. 2007). Other crucial miRNAs include miR-29 and miR-181 that target Tcl1, which is highly expressed in aggressive CLL (Pekarsky, Santanam et al. 2006). miR-34a has also been shown to target

E2F1 and B-Myb oncogenes in CLL as well as AML. v-Myb is found to be elevated in CLL patients that stimulate the miR-155 host gene. Increased miR-155 levels were associated with enhanced *ZAP70* expression and faster CLL progression (Vargova, Curik et al. 2011). Studies have shown that miR-155 is generally upregulated in most types of lymphomas, which include Burkitt's lymphoma, diffuse large B cell lymphoma, primary mediastina large cell lymphoma, and Hodgkin's lymphoma (Croce 2008). Recent murine studies have shown that miR-155 play a crucial role in B lymphocyte development (Kluiver, Poppema et al. 2005). Overexpression of miR-155 leads to abnormal proliferation of polyclonal pre-leukemic pre-B cells resulting in B cell malignancy. More recently miR-155 knockout studies have revealed the presence of defective dendritic cell functions, impaired cytokine secretion, and Th2 differentiation. In addition to its role in immunity, miR-155 has been shown to induce mediators of flogosis and is involved in responses to endotoxic shock (Thai, Calado et al. 2007).

The miR-17-92 cluster, located in 13q31-32, is highly overexpressed in lymphomas. He et al. group reported that this cluster is commonly amplified in B cell lymphoma patients (He, Thomson et al. 2005). They showed that miR-17-92 and cMYC act together to promote development tumors in mice, leading to lymphoproliferative disease, autoimmunity, and premature death (Xiao, Srinivasan et al. 2008). These studies are in concurrence with our studies in osteosarcoma emphasizing the function of the miR-17-92 cluster in various genomically chaotic cancers. The mice also showed enhanced proliferation of immature lymphocytes due to downregulation of *PTEN* and *BIM* and control apoptosis of B cell lymphocytes (He, Thomson et al. 2005). Upon detailed study

of this cluster, it was found that MYC binds and activates the expression of miR-17-92 while simultaneously activating E2F1 enabling a tightly controlled proliferative signal including lymphomas (O'Donnell, Wentzel et al. 2005). Thus, it is important to study such miRNA clusters that are deregulated in various cancers. Controlling the regulation of such clusters may help improve the future prognosis of the disease.

miRNAs in sarcomas

Sarcomas are a type of heterogeneous cancer that is mesenchymal in origin. They can be broadly stratified into bone and soft tissue sarcomas consisting of more than 50 subtypes. Current treatment strategies are not effective against many sarcoma types and ultimately lead to drug resistance. Therefore, a better understanding of sarcomas' pathobiology is required to develop better diagnostic and prognostic markers and more effective therapeutic treatments. We have performed an extensive review on various types of sarcomas (Subramanian, Lui et al. 2008). Recently, deregulations of miRNAs were identified in various types of sarcomas. Our studies show that there are unique miRNA expression signatures that can distinguish various sarcomas on the basis of histological types, reflecting difference in lineages and differentiation status of the tumors. The miRNA expression signatures can help improve the diagnosis and prognosis of soft tissue sarcomas. For instance, miR-210, a hypoxia-regulated miRNA, is positively correlated with the prognosis and age in a gender-specific manner in soft-tissue sarcoma patients (Greither, Wurl et al. 2012). Here, we discuss two major types of sarcomas,

osteosarcoma and rhabdomyosarcoma, and the implications of miRNAs induced dysregulation of various gene regulatory pathways.

Osteosarcoma is the most common bone cancer affecting young adolescents. Osteosarcoma affects 3-5 per million men and 2-4 per million women (Mirabello, Troisi et al. 2009). This will be discussed in detail in the next chapter.

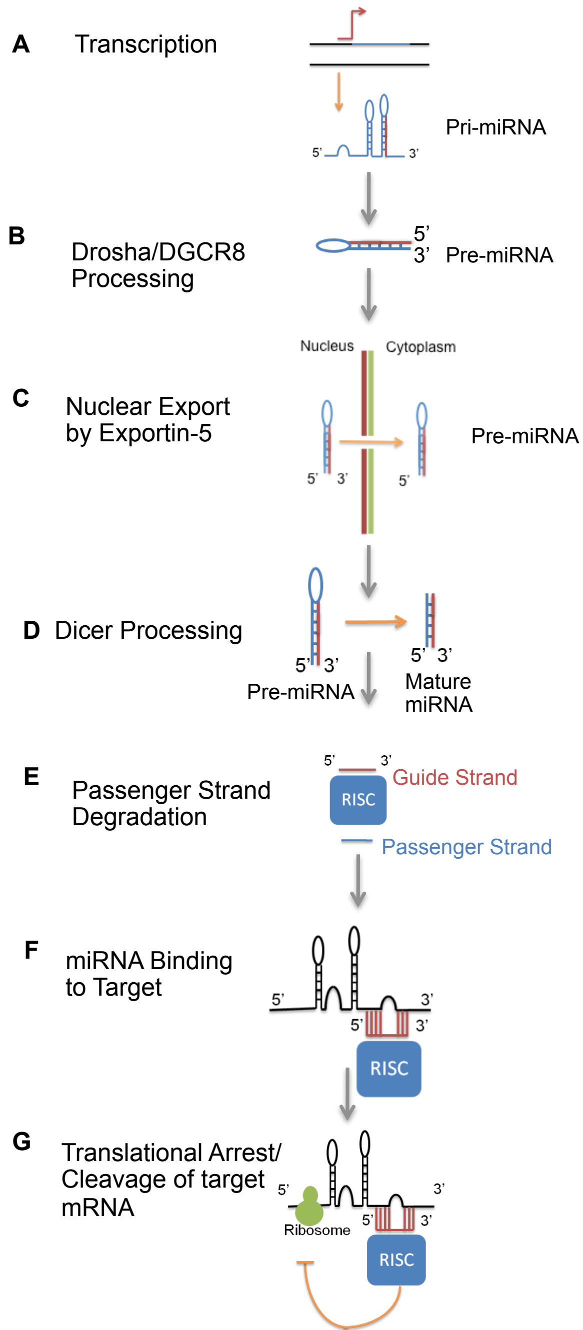
Another important type of sarcoma is rhabdomyosarcoma (Kluiver, Poppema et al. 2005). It is a skeletal muscle-derived tumor and accounts for 6-8% of all pediatric tumors(Linabery and Ross 2008). It consists of two histological subtypes, embryonic (EMRS) and alveolar RMS (Kluiver, Poppema et al. 2005, Linabery and Ross 2008). ARMS is more aggressive and has a poorer prognosis compared to EMRS (Qualman, Coffin et al. 1998). Most of the studies on RMS focus on myomiRs, such as the miR-1/miR-133/miR-206 family. These myomiRs play a crucial role in determining cell fate of myogenic precursors and maintaining muscle tissue homeostasis. All of the above mentioned myomiRs are significantly downregulated in RMS (Rao, Missiaglia et al. 2010). We also observed significant downregulation of myomiRs miR-1 and miR-133 in RMS compared to normal skeletal muscle (Subramanian, Lui et al. 2008, Li, Jin et al. 2012). Our data indicates that deregulation of these miRNAs stabilizes the expression of *PAX3* transcription factor and cyclin D2 in both ERMS and ARMS types (Subramanian and Kartha 2012). Interestingly, in ARMS *PAX3* forms a fusion transcript with forkhead homolog 1 (*FKHR*) and the subsequent loss of the PAX3 3′ UTR in the fusion transcript results in an oncogenic mechanism that evades miRNA-mediated regulation of *PAX3* (Subramanian and Kartha 2012) . In addition to the above miRNAs, miR-29 targets

E2F7, which plays a crucial role in cell cycle regulation. Overexpression of miR-29 in RMS cell types decreases the expression of various cell cycle genes and induces partial G1 arrest, leading to decreased cell proliferation. In summary, our data suggests that the RMS state occurs due to deregulation of multiple miRNAs and their target genes (Li, Sarver et al. 2012). Moreover, miR-29 can directly target histone deacetylase *HDAC4* during osteoblast differentiation causing global downregulation of DNA methylation (Li, Hassan et al. 2009). Thus, the role of miRNAs in epigenetic regulation in RMS makes it an attractive area of research to understand the RMS biology.

Conclusion

miRNAs are one of the many regulatory non-coding RNA types in eukaryotes. The role of miRNAs in cancer is crucial, but the presence of other regulatory RNAs should not be understated. Small interfering RNAs (siRNAs) are regulatory RNAs commonly generated by the breakdown of viral RNA and function similarly to miRNAs (Elbashir, Harborth et al. 2001). Another set of regulatory RNAs found in animals is Piwi-interacting RNAs, which are usually active in germline cells and play a crucial role in gametogenesis (Siomi, Sato et al. 2011). We have just begun to understand the role of these regulatory RNAs and more in-depth knowledge is required to fully understand the role of such regulatory RNAs in various biological processes. The recent discovery of RNAs that enhance the intricacy of gene regulation, called competing endogenous RNAs (ceRNAs), has added to our limited knowledge of the regulation of regulatory RNAs (Salmena, Poliseno et al. 2011). It was found that RNA transcripts could compete to bind

to common miRNAs. A known example is the *PTEN* ceRNA network, where PTEN's non-protein-coding pseudogene known as *PTENP1* is able to affect PTEN expression, its downstream PI3K signaling, and ultimately cell proliferation by directly competing for *PTEN*-targeting microRNAs (Poliseno, Salmena et al. 2010, Tay, Kats et al. 2011). Apart from the various mechanisms mentioned above, there is still a plethora of mechanisms by which these tiny RNAs control cell transformation and tumor progression (Ventura and Jacks 2009). For instance, the Croce et al. review focuses on various alterations and mechanisms that are involved in miRNA deregulation in cancer and how such dysregulation is involved in cancer initiation and progression in general (Croce 2009). Another review article by Garzon et al. discusses the rationale of using miRNAs as anticancer drugs and the strategies and challenges of such therapy (Garzon, Marcucci et al. 2010). With the knowledge gained by such investigations researchers have been able to develop miR-34 as a novel therapy in patients suffering from primary liver cancer or metastatic cancer with liver involvement. The treatment is currently in Phase-I clinical trials (Ben-Shushan 2013). Therefore, despite our limited knowledge about regulatory RNAs, we are certainly making progress toward understanding and revealing this level of complexity in gene regulation. It is clear that miRNAs play a crucial role in balancing various biological processes and any imbalance can have an outsized impact on the rest of the system. The next chapter describes the role of these crucial small RNAs in relation to osteosarcoma.


A
Transcription
Pri-miRNA
5'
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Drosha/DGCR8
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5'
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Degradation
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G
Translational Arrest/
Cleavage of target
mRNA
5'
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Ribosome
5'
3'
RISC

Figure 1. ***Schematic representation of miRNA biogenesis.*** A: Transcription of primary microRNAs (pri-miRNAs) by RNA polymerase II in the nucleus. B: Pri-miRNA is processed by Drosha and DGCR8 to form a precursor-miRNA (pre-miRNA) of about 70 nucleotides in length. C: Pre-miRNA is exported to cytoplasm by exportin 5. D: Final processing of pre-miRNA to mature duplex miRNA by RNase enzyme Dicer. E: Incorporation of mature duplex into RNA-induced silencing complex(Siomi and Siomi 2010) where miRNA* strand is selectively degraded. F: Binding of complex to the target mRNA guided by mature miRNA. G: Negative regulation of protein translation or degradation of the mRNA transcript based on the complementarity of the miRNA to the target sequence.

Chapter 2: Osteosarcoma, MicroRNAs, and Improving Treatment

Osteosarcoma is the most common primary bone malignancy affecting children, adolescents and young adults. Around 30% of patients with localized osteosarcoma and 70% of patients with metastasis will experience treatment failure within 5 years of diagnosis. Such dismal outcome has remained static for the past 20 years leading to an urgent need to identify novel targets and therapeutic agents that will improve patients overall survival and minimize immediate and long-term side effects of current chemotherapeutic agents. Additionally, osteosarcoma is commonly found in canines, with approximately 10,000 new cases each year in the US. The complex biology of osteosarcoma and tumor heterogeneity makes it very difficult to identify effective actionable targets and therapeutic agents. This chapter will highlight the recent developments in our basic understanding of microRNA mediated gene regulation in osteosarcoma and discuss the potential for development of microRNA-based prognostic biomarkers. Further, this chapter will analyze the recent development of preclinical evaluation of a novel agent Minnelide in the treatment of osteosarcoma and emphasize the prospects for combinatorial drug treatment in osteosarcoma.

Introduction

Osteosarcoma is an aggressive malignant cancer that arises from primitive cells that are mesenchymal in origin (Ottaviani and Jaffe 2009). Osteosarcoma is the eighth most commonly occurring pediatric cancer and consists of 20% of all bone cancers.

Osteosarcoma is the most commonly occurring primary bone sarcoma that affects children and adolescents (Ottaviani and Jaffe 2009). It also occurs in older adults, although rarely, making it have a bimodal onset. The incidence rates are estimated to be 5 per million cases per year in the United States (900 newly diagnosed cases per year). It is more commonly found in males (5.4 per million per year) than in females (4 per million per year) (Smith, Seibel et al. 2010).

Osteosarcoma usually occurs in the metaphyseal region of long bones, with higher predilection to femur (42%) than tibia (19%) and humerus (10%). The rest of all cases generally occur in the skull, jaw, and pelvis area (Mirabello, Troisi et al. 2009). Histologically, the characteristic feature of osteosarcoma is the presence of osteoid deposits within the tumor. The tumor cells appear to be very pleomorphic and may exhibit multinucleated, osteoclast-like giant cells (Klein and Siegal 2006).

Due to the chaotic karyotype and heterogeneous nature of osteosarcoma, understanding the molecular mechanisms of this cancer has been difficult (Lau, Harris et al. 2004). Osteosarcoma is considered to be a cancer that is associated with failure to properly differentiate and caused by genetic changes that can disrupt osteoblast development. Some osteosarcoma cases have a familial, inherited origin as in germline mutations in the retinoblastoma (*RB1*) gene. These cases usually show somatic deletion of the 13q14 region leading to inactivation of the remaining copy of *RB1* (Kivela, Tuppurainen et al. 2003). Li-Fraumeni syndrome is another cancer predisposition syndrome in which germline *TP53* mutation leads to osteosarcoma development (Overholtzer, Rao et al. 2003). There is an urgent need for better and more clear

understanding of the molecular mechanism of osteosarcoma development if targeted and effective therapies are to be developed.

In recent years, it has been discovered that regulatory RNAs such as microRNAs (miRNAs) play a major role in osteosarcoma development and progression (Jones, Salah et al. 2012). Given the need for better and clearer understanding of the molecular mechanism of osteosarcoma, it is essential to explore the role of regulatory RNAs such as miRNAs in osteosarcoma pathobiology. It is now estimated that more than 1,400 miRNA genes exist in the human genome. The function of miRNAs span from regulation of critical biological processes in normal cells to proliferation control in tumor cells. Moreover, these small non-coding miRNAs regulate more than 50% of the protein coding transcripts (Kusenda, Mraz et al. 2006).

There is evidence that supports the contention that miRNAs are differentially regulated in normal and tumor cells, and it has been revealed that miRNAs can play a crucial role in pathogenesis of cancer development and progression (Sassen, Miska et al. 2008, Vandenboom Ii, Li et al. 2008, Shi and Guo 2009). Additionally, miRNAs are highly conserved among various species, which suggests a potential role that miRNAs can play in diagnosis and prognosis of cancer, including osteosarcoma. For instance, in comparison with human osteosarcoma, dogs can more commonly acquire osteosarcoma, said incidence being more than 10,000 cases per year with exquisite breed predilection (Sarver, Thayanithy et al. 2013). The shorter life span, similar functioning and incidence of osteosarcoma, and extensive homology in genetic sequence and gene order between both humans and dogs, make dogs a great model to study and to help researchers further

understand the molecular mechanism in human osteosarcoma (Ostrander and Wayne 2005). Recent findings involving canine osteosarcoma will be discussed below as well.

This chapter will analyze and explore the following topics:

1. *Role of miRNAs in osteosarcoma*
2. *The perturbation of 14q32 miRNA/cMYC/miR-17-92 network:* This deregulated miRNA circuitry network is potentially a unifying molecular mechanism that could hold a better prognostic significance for osteosarcoma patients, which will be described in detail.
3. *Current osteosarcoma therapies:* This chapter will survey current osteosarcoma therapies and other methods and discuss the benefits and perhaps, at this point in their understanding of osteosarcoma, the necessity of a combinatorial approaches to treating osteosarcoma patients.
4. *Minnelide and osteosarcoma:* Lastly, I will discuss the recent preclinical findings of a novel agent Minnelide, a pro-drug of Triptolide, and its potential in treating osteosarcoma patients.

In summary this chapter will attempt to provide a clearer understanding of the current science surrounding the topics of osteosarcoma prognosis and diagnosis, the molecular mechanism at play in osteosarcoma, the role miRNAs play in relation to these topics, and of the path forward to developing better approaches to treating osteosarcoma, and for further researching osteosarcoma and other forms of cancer as well.

Role of miRNAs in osteosarcoma

The expression pattern of miRNAs can be clearly assessed by miRNA microarray approaches. Many studies have shown that there is differential miRNA expression patterns in various cancers, including osteosarcoma (Kobayashi, Hornicek et al. 2012, Namlos, Meza-Zepeda et al. 2012, Thayanithy, Sarver et al. 2012, Zhou, Shi et al. 2013). Sarver et al. generated miRNA expression profiles for over 300 sarcoma tissue samples that represent 22 different sarcoma subtypes (including osteosarcoma), and have developed a Sarcoma MircoRNA Expression Database (S-MED) (Sarver, Phalak et al. 2010, Sarver, Thayanithy et al. 2013). Interestingly, out of all the sarcoma analyzed, osteosarcoma stood out as a single cluster distinct from various other sarcomas.

