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MicroRNAs: Small but Critical Regulators of Cancer Stem Cells

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1. Introduction

Researchers have been attempting for decades to elucidate the molecular mechanisms at play within the cells that cause the development of cancer. There is now growing evidence that cancers may form from cancer stem cells. Somehow, a group of cells evolves that are immortal and can produce progenitor cells that can grow; however, the research community doesn't know how these cells lose control.

Recent studies have suggested the existence of a special small subpopulation of cancer cells that act as tumor-initiating cells or cancer stem cells. Cancer stem cells are implicated to maintain the self-renewal and unlimited growth capabilities of the cancer while only comprising a small fraction of the tumor. For this reason, cancer stem cells may be responsible for the tumor progression, drug/treatment resistance development, and metastasis.

Other studies have demonstrated that microRNAs (miRNAs) have a great deal to do with what genes are expressed/not expressed through their gene silencing capabilities. Interestingly, microRNAs might provide some new insight into the intricacies of cancer. These small RNA molecules could hold great potential therapeutically in the battle against cancer.

In this chapter, we discuss the functions of microRNAs and cancer stem cells and explore the link between these two topics. We also present methods to use in current and future research to study these topics and expound upon various molecular therapy options that could have implications in correcting cancer stem cell dysregulation and battling oncogenesis.

2. MicroRNAs

RNA interference is a vital system within cells that helps control which genes are active and to what extent they are activated. The two central small RNAs of RNA interference are small interfering RNA (siRNA) and microRNA (miRNA). Both are involved in gene silencing. siRNAs originate from the processing of a long, double-stranded RNAs and target mRNAs

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for degradation utilizing full complementary sequences (Zeng et al., 2003). In contrast, miRNAs are derived from the processing of short RNA hairpins and silence gene expression through mRNA degradation or translational repression of mRNAs with partially complementary target sequences (Zeng et al., 2003). However, there is a more important difference. siRNAs are often of exogenous origin, while miRNAs are endogenously encoded. Thus, miRNAs are naturally occurring in animal cells. For this reason, the use of miRNAs has interested many investigators because of their potential application to developing therapeutics to combat diseases such as cancer.

The biogenesis of miRNAs has been investigated and reviewed by many researchers and summarized in our recent review (DeSano and Xu, 2009). At the start, microRNAs are transcribed by the RNA polymerase II enzyme producing a long primary-miRNA (pri-miRNA) in the nucleus (Lee et al., 2004). Post-transcriptional modifications include a 5′ end cap structure and a 3′ end poly-adenylated tail that flank the pri-miRNAs (Cai et al., 2004). This suggests that pri-miRNAs are structurally and functionally similar to mRNAs. In addition to the 5′ cap and 3′ tail, pri-miRNAs contain specific hairpin-shaped stem-loop structures of ~70 nucleotides. These stem-loop structures are recognized and cleaved by an ~650 kDa nuclear microprocessor complex consisting of the RNase III endonuclease Drosha and the essential DiGeorge syndrome critical region gene 8 (DGCR8) binding protein, which yields a ~70 nucleotide hairpin intermediate (Qian et al., 2004). The resulting ~70 nucleotide hairpin intermediate (pre-miRNA) is transported into the cytoplasm from the nucleus by Exportin-5 and its cofactor Ran-GTP (Yi et al., 2003). While in the cytoplasm, the pre-miRNAs are further cleaved. This cleavage is carried out by a RNase III endonulease Dicer-1 and its essential transactivating response RNA binding protein (TRBP) (Haase et al., 2005). This produces a short imperfect double stranded miRNA duplex. Helicase then unwinds this imperfect miRNA duplex into a mature miRNA. Next, TRBP recruits the catalytic Argonaute 2 to the Dicer complex with the mature miRNA forming the RNA-induced silencing complex (RISC) (Chendrimada et al., 2005; Haase et al., 2005). The RISC subsequently regulates gene expression by mRNA degradation or translational repression via partially complementary sequences in the 3′-untranslated region (3′-UTR) of the targeted mRNA (Chekanova and Belostotsky, 2006; Croce and Calin, 2005; Zhang et al., 2007). In animals, microRNAs may also do this by targeting the coding regions of mRNAs (Rigoutsos, 2009). Therefore, miRNAs negatively regulate gene and protein expression via the RNA interference (RNAi) pathway.

Recently, miRNAs have been implicated to have a role in stem cell function. Stem cells are found throughout the human body and are essential to tissue development, replacement, and repair (Farnie and Clarke, 2007). This is because the level of expression of certain miRNAs is different in stem cells compared to normal tissues (Suh et al., 2004). Studies have analyzed miRNA expression profiles in undifferentiated human embryonic stem cells, partially differentiated embryoid bodies, and terminal differentiated cells. One analysis found that 104 miRNAs and 776 genes were differentially expressed among the three cells types (Ren et al., 2009). Another study found rapid regulation of certain miRNAs in response to differentiation (Stadler et al.). In addition to miRNA expression profiles, investigators have used Dicer-1 (*dcr*-1) mutants to confirm miRNAs' regulation of stem cell function. As discussed above, Dicer-1 plays an essential part in miRNA biogenesis; thus, a mutant *dcr*-1 would offer great insight into a proposed role of miRNAs in stem cells. Loss of *dcr*-1 resulted in early death in mouse models and depletion of stem cells in mouse embryos (Bernstein et al., 2003). This suggests that miRNAs do play a role in stem cell regulation

because a disruption of the miRNA pathway results in a decreased stem cell population. Another study (Kanellopoulou et al., 2005) found that mutated *dcr*-1 in embryonic mouse stem cells lead to reduced miRNA expression and severe defects in stem cell differentiation *in vitro* and *in vivo*; in addition, re-expression of Dicer-1 reversed these phenotypes. These dcr-1 mutants data demonstrate that miRNAs have a fundamental role in regulating stem cell function.

MicroRNAs also can function in stem cell biology through epigenetic regulation. Epigenetic regulation, including DNA methylation and histone modification is known to play vital roles in regulating stem cell proliferation and differentiation (Szulwach et al.). A DNA methyl-CpG-binding protein (MeCP2) has been shown to epigenetically regulate specific miRNAs in adult neural stem cells (Szulwach et al.). This is a rather interesting finding because the interaction (if any) between the miRNA and epigenetic pathways is not well understood. This results demonstrates that there is specific cross talk between epigenetic regulation and the miRNA pathway (Szulwach et al.). This cross talk could be significant to modulating stem cell function and differientation. Changes in DNA methylation and histone modification also are characteristic of cancers. These epigenetic changes result in dysregulation of gene expression profiles leading to the development and progression of disease states (Sharma et al.). MicroRNAs could be affected by these epigenetic changes due to the cross talk between the two pathways. There are widespread changes in miRNA expression profiles during tumorigenesis (Sharma et al.). Therefore, microRNAs' role in stem cell regulation and cancer formation and progression are an attractive area of research.

