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Molecular Pathways: MicroRNAs as Cancer Therapeutics

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Abstract

MicroRNAs (miRNAs) are approximately 18-25 nucleotides in length and affect gene expression by silencing the translation of messenger RNAs. Since each miRNA regulate the expression of hundreds of different genes, miRNAs can function as master-coordinators, efficiently regulating and coordinating multiple cellular pathways and processes. By coordinating the expression of multiple genes, miRNAs are responsible for fine-tuning the cell's most important processes like the ones involved in cellular growth and proliferation. Dysregulation of miRNAs appears to play a fundamental role in the onset, progression and dissemination of many cancers, and replacement of down regulated miRNAs in tumor cells results in a positive therapeutic response. Thus, in theory, inhibition of a particular miRNA linked to cancer onset or progression can remove the inhibition of the translation of a therapeutic protein – and conversely, administration of a miRNA mimetic can boost the endogenous miRNA population repressing the translation of an oncogenic protein. Although several basic questions regarding their biological principles still remain to be answered, and in spite of the fact that all data with respect to miRNAs and therapy are still at the preclinical level, many specific characteristics of miRNAs in combination with compelling therapeutic efficacy data have triggered the research community to start exploring the possibilities of employing miRNAs as potential therapeutic candidates.

BACKGROUND

Recent research has shown us that the non-protein-coding portion of the genome is crucial for gene expression regulation in a normal as well as diseased setting. The functional relevance of this fragment of the genome is particularly evident for a class of small non-coding RNAs called microRNAs (miRNAs). MiRNAs are a class of small, evolutionary conserved, non-coding RNAs of 18-25 nucleotides in length that post-transcriptionally control the translation of mRNAs (1). The number of miRNAs is growing rapidly and more than 700 miRNA genes have already been identified in the human genome alone, which approaches about ~3% of the number of all human genes (Sanger miRBase). MiRNAs are predicted to regulate the translation of more than 60% of protein-coding genes thus

coordinating many processes, including proliferation, development, differentiation and apoptosis (2). Thus, miRNAs constitute one of the most abundant classes of gene-regulatory molecules in animals. Due to their involvement in all cellular processes, the abnormal expression or alteration of miRNAs contributes to a range of human malignancies, including cancer. The biogenesis of miRNAs is a multi-step process that is closely related to their regulatory functions. The biosynthesis starts in the nucleus of the cell following transcription where a precursor miRNA (pre-miRNA) is produced through the action of Drosha, and further continues through the cytoplasm where Dicer processes it to the mature functional miRNA with the ability to silence target mRNA translation (Fig. 1; refs. 3, 4).

Interestingly, there is a wide range of miRNAs that map to regions of the human genome that are known to be frequently deleted or amplified in cancer (5). This finding suggested that miRNAs could contribute to the development of cancer and opened a new area of research for miRNA dysregulation in human cancer. Subsequently miRNAs were shown to be differentially expressed in cancer cells, in which they formed unique miRNA expression patterns (6). Dysregulation of miRNAs in cancer can occur through epigenetic changes and genetic alterations, which can affect the production of the pri-miRNA, their processing to their mature miRNA form and/or interactions with mRNA targets. The recent findings of genetic defects in cancer associated genes of the miRNA processing machinery, such as TARBP2 (7), Dicer (8) and XPO5 (9), has strongly highlighted the relevance of these pathways in cellular transformation, where such defects contribute to a global dysregulation of miRNAs in cancer. Although miRNAs have been categorized as oncogenic or tumor suppressive, the miRNA expression profile of human tumours is characterized by the impairment of miRNA production that results in global miRNA downregulation (6- 10). The first high-throughput study accessing miRNA expression profiles in cancer was done in 334 patient samples of various cancer types, and showed that miRNA expression profiles can distinguish the developmental lineage and differentiation state of the tumors (6). In fact, miRNA profiling can be more accurate at classifying tumors than mRNA profiling because miRNA expression correlates closely with tumor origin and stage (6). Further studies were able to identify the tissue of origin of metastatic tumors with unknown primary origin based on the miRNAs expression profiles (6). Nonetheless the detection of global miRNA expression patterns for the diagnosis of cancer has not yet been proved in spite of the promising results of some individual or small groups of miRNAs, like the combination of high miR-155 and low let-7 expression in non-small cell lung cancer that correlates with poor prognosis (11).