There are various other studies that have investigated the role of miRNAs in osteosarcoma using miRNA expression profiles (Kusenda, Mraz et al. 2006, DeSano and Xu 2009, Gattolliat, Thomas et al. 2011, Kobayashi, Hornicek et al. 2012, Kelly, Haibe-Kains et al. 2013). An early study, Maire et al., found differential miRNA expression profiles in 7 osteosarcoma samples that could target specific pathways compared to the normal bone (Maire, Martin et al. 2011). Recently, Lulla et al. and Stabley et al. showed that there are more than 22 differentially expressed miRNAs as compared to osteoblasts (Stabley 2010, Lulla, Costa et al. 2011). The miRNAs that are highly expressed include miR-135b, -150, -370, -542-5p, -652, and -654. For instance, miR-206 and -286 play an important role in osteoblast differentiation. Some of these miRNAs, such as miR-370, can target the insulin receptor substrate-1 (*IRS1*) gene that interacts with the insulin-like

growth factor 1 protein, which is commonly overexpressed in osteosarcoma, suggesting miRNAs can directly influence key signaling pathways and differentiation of osteoblasts, resulting in osteosarcoma.

miRNAs can also target various tumor suppressor genes and affect their expression. For example, *TP53*, a common tumor suppressor that is expressed during DNA damage leading to cell cycle arrest or apoptosis by dissociation from MDM2, is found mutated in more than 20% of osteosarcoma cases (Overholtzer, Rao et al. 2003). Among the several miRNAs that can target *TP53*, the highly conserved miR-34 family (miR-34a, -34b, and -34c) is one of the important components that induce G1 arrest and apoptosis via *CDK6*, *E2F3*, *Cyclin E2*, and *BCL2* in a TP53 dependent manner in osteosarcoma cells (Chang, Wentzel et al. 2007, Mandke, Wyatt et al. 2012). According to various studies, miR-34 is significantly downregulated due to genetic and epigenetic alterations in primary osteosarcoma samples (Nalls, Tang et al. 2011, Doridot, Houry et al. 2013).

Recently, our group identified a significant downregulation of more than 40 miRNAs located in the 14q32 locus in various human osteosarcoma samples (Thayanithy, Sarver et al. 2012) and the miRNAs in this locus was conserved in canine and mice as well. Additionally, our group and various other studies found overexpression of cluster of miR-17-92 and its paralog miR-106b cluster that are located within introns of c13orf25 and *MCM7* respectively, suggesting an oncogenic role in osteosarcoma. Interestingly, transcription factors such as *E2F1, E2F2, E2F3* (components of the TGF-B pathway) and c*MYC* can activate both these miRNA clusters, and these transcription

factors are amplified in the osteosarcoma as well (Sylvestre, De Guire et al. 2007, Olive, Jiang et al. 2010, Li, Li et al. 2011).

A strong inverse correlation has also been identified between *PTEN*, and other tumor suppressor genes, and several members of the miR-17, -19, -130/301, and -26 families in osteosarcoma, when compared to normal bone (Song, Salmena et al. 2012). This information is crucial because PTEN antagonizes signaling through the PI3K/AKT pathway that promotes cell proliferation and inhibition of apoptosis.

Together, the above observations suggest that miRNAs play a crucial role in osteosarcoma progression, and they can behave either as a tumor suppressor or oncogenic that can affect various target genes and contribute to development of osteosarcoma cancer.

The perturbation of 14q32 miR-cMYC-miR-17-92 network in osteosarcoma

Osteosarcoma is characterized by of various genetic and chromosomal aberrations. For instance, many tumors show DNA copy number amplification of *CD5L* and *RUNX2* at the 6p12 region and *cMYC* amplification at the 8q24 region, which are responsible for osteoblast differentiation (Martin, Zielenska et al. 2011, Han, Wang et al. 2012, Lucero, Vega et al. 2013). Other common gene aberrations include mutations in *RB, TP53, BMPs,* and *MMPs* (Tang, Song et al. 2008, Broadhead, Clark et al. 2011). But even with this information, little is known about the pathobiology of the disease itself.

Earlier work from our lab wanted to investigate the role of miRNAs in osteosarcoma and then identify any unifying mechanism that could hold a better

prognostic and diagnostic significance. The studies started with assessing *cMYC* expression in various osteosarcoma samples, and they made some unique and potentially significant findings and conclusions.

The *cMYC*, proto-oncogene is amplified in about 30% of osteosarcoma patients, however, the majority of osteosarcoma cases is characterized by overexpression of cMYC. The authors reasoned that *cMYC* expression was regulated by certain miRNAs. They identified a unique miRNA network involving 14q32 miRNAs, *cMYC*, and the miR-17-92 cluster. This network is novel in the sense that it has not been identified and/or analyzed before. The group performed a comprehensive expression profile of miRNAs on around 22 osteosarcoma tissues, and they observed that around 40 miRNAs in the 14q32 region were significantly downregulated (5 to 6 fold). In contrast to the 14q32 miRNAs cluster, the miR-17-92 cluster—especially miR-19a and -19b at the 13q32 region—showed significantly higher expression when compared with normal bone tissue samples. They validated such similar miRNA differential expressions in various osteosarcoma cell lines such as Saos2, U2OS, and HOS, as compared to osteoblasts. Also, the team noticed that expression of 14q32 miRNAs were higher in differentiated osteoblasts (up to 4-fold) than the osteosarcoma cell lines **(Fig. 2)**.

To understand the function of the upregulated miR-17-92 cluster, the team performed series of knockdown experiments. They found that blocking miR-18a and -20b with anti-miRs in the cluster caused apoptosis, as compared to controls. Similar results were observed when they blocked *cMYC* expression in osteosarcoma cell lines. These results suggest that osteosarcoma is oncogenically addicted to *cMYC* and the miR-17-92

expressions are essential for osteosarcoma cell proliferation. Also, restoration of the expression of 14q32 miRNAs (*miR-544, -369-3p, -134, and -382),* or a reduction of the miR-17–92 cluster by ectopic means resulted in induction osteosarcoma cell apoptosis. The foregoing observations suggest a potential tumor suppressor role of 14q32 miRNAs and the regulation of *cMYC* and the miR-17-92 cluster with these 14q32 miRNAs. To validate the interaction between the 14q32 miRNAs (miR-544, -369-3p, -134, and -382) and *cMYC*, the team performed luciferase reporter assays with *cMYC* 3'UTR reporter construct, and they observed that the 14q32 miRNAs acted synergistically and suppressed reporter transcripts having the *cMYC* 3'UTR, and also that overexpression of the 14q32 miRNAs decreased cMYC protein expression.

Various studies have shown that *cMYC* transactivates the miR-17-92 cluster miRNAs that is an important component of many cancers including colon cancer, hepatoma, and leukemia. Investigating the *cMYC-miR-17-92* cluster in human osteosarcoma tissues further, the authors' group found that overexpression of the 14q32 miRNAs in osteosarcoma cells decreased transcript levels of miR-17-92 miRNAs leading to apoptosis. Further they showed that the pro-apoptotic effects of the 14q32 miRNAs are attenuated by exogenous expression of miR-17-92 or *cMYC* without the 3'UTR. Next, they wanted to investigate the mechanism of significant downregulation of these 14q32 miRNAs. The comparative genomic hybridization analyses showed no DNA copy number change at the 14q32 region, which led to speculation of imprinting changes of this region in osteosarcoma. Previous studies in mice have shown that the homologous region of the *14q32* miRNA cluster is maternally expressed and related to the

surrounding imprinted genes such as *Dlk1, Gtl2,* and *Rtl1* (Hagan, O'Neill et al. 2009). The *DLK-MEG3* region seems to be important, as it is also involved in Prader-Willi/Angelman syndromes (pUPD12 and pUPD14) (Hagan, O'Neill et al. 2009). In humans, three differentially methylated regions presumably control the corresponding DLK1-GTL2 region. *MEG3*, one of the components in this region, is maternally expressed, which is located upstream to the 14q32 miRNA clusters. *MEG3* was also expressed at significantly lower levels in osteosarcoma as compared to normal bone, suggesting a shared transcriptional mechanism that may regulate the expression of genes and miRNAs at the 14q32 locus. Also, *MEG3* can activate and stabilize tumor suppressor *TP53*, so it is likely that reduced *MEG3* expression may reduce the endogenous levels of *TP53* in osteosarcoma (Zhou, Zhang et al. 2012). Based on this knowledge the authors' group investigated the possibility that downregulation of the 14q32 miRNAs was epigenetically controlled through DNA methylation and histone acetylation. They analyzed DNA methylation at the CpG regions of selected upstream 14q32 miRNAs (miR-127, -411, -431, and -432) using combined bisulfite restriction analyses (COBRA). The results suggested that there was no change in methylation patterns or relative hypomethylation patterns in osteosarcoma tumor samples as compared to normal bone. This suggests that DNA methylation may not be the primary reason of significant downregulation of these 14q32 miRNAs. Subsequently they assessed the histone acetylation in osteosarcoma cells (Saos2) via ChIP assays. The ChIP arrays interpretation demonstrated significant deacetylation in histone (H3) proteins in samples that included human osteosarcoma cell lines as well as tissues.

Together, above experiments revealed a mechanistic role of 14q32 miRNAs and their tumor suppressor role in osteosarcoma, and how their loss may induce transformation of osteoblasts to osteosarcoma cells. These results suggest that downregulation of 14q32 miRNAs are necessary for apoptotic escape and thus for sustaining tumorigenesis in osteosarcoma. Additionally, DNA copy number analysis of osteosarcoma patient genomes showed that decreased expression of the 14q32 miRNAs in osteosarcoma is not due to the loss or deletion of the loci thereof, and hence may involve transcriptional and or epigenetic regulation. Further characterizations of these networks show decrease in histone acetylation. Also, the group identified a unifying mechanism that can potentially explain the regulation of *cMYC* in osteosarcoma. Therefore, the 14q32 miRNAs may not only be prognostically significant, but may also provide suitable targets for the development of more effective treatments of osteosarcoma.

Prognostic markers in osteosarcoma

There are various factors that can contribute to the prognosis of osteosarcoma (Bakhshi and Radhakrishnan 2010). Some of the factors include anatomical site, tumor size, age, stage, and histology, among others. Each of these factors and how it correlates with prognosis of osteosarcoma is discussed below.

It has been established that total surgical resection with negative margins is essential for achieving cure of non-metastatic osteosarcoma (Gonzalez-Herranz, Burgos-Flores et al. 1995, Bruland and Pihl 1997, Geller and Gorlick 2010). This gets quite

challenging to achieve with axial osteosarcoma involving the spine, ribs, and pelvis due to anatomical constraints. Hence, axial tumors present with a worse prognosis (survival rate of 20-40%) than osteosarcoma of the extremity (survival rate of 60-70%), even if they are of comparable grade and stage (del Prever, Fagioli et al. 2005, Lee, Kim et al. 2010).

Another important factor that governs better prognosis of osteosarcoma is smaller tumor size, measured in terms of the gross tumor length, volume, or surface area. Studies have demonstrated that a tumor size of more than 10 centimeters has been consistently associated with poor outcome (Bieling, Rehan et al. 1996, Jaffe and Jaffe 1996). However, due to variability in measuring tumor size, a single minimum cutoff value beyond which tumor size becomes significant is difficult to determine. In addition, it also becomes difficult to compare studies on tumor size because of use of different imaging modalities such as conventional radiography, CT scan, and MRI.

Age as a prognostic factor in osteosarcoma has been a very ambiguous subject to study. Two large retrospective studies showed that patients younger than 14 years and older than 40 years had inferior outcomes. In contrast to these studies, age was not a prognostic marker in the recently reported retrospective analysis on 2680 patients in ten centers worldwide (Endo, Yoshida et al. 2013, Nahar, Tague et al. 2013, Sternberg, Pondenis et al. 2013, Ueki, Maeda et al. 2013). This suggests that with better supportive care, age will no longer be an important prognostic factor.

The clinical stage in osteosarcoma is one of the strongest predictors of survival rate of osteosarcoma patients. There are two widely accepted staging systems in

osteosarcoma: (1) the Musculoskeletal Tumor Society Staging System developed by Enneking et al. (Ponce, Thompson et al. 2013) and the American Joint Committee on Cancer (AJCC) staging system (Saeter 2003, Saeter, Kloke et al. 2005, Saeter 2007, Bielack, Carrle et al. 2008, Bielack, Carrle et al. 2009, Jawad and Scully 2010). Various studies have shown that patients with metastatic disease have a worse outcome in comparison with patients with localized disease. Also, patients with pulmonary metastasis have a better outcome than patients with non-pulmonary metastasis. Patients with skip metastases (discontinuous lesions occurring in the same bone as the primary lesion) are associated with poor prognosis for as yet unknown reasons (San-Julian, Diaz-de-Rada et al. 2003, Bacci, Longhi et al. 2006). It is worth noting that patients with stage 3 osteosarcoma (no metastasis, as per the AJCC classification), have an outcome similar to patients with stage 4 osteosarcoma (pulmonary metastasis). Historically, it has been shown that chondroblastic osteosarcoma has a poor response to chemotherapy in comparison with other subtypes, and thus is associated with a poor outcome (Sanerkin 1980).

Patients who are eligible for limb salvage surgery have a higher risk of local recurrence but have osteosarcoma comparable with patients who undergo amputation (Picci, Bacci et al. 1997, Pan, Chan et al. 2012). Patients who undergo amputation have large tumors and/or involvement of major neurovascular structures. These patients have inferior outcomes when compared with patients who underwent limb salvage surgery, due to the tumor size, aggressiveness, and difficulty in obtaining a negative margin,

rather than due to the choice of amputation as a procedure. Therefore, limb salvage surgery is currently the preferred surgical procedure.

Elevated pre-therapy serum lactate dehydrogenase (LDH) levels were associated with decreased 5-year disease-free survival (DFS), when compared with patients with normal LDH (39.5% vs. 60%, respectively) (Gavhed, Akefeldt et al. 2009, Ghali, Wikstrand et al. 2009, Wang, Park et al. 2013).

Serum alkaline phosphate (SAP) levels are found elevated in increased osteoblastic activity as seen with fractures, physiological growth and osteosarcoma (Nagoya, Uede et al. 1991, Troseid, Arnesen et al. 2009). Bacci et al. observed in extremity osteosarcoma that elevated SAP was found in 258 out of 560 patients and significantly correlated with poor DFS and osteosarcoma ($p = 0.002$) (Bacci, Picci et al. 1993).

Another strong predictor to survival is response to chemotherapy. A tumor necrosis of more than 90% has been proposed as a cutoff for accepting a good response to chemotherapy and a better survival outcome (1988, Morello, Buracco et al. 2001, Holzer, Krepler et al. 2003, Bacci, Longhi et al. 2006). Approximately 20% of osteosarcoma patients in the West are presented with macrometastatic disease. Out of the remaining 80% of the osteosarcoma patient population, only 20% would be expected to survive if they are treated with surgery alone, suggesting that a significant number of non-metastatic patients have micrometastatic disease

High-dose methotrexate (HDMTX) is an important component of various regimens used for treating osteosarcoma (Yoshikawa, Takaoka et al. 1985, Wagener, van

Oosterom et al. 1986, Ripamonti, Avella et al. 1993, Ambroszkiewicz, Gajewska et al. 2010). The use of chemotherapy regimens that lack anthracyclines (doxorubicin) or platinum (cisplatin) has been shown to be associated with inferior outcomes, confirming the important role played by these two agents in achieving cure of osteosarcoma.

Tumor angiogenesis can play a major role in reflecting guarded prognosis (Yang, Wang et al. 2013). Using antibodies against endothelial antigens such as CD31, CD34, factor 8, and VEGF can measure tumor angiogenesis. For instance, a study by Kaya et al. analyzed the serum VEGF level in patients with osteosarcoma and observed that a serum VEGF level more than 1000 pg/l was associated with worse osteosarcoma (Kaya, Wada et al. 2000). Also, a higher incidence of pulmonary metastasis has been shown to correlate with increased angiogenesis in osteosarcoma. However, there is no evidence that shows correlation between angiogenesis and outcome in osteosarcoma. Additionally, Bajpai et al. observed that the degree of tumor angiogenesis in osteosarcoma correlated with the tumor grade and histological necrosis, which suggests that angiogenesis can be used as a surrogate marker for predicting tumor aggressiveness and response to chemotherapy (Bajpai, Gamanagatti et al. 2010).

14q32 miRNAs are potential prognostic markers for osteosarcoma

In addition to the above-mentioned factors for determining prognosis in osteosarcoma cases, miRNAs can also play a crucial role in determining outcome of the disease. Our lab compared miRNA expression data in human tissues with an additional 16 human osteosarcoma tissue samples and a detailed clinical follow up with 37 canine

osteosarcoma samples. As mentioned earlier, they observed a significant downregulation of 14q32 miRNAs compared to the other sarcoma types presented in the S-MED.

Additionally, they verified that these 14q32 miRNAs were highly correlated with each other using a correlation-based network analyses that was based on the miRNA expression levels as determined by microarray analyses. The high correlation of these 14q32 miRNAs suggests that an individual miRNA from this locus could represent the expression levels of most of the 14q32 miRNAs. On profiling the mRNA transcript levels of tumor samples that had miRNA profiles, they found expression changes in around 385 genes due to expression changes of miR-382. These gene expression level changes correlated with the poor survival rate of the human patients.

This means that a decrease in miR-382 expression led to increased expression of genes responsible for metastasis and lower survival rate of osteosarcoma patients **(Fig. 3)**. Similar results were observed in the orthologous miRNA 14q32 locus, CFA 8 in canine that was identified using the Entrez gene database. After mapping the miR-382 expression in 16 canine osteosarcoma samples, Sarver et al observed that canine tumors with lower miR-382 levels correlated with increase gene expression of gene related to metastasis and shorter survival rates. These results suggest that 14q32 miRNAs are conserved and have a potential prognostic significance in both human as well as canine osteosarcoma.

According to Dr. Fuchs, 10% of osteosarcoma patients require limb amputation, and currently the use of limb salvage is performed as a surgical alternative to amputation (Yin, Liao et al. 2012). Limb salvage aims to extract cancerous cells while keeping the limb function intact (Mito, Ferrer et al. 2012). However, the challenge of decreasing the recurrence of osteosarcoma remains unaltered. Osteosarcoma recurrence rates have been static since the 1970s, ranging between 10% and 20% (Allison, Carney et al. 2012). The big question that yet remains to be answered is: Why it is still so challenging to combat metastatic osteosarcoma? Perhaps one of the reasons is that the osteosarcoma tumor is extremely heterogeneous, which makes testing new drugs extremely difficult.