3. Self-renewal of cancer stem cells

Stem cells are defined by their multi-lineage differentiation and their ability to undergo self-renewal (Dontu et al., 2003). This self-renewal can be either asymmetric or symmetric. Self-renewal is unique from other proliferative processes in that at least one of the progeny is identical to the initial stem cell. In all other replicative processes, the progeny of division undergo a series of differentiation events. In asymmetric stem cell self-renewal, one of the two progeny is identical to the initial stem cell, whereas the other cell is a committed progenitor cell, which undergoes cellular differentiation (Al-Hajj and Clarke, 2004). Since one stem cell is a product of asymmetrical self-renewal division, the stem cell number is maintained. However, in symmetrical self-renewal, two stem cells are produced, resulting in stem cell expansion. Both the self-renewal and differentiation of stem cells are regulated by the stem cell niche, which is the microenvironment surrounding the stem cell (Wicha, 2006).

Recently, evidence has emerged that suggests that a small subset of cancer cells in tumors have stem cell properties. The cancer stem hypothesis states that cancers are derived from a small fraction of cancer cells that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and initiate/maintain the tumor (Papagiannakopoulos and Kosik, 2008). According to this cancer stem cell hypothesis, cancer stem cells are tumor-initiating cells that proliferate uniquely through self-renewal. Cancer stem cells are thought to only constitute a small fraction of the tumor, but may be responsible for tumor outgrowth, progression, metastasis, and treatment-resistance (Wicha, 2007). Thus, it has been hypothesized that to be maximally effective, cancer therapy should be directed against these cancer stem cells (Rich and Bao, 2007).

This self-renewal capability has also been demonstrated by examining the ability of subpopulations of tumor cells identified by cell surface markers to form tumors when transplanted into immunosuppressed NOD/SCID mice *in vivo*. This approach was first successfully used to demonstrate the existence of leukemic cancer stem cells (Bonnet and Dick, 1997). A similar approach has been utilized to identify a subpopulation of human mammary cancer cells that bear the $CD44^{+}CD24^{-}ESA^{+}$ $Lineage^{-}$ that have the properties of breast cancer stem cells (Al-Hajj et al., 2003). After isolation from primary human breast cancer carcinomas or metastatic lesions, less than 100 of these cells are able to form tumors reproducibly, while tens of thousands of phenotypically distinct cancer cells are unable to generate tumors (Al-Hajj et al., 2003). Thus, the central feature of cancer stem cells is this relatively unlimited asymmetric self-renewal (Al-Hajj and Clarke, 2004).
In addition, an *in vitro* mammosphere assay has been developed to demonstrate that only a minority of cells in human cancers are capable of self-renewal. Using this mammosphere method, it was found that secondary mammospheres from the human breast cancer cell group bearing $Lin^{-}CD29^{H}CD24^{H}$ were larger in size and number compared with all other subpopulations of tumor cells (Zhang et al., 2008a). This suggests that these cells are tumor-initiating and undergo self-renewal. Thus, a certain subpopulation of cancer cells is able to self-renew and initiate tumor formation, supporting the term "cancer stem cells".
Self-renewal of cancer stem cells is thought to be a likely cause of the resistance seen of current cancer treatment and relapse in cancer patients. Recently, we have been provided with the first clinical evidence that implicates that a glioma stem cell/self-renewal phenotype is responsible for the treatment resistance seen in glioblastoma patients (Murat et al., 2008). Strong arguments can be made that genetic alterations cause cancer stem cell dysregulation, which results in unlimited self-renewal. It is believed that abnormal stem cell self-renewal is a likely necessity for cancer initiation, formation, and resistance to current therapies.

4. Signaling pathways of cancer stem cells

The question then becomes – How does irregular self-renewal capabilities occur in cancer stem cells? There is growing evidence that many pathways that have characteristically been connected to cancer also regulate normal stem cell development (Murat et al., 2008). This evidence suggests that these signaling pathways play a significant role in dysregulating stem cell genes in cancer stem cells leading to the formation and growth of tumors. The pathways of Bcl-2, Wnt, Hedgehog, Notch, Bmi-1, HMGA2, and CD44 have been found to be involved in the survival, self-renewal, and differentiation of cancer stem cells.

4.1 Bcl-2

Bcl-2 has been investigated rigorously because of its status as a proto-oncogene. It has been shown to be over expressed in many cancers and exhibits an anti-apoptotic effect in these cancers. Bcl-2 over-expression leads to increased number of stem cells and cancer stem cells, suggesting a role in the stem cell niche (Domen et al., 1998; Ji et al., 2009). Thus, Bcl-2 has been connected to the survival of stem cells and cancer stem cells because of its over expression in cancers.

4.2 Wnt

Wnt signaling is the next pathway. The presence of Wnt activates the Wnt receptor, causing a downstream accumulation *of β-catenin* in the cytoplasm. This accumulation of *β-catenin* is

translocated to the nucleus and activates the expression of many genes associated with self-renewal. The Wnt pathway has been implicated in oncogenesis. Over-expression of *β-catenin* enlarges the pool of stem cells (Reya et al., 2003). Activation of *β-catenin* enhanced the self-renewal potential in leukemic stem cells (Jamieson et al., 2004). Therefore, Wnt signaling is involved in the self-renewal capability of cancer stem cells.

4.3 Hedgehog

The Hedgehog pathway is also important in the dysregulation of caner stem cells self-renewal potential. When Hedgehog is present, its receptor Patched is activated. This results in activation of Smoothened and later Gli transcription factors, which are translocated into the nucleus and regulates the transcription of certain genes including those that regulate self-renewal. Increased self-renewal has been shown to occur upon Hedgehog stimulation in hematopoietic stem cell populations (Bhardwaj et al., 2001). Many human cancers have activated levels of Hedgehog signal transduction (Xie et al., 1998). This suggests that dysregulation of self-renewal properties of cancer stem cells due to increased Hedgehog signaling could form cancer in humans.

4.4 Notch

The Notch pathway is significant as well. Notch is a transmembrane receptor that binds the ligand Delta. When Delta is present, an extracellular protease TACE cleaves the extracellular domain of Notch. This leads to cytoplasmic domain of Notch to be cleaved by γ-secretase. This newly liberated cytoplasmic portion of Notch is translocated into the nucleus where it binds to DNA-binding proteins of the CSL family. This activates transcription of genes utilized during development and renewal of adult tissues. Atypical Notch signaling has been demonstrated to promote self-renewal of mammary stem cells, as well as aids in the development of invasive breast cancer (Dontu et al., 2004; Farnie and Clarke, 2007). These findings suggest that Notch signaling transduction could lead to the dysregulation of self renewal in cancer stem cells.