Much of what we have learnt concerning the functional contribution of specific miRNAs to cancer development is based in studies using germline transgenic and knockout mice. Several strains of mice silencing or overexpressing cancer-associated miRNAs have been developed and characterized in an *in vivo* context recapitulating the behavior of some human malignancies, what can be extremely useful to evaluate therapeutics. Perhaps the most exciting fact that has urged from our understanding of miRNA biology is the potential use of miRNA mimics or antagonists as therapeutics. Because miRNA expression is often altered in cancer cells, agents that modulate miRNA activity could potentially produce cancer-specific effects. However, to evaluate the potential of small RNAs in cancer therapy, an

appreciation of how extensively they are tied to the normal cell's gene regulation networks is essential. Overexpression or inhibition of miRNAs can be achieved in several ways. Synthetic miRNA mimics include siRNA-like oligoribonucleotide duplex and chemically modified oligoribonucleotide (12, 13). Conversely, miRNAs can be inhibited by various modified antisense oligonucleotides such as 2'-O-methyl antisense oligonucleotide and antagomirs. As the first successful tool for knockdown of a miRNA *in vivo*, antagomirs are of special interest as they appear to be delivered to all tissues (except brain) after tail vein injections into mice (14, 15). Delivering tumor-suppressive miRNAs and silencing oncogenic miRNAs with antagomirs as been successful in several mouse models and as progressed to delivering miRNA-based molecules intranasally, intratumorally or systemically. Nonetheless the therapeutic value of miRNA mimics and antagomirs would be greatly enhanced by technical improvement for selective tumor-specific or tissue-specific delivery. In the same regard, miRNAs are also being evaluated for their ability to sensitize cancers to chemotherapy.

CLINICAL-TRANSLATIONAL ADVANCES

Therapies that Inhibit Oncogenic MiRNA Function

In spite the overall downregulation of miRNAs observed in human cancer some specific upregulated miRNAs with oncogenic potential (oncomiRs) are potential therapeutic targets for inhibition. Oncogenic miRNAs can be inhibited by using antisense oligonucleotides, antagomirs, sponges or locked nucleic acid (LNA) constructs (16). The effectiveness of these antisense oligonucleotides has demonstrated promising results in certain cases.

One of such cases was described in a breast cancer mouse model where the systemic delivery of antagomir-miR-10b to tumor-bearing mice reduced miR-10b levels and prevented the onset of metastasis, suppressing dissemination to the lungs (17). This work suggests that antagomir-miR-10b may prevent metastasis in highly invasive cancers containing elevated miR-10b levels.

Likewise, the use of LNA constructs has demonstrated great success in the treatment of hepatitis C in non-human primates, inhibiting the virus replication through inhibition of miR-122 using an LNA construct systemic delivered with no toxicity observed (18). The inhibition of miR-122 in patients who are hepatitis C-positive may decrease the risk associated of developing hepatocellular carcinoma.

However, cancer cells often exhibit multiple miRNA dysregulation, therefore silencing of a single miRNA might not be sufficient for use in cancers where more than one oncomir is being overexpressed. Recent research suggests that several miRNAs can be simultaneously inhibited using one antisense oligonucleotide that targets multiple miRNAs: multiple-target anti-miRNA antisense oligodeoxyribonucleotide – MTg-AMO (19). The authors have described an MTg-AMO that is able to inhibit at the same time three very well described oncomirs that are often overexpressed in many tumors, miR-21, miR-155 and miR-17-5p (19). The use of this MTg-AMO resulted in an enhanced suppression of cancer growth compared with individual inhibition of the miRNAs (19). This technique will allow for the

simultaneous inhibition of several miRNAs involved in various aspects of cancer biology like angiogenesis, invasion or metastasis.