This deficiency in osteosarcoma therapies urgently calls for a better and clearer understanding of the molecular mechanism and progression of osteosarcoma tumors. This will necessarily involve understanding of the crosstalk between different types of cells within the bone microenvironment and the interaction between tumor cells and stromal cells, endothelial cells, and macrophages (Andrews 2013).

Another important aspect of this disease is to understand the cell that leaves the primary niche and metastasizes. For instance, genes involved with the macrophage activity play a crucial role in osteosarcoma metastasis (Buddingh, Kuijjer et al. 2011). The involvement of macrophages in metastasis could explain the beneficial effects of muramyl tripeptide (MTP) (Meyers, Schwartz et al. 2008). MTP improved 6-year survival rates from 70% to 78% by activating macrophages in osteosarcoma (Chou, Kleinerman et al. 2009). Another immunomodulatory approach is using GM-CSF, which

can also stimulate macrophages that can prevent pulmonary recurrence (Arndt, Koshkina et al. 2010).

Drugs that target other pathways fall under the class of SRC inhibitors, mTOR inhibitors, tyrosine kinase inhibitors, and RANKL inhibitors (Chawla, Staddon et al. 2012, Ebb, Meyers et al. 2012, Yap, Arkenau et al. 2013). Saracatinib, a SRC inhibitor, could prevent adhesion and migration of osteosarcoma cells in vitro but had no effect on pulmonary metastases *in vivo* (Hingorani, Zhang et al. 2009). Other tyrosine kinase inhibitors include: Trastuzumab, an inhibitor of HER-2 receptors; Cituximab, an inhibitor of IGF1 receptors; sorafenib, an inhibitor of VEGF receptors; and osi-930, an inhibitor of multikinase receptors.

Another new emerging class of drugs focuses on inhibiting osteoclast activity that plays a crucial role in the bone microenvironment (Rousseau, Escriou et al. 2011). A recent phase 2 study of the receptor activator of a nuclear factor-kb ligand (RANKL) inhibitor called denosumab could prevent osteolysis and enhanced tumor response to other chemotherapeutic agents such as ifosfamide (Thomas, Henshaw et al. 2010). Bisphosphonates constitute another class of drugs that tends to increase apoptosis in osteoclasts, which brings a correct balance in osteoblasts/osteoclast ratio and eventually inhibits osteoclast-mediated angiogenesis (Meyers, Healey et al. 2011).

In summary, the aforementioned drug studies suggest that one drug would not be able to cure osteosarcoma. The future therapeutics has to be designed in such a way that they target multiple pathways that can cause, contribute to, or otherwise result in osteosarcoma. And to that end, combinatorial drugs and treatments are essential.

Chromatin modifying drugs in combination treatment

Given the current understanding of the molecular mechanism of osteosarcoma, as well as the various shortcomings with standalone osteosarcoma drug therapies and treatments, it is apparent that there is an urgent need for combinatorial treatment of osteosarcoma. The authors' previous work, discussed above, has shown that there is an inverse correlation between expression of 14q32 miRNAs and *cMYC*. Furthermore, their work illustrated that the significant downregulation of 14q32 miRNAs is due to decreased histone acetylation.

Another study conducted by the same group demonstrated that using chromatin modifiers led to an increase in acetylation and an increase in 14q32 miRNAs, which were confirmed by western blots and ChIP (Thayanithy, Park et al. 2012). Not only did the combination of the chromatin modifiers (5-Aza and 4-PBA) restore the expression of 14q32 miRNAs, but also they significantly changed the gene expression. On performing gene expression analyses of 13 osteosarcoma patient samples with 4 normal bones, and comparing this with the gene expression profile of drug treated and untreated Saos2 cells, the microarray data identified 265 genes that were differentially expressed (greater than a 2-fold change, with p values less than 0.001). Also, the authors' group could replicate the same hypothesis using FDA approved chromatin modifiers such as SAHA (histone deacetylase inhibitor) and Zeb (DNA methylation inhibitor). Next, they wanted to see if this same effect is also observed in canine osteosarcoma, as it was previously observed (discussed above) that 14q32 miRNAs are significantly downregulated in canines.

Interestingly, they found that canine osteosarcoma cell lines with more “aggressive” gene expression profiles and shorter doubling times from both species were more sensitive to the effects of both compounds (Scott, Sarver et al. 2011). Thus, the results confirmed to authors’ prediction that sensitivity to chromatin modification is directly related to specific patterns of genome-wide gene expression and downregulation of 14q32 miRNAs.

Minnelide and osteosarcoma

Knowing that osteosarcoma is highly heterogenetic, it is necessary to find a novel drug that can target the proposed molecular mechanism of osteosarcoma. Triptolide, a diterpene epoxide synthetic derivative from the Chinese plant *tryptergium wilfordii*, was identified in a small screen, as a regulator of heat shock gene transcription. This drug has been shown to inhibit cell proliferation and induce apoptosis mainly by downregulation of HSP70 levels (Phillips, Dudeja et al. 2007). However, in spite of these promising results, Triptolide was relatively ineffective for in vivo models, due to it’s being sparsely soluble in the body.

Resultantly, a group led by Saluja developed a water-soluble prodrug of triptolide called Minnelide, and proceeded to demonstrate its true effectiveness in various preclinical models of pancreatic cancer, and has at present reached into phase 1 trials (Chugh, Sangwan et al. 2012). The authors’ group tested the effects of Triptolide and Minnelide *in vitro* and *in vivo* osteosarcoma models. The reason to test these two drugs was that there is a high elevation of HSP70 in osteosarcoma along with dysregulation of

cMYC and *NF-κB* transcription factors responsible for osteosarcoma progression (Banerjee, Thayanithy et al. 2013).

Previous work from our lab observed that triptolide at doses as low as 100nM could induce cell death mediated by apoptosis in osteosarcoma cell lines, without any significant cell death in osteoblasts. These studies were confirmed by cell viability assays and caspase activity assays. Additionally, they observed that Triptolide (at 100nM) significantly reduced the levels of osteosarcoma survival genes such as *cMYC*, *β-catenin*, *cyclin D1*, *survivin*, and *RUNX2*. This suggests that triptolide has a potential role in inhibiting osteosarcoma cell proliferation by targeting various cell survival genes. In further analyses of Minnelide (the soluble drug form of triptolide) in an osteosarcoma orthotropic mouse model (n=12), the authors' group observed significant decrease in tumor burden with insignificant presence of lung micrometastases compared to the control group (n=12) **(Fig. 4)**. They also observed a significant decrease in the *NF-κB* activity in Minnelide mice tumor tissues and osteosarcoma cells, as compared to the untreated tissues and osteosarcoma cells.

Next, they assessed the role of Minnelide in osteosarcoma progression and metastasis in lung colonization mice models. Six weeks after starting Minnelide treatment in these mice, they noticed that the number of metastatic nodules in the treated group was significantly lower than the untreated group, and also that 3 out 8 mice did not exhibit any lung nodules.

Therefore, the authors propose that Minnelide can be a novel therapeutic agent in osteosarcoma cases with the potential to increase survival rate by significant reduction of

metastasis with only limited side effects. They consider this drug to have a potential therapeutic role in treating human osteosarcoma.

Long non-coding RNAs in cancer and osteosarcoma

A remaining layer of complexity is generated by larger regulatory RNAs known as long noncoding RNAs ("lncRNAs"), which are longer than 200 nucleotides and fulfill a range of functions involving transcription, post-transcription, epigenetic regulation, both in the nucleus and the cytoplasm. It is now known that only one-fifth of transcription across the human genome is associated with protein-coding genes, which indicates that there are at least four times more lncRNAs than coding RNA sequences (Kapranov, St Laurent et al. 2010). Moreover, the larger scale DNA sequencing projects such as FANTOM (Functional Annotation of Mammalian cDNA) are being undertaken to reveal the complexities of such transcription (Carninci, Kasukawa et al. 2005). For instance, the FANTOM3 project has identified about 5,000 non-coding transcripts from around 10,000 distinct loci that contains various miRNA signatures such as 5' capping, splicing, and polyadenylation, but have insignificant or no open reading frame (ORF) (Carninci, Kasukawa et al. 2005).

Recently, it has been shown that there are enhancers that could express lncRNAs, and these RNAs depicted an increase in concentration in a stimulus-dependent manner, suggesting that they might confer an additional layer of regulation in gene expression. The two studies that provided substantial evidence for a causal role in transcriptional activation for enhancer-associated lncRNAs were based on knockdown approaches to

deplete the levels of several lncRNAs in human cells (Wang, Arai et al. 2008, Orom, Derrien et al. 2010). Depletion of specific lncRNAs resulted in repression of neighboring protein-coding genes in a *cis* regulatory manner. Moreover, the above results of transcriptional activation could be reiterated in classical enhancer assays using heterologous reporters (Orom, Derrien et al. 2010). However, identifying such lncRNAs within various cDNA libraries still remains challenging, since it is difficult to distinguish protein-coding transcripts from non-coding transcripts.

We have just begun to identify such lncRNAs in osteosarcoma and the regulation thereof. One of the two recent discoveries of lncRNAs performed an expression profile of lncRNAs in osteosarcoma and compared it with paired adjacent noncancerous tissue using microarray analysis. It was found that there are 25,733 lncRNAs that were expressed in osteosarcoma; 403 lncRNAs were consistently over-regulated and 798 lncRNAs were consistently under-regulated in all samples analyzed (>2.0-fold, $p < 0.05$) (Li, Liu et al. 2013). The other study described the role of one of the many lncRNAs, TUG1 and its associated transcript variants (n377360), in proliferation and apoptosis in one of the osteosarcoma cell lines (U2OS) (Zhang, Geng et al. 2013). These findings suggest that the role of such regulatory RNAs is still to be explored in depth.

Conclusion

The discovery of miRNAs sheds new light on our basic understanding of gene regulation. It is now well established that miRNAs can fine-tune gene expression and maintain the functional balance of various gene networks. A single miRNA can regulate

multiple (tens to hundreds) miRNA targets and, vice versa, several miRNAs can regulate a single miRNA (Eulalio, Huntzinger et al. 2008, Bartel 2009, Friedman, Farh et al. 2009, Ebert and Sharp 2012). Several studies have demonstrated the complexity of miRNA regulation of various genes interacting with multiple networks (Hammond 2007, Yang, Lin et al. 2007, Sarver, Li et al. 2010).

The complexity in gene regulation does not end in identifying and understanding the role of miRNAs. There are also other regulatory RNAs such as long non-coding RNAs, competing endogenous RNAs, and piwi-interacting RNAs that, along with miRNAs can affect the gene regulation and eventually various cellular networks and can lead to a disease, including cancer. To emphasize, gene expression can either be regulated in *cis* by enhancer sequences, or in *trans* by genes that encode transcription factors (that act as activators or repressors) or RNA-binding proteins (Wang, Arai et al. 2008, Orom, Derrien et al. 2010). Recently, several studies have demonstrated that non-coding RNA molecules can regulate gene expression in *trans* by acting as sponges of small regulatory RNAs such as miRNAs (Karreth, Tay et al. 2011, Salmena, Poliseno et al. 2011, Tay, Kats et al. 2011). Together, these RNA molecules are called competing endogenous RNAs (ceRNAs) that constitute a major proportion of gene regulators.

This concept of competitive target inhibition of miRNAs within a cell was first demonstrated by Phil Sharp's laboratory (Ebert, Neilson et al. 2007). The identification of this level of gene regulation can explain the correlation between genome size and increase in species complexities. The extent of miRNA inhibition in such cases depends on the relative production and turnover rates of the miRNA and sponge RNA (Chitwood

and Timmermans 2007). This idea of competitive target inhibition depicts another kind of unanticipated complexity in the network of RNA regulatory interactions.

Following this discovery, several other miRNAs were identified that could competitively inhibit regulatory small RNAs in prokaryotes (Figueroa-Bossi, Valentini et al. 2009, Mandin and Gottesman 2009, Overgaard, Johansen et al. 2009). Additionally, miRNA recognition elements ("MREs") that are present in coding and non-coding transcripts help in forming cross talk hubs for gene interactions, affecting the expression levels and activities of different ceRNAs. Decoding such cross talk between MREs and transcripts is critical to demarcate the intricacies in gene regulation, and we have just begun to unravel this complexity.

The next step in understanding the osteosarcoma biology is to apprehend the heterogeneity and aggressiveness of such cancer. Researchers already know that commercially available cell lines reflect different types of aggressiveness in vivo. A recent study led by Myklebost et al., demonstrated that osteosarcoma cell lines exhibited cancer-related phenotypes ranging from indolent to very aggressive (Lauvrak, Munthe et al. 2013). Along with such observations, they discovered that several miRNAs were differentially expressed in highly aggressive osteosarcoma cell lines when compared with non-aggressive osteosarcoma cell lines. Notably, four genes (*COL1A2*, *KYNU*, *ACTG* and *NPPB*) were highly expressed in aggressive osteosarcoma cell lines (HOS-143b), suggesting their role in osteosarcoma tumorigenesis. Also, in the same aggressive cell lines they found significant upregulation of miRNAs such as miR-135-5p, -146-5p and -155-5p which are predicted to be linked to have metastatic capacity in osteosarcoma

(Lauvrak, Munthe et al. 2013). This suggests that we, as scientists cannot base our studies on just looking into one of the osteosarcoma cell lines.

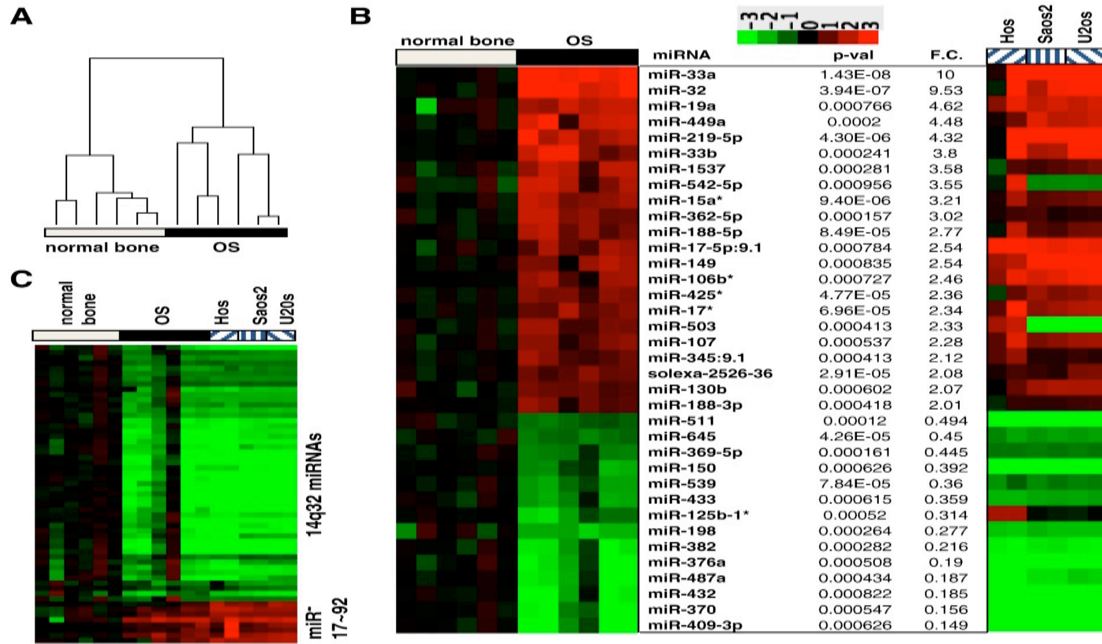
This brings us to another aspect of studying osteosarcoma: studying animal models. The challenge here is to find a model that can replicate human osteosarcoma's heterogeneity and aggressiveness. Dog models are among the best models to study osteosarcoma as they spontaneously develop the disease that closely resembles human osteosarcoma. The authors have shown that canine osteosarcoma shares similar genetic homology in terms of various genes and miRNAs as human osteosarcoma. Similarly many mouse models such as orthotopic mouse models, lung colonization models have also proven efficient in both mechanistic biological understanding of pathogenesis and preclinical evaluation of medical interventions in osteosarcoma. Most of these mouse models have been xenografts of human cells into immunocompromised mice that do not represent the right condition of the human body. Recent advances have been made in generating genetic mouse models that have been made able by genetic manipulation resulting in spontaneous occurrence and screening of the disease (Jones 2011). In collaboration with other researchers at the University of Minnesota, have generated osteosarcoma mouse models using sleeping beauty based transposon system that causes random insertional mutagenesis in the mouse genome, specifically in osteoblasts, resulting in spontaneous osteosarcoma development. This model also helps us identify various driver genes that are oncogene or tumor suppressor in nature by using high throughput forward genetic screens. Therefore, such models can help us better understand the development of osteosarcoma tumor and metastasis.

The final portions of the foregoing chapter discussed the current and prospective therapeutics in osteosarcoma. As it is already known, in spite of use of conventional therapies such as surgical intervention and chemotherapy, 70% of osteosarcoma patients will succumb to death. This tells us that there is a need to understand and make use of unconventional medicine, one such practice being personalized medicine. Looking into and understanding individual cancer genomics through DNA sequencing and patient tumor analysis, researchers should aim to advance personalized medicine. This will help us differentiate patients on the basis of osteosarcoma subtypes and eventually lead to better survival rates. Such type of medicine is already being undertaken in various other cancers. For instance, the drug imatinib (Gleevec) is designed to inhibit an altered enzyme that is produced by a fused version of two genes found in chronic myelogenous leukemia (Fausel 2007). Therefore, the future of cancer therapeutics most definitely relies on the type of genomic information generated by cancer genomics projects such as TCGA, which will drive research to develop similar treatment strategies that will be most effective for a given set of genomic changes (Chang, Creighton et al. 2013).