4.5 Bmi-1

Bmi-1 signaling has been implicated in this discussion because of its effects on cancer stem cell self-renewal potential. Loss of Bmi-1 resulted in a decrease in stem cell differentiation and self-renewal (Zencak et al., 2005). Aberrant levels of Bmi-1 have also been demonstrated to generate cancers (Sparmann and van Lohuizen, 2006). Bmi-1 activation was found in $CD44^{+}CD24^{-/low}Lin^{-}$ human breast cancer stem cells (Liu et al., 2006). In addition, modulation of Bmi-1 expression alters the mammosphere-initiating cell number and size (Liu et al., 2006). This suggests a role in the dysregulation of self-renewal properties in cancer stem cells and future research is needed to gain insight into the Bmi-1 pathway.

4.6 HMGA2

HMGA2 has been associated in the self-renewal potential and survival of cancer stem cells. HMGA2 is thought to regulate gene expression by modulating macromolecule complexes that are involved in many biological processes. HMGA proteins are expressed during development; specifically, HMGA2 has been suggested to control growth, proliferation, and differentiation (Fusco and Fedele, 2007). In addition, HMGA2 has been found to be over-expressed in lung and pancreatic carcinomas and metastasis (Abe et al., 2003; Fusco and

Fedele, 2007; Meyer et al., 2007). Thus, excessive HMGA2 signaling could dysregulate cell survival and self-renewal in cancer stem cells.

4.7 CD44

CD44 is another intriguing pathway being implicated with cancer stem cells. So far, there is no specific cellular marker for CSC. We and many others have found that pancreatic cancer stem cells from cell lines or primary tumors are enriched in CD44+ population; p53 directly regulates CD44; pancreatic cancer cells lacking functional p53, especially cancer stem cells, have high *CD44*, low miR-34 and high *Bcl-2/Notch* expression. Recent studies indicate that CD44 molecules activate down-stream Nanog that in turn activate Sox2 and Rex1 (Bourguignon et al., 2008; Kasper, 2008), and these transcription factors have been implicated in stem cell maintenance. Besides being a cellular marker for CSC, CD44 has recently been functionally linked to cancer stem cell maintenance, growth and resistance (Bourguignon et al., 2008; Godar et al., 2008; Peterson et al., 2007; Pries et al., 2008). Anti-CD44 antibody treatment markedly reduced leukemic repopulation by targeting CD44+ leukemic stem cells (Jin et al., 2006). A recent study shows that CD44 downstream signaling CD44—Nanog—Sox2/Rex1 and CD44—Nanog—Stat3--MDR1/P-gp are involved in CD44+ tumor cell resistance and progression (Bourguignon et al., 2008). We have observed that anti-CD44 mAb H4C4 inhibits MiaPaCa2 tumorspheres, reduces CD44+/CD133+ CSC number and blocks tumor-initiation, accompanied by CD44 downstream signaling inhibition (Hao, et al, manuscript in preparation). Therefore, aberrant CD44 signaling could be rather important in the dysregulation seen in cancer stem cells that results in oncognesis, tumor progression, metastasis, resistance to treatments, and relapse in cancer patients.

5. Examples of MicroRNAs regulating cancer stem cells

Over the past couple of years, cancer research has focused on miRNAs and the possibilities of the cancer stem cell hypothesis. Investigators have shown that cancer stem cells have aberrant levels of specific miRNAs, which results in dysregulation of the self-renewal potential through the signaling pathways described above in these cancer stem cells. This dysregulation is a very plausible explanation to the initiation, formation, and sustainment of tumors.

MicroRNAs in cancer cells can acts as oncogenes or tumor suppressors (DeSano and Xu, 2009). Oncogenic miRNAs are often called oncomiRs. They are usually a dominant, gain-of-function mutation. As a result, they are up-regulated in cancer cells. Specific miRNAs like miR-21, miR-17-92 cluster, miR-135, and miR-294 have been shown to be oncogenic miRNAs.

5.1 miR-21

The microRNA miR-21 has been shown to be overexpressed in tumor tissues (Gao et al.). It has been shown to function as an oncogene in breast cancer through the modulation of Bcl-2 and Programmed Cell Death 4 (PDCD4) (Asangani et al., 2008; Frankel et al., 2008). It has also been shown to play a pivotal role in gastric cancer pathogenesis and progression (Zhang et al., 2008b). Thus, over-expression of miR-21 leads to dysregulation of Bcl-2 and modulation the cancer stem cell environment, which results in increased turmor growth and decreased apoptosis.

5.2 miR-17-92

The miR-17-92 cluster consists of seven miRNAs. This cluster is significantly over-expressed in lung cancers (Hayashita et al., 2005). It does act as an oncogenic miRNA. It has been shown that an introduction of miR-17-92 into hematopoietic stem cells drastically accelerates the formation of lymphoid malignancies (Hayashita et al., 2005). Interestingly, miR-17-92 is connected to the Hedgehog pathway. In engineered medulloblastomas, miR-17-92-induced tumors were found to activate the Hedgehog signaling pathway (Uziel et al., 2009). This implicates a result of increased self-renewal potential through the modulation of the Hedgehog pathway in cancer stem cells.

5.3 miR-135

The microRNA miR-135 also regulates cancer stem cells through its oncogenic properties. The miR-135a and miR-135b miRNAs were found to be greatly up-regulated in colorectal adenomas and carcinomas, functioning to down-regulate APC gene expression, which is part of the Wnt signaling pathway (Nagel et al., 2008). If APC is not expressed at the correct levels, *β-catenin* will accumulate, leading to the activation of self-renewal genes. Thus, miR-135 plays an oncogenic role in modulating Wnt signaling transduction, resulting in dysregulation of cancer stem cells.

5.4 miR-29a

Recent research has found that miR-29a plays a vital role in cancer stem cells. It has been shown that miR-29a is highly expressed in hematopoietic stem cells and acute myeloid leukemia (Han et al.). This expression of miR-29a results in the acquisition of aberrant self-renewal capacity (Han et al.). This data suggests that miR-29a initiates cancer formation through the dysregulation of self-renewing leukemia stem cells. Over-expression of these oncomiRs leads to further cancer progression and resistance to treatment.

5.5 miR-294

The microRNA miR-294 is particularly interesting because it is a representative member of the embryonic stem cells cell cycle regulating (ESCC) miRNAs. In DGCR8-/- knockouts, the introduction of miR-294 activates numerous self-renewal genes, such as *Myc*, Oct4, Sox2, Tcf3, and Nanog (Melton et al.). This data suggests that miR-294, and possibly other ESCC miRNAs, modulates the self-renewal potential through regulating many different pathways that are important in stem cells. A role in cancer stem cells needs to addressed in the future and could add some serious insight into the intricacies of cancer stem cell self-renewal and differentiation.