The next "big thing" in the field of unconventional cancer therapy would be combinatorial treatment. As discussed above, we have found that there are more profound and effective therapeutic effects when two different drugs are combined. And such combinatorial therapies can become more robust when the drugs target two different pathways that are deregulated in osteosarcoma. We are attempting to make use of such combinatorial therapeutics by combining the chromatin modifiers and Minnelide, as they

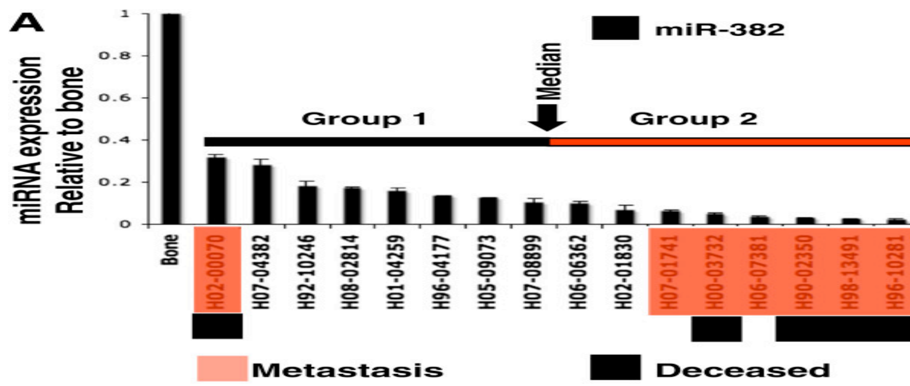
correct epigenetic deregulation and *cMYC* overexpression in osteosarcoma, respectively **(Fig. 5)**.

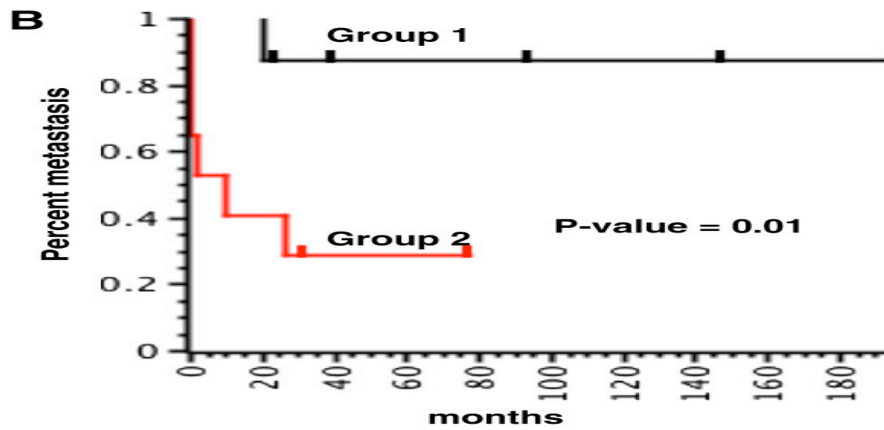
Therefore, the future of osteosarcoma therapy lies in understanding the genomics of each individual patient and using drug therapy or the combination of drugs with conventional therapies that target these subtle but impactful mutations, with the end goal of course being the prevention of individual death. The above information has laid a strong foundation for my thesis where I delve into understanding the role of miR-17-92 cluster in relation to osteosarcoma development and progression that I will be discussing in the next chapter.



miRNA	p-val	F.C.
miR-33a	1.43E-08	10
miR-32	3.94E-07	9.53
miR-19a	0.000766	4.62
miR-449a	0.0002	4.48
miR-219-5p	4.30E-06	4.32
miR-33b	0.000241	3.8
miR-1537	0.000281	3.58
miR-542-5p	0.000956	3.55
miR-15a*	9.40E-06	3.21
miR-362-5p	0.000157	3.02
miR-188-5p	8.49E-05	2.77
miR-17-5p:9.1	0.000784	2.54
miR-149	0.000835	2.54
miR-106b*	0.000727	2.46
miR-425*	4.77E-05	2.36
miR-17*	6.96E-05	2.34
miR-503	0.000413	2.33
miR-107	0.000537	2.28
miR-345:9.1	0.000413	2.12
solexa-2526-36	2.91E-05	2.08
miR-130b	0.000602	2.07
miR-188-3p	0.000418	2.01
miR-511	0.00012	0.494
miR-645	4.26E-05	0.45
miR-369-5p	0.000161	0.445
miR-150	0.000626	0.392
miR-539	7.84E-05	0.36
miR-433	0.000615	0.359
miR-125b-1*	0.00052	0.314
miR-198	0.000264	0.277
miR-382	0.000282	0.216
miR-376a	0.000508	0.19
miR-487a	0.000434	0.187
miR-432	0.000822	0.185
miR-370	0.000547	0.156
miR-409-3p	0.000626	0.149

Figure 2. miRNA expression patterns in osteosarcoma. (A) Unsupervised hierarchical clustering of miRNA expression in osteosarcoma relative to normal bone tissues. (B-C) Differentially expressed miRNAs between normal bone tissue and osteosarcoma. The miRNA expression data for the OS cell lines HOS, Saos-2 and U2OS relative to the expression in human osteoblasts is shown in the far right panel.


A
miR-382
miRNA expression
Relative to bone
Median
Group 1
Group 2
Bone
H02-00070
H07-04382
H92-10246
H08-02814
H01-04259
H96-04177
H05-09073
H07-08899
H06-06362
H02-01830
H07-01741
H00-03732
H06-07381
H90-02350
H98-13491
H96-10281
Metastasis
Deceased


B
Group 1
Percent metastasis
Group 2
P-value = 0.01
months

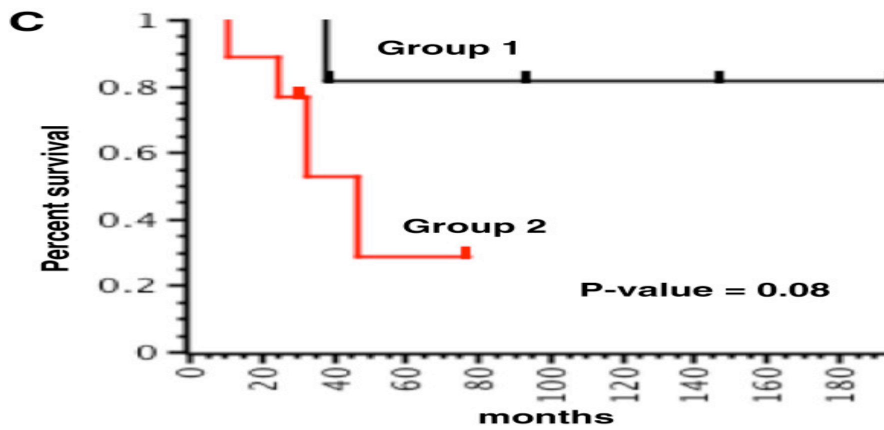
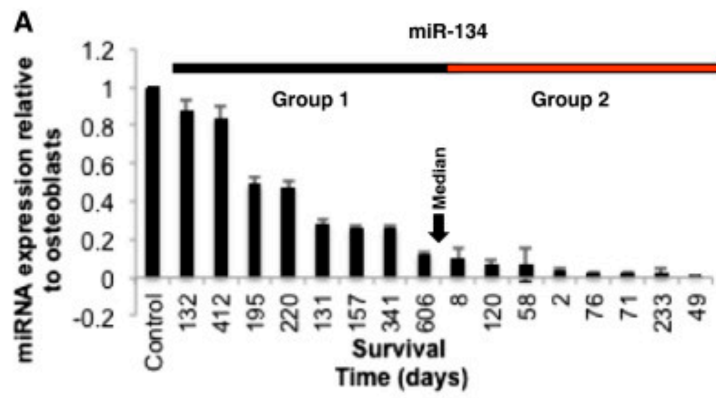
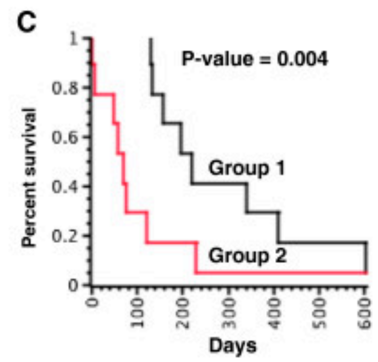
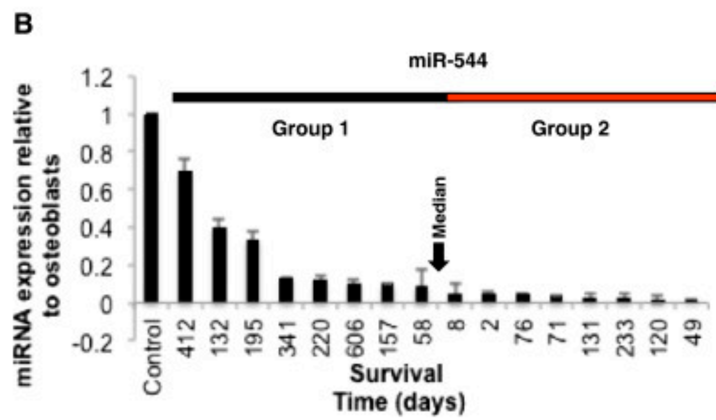

C
Group 1
Percent survival
Group 2
P-value = 0.08
months

Figure adapted from Sarver et al. 2013.

Figure 3. 14q32 miRNA expression level, metastasis, and outcome in human osteosarcoma. (A) miR-382 expression levels in osteosarcoma primary tumor samples with clinical follow-up information. (B and C) Kaplan-Meier analysis of metastasis in osteosarcoma patients based on miR-382 expression. Patients with lowest levels of miR-382 expression showed increased likelihood of metastasis and decreased likelihood of survival.


A
miR-134
Group 1
Group 2
Median
miRNA expression relative to osteoblasts
Survival Time (days)
Control
132
412
195
220
131
157
341
606
8
120
58
2
76
71
233
49


C
P-value = 0.004
Group 1
Group 2
Percent survival
Days


B
miR-544
Group 1
Group 2
Median
miRNA expression relative to osteoblasts
Survival Time (days)
Control
412
132
195
341
220
606
157
58
8
2
76
71
131
233
120
49

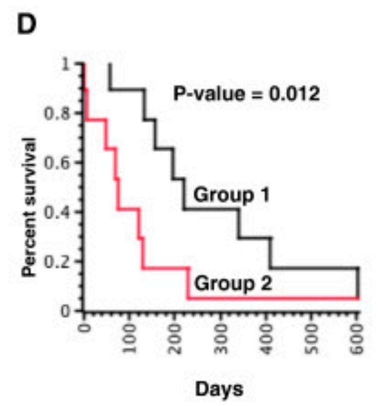
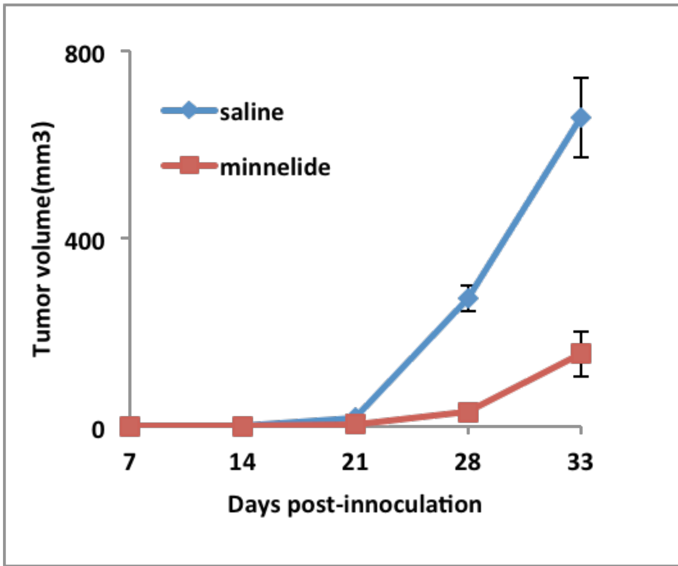
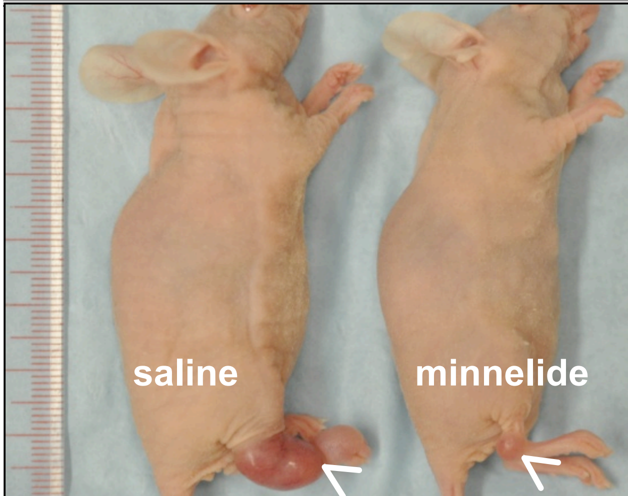
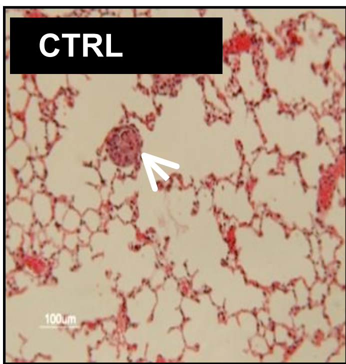

D
P-value = 0.012
Group 1
Group 2
Percent survival
Days

Figure 4. 14q32 orthologous region transcript levels and outcome in canine osteosarcoma. (A and B) Expression levels of miR-134 and miR-544 in canine osteosarcoma primary tumor samples with clinical follow-up information. (C and D) Kaplan-Meier analysis of survival in osteosarcoma patients showing that dogs with the lowest levels of miR-134 or miR-544 expression showed decreased likelihood of survival.


800
400
0
Tumor volume(mm3)
saline
minnelide
7
14
21
28
33
Days post-innoculation


saline
minnelide


CTRL

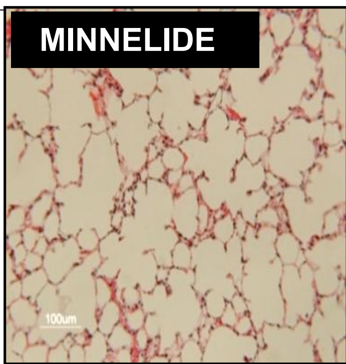

MINNELIDE

Figure adapted from Banerjee et al. 2013.

Figure 5. Minnelide treatment reduces tumor burden in an orthotopic mouse model of osteosarcoma. (A and B) Mice treated with Minnelide showed significantly decreased tumor size, late tumor onset, and significantly reduced tumor progression. (C) HandE staining of lung tissue (10X mag). Minnelide decreased the formation of pulmonary micrometastases.

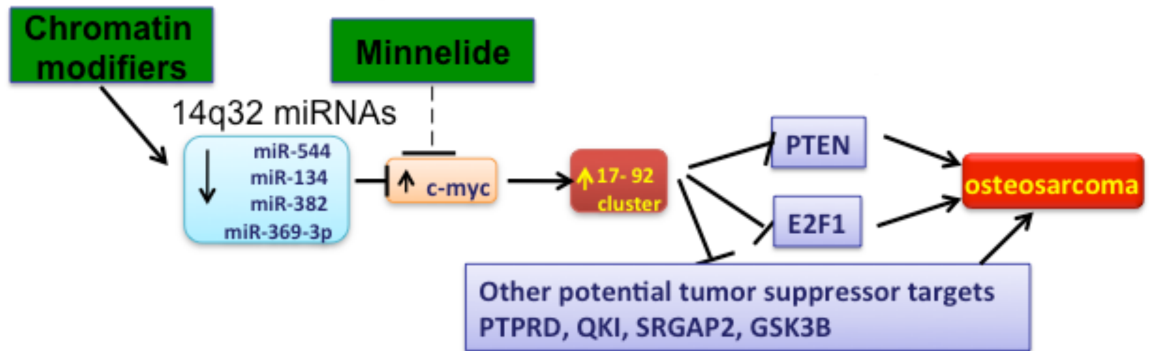


Figure 6. Schematic representation of 14q32 miRNA-cMYC-miR-17~92 gene regulatory network in OS. Downregulation of 14q32 miRNAs can potentially stabilize cMYC levels, leading to activation of miR-17-92 cluster miRNAs that in turn can target tumor suppressor genes and contribute to osteosarcoma.

Chapter 3: Expression of the miR-17-92 cluster at high levels controls diverse biological processes to induce osteosarcoma malignant behavior

Abstract

Osteosarcomas are aggressive bone cancers with poor outcomes. Treatment has remained static for 30 years due to a paucity of information on genetic changes that cause osteosarcoma. We have found that many osteosarcomas are characterized a by massive upregulation of a microRNA cluster, miR-17-92, that simultaneously silences a suite of key tumor and metastasis suppressors. We identified two novel potential tumor suppressor genes, *PTPRD* and *SRGAP2* that are targeted by miR-17-92. Furthermore, we found a small molecule, triptolide, which decreases levels of miR-17-92, tumor burden, and metastasis in osteosarcoma *in vivo*. These data have implications for understanding osteosarcoma's aggressiveness and development and suggest a new way to treat cancer via the targeting of microRNAs.

Introduction

Osteosarcoma (OS) is the most common malignant primary bone tumor, with peak a incidence in adolescence (Anderson 2008). Progress made more than three decades ago using surgical advancements and adjuvant chemotherapy improved the 5-year survival rate of patients without metastasis to the range of 60-70% (Bacci, Ruggieri et al. 1995, Kudawara, Aoki et al. 2013). Certain tumor suppressor genes are frequently mutated in OS, including most notably *TP53* and *RB1* (Miller, Aslo et al. 1996). Few recurrently activated proto-oncogenes have been identified with certainty in human OS, although a subset have an amplification of *MYC*, a genomic feature likely contributing to some OS cases (Han, Wang et al. 2012). Rare examples of oncogenic mutations in *RAS* or *PI3K* have been described in human OS (Choy, Hornicek et al. 2012). However, due to the high level of genetic instability in these tumors, including chromothripsis and kataegis, many OS drivers may be inconspicuous, consisting largely of elements altered at the gene copy number level or other complex genomic rearrangements (Stephens, Greenman et al. 2011). This has left few clear, targetable mutations to serve as the basis for new therapies and all recent clinical trials have proved disappointing (Ferrari and Palmerini 2007). Thus, there is an urgent need to identify better possible targets for therapeutic intervention in OS, especially in aggressive cases where the tumor has already metastasized. Non-coding RNAs may provide such targets in OS.

It is now clear that non-coding genes, miRNAs, can play critical roles in cancer development and maintenance (Hayes, Peruzzi et al. 2014). miRNAs are small non-

coding RNAs that post-transcriptionally regulate at least 60% of protein coding genes by interfering with ribosomal activity and mRNA stability (Friedman, Farh et al. 2009). A variety of miRNAs have been implicated in osteosarcomagenesis, including some that are recurrently lost or silenced in OS, and others that are often expressed well above philologically normal levels (Novello, Pazzaglia et al. 2013). Expression of the miR-17-92 cluster at high levels, identified using array hybridization or reverse transcription-quantitative polymerase chain reaction (qRT-PCR), has been reported in a subset of human OS and portends a poor prognosis (Li, Yang et al. 2014). The miR-17-92 cluster encodes 6 individual miRNAs and was the first cluster of oncogenic miRNAs identified in human cancer (He, Thomson et al. 2005). Studies have shown that miR-17-92 can induce transformation in model systems and is required to maintain cancer cells in some settings (Ji, Rao et al. 2011). Most work to identify putative miR-17-92 target genes and pathways has been investigated in lymphoid malignancies and developing mouse embryos. To this point, however, the purported oncogenic effects of miR-17-92 in OS have not been well described.

We used small RNA sequencing to define miR-17-92 encoded miRNA levels in human OS, normal bone, OS cell lines, and normal osteoblasts. We performed extensive loss-of-function and gain-of-function studies to elucidate some functions of the miR-17-92 cluster in OS. We developed a robust assay for miR-17-92 transformation in a poorly aggressive OS cell line, which revealed overexpression of the cluster alone had the ability to induce tumorigenicity and metastatic capacity.

We further performed detailed transcriptome analysis to understand this enhanced aggressive behavior and to identify target genes and pathways of the cluster. The analysis highlighted how overexpression changes the mRNA landscape, enriching pathways that are crucial in several biological processes thought to influence EMT and oncogenesis. We also identified two novel potential tumor suppressor genes targeted by miR-17-92, *PTPRD* and *SRGAP2*. Finally, we discovered that miR-17-92 expression at high levels sensitizes OS cells to a small molecule called triptolide. Triptolide induced a dramatic downregulation of miR-17-92 expression, with a concomitant increase in the expression levels of PTPRD and SRGAP2. Our study highlights an in-depth understanding of the role of the miR-17-92 cluster in OS, identifies two novel potential tumor suppressor gene targets of miR-17-92, and reveals a drug that could be developed to treat poor prognosis, miR-17-92 high OS cases.

Materials and Methods

miRNA and mRNA RNA-Seq Expression Profiling

To perform high-resolution quantification of mRNA and miRNA expression using RNA-Seq, we used at least 1 μg of each pool of total RNA according to the miRvana RNA isolation kit (Ambion, Austin, TX, USA). Small RNA libraries were sequenced (8-12 samples per lane, each sample had 10 million reads) on a High Seq 2000 (Illumina, San Diego, CA, USA). Need to verify chemistry details for the mRNA sequencing. Raw sequence FASTQ files were quality checked using FastQC before and after adapter trimming with Trimmomatic [Bolger 2014]. These were then aligned to the Mouse Dec.

2011 (GRCm38/mm10) Assembly genome (UNCLEAR) using the Bowtie2 algorithm [Langmead 2012]. The miRNA aligned reads were then quantified against the miRBase 21 transcript annotations for mature miRNAs [Kozomara 2014] using HTSeq-count [Anders 2015]. Within each sample, reads from miRNA genes were normalized and scaled to counts per million mapped miRNA reads. Normalized counts were $\log_2$ transformed and mean-centered. Statistical analysis and clustering was performed using the Partek Genomics Suite software package (Partek Inc., St. Louis, MO, USA).

Functional, Pathway, and Network Analyses

Network and pathway analyses, Functional Analysis, and microRNA Targeting Analysis were performed using Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA, USA). IPA employs a right-tailed Fisher exact test to calculate a P value corresponding to the probability that a biologic function not relevant to the input dataset is falsely identified as relevant. A Benjamini–Hochberg false discovery rate of 0.05 was used to correct such *P* values. Gene Set Enrichment Analysis (GSEA) was performed by the JAVA program (http://www.broadinstitute.org/gsea) using MSigDB Hallmark curated gene set collection. A Pearson metric was applied to rank genes and gene sets enriched at a nominal $P < 0.05$ and a false discovery rate < 0.25 [Subramanian, 2005]

Reverse transcription-quantitative polymerase chain reaction (qRT-PCR)

cDNA was quantified with the miRscript SYBR green detection kit (Qiagen) using an miRNA-specific forward primer and a universal primer, following the manufacturer's

instructions, in an ABI 7500 optical cycler (Applied Biosystems). The oligonucleotide primer sequence used for analysis is provided in Supplementary Table 2. *U6 snRNA and* β *Actin* were used as controls for miRNA and mRNA RT-PCR analyses, respectively. Threshold cycle (Ct) values calculated by the SDS v1.2.1 software (Applied Biosystem, USA) were exported and subjected to statistical analysis. Cycle threshold values obtained from duplicate reactions were subjected to statistical analysis, and expression was calculated following the comparative Ct method ®. RT-PCR reactions were carried out in triplicates and the average values were plotted as mean +SD values.

Flow cytometry

For Annexin V analysis, osteosarcoma cell lines were dissociated from culture plate 48 hours after transduction and washed twice with cold 1X PBS. Cell pellets were resuspended in 1X Binding Buffer (BD Biosciences 556454) and incubated with propidium iodide (BD Bioscience 556463) and Annexin V-APC (BD Biosciences 550474) for 15 minutes. 400 uL 1X Binding Buffer was added to samples. For cCas3 and cPARP analysis, osteosarcoma cell lines were dissociated from culture plate 48 hours after transduction and fixed in 2% paraformaldehyde for 10 minutes at room temperature then permeabilized in 90% methanol for 15 minutes at 4 °C. Permeabilized cells were washed with 1X PBS and then incubated with directly conjugated antibodies against cCas3 (Cell Signaling Technologies 9602) or cPARP (Cell Signaling Technologies 6987) at a concentration of 1:50 for 45 minutes at room temperature. Samples were washed with 1X PBS and stored at 4 °C until analysis.

Flow cytometry was performed using standard protocol on LSRII analyzer (Becton Dickinson) in the Flow Cytometry Core Facility at the University of Minnesota and FCS files were analyzed using FlowJo software (Tree Star).

Soft agar colony forming assay

Cells at the exponential growth phase were harvested with trypsin and counted using a Countess II automated cell counter (Life Technologies). Cells were suspended in medium with 0.48% agarose (Lonza, 50100, Rockland, ME, USA) and 1×10^4 cells were overlaid onto a solidified layer of medium-containing 0.8% agarose in 6-well plates. The plates were incubated at 37°C in a humidified CO2 incubator for 11-21 days with 1mL of media over the soft agarose layer being replaced every 5-7 days. For analysis, the media was removed and plates were fixed and stained with 0.005% crystal violet in PBS containing 4% formaldehyde for 1 h and the colonies were imaged under a dissecting microscope. Colony counts were obtained using ImageJ with thresholds to exclude single cells and cells settled on the bottom layer of 0.8% agarose.

Tissue fixation, histopathology, and immunohistochemistry

Tumors and tissue specimens were fixed in 10% neutral-buffered formalin for 24 hours and then routinely processed and embedded in paraffin. Histologic sections were cut at 4 µm and stained with hematoxylin and eosin (HE) or prepared for immunohistochemistry.

Four µm formalin-fixed, paraffin-embedded sections of tumor were deparaffinized and rehydrated, and antigen retrieval was performed using 10mM citrate buffer pH 6.0 in a steamer. After endogenous peroxidase blocking, a protein block was applied. Immunohistochemistry for PTPRD, SRGAP2, and β-actin was performed using 1:100, 1:50, 1:200 primary antibodies on a Dako Autostainer. Detection was achieved using a with diaminobenzidine (Dako) as the chromogen. Mayer's Hematoxylin (Dako) was used as the counterstain. A tumor with Western blot-confirmed expression of all three target proteins was used as a positive control tissue. For negative control slides the primary antibody was substituted with appropriate isotype control antibody.

Imaging

Tissue sections were imaged on a Nikon E800M microscope at 20X, 40X, 200X, and 400X magnification using a Nikon DSRi2 camera and Nikon Elements D Version 4 software. Each section was imaged using the same white balance and shading correction settings. Whole image sharpness was uniformly adjusted and minor background blemishes were removed with Photoshop Elements version 11 software.

Western blot analysis

Proteins were isolated from cell pellets vortexed at 2000 rpm for 15min at 4°C in complete RIPA buffer with protease and phosphatase inhibitors (Sigma-Aldrich, COEDTAF-RO, P5726, and P0044, St. Louis, MO, USA). Total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., #23225,

Waltham, MA, USA) according to the manufacturer's protocol. Protein samples were denatured at 95°C for 10 minutes with NuPAGE LDS Sample Buffer and Reducing Agent (Life Technologies, NP0007 and NP0009). Samples were run on either NuPAGE 10% or 4-12% Bis-Tris gels, (Life Technologies) and transferred to PVDF membranes (Bio-Rad, #162-0177, Hercules, California, USA) at 12V overnight. Membranes were blocked using 5% non-fat milk (Bio-Rad, #170-6404) in PBST for 1h at room temperature. Membranes were incubated at 4°C overnight with primary antibodies against B-actin (1:2000; Santa Cruz, sc-1616, Dallas, Texas, USA), APC (1:200; Santa Cruz, sc-896), PTPRD (1:100; Abcam, ab103013, Cambridge, MA, USA), srGAP2 (1:1000; Abcam, ab121977), and Pten (1:1000; Cell Signaling,138G6, Danvers, MA, USA). Blots were washed and then incubated with the HRP conjugated secondary antibodies against rabbit or goat IgG for 1h (1:5000; Santa Cruz, sc-2313 and sc-2020). Visualization of proteins was performed using the WesternBright Quantum kit (Advansta, K-12042-D20, Menlo Park, CA, USA) according to the manufacturer's protocol and imaged using a LICOR Odyssey (LI-COR Inc., Lincoln, Nebraska, USA). Membranes were stripped using NewBlot PVDF Stripping Buffer (LI-COR Inc.) according to the manufacturer's protocol.

Proliferation assay

Proliferation assays were performed in 96-well plates. Five thousand cells were seeded per well in ten wells and measured at 24, 48, and 72 hours. Proliferation was assessed

using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) luminescent cell viability assay (Promega) according to the manufacturer's protocol.

Mouse Methods

The animal research described in this article was approved by the Institutional Animal Care and Use Committee (IACUC). NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) and NOD.*Cg-Rag1tm1Mom Il2rgtm1Wjl*/SzJ (NRG) mice 6 to 12 weeks old were used to study the growth of transgenic human osteosarcoma cell lines. Mice were transplanted at 6 to 7 weeks of age. For subcutaneous xenographs, cells were trypsinized and resuspended at $1x10^6$/100uL in PBS and kept on ice. Immediately before injection, cells were mixed 1:1 with Matrigel and 200uL ($1x10^6$cells) of this mixture was injected into the right flank. Once they could first be palpated, tumors were measured every two days using digital calipers (OriginCal). Tumor volume was calculated according to the formula $[(width)^2x(length)]/2$. Mice were sacrificed and tumors and organs collected when the largest reached 2 cm^3. Tail vein injections were preformed with $2x10^6$cells suspended in PBS.

Results

The miR-17-92 oncomiR cluster is expressed at high levels in a subset of human OS.

To identify potentially oncogenic microRNAs in OS, we performed small RNA sequencing analysis on miRNA libraries generated from 24 primary human osteosarcoma tumor samples and 4 morphologically normal human bone tissue samples. We observed that the miRNAs in the miR-17-92 cluster were significantly upregulated in half (12/24) of the OS tumors compared to the normal bone samples (Fig. 7A). miR-19a-5p showed the most distinct expression pattern. Interestingly, miR-92a-1, which has previously been shown to be post-transcriptionally processed separately from the rest of the cluster members (Du, Wang et al. 2015), was the only miRNA in this cluster to show significant expression in a subset of both normal bone and OS tissue samples. Additionally, we performed qRT-PCR to assess the levels of miR-17-92 in various human OS cell lines and compare them with human osteoblasts. All four OS cell lines showed significantly higher expression of the miRNAs within the cluster compared to osteoblasts, with U2OS showing the least expression among the OS cell lines (Fig. 7B). These results indicate that miR-17-92 cluster is potentially significant oncomiR cluster in OS, which functions by targeting important OS tumor suppressor gene (TSG) mRNAs. Previous work by our group utilized a *Sleeping Beauty* (SB) transposon screen to identify the genetic drivers of osteosarcoma (Moriarity, Otto et al. 2015). MicroRNA targeting analysis of the 3'-untranslated regions (UTRs) of TSG identified in this screen predicted the miR-17-92 cluster to be a common upstream regulator. Roughly 60% (170/281) of the TSGs

identified by the SB screen contain 1 or more predicted binding sites for 1 or more of the miR-17-92 cluster members (Fig. 7C). Roughly 40% (70/170) of these TSG 3'-UTRs contain predicted binding sites for 2 or more miR-17-92 miRNAs.

Transient and stable knockdown of miR-17-92 cluster affect cell viability, proliferation, and migration in human OS cell lines.

To determine the role of miR-17-92 encoded microRNAs in human OS maintenance and tumorigenicity, we used miRZip vectors to specifically knock down individual miRNAs in the miR-17-92 cluster (miRZip17, miRZip19a/b, miRZip20). We confirmed the knockdowns of specific miRNAs via qRT-PCR (Supplementary Fig. S1). We transduced both individual and combinations of miRZips in 143B and MG63 OS cells and performed multiparameter flow cytometry to detect early apoptotic markers. OS cell lines transduced with the miRZips showed increased early apoptotic marker levels, including cleaved caspase 3 and cleaved PARP, as well as a destabilized cell membrane indicated by increased Annexin V staining. MiRZip-20 caused the greatest increase in apoptosis across cell lines (Fig. 8A-C). To further study the cluster as a whole, we generated a tetracycline inducible anti-miR-17-92 lentiviral construct that targets the primary transcript of the 13q32 chromosome region from which the mature miR-17-92 family of miRNAs is generated. We transduced 143B, SAOS-2, and MG63 cells with the construct, along with a vector control, and generated stable lines that showed a significant decrease in the expression of the miRNAs when treated with doxycycline. We performed a wound healing assay in these stable OS cell lines and observed that migration was

significantly reduced in OS cells where miR-17-92 was knocked down compared to the vector control cells (Fig. 8D). MG63 and SAOS-2 OS cell lines gave similar observations (data not shown). These data suggest that the miR-17-92 cluster is a potential oncomiR in OS and loss-of-function of the cluster reveals that miR-17-92 targets various apoptotic genes and migration of OS cell lines.

Inhibition of miR-17-92 cluster miRNA expression reduces OS tumor size and cellular density, without affecting cell morphology or mitotic index

To validate the observations in *in vitro* we injected 143B cells stably expressing either anti-miR-17-92 or a control vector subcutaneously into immunodeficient (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ [NSG]) mice to observe the effect on xenograft tumor formation (n=4 per group). Tumor formation in mice with anti-miR-1792 expressed in the presence of a doxycycline diet was significantly reduced compared to the controls ($p<0.001$) (Fig. 8E). Further analysis revealed the subcutaneous tumors derived from the parent cell line had very dense cellularity, extensive areas of necrosis, and invaded deeply into the panniculus (data not shown). Tumors derived from the control were slightly smaller than parental cell-line tumors but displayed similar cellular density, tissue invasion, cell morphology, and mitotic index (Fig. 8F). Compared to vector control (ECV), tumors derived from anti-miR-17-92 group mice on a doxycycline diet (1792DOX) were markedly smaller with decreased cellularity and limited tissue invasion (Fig. 8F). The cell and nuclear morphology were similar to ECV and the mitotic index remained high with 8-12 mitotic figures per high magnification (400X) field. In addition,

nuclear Ki67 expression was not significantly different between ECV and 1792DOX (data not shown). Tumors derived from anti-miR-17-92 cells without doxycycline induction displayed a slight decrease in size and cellular density when compared to empty vector control.

The enforced expression of miR-17-92 at high levels leads to colony formation *in vitro* and metastatic tumor formation *in vivo* in a poorly aggressive OS cell line.

To determine whether the miR-17-92 cluster is important in OS proliferation and aggressiveness, we chose to overexpress miR-17-92 in an OS cell line, which normally expresses low levels of miR-17-92 miRNAs (Fig. 7B). U2OS is one of the least aggressive OS cell lines, barely forming any colonies in soft agar or xenograph tumors in mice (Grossel, Baker et al. 1999, Zhang, Morita et al. 2001, Carvajal, Hamard et al. 2012). We stably overexpressed the miR-17-92 cluster using lentiviral transduction in U2OS cells and confirmed increased levels of miR-17-92 via qRT-PCR (Fig. 9A). U2OS cells stably overexpressing miR-17-92 were able to grow in an anchorage-independent manner forming robust colonies *in vitro*. (Fig. 9B). However, there was no change in the proliferation rate between U2OS overexpressing miR-17-92 and the control groups on plastic. Additionally, we performed similar assays in 143B and saw quantitative increases in the transformed phenotype of these cells after miR-17-92 lentiviral vector transduction.

U2OS cells stably overexpressing miR-17-92 and a control vector were injected subcutaneously and via the tail vein into immunodeficient NRG mice (n=4) (Fig. 9C and D). We observed injection site tumors and widespread metastasis formation in mice with

U2OS overexpressing miR-17-92 via subcutaneous and tail vein injections respectively. No tumors or metastases were observed in any of the control groups, showing that high levels of miR-17-92 encoded microRNA can confer an aggressive phenotype on OS cells *in vivo*, may play an important role in disease progression, and may be a suitable target for therapy.

miR-17-92 cluster overexpression results in distinct transcriptome changes

To globally characterize miRNA and mRNA transcriptional changes resulting from miR-17-92 cluster overexpression we performed comparative RNA-Seq analyses on miRNA and mRNA libraries generated from miR-17-92 overexpressing cells (1792 OEXP), parental U2OS cell lines, and U2OS cells transfected with the control vector. To identify the miRNAs and genes regulated directly or indirectly by the miR-17-92 cluster we performed two-group t-tests comparing the 1792 OEXP results with the parental and vector control cells. One hundred and seventy one miRNAs were significantly dysregulated ($P < 0.05$, fold-change >= 2) including the miR-17-92 cluster (Fig. 10A). Two thousand nine hundred and thirty eight mRNAs were significantly dysregulated ($P < 0.05$, fold-change >= 2) including MYC (Fig. 10B-C), a transcription factor with a well-described feedback loop involving the miR-17-92 cluster.

miR-17-92 cluster overexpression promotes critical pro-cancer signaling pathways

Functional enrichment analysis of the full set of gene FPKM values using the curated MSigDB Hallmark gene sets was performed [REF:Liberzon]. This analysis

determined that the 1792 OEXP cell expression profiles were significantly (FDR q-value < 0.25) functionally enriched for oxidative phosphorylation, the MYC signaling pathway, unfolded protein response, the Wnt/β-catenin signaling pathway, and epithelial mesenchymal transition (EMT) signaling (Fig. 10C). We further validated several of these upregulated gene products via western blot and flow cytometry (Fig. 10D). Similar results were obtained using Ingenuity Pathway Analysis (IPA) functional analysis (data not shown).