Nevertheless, not all miRNAs act as oncogenes. The expression of some miRNAs is decreased in cancer cells. These miRNAs are tumor suppressor miRNAs and sometimes called TSmiRs. They are usually a loss-of-function, recessive mutation. TSmiRs, when normally expressed, prevent tumor formation and development; however, in cancer, their expression is down-regulated, allowing increased disease progression.

5.6 miR-128

The first example of tumor suppressor miRNAs that play a role in cancer stem cells is miR-128. Levels of miR-128 were drastically reduced in high grade gliomas (Godlewski et al., 2008). This suggests that miR-128 is a tumor suppressor. Upon introduction of miR-128, the

proliferation and growth of glioma cells were inhibited (Godlewski et al., 2008). Researchers were able to elucidate the mechanism involved. Expression of miR-128 down-regulated Bmi-1 signal transduction (Godlewski et al., 2008). Therefore, miR-128 blocked the self-renewal of glioma cancer cells via Bmi-1 modulation. This demonstrates the importance of miR-128 in regulating the self-renewal potential of cancer stem cells.

5.7 miR-199b-5p

Another intriguing miRNA is miR-199b-5p. It is a tumor suppressor miRNA. In metastatic cancer patients, its expression is lost (Garzia et al., 2009). This miR-199b-5p was discovered to down-regulate the expression of a transcription factor of the Notch signaling pathway. Upon introduction of miR-199b-5p, the Notch signaling was blocked and the subpopulation of medulloblastoma stem-cell-like cells decreased (Garzia et al., 2009). Thus, miR-199b-5p leads to a decrease of the self-renewal properties of cancer stem cells.

5.8 Let-7

Let-7 is a tumor suppressor miRNA that has garnered much interest in the cancer research community. Let-7 expression levels are reduced in various cancers relative to normal tissues (Johnson et al., 2007). Let-7 is not expressed in breast-tumor initiating cells (Yu et al., 2007). Upon expression of let-7 in breast tumor-initiating cells, it was shown that let-7 regulates the self-renewal *in vitro*, multipotent differentiation, and the ability to form tumors (Yu et al., 2007). These are the key features of cancer stem cells. It has been found to play a role in many pathways. Expression of let-7 has been shown to down-regulate HMGA2, RAS, Lin28, Sall4, and Myc (Johnson et al., 2005; Mayr et al., 2007; Melton et al.). All of these let-7 targets help regulate self-renewal. Thus, let-7 is a tumor suppressor miRNA that negatively regulates many targets in different pathways that all dysregulate the self-renewal capability of cancer stem cells.

5.9 miR-34

Another miRNA of great interest is miR-34. This TSmiR is down-regulated in various types of cancer, suggesting its tumor suppressor properties (He et al., 2007). We have researched this TSmiR rigorously. We used various assays to determine miR-34's role in cancer stem cells. In p53-deficient human gastric and pancreatic cancer cells, restoration of miR-34 inhibited cell growth and induced G1 phase block and apoptosis (Ji et al., 2008; Ji et al., 2009). This indicated that p53 function may be restored my miR-34. Restoration of miR-34 inhibited tumorsphere growth *in vitro* and tumor initiation *in vivo*, which is implicated to be correlated to the self-renewal potential of cancer stem cells (Ji et al., 2008; Ji et al., 2009). MicroR-34's mediated suppression of self-renewal seems to be through the direct modulation of its downstream targets of Bcl-2, Notch, and HMGA2 (Ji et al., 2008; Ji et al., 2009). This indicates that miR-34 is involved in the gastric and pancreatic cancer cells' self-renewal/differentiation decision making. Therefore, miR-34 is a rather significant tumor suppressor miRNA of cancer stem cells by regulating both apoptosis and self-renewal capabilities. Decreased expression of TSmiRs like these discussed above leads to cancer initiation and further tumor progression. **Figure 1** provides an overall schematic review of the stem cell miRNAs discussed concerning their interactions with stem cell signaling pathways in cancer stem cells.