Gain of function studies using miR-17-92 vector mutants reveals a critical role for miR-17-92 in conferring OS aggressiveness

To understand the role of individual miR-17-92 encoded microRNAs in OS aggressiveness we generated miR-17-92 mutant constructs by deleting individual miRNAs from our overexpression vector (Fig. 11A). We generated stable U2OS lines with each of the overexpression mutant construct and performed soft agar assays. This revealed that deletion of either miR-17 or miR-18 from the overexpression vector led to a significant increase in colony formation ($p<0.05$) indicating that miR-17/18 may be potential tumor suppressive miRNAs within the cluster (Fig. 11B). However, there were no significant changes observed with any of the other miRNAs within the cluster, suggesting multiple miR-17-92 encoded microRNAs contribute to OS aggressiveness.

miR-17-92 cluster miRNA levels are inversely correlated to the levels of candidate OS tumor suppressor proteins in OS cell lines and xenografts.

Out of the SB identified TSGs we decided to investigate two of the top candidates, *PTPRD* and *SRGAP2* (Fig. 12A). To analyze whether these genes are indeed targets of miR-17-92, we performed western blots on 143B OS cells containing our inducible anti-miR-17-92 construct. We observed both proteins to be present at significantly higher levels when miR-17-92 was knocked down compared to the controls (Fig. 12B). To further study the relation between miR-17-92 and PTRPD and SRGAP2 we transiently overexpressed PTRPD and SRGAP2 individually and in combination and performed colony forming assays in 143B cells (Fig. 12C-D). We observed that SRGAP2 and PTPRD alone or combined significantly reduce the number of colonies as well as the size of the colonies in 143B cells compared to controls. These data strongly suggest that miR-17-92 directly or indirectly targets PTPRD and SRGAP2 in OS.

Additionally, IHC was performed on two ECV- and two 1792DOX-derived tumors to evaluate changes in PTPRD and SRGAP2 protein levels. Compared to empty vector control, tumors derived from anti-miR-17-92 expressing cells displayed marked and diffuse increases in both PTPRD and SRGAP2 immunoreactivity (Fig. 12E). No changes were observed in β-actin immunoreactivity (data not shown). These results correspond with expression changes observed in cell culture lysates analyzed by western blot (Fig. 12B).

We performed western blots to assess levels of PTPRD and SRGAP2 in U2OS cells stably overexpressing miR-17-92 (Fig. 12F). We observed that PTPRD and SRGAP2 levels are significantly decreased in the U2OS cells overexpressing miR-17-92.

Collectively, this data suggests that miR-17-92 regulates these novel potential tumor suppressor genes in OS.

The natural product triptolide decreases miR-17-92 cluster expression, increases OS tumor suppressor protein expression and inhibits viability and proliferation in OS cells

To explore potential therapeutic avenues to regulate high miR-17-92 expression and activity in OS, we chose to use triptolide, a small molecule inhibitor shown to have promising effects in multiple tumor types, including OS (Li, Chu et al. 2012). Previously, it has been shown that triptolide is able to decrease levels of MYC, a well-studied transcription factor and known transactivator of the miR-17-92 cluster (Wong, Dong et al. 2011). We hypothesized that triptolide decreases miR-17-92 levels in OS. We performed a dose response curve over a wide range of triptolide concentrations (data not shown). We found that triptolide is able to significantly reduce the transcript levels of miRNAs in the miR-17-92 cluster ($p<0.05$) in a dose dependent manner in 143B cells (Fig. 13A). Similar reductions were observed in MG63 and SAOS-2 cells (data not shown). Western blot data revealed that concentrations of triptolide as low as 12.5nM led to an increase in the levels of miR-17-92 targets PTPRD and SRGAP2 in 143B cells (Fig. 13B). We performed a rescue experiment to further validate the effect of triptolide on miR-17-92 using U2OS cells overexpressing miR-17-92. After colonies began to form, we treated each group of cells with low doses of triptolide for one week. Treatment at 12.5nM or 25nM triptolide caused a significant decrease in the size but not the number of

colonies (Fig. 13C-E). Additionally, we wanted to see whether triptolide specifically affects OS cells overexpressing miR-17-92 to determine the sensitivity and selectivity of the drug. Our viability assays reveal that triptolide affects OS cells overexpressing miR-17-92 viability significantly more than the control group (Fig. 13F). The data indicates that triptolide potentially targets miR-17-92 in OS and is an important mechanism of the drug's action *in vitro*.

Discussion

miR-17-92 was the first microRNA cluster to be implicated in cancer and has been extensively studied in relation to various types of cancer (Takakura, Mitsutake et al. 2008, Diosdado, van de Wiel et al. 2009, Robaina, Faccion et al. 2016, Zhou, Ma et al. 2016). It is a complex locus encoding multiple individual microRNAs that are highly conserved among species. Knockout studies in mice indicate miR-17-92 encoded microRNAs play a variety of roles in normal development and influence many biological processes and pathways (Cloonan, Brown et al. 2008, Olive, Li et al. 2013). And while more research has been done to study miR-17-92 in other cancers, the role of the cluster is not well understood in OS. This report is the first to describe small RNA sequencing to assess dysregulation of miRNAs in human primary OS tumors and OS cell lines. We observed that roughly 50% of OS present with highly elevated levels of expression of miR-17-92 encoded microRNAs. A recent study showed an association between high miR-17-92 expression levels and worse recurrence-free and overall survival for OS (Li, Yang et al. 2014). Our study is consistent with the presence of a miR-17-92 high subset

of osteosarcomas. While other miR-17-92 encoded microRNAs were coordinately upregulated in a subset of OS, miR-92 was present at similar levels in all OS tumors, levels similar to those measured in normal bone. While each miRNA within the cluster is produced from a single transcript, there is an additional post-transcriptional regulatory stability mechanism at the miRNA level for miR-92. A study led by Duo et al. provided additional evidence of post-transcriptional 'uncoupling' of miR-92a from the rest of the miR-17-92 cluster (Du, Wang et al. 2015). It is not yet clear when in the development or progression of OS miR-17-92 becomes upregulated, or by what mechanism. However, we believe this event could be related to high levels of MYC, which has been reported to transactivate the miR-17-92 promoter (Ji, Rao et al. 2011, Li, Li et al. 2011). We decided to investigate the function of the miR-17-92 locus in OS to determine if the association we observed with poor prognosis could be a direct effect of this cluster. To do so we performed detailed loss-of-function and gain-of-function experiments to understand the role of miR-17-92 in OS.

Our loss of function data suggests that miR-17-92 encoded microRNAs are required for OS cell maintenance. Aggressive OS cell lines like MG63 and 143B demonstrate an "oncogene addiction" phenotype when miR-17-92 levels are reduced, including the induction of apoptosis in the setting of acute miRNA suppression. Therefore, therapies that target miR-17-92 function could be used to eliminate aggressive OS cells in patients, even without full attenuation of the cluster. Indeed we generated an inducible vector that reduced levels of the miRNAs expressed from the cluster and created stable knockdowns

in 143B and MG63. Xenographs of these stable cell lines show significant decreases in tumor burden compared to controls.

Prior data on miR-17-92 is consistent with a role in tumor maintenance. The role miR-17-92 has been studied in many types of cancer, but most intensively in lymphoid malignancies, where gene copy number increases are frequently found (reviewed in (Dal Bo, Bomben et al. 2015)). Abundant gain-of-function studies implicate miR-17-92, and specific encoded microRNAs, but fewer studies report loss-of-function experiments. However, in a transgenic mouse model of MYC-driven lymphomagenesis, it was found that miR-19a and miR-19b were required to suppress lymphoma apoptosis and recapitulate the oncogenic properties of the entire cluster (Mu, Han et al. 2009). In a CLL-like cell line, inhibition of miR-17 reduced proliferation and tumorigenicity (Dereani, Macor et al. 2014). Similarly, targeting miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92 induced apoptosis (Matsubara, Takeuchi et al. 2007). Consistent with these results we found that inhibition of miR-20 induced the most robust apoptosis in our OS cell lines.

To further understand the role of the miR-17-92 cluster in OS genesis, we performed gain-of-function assays in a commonly used, poorly aggressive OS cell line, U2OS. This led to development of a very valuable new model system to gain insight into the function of the miR-17-92 cluster in OS progression. U2OS is a unique OS cell line, as unlike any other OS cell line, it does not carry mutated copies of the TP53 or RB1 genes (Hellwinkel, Muller et al. 2005). It also maintains a relatively low expression level of

miR-17-92, which is more comparable to the levels found in normal osteoblasts. We found that expression of miR-17-92 could induce anchorage independent growth, tumorigenicity *in vivo*, and metastatic capacity. These data reveal that expression of miR-17-92 at high levels can coordinately confer a suite of new phenotypes to an OS cell, critically including its ability to metastasize. Thus, we suspect that miR-17-92 expression is a key driver of OS aggressiveness and mortality in patients.

To determine how the miR-17-92 cluster can confer this suite of cancer hallmarks, we performed a global transcriptome analysis in U2OS cells overexpressing miR-17-92 and controls (vector control group and the parent line). Our purpose was to gain insight into the multiple direct and indirect targets that are regulated by the cluster. We found that miR-17-92 overexpression causes dysregulation of distinct sets of genes in the context of OS cells. Using GSEA analysis, we found that these transcripts are enriched in certain biological processes and predict specific effects on OS cells that we validated *in vitro*. We predict that these pathways govern the aggressiveness of certain osteosarcomas and the poor prognosis observed in some OS patients. Since poor prognosis is ultimately associated with metastatic disease, we predict that these miR-17-92 cluster controlled biological processes will be directly involved in the ability of OS cells to survive and proliferate in the metastatic niche. Some of the top ranked enriched pathways includes EMT, oxidative phosphorylation, unfolded protein response, and β-catenin. All of these pathways have become established spokes in the map of cancer maintenance and progression (Mortus, Zhang et al. 2014, Nogalska, D'Agostino et al. 2015, Wang, Jia et al. 2015).

Additionally, our enrichment analysis revealed that MYC is one of the most enriched pathways activated by miR-17-92 in OS cells. Interestingly, miR-17-92 encode microRNAs may have opposing effects on MYC. Previous data on B cell lymphomagenesis indicated cooperation between MYC and miR-17-92 via miR-19 suppression of PTEN and MYC induced apoptosis. A recent paper reported that miR-19b represses MYC translation via blockade of Chek2 and subsequently increased recruitment of HuR/RISC to MYC transcripts. They proposed that miR-17-92 fine tunes c-MYC activity and aids in maintaining cancer cell homeostasis (Mihailovich, Bremang et al. 2015). Our data clearly indicates enhanced MYC transcript and protein levels occur as a consequence of enforced expression of miR-17-92 at high levels. As both a target of MYC and inducer of MYC, we propose that miR-17-92 plays a crucial role in a feed-forward loop maintaining high MYC levels and suppressing apoptosis, among many other biological effects.

It is likely that specific miRNAs from the miR-17-92 cluster govern specific traits. We generated miR-17-92 overexpression cluster mutants where we deleted individual miRNAs within the cluster. We established stable U2OS lines that were transduced with the overexpression constructs that had individual miRNAs deleted. We performed soft agar assays to investigate the transformative ability of each of the constructs. To our surprise our assays suggest that no one specific miR-17-92 encoded miRNA is critical for OS transformation and instead several of these miRNAs must independently contribute to transformation. Interestingly, miR-17 and miR-18 deletion overexpression constructs significantly increased the number of colonies formed. This data corresponds to recent

literature showing that miR-17 and miR-18 have tumor suppressive functions in this cluster (Hossain, Kuo et al. 2006, Liu, Liu et al. 2015). Our data of osteosarcomas also shows that unlike in B cell lymphomagenesis, miR-19a and miR-19b are not absolutely required for conferring a transformed phenotype in the context of OS.

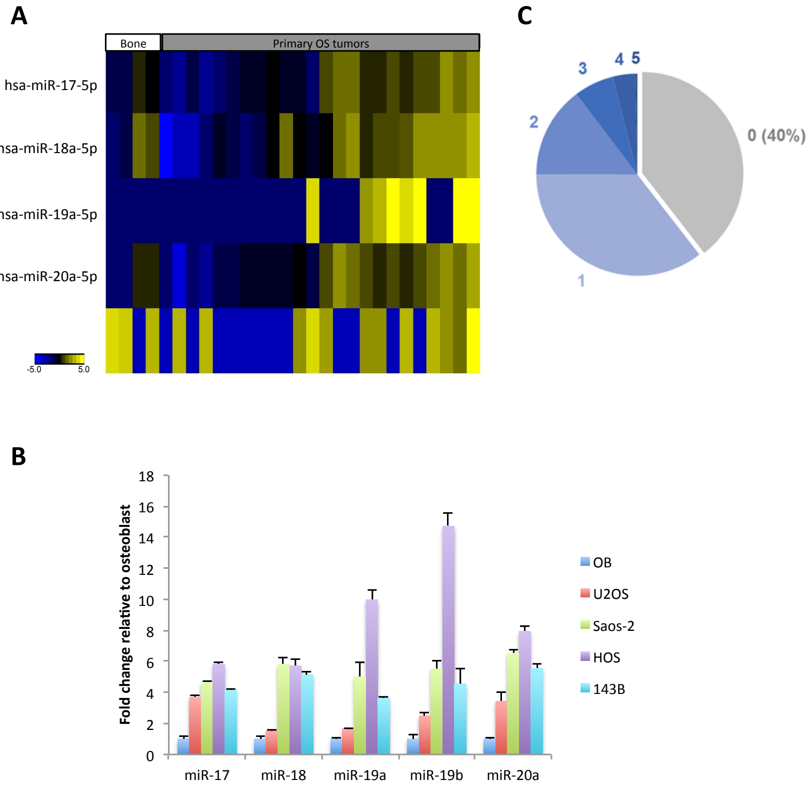
To identify novel TSG targets of the miR-17-92 cluster we used a comparative oncogenomics approach. It is challenging to discover functional targets of miR-17-92 encoded microRNAs as thousands of genes harbor potential binding sites within their 3' UTRs (Kanzaki, Ito et al. 2011). Thus, to identify and verify a list of target genes in a biological system can be a time consuming process. Therefore, we took a unique approach where we exploited a large set of candidate OS TSGs, identified at common insertion sites (CIS) by a transposon-based forward genetic screen in mice (Moriarity, Otto et al. 2015). Prior analysis of commonly mutated TSG candidates in that screen identified mir-17-92 as one of the most enriched upstream regulators of around 121 CIS-associated genes. Our analysis of predicted targets led to two top potential tumor suppressor genes, PTPRD and SRGAP2, as potential functional targets of miR-17-92 encoded microRNAs. PTPRD is a tyrosine phosphatase targeting STAT3 (Laczmanska and Sasiadek 2011), and is mutated in multiple cancer types including lung cancers, glioblastomas, and others (Veeriah, Brennan et al. 2009). Very little is known about SRGAP2, a Rho family GTPase activating protein (GAP) and regulator of membrane protrusion, cell spreading and migration; especially its role as a potential tumor suppressor. Enforced expression of these genes at high levels in OS cells revealed a reduction in soft agar colony formation. Additionally, the knockdown of miR-17-92 in

OS cells significantly increased the expression of PTPRD and SRGAP2 proteins showing they could be direct targets. A similar inverse correlation was observed in our gain-of-function experiment. This suggests that PTPRD and SRGAP2 are potential functional targets of miR-17-92 whose repression is responsible for aspects of OS progression.

OS is a genetically complex, heterogeneous cancer. There has been no significant change in the standard of care therapy in the past 30 years. Our data reveal that the miR-17-92 locus is an attractive drug target. Triptolide is a diterpene triepoxide isolated from a traditional Chinese herb that potentially has anti-inflammatory, immunosuppressive, and antitumor activity (He, Yu et al. 2007, Lin, Chen et al. 2007, Liu, Chen et al. 2007, Phillips, Dudeja et al. 2007, Wang, Yang et al. 2007, Westfall, Nilsson et al. 2008). However, The molecular mechanism of action has remained largely elusive to date. It has been shown that triptolide binds to XPB, a subunit of a transcription complex that prevents its DNA-dependent ATPase activity, which leads to the inhibition of RNA polymerase II–mediated transcription (Titov, Gilman et al. 2011). Recently, minnelide, a prodrug of triptolide was shown to decrease tumor burden and metastases in OS by specifically targeting MYC (Banerjee, Thayanithy et al. 2013). Knowing MYC transactivates mir-17-92 levels; we investigated triptolide's effect on the cluster. We observed that triptolide significantly reduces the level of the miRNAs and in turn increase the levels of PTPRD and SRGAP2. These findings suggest another possible mechanism of action for triptolide, especially in OS.

In summary our work reveals the miR-17-92 cluster is a central player in OS progression. We discovered the enrichment of a transformative mRNA landscape in OS cells overexpressing miR-17-92. We were successful in associating colony formation with the cooperative role of the miRNAs within the cluster. We identified and established PTPRD and SRGAP2 as potential tumor suppressors that are regulated by the cluster. Finally we found that triptolide potentially targets miR-17-92 transformed OS cells. The selective lethality of miR-17-92 high OS cells by triptolide suggests these poor prognosis cases may benefit from treatment with minnelide.

Figure 7. miR-17-92 is highly overexpressed in OS.