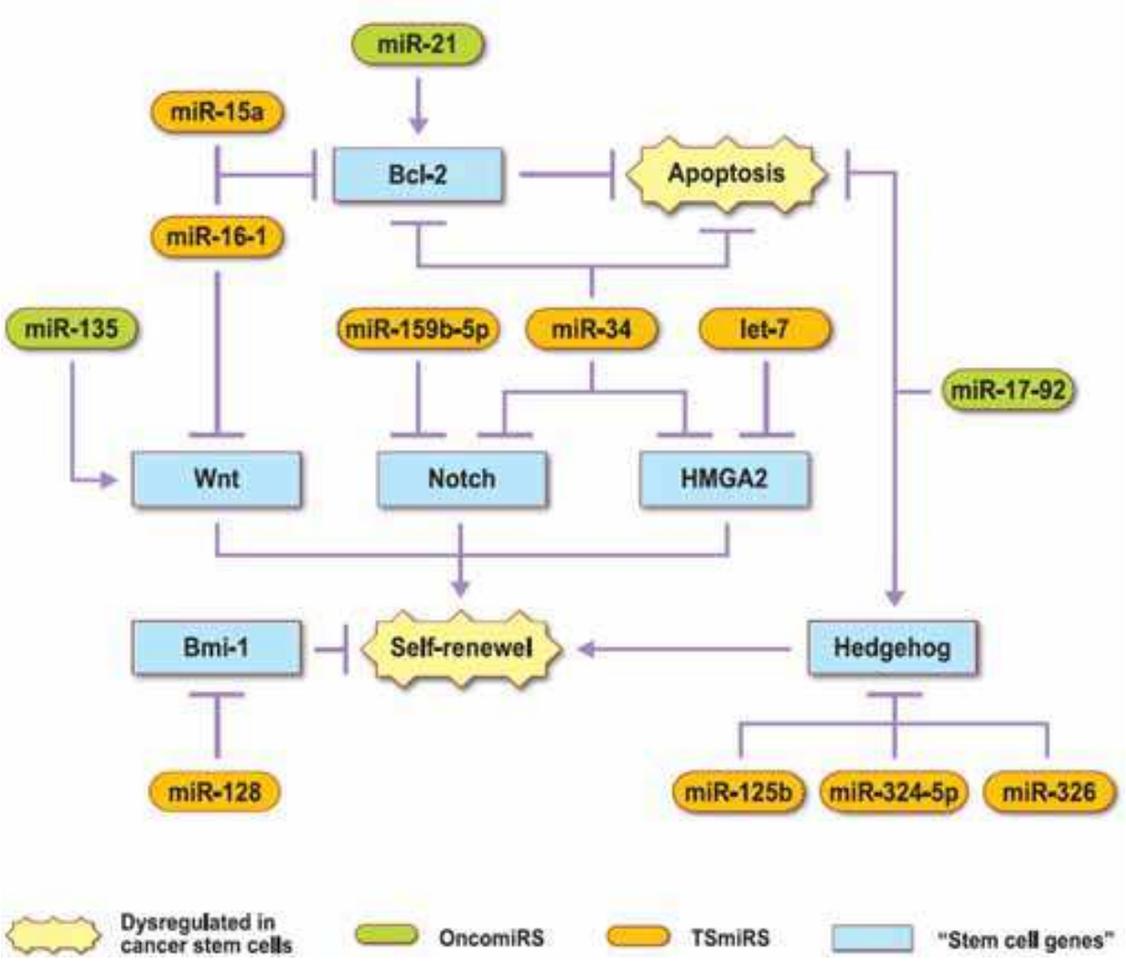

Fig. 1. **Potential "stem cell miRNAs" that modulate "stem cell genes" related to cancer stem cells.** Certain miRNAs have been shown to be aberrantly expressed in cancer. OncomiRs, which initiate cancer development, are over-expressed. TSmiRs, which prevent tumor development, are decreased. These miRNAs regulate genes that are implicated in stem cells. The aberrant expression of these potential "stem cell miRNAs" in cancer suggests that dysregulation of "stem cell genes" leads to increased levels of self-renewal and decreased levels of apoptosis within cancer stem cells. This results in further cancer progression. (Modified from DeSano and Xu, "MicroRNA regulation of cancer stem cells and therapeutic implications." AAPS J, 2009; 11(4):682-692 (DeSano and Xu, 2009). With permission.)

6. Cancer stem cells and miRNA connection in support of oncogenesis

There are aberrant expression levels of miRNAs in cancer. Tumors analyzed by miRNA profiling have been found to have significantly different miRNA profiles compared to normal cells from the same tissue (Calin et al., 2006). In addition, miRNAs have been found with rather convincing evidence to be important factors in stem cell biology. Using cDNA cloning, multiple miRNAs have been found to be uniquely expressed in human embryonic stem cells compared to their differentiated counterparts (Suh et al., 2004). Based on these

findings, it is rather intriguing that undifferentiated stem cells exhibit expression profiles of miRNAs that are reminiscent of cancer cells (Papagiannakopoulos and Kosik, 2008).

Still further research has allowed us to merge this obvious parallel even further. Recent evidence shows that there is a distinct subpopulation of cancer cells acting as cancer stem cells within tumors that have the ability to self-renew - thus initiating, maintaining, and progressing the cancer. Aberrant gene expression and function are hallmark characteristics of cancer. As a result of this, it is thought that genetic alterations from acquired epigenetic abnormalities cause dysregulation of genes within cancer stem cells (Zhao et al., 2008). The cancer stem cells are allowed to escape the restrictions of the stem cell niche because of this dysregulation. This results in self-renewal potential. Microenvironmental signals or factors are believed to account for the cancer stem cells' epigenetic abnormalities, resulting in the interference or silencing of certain genes. Thus, an underlying sub-cellular process must account for the cancer stem cell dysregulation.

Knowing that cancers exhibit aberrant expressions of miRNAs and miRNAs in general work through negatively regulating gene and protein expression, miRNAs can be this sub-cellular process. It is suggested and supported by recent findings that miRNAs cause gene dysregulation in cancer stem cells that leads to oncogenesis and further disease progression. All of the miRNA examples discussed have showcased this link between cancer stem cells and miRNAs. Yet, the question remains – how does this link translate and occur within the cancer stem cells themselves?

Most researchers believed and thus previous research has focused on the conventional miRNA hypothesis – that one miRNA is up-regulated or down-regulated, leading the activation of stem cell gene signaling pathways, which results in the cancer stem cell self-renewal and disease progression. This hypothesis is supported by the many oncogenic and tumor suppressor miRNA examples outlined. It is a rather straight forward hypothesis and data has been generated that has demonstrated these effects. However, could it be this simple? Could more be going on sub-cellularly?

A new possibility has emerged from the latest research. This new possibility proposes that the dysregulation in cancer stem cells is a result of an antagonism network between different miRNAs that stabilizes the switch between self-renewal ability and differentiation (Melton et al.). These different miRNAs could have oncogenic or tumor suppressor characteristics like the conventional hypothesis states. Nevertheless, this new possibility of an antagonism network implicates that miRNAs can regulate other miRNAs, initiating downstream dysregulation of cancer stem cell self-renewal potential. Researchers have found that the let-7 and the embryonic stem cells cell cycle regulating (ESCC) miRNAs like miR-294 have opposing effects of embryonic stem cell self-renewal and proposed that these miRNAs act in self-reinforcing loops to maintain self-renewal states versus differentiated states (Melton et al.). In the self-renewing state, ESCC miRNAs indirectly increase expression of Lin28 and c-Myc, and Lin 28 functions to block the maturation of let-7 (Melton et al.). Upregulated c-Myc forms a positive feedback loop in which c-Myc, N-Myc, Oct4, Sox2, and Nanog bind and activate ESCC miRNA expression (Melton et al.). This keeps the cells in a self renewal capable state. Thus, ESCC miRNAs like miR-294 prevent co-expression of let-7 miRNAs. Oncogenic miRNAs could regulate and block co-expression of tumor suppressor miRNAs causing cancer stem cell dysregulation.

In order to differentiate, Oct4, Sox2, and Nanog expression are down-regulated, resulting in the loss of Lin28 expression (Melton et al.). Losing Lin28 expression means that let-7 expression increases. This is even enhanced by a new positive feedback loop where let-7

suppresses the expression of its own negative regulator Lin28 (Melton et al.). This causes a loss of self-renewal potential and differentiation of the stem cells. In the differentiated state, let-7's down-regulation of Myc expression prevents co-expression of the ESCC miRNAs (Melton et al.). In this instance, tumor suppressor miRNAs regulate and prevent co-expression of oncogenic miRNAs resulting in dysregulation of cancer stem cells.

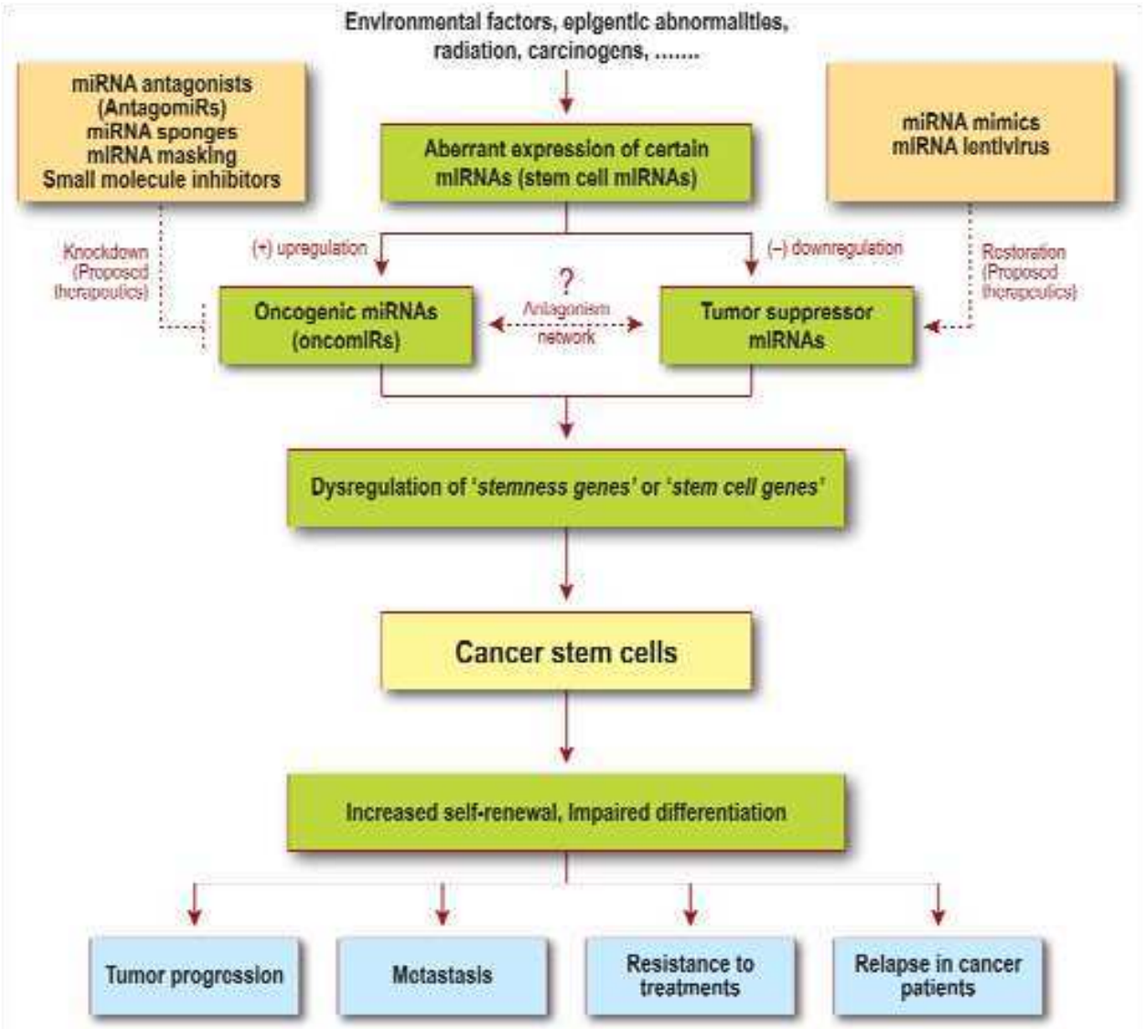

Fig. 2. **Link between miRNAs and cancer stem cells.** Aberrant expressions of miRNAs, either as oncogenic or tumor suppressor miRNAs, can lead to dysregulation of stem cell genes, causing increased self-renewal potential and impaired differentiation in cancer stem cells. This dysregulation subsequently results in carcinogenesis and oncogenesis. It is proposed that miRNA antagonists can knockdown the effects of oncogenic miRNAs, and miRNA mimics can restore the capabilities of tumor suppressor miRNAs. Therefore, miRNA could be a vital tool in addressing cancer stem cell dysregulation. MicroRNA-based molecular therapy could hold great therapeutic potential against cancer progression, resistance, and relapse. (Modified from DeSano and Xu, "MicroRNA regulation of cancer stem cells and therapeutic implications." AAPS J, 2009; 11(4):682-692 (DeSano and Xu, 2009). With permission.)

Thus, an antagonism network of miRNAs that stabilizes the switch between self-renewal and differentiation could be a possible sub-cellular mechanism that could explain the dysregulation of stem cell genes seen in cancer stem cells. This new antagonism network hypothesis is intriguing and needs to be further developed as well as the conventional miRNA hypothesis. Recent research has established and **Figure 2** outlines a rather convincing link between miRNAs and cancer stem cell dysregulation (DeSano and Xu, 2009). This dysregulation leads to increase self-renewal, resulting in tumor initiation and progression, metastasis, resistance to treatments, and relapse in cancer patients (Ji et al., 2010). Still, the underlying mechanism has evaded researchers. Studying and performing experiments that support or debunk either of these hypotheses will help the oncology community gain great insight into what is going on sub-cellularly in these terrible diseases and will allow us to attack the cancer with greater efficacy. Therefore, confronting abnormal miRNA expression levels with molecular miRNA therapy can be a promising and powerful tool to tackle oncogenesis (DeSano and Xu, 2009; Ji et al., 2009; Ji et al., 2010).

7. Potential miRNA-based molecular therapeutics

The distinct and clear connection between aberrant expression levels of certain miRNAs and dysregulation of cancer stem cells offers the scientific community an unique opportunity to fight cancer initiation and sustained development through the use of molecular miRNA therapies that target oncogenic or tumor suppressor miRNAs. In theory, molecular miRNA-based cancer therapy should eradicate the cancer stem cells' self-renewal potential, significantly reduce the cancer's resistance to current cancer treatment, and hinder relapse in sick patients.

For this reason, the development of miRNA-based molecular therapeutics has been at the forefront of oncology research recently. Still, there are many critical experimental steps that are required. The development of miRNA/RNAi-based therapeutics must include miRNA profiling of cancer compared to healthy tissue (specifically cancer stem cells compared to differentiated cells), functional analysis of dysregulated miRNAs, and *in vitro* followed by *in vivo* studies that include the use of differing RNA-based therapeutic techniques that address the aberrant miRNA expression levels (Papagiannakopoulos and Kosik, 2008). For oncogenic miRNAs, a therapeutic knockdown effect is needed because these miRNAs cause cancer when over-expressed. Potential therapies include antagomiRs, miRNA sponges, miRNA masking, and small molecule inhibitors. For tumor suppressor miRNAs, a therapeutic restoration is necessary because their expression levels are knockdown or non-existent in cancerous tissues. MicroRNA mimics or lentiviruses are possible methods that can re-establish the tumor suppressor capabilities of these miRNAs. All of these molecular therapeutic possibilities have the distinct purpose of regulating aberrant miRNA levels, which causes cancer stem cell dysregulation and disease progression. They could have a powerful impact on clinical cancer research.