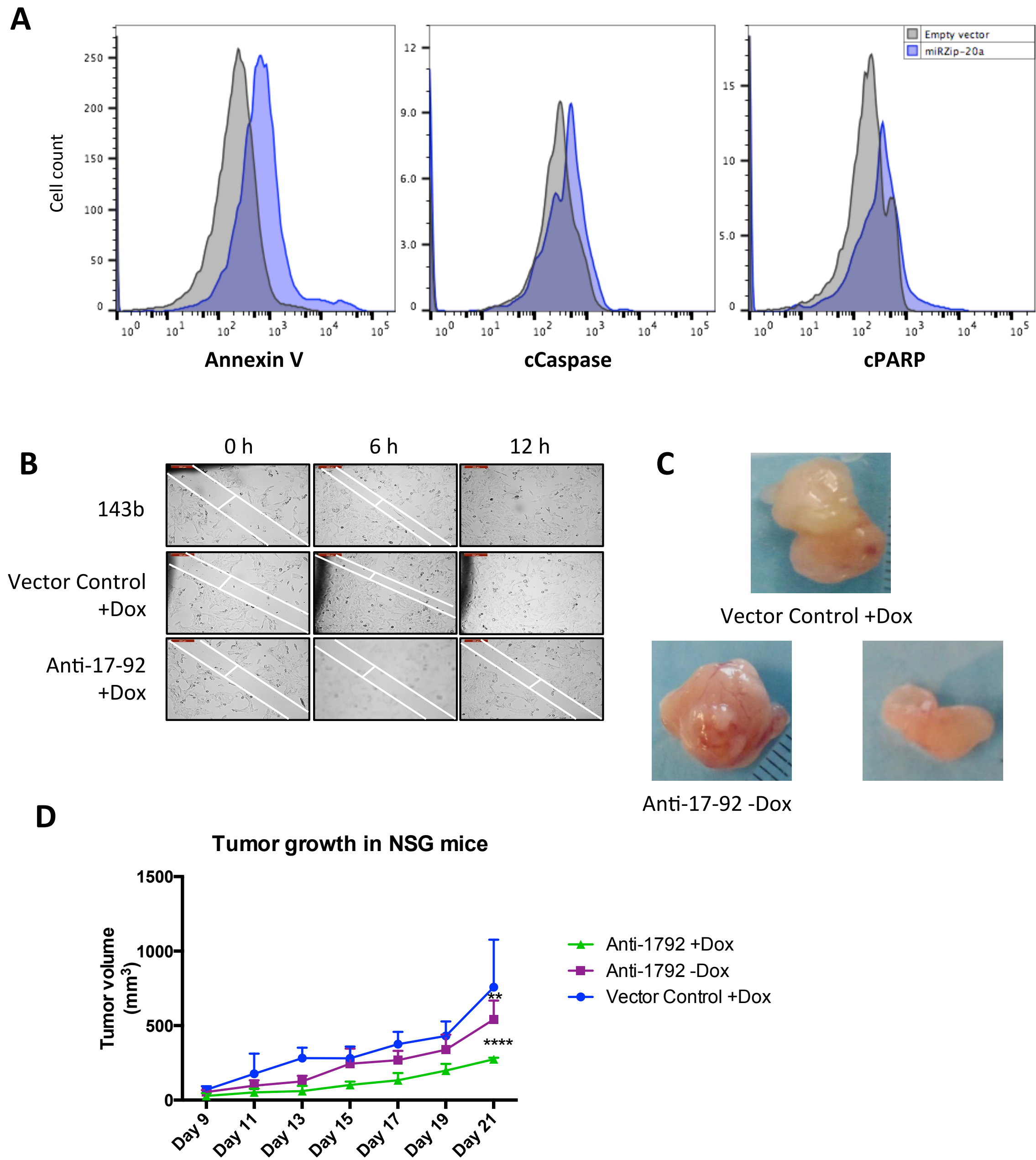


A) miR-17-92 miRNA cluster member expression in 24 primary human osteosarcoma and 4 morphologically normal human bone tissue samples showed that in a subset of tumors, the miR-17-92 cluster was significantly upregulated.

B) miR-17-92 transcript levels were significantly higher ($p<0.01$) in four of the OS cell lines compared to osteoblast cell lines (OB) via qRT-PCR.

C) Tumor suppressor genes (n=281) identified by SB screen CIS analysis grouped by the number of predicted 3'-UTR binding sites for miR-17-92 miRNAs.

Figure 8. Knockdown of miR-17-92 decreases tumor burden in mice



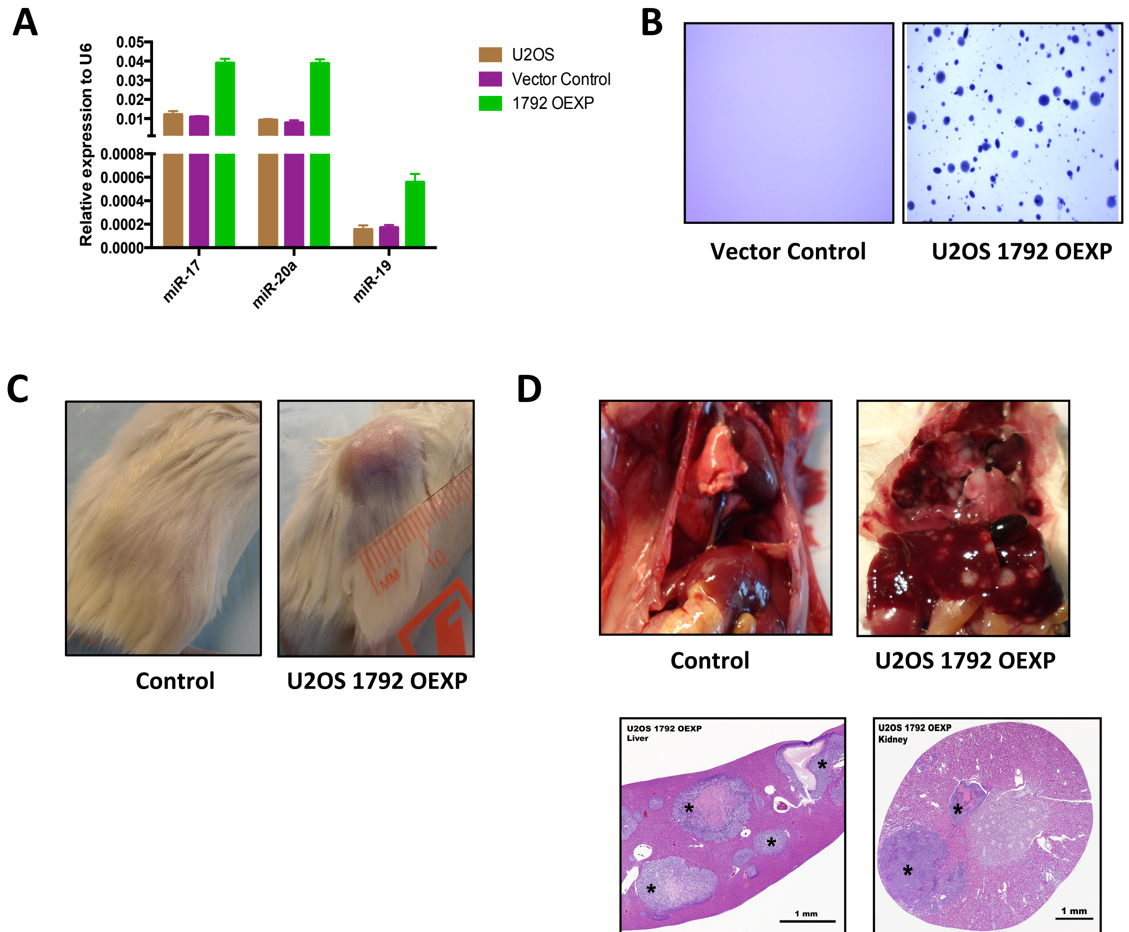
A) 143B cells were collected after transduction of MZs (48 hours) and apoptotic markers Annexin V, cleaved Caspase3 (cCas3), and cleaved Parp (cPARP). MZ-20 had the most significant increases in the apoptotic markers compared to the vector control (empty vector).

B) Transient transfection of doxycycline inducible anti-miR-17-92 in 143B cells reduced the migration of 143B cells compared to the controls (Vector Control+Dox, 143B alone).

C) 143B stably expressing anti-17-92 injected subcutaneously into mice treated with doxycycline showed significantly less tumor burden in NSG mice (n=4) compared to the control groups (n=4 each).

D) H&E reveals that there is less cellular density in xenograft sections expressing anti-17-92 compared to the control groups.

Figure 9. Overexpression of miR-17-92 in U2OS leads to tumor formation and progression.



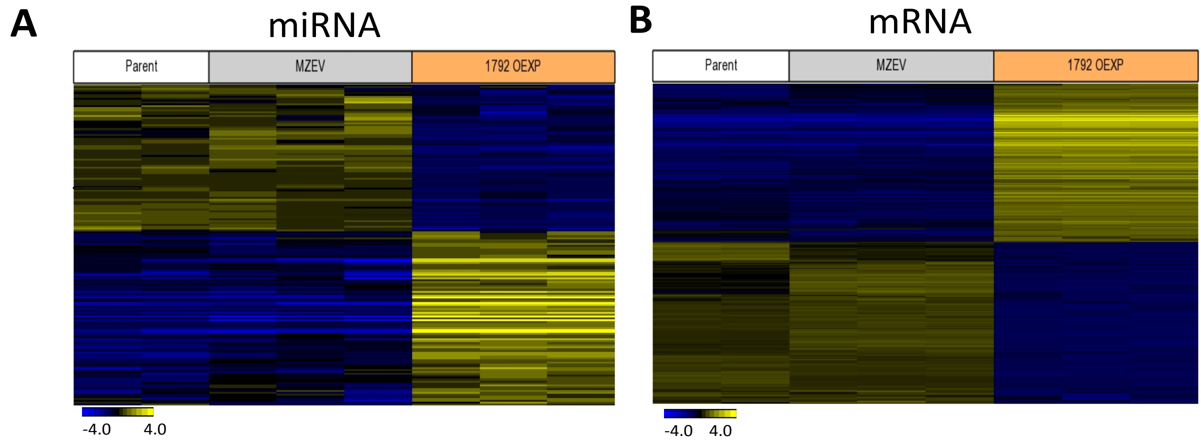
A) U2OS stably overexpressing miR-17-92 confirmed via qRT-PCR (triplicates).

B) Colony forming assays reveal that only U2OS cells overexpressing miR-17-92 formed anchorage independent colonies and the control groups did not form any colonies.

C) Subcutaneous injections of U2OS overexpressing miR-17-92 form tumors in NRG mice (n=3). One of the mice showed liver metastasis in the same group. No tumor formation was seen in U2OS vector control group (n=3).

D) Tail vein injections of U2OS miR-17-92 cells in NRG mice led to numerous macro and micro metastases (n=4). No macro or micro metastases were observed in the control group (n=4).

Figure 10. miR-17-92 regulates several cancer-related functions in OS cells

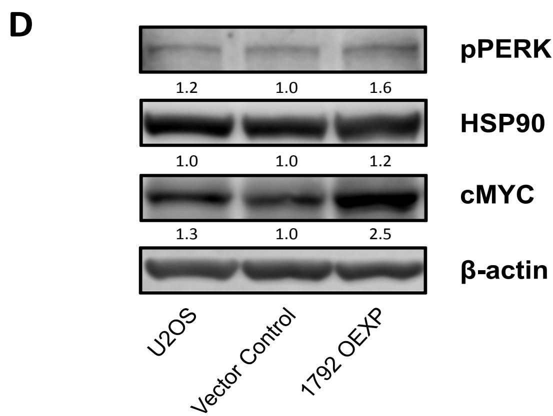


C

Enriched in miR-17-92 overexpressing cell line

Hallmark gene sets	NES	FDR q-value
Oxidative phosphorylation	1.90	0.001
MYC targets v1	1.73	0.007
Unfolded protein response	1.54	0.031
Pancreas β-cells	1.52	0.031
MYC targets v2	1.48	0.036
TNFα signaling via NFκB	1.37	0.088
Wnt/β-catenin signaling	1.31	0.138
Epithelial mesenchymal transition	1.23	0.236

D



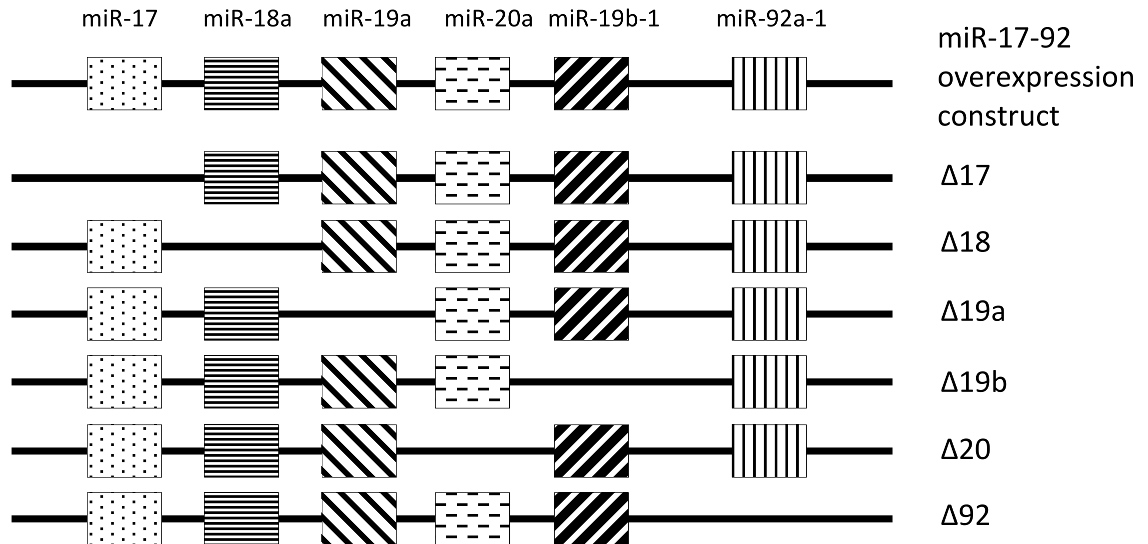
A) RNA-Seq analysis revealed that overexpression of the miR-17-92 cluster resulted in distinct changes in global miRNA expression patterns. The red bar indicates the location of the miR-17-92 miRNA cluster.

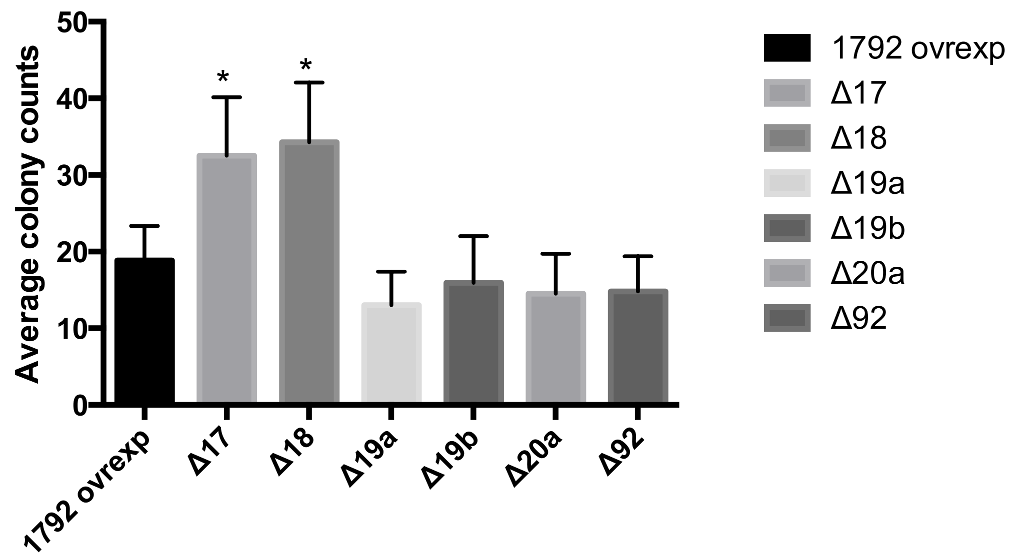
B) Differentially expressed mRNAs in miR-17-92 overexpressing cells compared with parental and control-plasmid (Vector Control) cells, identified using a two-group t-test ($P < 0.05$, fold-change > 2).

C) Several gene sets were significantly enriched in miR-17-92 overexpressing cells compared to parental and control cells identified in a GSEA analysis using the MSigDB Hallmark gene sets [REF:Liberzon].

D) Protein lysates from U2OS overexpressing miR-17-92 show increased levels of cMYC, pPERK compared to the vector control or U2OS alone.