For oncogenic miRNAs, an antagomiR (anti-miRNA oligonucleotide) can be used to block the effects of the oncomiR. The antagomiR uses competition to block the oncogenic interaction between the upregulated miRNA and its target mRNA, resulting in cancer suppression (Weiler et al., 2006). For example, an anti-miR-21 oligonucleotide was transfected into breast cancer MCF-7 cells and it was shown that this antagomiR suppressed cell growth in vitro and tumor growth in vivo by increasing apoptosis and decreasing cell

proliferation (Si et al., 2007). Thus, antagomiRs are a promising molecular therapeutic targeting oncogenic miRNA-initiated cancer stem cell dysregulation.

Another potential therapy against oncogenic miRNAs is miRNA sponges. A miRNA sponge is a synthetic mRNA, which contains multiple binding sites for an endogenous miRNA (Li et al., 2009). The sponge, in effect, competitively "soaks" up the oncogenic miRNA. This prevents the interaction between the miRNA and its specific mRNA targets that cause cancer stem cell dysregulation through the activation of stem cell genes. A single miRNA sponge could be used to stifle an entire miRNA family because of its multiple binding sites. These miRNA sponges inhibited miRNAs as effectively as antagomiRs *in vitro* (Ebert et al., 2007). However, the efficacy of these miRNA sponges need to be evaluated *in vivo* (Li et al., 2009). Still, miRNA sponges have great potential as a molecular therapy targeted against oncogenic miRNAs.

MicroRNA masking could be used to fight cancer initiation and progression through its regulation of aberrant miRNA expression levels. Each miRNA may regulate tens if not hundreds of genes, and a single gene can be regulated by multiple miRNAs (Li et al., 2009). The potential molecular therapies discussed above are only sequence-specific, which produces many obstacles like off-target side effects and undesired toxicity that researchers must confront. MicroRNA masking is instead gene-specific. MicroRNA masking employs the strategy of designing a sequence with perfect complementarity to the binding site in the target gene for an endogenous miRNA, which can form a duplex with the target mRNA with higher affinity (Li et al., 2009; Xiao et al., 2007). This miRNA masking effectively blocks access of the miRNA to its binding site without any possible side effects because it is gene-specific instead of sequence-specific like the antagomiRs or sponges. MicroRNA masks that were complementary to cardiac pacemaker channel genes HCN2 and HCN4 significantly enhanced HCN2/HCN4 expression and function by inhibiting the suppressive actions of endogenous miR-1 and miR-133 (Xiao et al., 2007). These results demonstrate that miRNA masking can be an important molecular miRNA-interfering therapeutic strategy that is gene-specific and can be directed against aberrant oncogenic miRNA expression levels that activate self-renewal genes in cancer stem cells.

Oncogenic miRNAs can be down-regulated and even knocked out through the use of small molecule inhibitors. Since oncogenic miRNAs cause cancer stem cell dysregulation and disease progression when over-expressed, the small molecule inhibitors must block the formation or generation of these miRNAs. Thus, small molecule inhibitors that target the steps in the biogenesis of miRNAs could hold much promise. Azobenzene has been shown to be a specific and effective inhibitor of the biogenesis of miR-21 (Gumireddy et al., 2008). In an experiment that utilized miRNA array analysis, it was demonstrated that there was a rapid alteration of miRNA levels in response to the potent hydroxamic acid HDACi LAQ824 in the breast cancer cell line SKBr3 (Scott et al., 2006). In addition to blocking miRNA formation and function, small molecule inhibitors of the miRNA pathway could be promising tools used to boost patient response to existing chemo- and radiotherapies (Gumireddy et al., 2008). This can be seen in data from our lab. We have employed multiple small molecule inhibitors – Gossypol, SH-130, Celastrol, and Embelin – and demonstrated that they all can sensitize cancer cells to ionizing radiation therapy and induce apoptosis *in vitro* and *in vivo* (Dai et al., 2009; Dai et al., 2008; Lian J, 2010; Meng et al., 2008; Wu et al., 2010). Therefore, small molecule inhibitors have great potential in addressing oncogenic miRNAs that cause dysregulation of cancer stem cells. They could fight against tumor initiation and progression, metastasis, resistance to treatments, and relapse in cancer patients.

Nevertheless, not all miRNAs that cause cancer are oncogenic/up-regulated. Many tumor suppressor miRNAs are down-regulated in cancer tissues. Thus, their expression needs to be reinstated in order to fight the disease. The first way that we can therapeutically attack the down-regulated tumor suppressor miRNAs is through the introduction of miRNA mimics. MicroRNA mimics are small, chemically modified, double-stranded RNA molecules that mimic endogenous mature miRNA molecules (Li et al., 2009). These miRNA mimics are simply just re-introducing RNA molecules that can pose and fill the role of the missing endogenous miRNA molecules that were down-regulated due to mutation, etc. For example, we introduced miR-34 mimics into cancer cells by transfection. These miR-34 mimics were found to arrest the cell cycle in the G1 phase, significantly increase the activation of caspase-3 and apoptosis, and decrease the expression of its downstream targets of bcl-2, Notch, and HMGA2 (Ji et al., 2008). The use of this mimic restored miR-34 with its tumor suppressor potential and capabilities. Thus, the use of miRNA mimics as a therapy to restore the expression of tumor suppressor miRNAs could help in defeating the aberrant miRNA expression profiles that cause cancer stem cell dysregulation.

However, miRNA mimics might not be the greatest molecular therapy for tumor suppressor miRNAs because the transfection of mimics can only last a couple of days and thus, the long-term biological effects cannot be examined. Nevertheless, there are ways to overcome this obstacle. One of these ways is viral vector-based gene restoration (Li et al., 2009). Researchers have been able to engineer lentiviral vector systems. Cells can be infected with a lentivirus that expresses a certain miRNA. This infection re-establishes the tumor suppressor miRNA back into the cell, and this lentiviral vector system generates stable cells that continue to express the miRNA. These stable tumor suppressor miRNA-expressing cells can be analyzed for a long period, which solves the dilemma posed by the miRNA mimic therapy. In our lab, we infected gastric cancer cells with a lentivirus that expressed miR-34a. This produced stable cancer cells that expressed miR-34a. This lentivirus was found to inhibit cell growth and tumorsphere formation (Ji et al., 2008). These results showed the promise of the lentiviral system *in vitro* and *in vivo*. We also tested this lentiviral system in pancreatic cancer stem cells and observed the same effects (Ji et al., 2009). The lentivirus vector system was able to restore the tumor suppressor ability of miR-34. Therefore, viral vector-based miRNA restoration has potential to reinstate tumor suppressor miRNAs that have been down-regulated or knocked out, resulting in cancer stem cell dysregulation and tumor development.