Figure 11. Generation of miR-17-92 mutant overexpression constructs

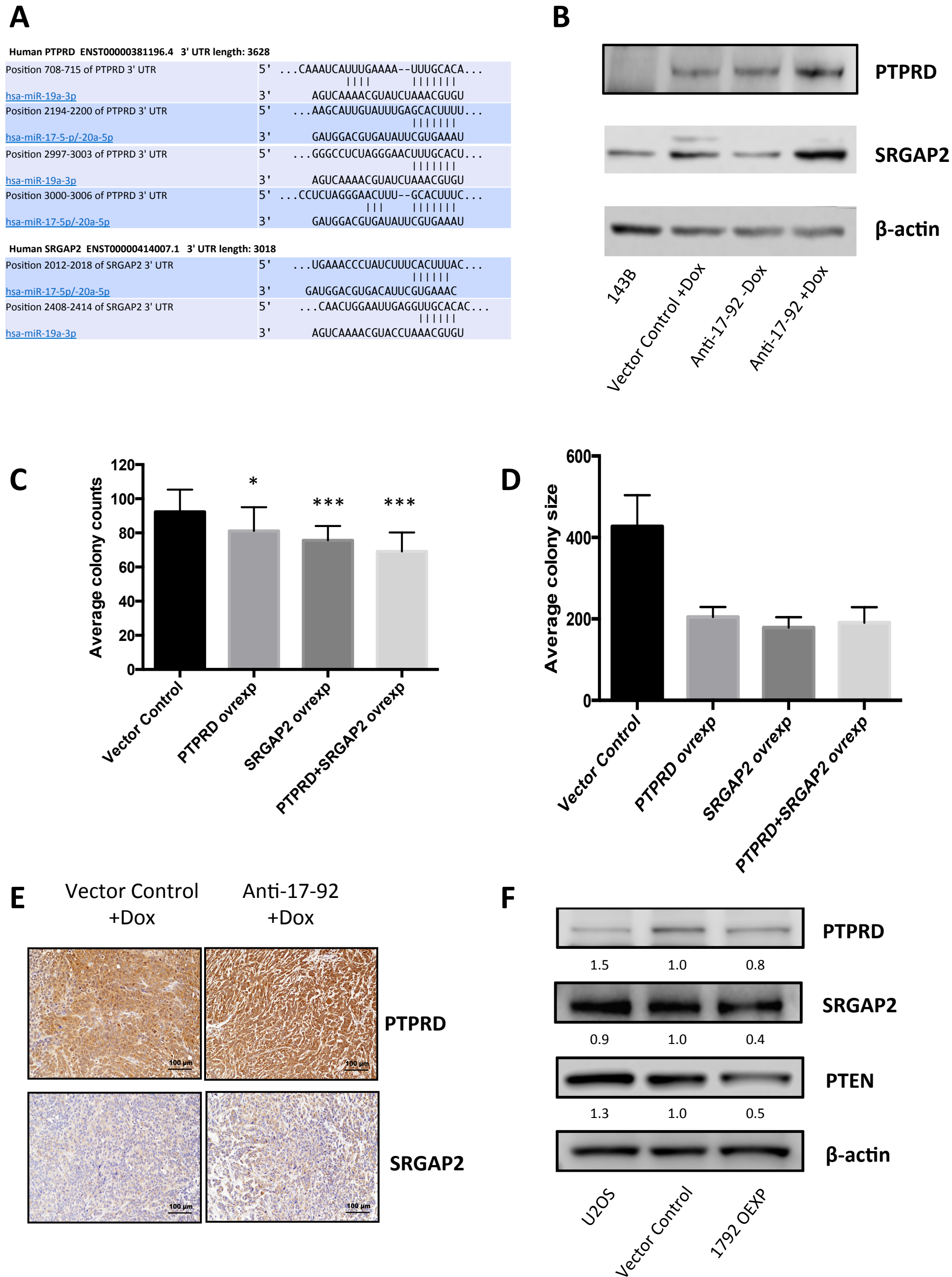




A) Schematic of miR-17-92 overexpression mutant constructs, total of 6 different deletion mutants.

B) Colony forming assay with the deletion mutants reveal that there is significant increase in the colony formation in delta 17 and 18 overexpression vectors in U2OS cells (*$p<0.05$).

Figure 12. Novel tumor suppressor genes PTPRD and SRGAP2 identified as targets of miR-17-92.



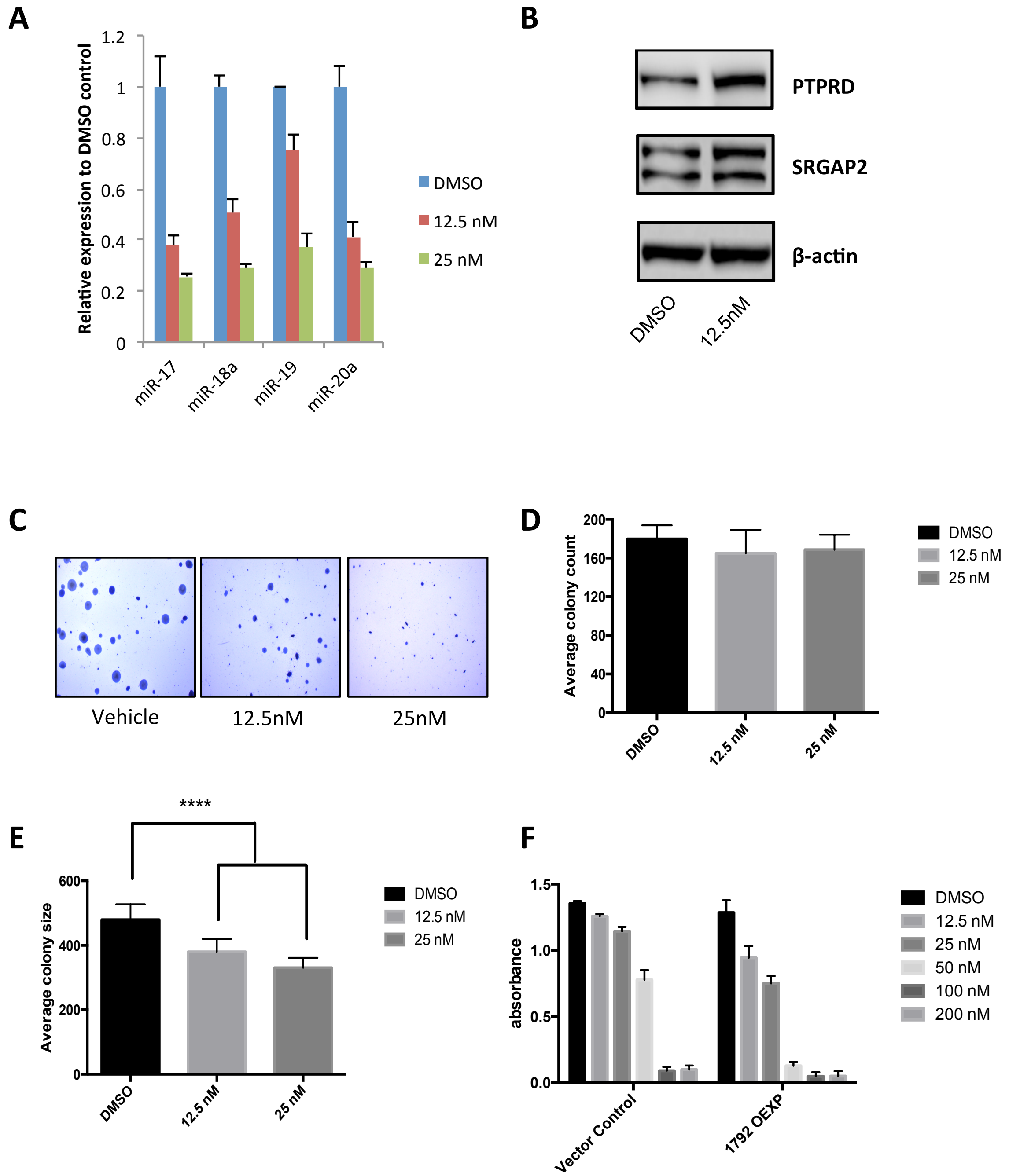
A) Increase in the protein levels of PTPRD and SRGAP2 in anti-17-92 +Dox compared to the absence of doxycycline (anti-17-92 –Dox), vector control and 143B.

B-C) Colony forming assays revealed that overexpression of either SRGAP2 or PTPRD or both in 143B cells (four replicates each) significantly decreased colony numbers and colony size compared to the vector control (*$p<0.05$, ***$p<0.001$)

D) IHC on the same xenograft sections (n=2) suggest that there is an increase in the levels of PTPRD and SRGAP2 proteins in the presence of doxycycline compared to the controls.

E) The protein lysates from U2OS overexpressing miR-17-92 shows reduced levels of PTPRD, SRGAP2, and established cluster target PTEN compared to the vector control or U2OS alone.

Figure 13. Triptolide potentially targets miR-17-92.



A) Triptolide decreases the transcript levels of miR-17-92 in a dose dependent manner in 143b cells.

B) 143b protein lysates after 48 hours of triptolide treatment show increased levels of PTPRD and SRGAP2 via western blotting.

C-E) Treatment of U2OS overexpressing miR-17-92 with triptolide revealed a significant decrease in the colony size (****$p<0.0001$) compared to the vehicle control in colony forming assays.

F) Viability assays revealed that triptolide significantly reduces the viability of OS cells overexpressing miR-17-92 compared to vector control.

Chapter 4: Summary and future directions

Our findings show that the miR-17-92 cluster encodes miRNAs that are expressed at high levels in a subset of human OS cases and can induce, in cell autonomous manner, characteristic hallmarks of OS aggressiveness. Our data suggest that miR-17-92 encoded miRNAs play a crucial role in the acquisition of aggressiveness in OS and renders cells sensitive to the small molecule triptolide. Further work would entail detailed analyses of the underlying mechanism of the cluster in OS progression. Several important questions remain unanswered and experimental approaches unexplored. This includes proof of miR-17-92's role in OS in an autocthounous model *in vivo* using gain and loss of function approaches. Also important would be a thorough identification of the direct targets of miR-17-92 encoded miRNAs using proteomic and RNA-based methods. Such in-depth analysis of targets' identification and the biological processes they control will help us understand the still opaque metastasic process. My data shows that the small molecule triptolide has a particular selectivity for OS cells that expresses miR-17-92 at higher levels. The biochemical basis for this selectivity should be explored and efforts made to leverage this information to create a useful novel targeted therapy for OS.

Characterization of bone-specific miR-17-92 knockout (KO) mice.

One way to further elucidate the role of miR-17-92 in OS tumor formation would be to generate a miR-17-92 conditional loss of function or knockout (KO) mouse, specifically in cells that are targets for osteosarcomagenesis. This would allow us to determine if

these genes play a role in various stages of OS development, all of which can be modeled in *Osx1-Cre; Trp53*$^{Flox/Flox}$*; Rb1*$^{Flox/Flox}$ or *Osx1-Cre; LSL-Trp*^{53R270H}/+ mice. The experimental plan would be to cross mice harboring a floxed *miR-17-92* allele with transgenic mice expressing Cre under the control of the Osterix gene (*Osx*) promoter and the conditional *LSL-Trp53*^{R270H} allele (17-92 OB KO^{OsxTRP53})(Moriarity, Otto et al. 2015, Chamorro-Jorganes, Lee et al. 2016). To assess the level of inactivation of the miR-17-92 cluster, one could isolate mesenchymal stem cells from the bone marrow of 17-92$^{fl/fl}$, *Trp53*^{R270H} Osx-Cre (17-92 OB KO^{Osx}), and 17-92 OB KO^{OsxTRP53} culture, differentiate them into osteoblasts (OBs), and assess miR17-92 cluster components by qRT-PCR. The loss of miR-17-92 in OBs should not be embryonic lethal; however, OB-specific deletion in developing osteoblasts may have a skeletal phenotype and mice should be closely monitored for any sign of this. It may be necessary to delete miR-17-92 and activate the *LSL-Trp53*^{R270H} allele in a subset of osteoblasts for this reason. The 17-92 OB KO^{OsxTRP53} mouse model will potentially determine whether miR-17-92 loss delays or inhibits the tumor formation compared to the Osx-Cre; *Trp53*^{R270H} animals.

Characterization of osteoblast-specific miR-17-92 driven mice.

To test the function of miR-17-92 in OS development *in vivo* using a gain of function approach, one could overexpress the whole cluster and individual miRNA families (miR-17, miR-18, miR-19, miR-92 families) within the cluster in the transgenic animal and cross it with either Osx-Cre or P53 ^{R270H}-Osx-Cre mouse model as described above. Such crosses would generate five different mouse models with either Osx-Cre

alone or $P53^{R270H}$-Osx-Cre. Such mouse models will help us understand the role of miR-17-92 as whole as well as specific families within the cluster.

Identification of miR-17-92 specific targets

To identify miR-17-92 targets we will use of a systems biology approach that integrates *in silico* miRNA-target prediction with quantitative proteomics and transcriptomics analysis on acute induction of the cluster (Mihailovich, Bremang et al. 2015). We would implement SILAC-based quantitative proteomics to determine the impact of miRNAs on global protein output (Mann 2006). We would perform this methodology in OS cells overexpressing miR-17-92 constructs (as described in chapter 3) and control groups (Vector and Parental OS line). This approach will provide a comprehensive picture of fluctuations in protein levels that resulted from modulation of miR-17-92 expression. We should be able to identify and quantify over 4,500 proteins with high precision and statistical confidence. We will confirm the results obtained by SILAC using western blots.

It's quite challenging to identify the most relevant mRNA targets of miRNAs as they have the capability to regulate thousands of transcripts. Biochemical approaches have been developed to help overcome the challenges of finding direct miRNA targets. The miR-17-92:mRNA target pairs can be purified by the immunoprecipitation of the RISC components, Argonaute (AGO), or TNRC6 (Chi, Zang et al. 2009, Hafner, Landthaler et al. 2010). Any mRNAs under direct regulation of miR-17-92 would be co-immunoprecipitated along with the RISC and transcripts could be isolated and identified

by microarray or deep sequencing and compared them to mock samples. This technique has been quite successful in identifying direct targets for miR-1 and miR-124 (Karginov, Conaco et al. 2007, Hendrickson, Hogan et al. 2008). One shortcoming with our study is that the cluster has various miRNAs. To perform such studies, we will have to dissect each miRNA from the cluster, which could require some effort. Another drawback of the Ago co-immunoprecipitation approach is that it may not necessarily emulate *in vivo* interactions between miRNA:mRNA and RNA binding proteins following cell lysis. There is a potential for loss of targets during this process. However, we are certain we would be able to capture many of the targets knowing that there are has been success in earlier studies. A new advancement to improve the technique involves employing UV irradiation to crosslink RNA to RNA-binding proteins prior to immunoprecipitation (Fig. 14) This technique is known as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP), or crosslinking immunoprecipitation (CLIP)-seq. Performing HITS-CLIP on cells with and without treatment with specific miRNA antisense inhibitors is likely to provide a powerful method for identification of specific miRNA targets.

Specific role of miR-17-92 in OS metastasis

Due to a lack of understanding of OS metastasis,, it becomes necessary to delve into the importance of miR-17-92 in its role in tumor progression and metastasis. As described earlier, we took a comparative oncology approach and utilized data from a Sleeping Beauty (SB) transposon-driven OS model to identify the top CIS genes that are

potential tumor suppressor driver gene in the spontaneous metastatic SB OS model. We will further validate these genes and confirm their regulation by miR-17-92. We are planning to generate CIS gene specific mouse models that would be crossed with Osx-Cre and TRP53 ^{R270H} transgenics to test the effect on metastasis formation in mice.

miRNA Therapeutics – development of triptolide into an "effective drug" in miR-17-92 high OS patients

We have known about miRNAs and their role in mRNA regulation for over a decade; however, we still do not entirely understand the molecular mechanisms of how miRNAs regulate gene expression nor have we identified and established all the miRNA targets in the human genome. Thus, it becomes essential to understand the function and targets of miRNA by inhibiting the miRNA in a biological process. We have shown that triptolide targets in a specific manner in OS cells that are overexpressing miR-17-92. However, there are still some caveats to this study. We yet have to determine the mechanism of triptolide inhibition of the cluster. Understanding how triptolide regulates these miRNAs could have implications both in research as well as therapeutic applications. Recently, Jamal et al. have been successful in creating predictive computational models for small molecules that have the potential to bind and inhibit miRNA action using sophisticated algorithms and chemical descriptors (Jamal, Periwal et al. 2012). This is a useful step forward for contextualizing studies such as ours where we observe a significant inhibition of miRNAs due to a small molecule. In addition, they have shown that the methodology is highly accurate and can be used as a computational

screen prior to performing high throughput drug screens. Such approaches will help us identify the underlying mechanism of triptolide and its selectivity for miR-17-92.

The next goal of this drug based study would be to identify an efficient way to deliver this small molecule to make it tumor specific as triptolide has been shown to affect RNA Pol II and may have a global transcriptional effects that could potentially lead to toxicity in patients. Nanoparticles may play an important assisting role in such cases. Using nanoparticles for the delivery of small molecules in anticancer therapy is a rapidly growing area of research (Chen 2010). The advantages of using therapeutic nanoparticles (TNPs) for drug delivery include (a) improved the solubility of anticancer agents; (b) enhanced circulation time of anticancer agents in the blood vessels; (c) increased accumulation of anticancer agents in targeted tumor tissues; (d) uptake by tumor cells through endocytosis, resulting in increased intracellular drug concentrations; (e) controlled and stable drug release; and (f) minimized efflux pump-mediated drug-resistance, as TNPs are not substrates for ATP-binding cassette proteins. Such strategies may make small molecules such as triptolide an attractive drug for OS, specifically in patients presented with higher levels of miR-17-92.

To conclude, my doctoral thesis is a major attempt to understand the role of miR-17-92 in OS development and progression. With the establishment of biological functional assays that show the crucial role of miR-17-92 in driving OS tumorigenesis, identification of two novel potential tumor suppressor genes, and a small molecule that targets the cluster, I am confident my work has laid a key groundwork for future research on the miR-17-92 cluster and it's significance as a prognostic marker in OS patients as

wells as a potential for development of targeted therapy for “miR-17-92 high” OS patients.

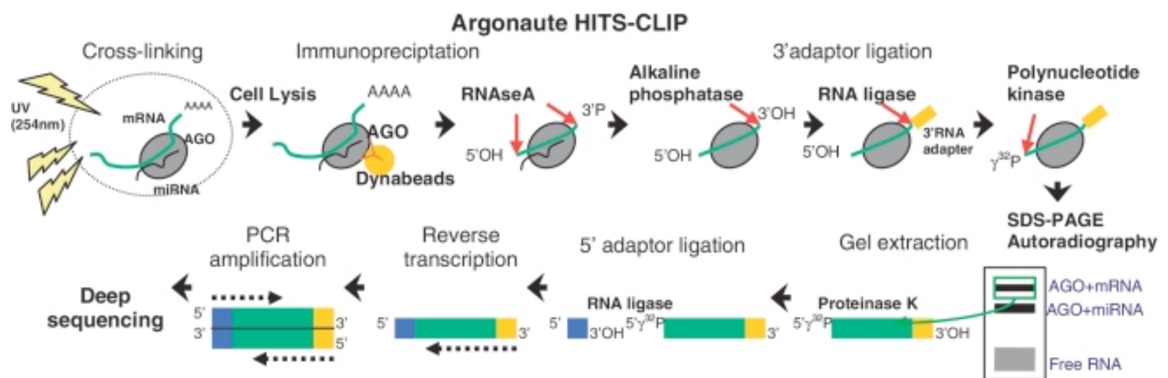


Fig 14. A step-wise protocol of argonaute high throughput sequencing of cross-linking immunoprecipitation (HITS-CLIP).

Adapted from Thomson, Daniel W., Cameron P. Bracken, and Gregory J. Goodall. "Experimental strategies for microRNA target identification." *Nucleic acids research* 39.16 (2011): 6845-6853.

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