8. The seemingly never solved problem – delivery, delivery, delivery

To achieve a strong therapeutic effect with any of these potential molecular therapies, we must be able to translate our research from our labs to the clinics. However, in order to be clinically ready, the miRNA-based therapeutics must be effectively, efficiently, and functionally delivered to the cancerous tumor. This has been a great challenge for researchers since the beginning of cancer therapy.

A new exciting field has emerged that has focused on nanotechnology for systemic delivery of therapeutics *in vivo*. In theory, the nanoparticle would encompass the miRNA-based therapeutic, target it to the cancerous tumor, and effectively and efficiently deliver it to the cancer cells while bypassing (and not affecting) the normal, healthy cells in the body. Nanoparticle technology could be essential to delivering a wide variety of therapies to various yet specific cells in the body. It could be a part of breakthrough treatments for many

diseases that could link *in vitro* and *in vivo* studies. Researchers have tirelessly attempted to develop a nanoparticle system that would allow this to happen.

Many attempts have failed; however, some strategies have been proven to be rather successful. The human transferrin protein receptor (TFR) has been known to be up-regulated in malignant cancer cells. Using patients with metastatic melanoma, it was shown that a synthetic nanoparticle delivery system that contains a linear, cycodextrin-based polymer, a human transferrin protein (TF) targeting ligand on its exterior to engage TF receptors, a hydrophilic polymer used to promote nanoparticle stability in biological fluids, and siRNA designed to reduce the expression of RRM2, reduced RRM2 mRNA levels as well as RRM2 protein levels (Davis et al.). Tumor biopsies from melanoma patients obtained after treatment showed the presence of intracellularly localized nanoparticles in amounts that correlate with dose levels of the nanoparticles administered (Davis et al.). This is the first in-human phase I clinical trial involving the effective systemic administration of siRNA to patients with solid tumors using a targeted, nanoparticle delivery system (Davis et al.). This study demonstrates that systemic administration of siRNA to a human can produce an inhibition effect of a specific gene by an RNAi mechanism. This is rather exciting and because siRNA was successfully targeted and delievered one can infer that a systemic administration of miRNA-based therapeutics can be effectively delivered to cancer cells via nanoparticles.

We have developed a tumor-specific, ligand-targeting, self-assembled, nanoparticle-DNA lipoplex systems designed for systemic gene therapy of cancer (US Patents No. **6,749,863** and **7,479,276**) (Xu et al., 2002a; Xu et al., 2002b). These nanovector systems employ transferrin or scFv against transferrin receptors as tumor-targeting ligands (Xu et al., 2002a; Xu et al., 2002b). When using Tf as a targeting ligand, we obtained the self-assembled nanovectors at the sizes of 50-90nm, with highly compact structure and favored surface charge (Xu et al., 2002a,b). These nanovectors have novel nanostructures that resembles a virus particle with a dense core enveloped by a membrane coated with Tf molecules spiking on the surface (Xu et al., 2002a,b). This nanovector system demonstrates promising efficacy and specificity in targeted delivery of various genes and anti-sense oligonucleotides like p53 to cancer *in vivo* compared to normal tissues (Xu et al., 1997; Xu et al., 1999). This nanovector system shows promising efficiency and specificity in targeted delivery of various genes and anti-sense oligonucleotides to cancer but not normal tissues *in vivo*. In the AACR 101th Annual Meeting, Washington, DC, April 17-21, 2010, at the Late-breaking Oral Presentation session on clinical trials, Drs. Pirollo and Chang reported the success of a first-in-man, Phase I trial of this nanovector, TfRscFv-nano-p53 (SGT-53, NCT00470613, ClinicalTrials.gov). The nanovectors are well tolerated in humans and already showed early responses. The exogenous p53 expression was observed in human cancer tissues in a SGT-53 dose-dependent manner, but *not* in normal tissues. The study demonstrates that the nanovectors are safe and effective to deliver gene therapeutics to both primary tumors and metastatic lesions. These unprecedented findings in cancer gene therapy trial subjects represent a major breakthrough in the field and suggest that delivery of genes to tumors with selectivity is indeed possible (Pirollo, *et al*, LB-172, www.aacr.org). The success of these nanovectors provides a potential and rather promising tumor-targeted delivery system for novel RNAi-based therapeutics. This is a thrilling possibility because packaging miRNA-based therapeutics discussed above into nanoparticles that can be effectively and efficiently targeted and delivered to cancerous tumors could remedy aberrant miRNA expression levels that are responsible for cancer stem cell dysregulation and subsequent oncogenesis.

9. Conclusion

In this chapter, we explore the connection between microRNAs and cancer stem cells. Abnormal miRNA expression profiles of oncogenic and/or tumor suppressor miRNAs are linked to the activation of stem cell signaling pathways in cancer stem cells. This dysregulation of cancer stem cells leads to disease initiation, development, progression, metastasis, resistance to current treatments, and relapse in patients. Accordingly, the development and use molecular miRNA therapies are imperative to addressing oncogenesis. In addition and maybe more importantly, effective and efficient packaging, targeting, and delivery of these miRNA-based therapeutics needs to be addressed. Nanoparticle technology could hold the key to accomplishing this. For this reason, future research needs to be aimed at developing nanoparticle delivery systems as well as uncovering the subcellular intricacies of miRNA regulation of cancer stem cells' self-renewal potential and capabilities. Defeating cancer stem cell dysregulation through molecular miRNA therapies could aid in the fight against cancer progression, resistance, and relapse.

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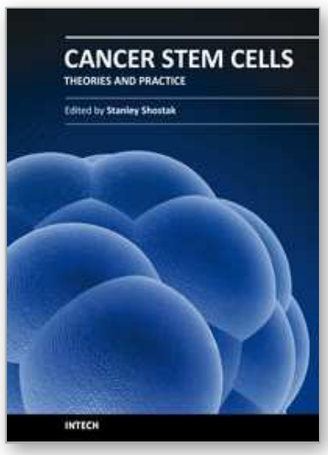

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Cancer Stem Cells Theories and Practice does not 'boldly go where no one has gone before!' Rather, Cancer Stem Cells Theories and Practice boldly goes where the cutting edge of research theory meets the concrete challenges of clinical practice. Cancer Stem Cells Theories and Practice is firmly grounded in the latest results on cancer stem cells (CSCs) from world-class cancer research laboratories, but its twenty-two chapters also tease apart cancer's vulnerabilities and identify opportunities for early detection, targeted therapy, and reducing remission and resistance.

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