The success of the inhibition of miRNAs for therapy will depend on their pharmacokinetic behavior as this will serve as a basis for predicting how the compound will behave in humans. Most anti-miRs distribute broadly but tend to accumulate in a characteristic pattern with most in liver and kidney and each chemical modification alter this characteristic pattern. Thus it becomes important to characterize each one of the molecules used in animal models for prediction in humans. Also plasma anti-miRs are cleared from plasma within hours by uptake into tissues but, once inside cells, the anti-miRs are very metabolically stable so their clearance is slow and half-lives in tissues are often in the order of weeks, providing therapeutic benefit long after blood levels are near zero (20, 21).

Therapies that Restore Tumor-Suppressive MiRNA Function

miRNA replacement therapy has been strategically used to name the therapy that aims to restore miRNAs with tumor-suppressive functions. MiRNA let-7 is one of the best-described tumor-suppressive miRNAs (22). In xenograft models tumor burden was reduced by intra-tumoral delivery of let-7b, as well as the burden of KrasG12D/+ lung tumors through the intranasal delivery of let-7a using lentivirus or by the systemic delivery of let-7b (23, 24). Let-7 miRNA is downregulated in multiple cancers hence their oncogenic targets are overexpressed enhancing tumor proliferation, invasion and metastasis, what makes this cancers perfect targets for the use of let-7 miRNA mimetics as therapeutic tools.

Oncogenic KRAS is targeted by several different miRNAs, but it also inhibits the transcription of a miRNA cluster containing miR-143 and miR-145, through the activation of RAS-responsive element binding protein 1 (RREB1; refs. 25-27). In a very sophisticated mechanism miR-143 and miR-145 target RREB1 and KRAS respectively, inhibiting their translation (26). Systemic intravenous delivery of miR-143 and miR-145 to subcutaneous and orthotopic xenografts downregulated RREB1 and KRAS levels (28). A high throughput analysis of 744 cancer samples has revealed miR-143~145 cluster as frequently deleted. Therefore these tumors, as well as tumors with KRAS overexpression, would be good candidates for therapy using miR-143 and miR-145 miRNA restoration therapy.

P53 protein has been found to affect microRNA processing by enhancing the maturation of several growth suppressive miRNAs — its third known anti-tumour activity (29). Most cancerous p53 mutations affect the domain that interferes with miRNA processing and thus may abolish all tumour-suppressor functions. One of the best-studied miRNAs whose processing is enhanced by p53 and it is downregulated in various cancerous, is miR-34 that stimulates apoptosis or cellular senescence, induces G1 arrest and prevents migration (30). Recent research has demonstrated that miR-34 replacement therapy would be of great therapeutic benefit. Delivery of miR-34 mimic either intra-tumorally or systemically impaired tumorigenesis on a xenograft model of non-small cell lung cancer; in the same regard systemic delivery of miR-34 reduced tumor growth of KrasLSL-G12D/+ lung tumors (31, 32). Knowing that a high percentage of prostate as well as pancreatic cancers contain p53 mutations and/or attenuated miR-34 expression, miR-34 should be also considered as a good therapeutic approach for these types of cancers (28, 32).

A recent study reports the restoration of miR-26a expression in a hepatocellular carcinoma mouse model (33). The treatment has resulted in suppression of proliferation and induction of apoptosis, inhibiting cancer progression (33). In this model an adenoviral vector was used to systemic deliver the miR-26a mimic what can be very limiting due to the challenges it presents. The use of these molecules as therapeutics would undoubtedly impact cancer treatments by allowing the restoration of miRNAs that in most cases target oncogenic transcription factors that are difficult to inhibit through traditional medicinal chemistry (34). Also, miRNA-mimetics present the same pharmacokinetic characteristics described for anti-miRs what renders them good candidates for use in humans (20, 21).

In spite the promising therapies that aim to restore the function of one miRNA we are still far from restoring the “normal” expression of miRNAs in tumors. A large body of evidence shows that human tumors are characterized by impaired miRNA processing that lead to global miRNA downregulation. Some chemical compounds alter the expression of a group of miRNAs; therefore it may be possible to screen for drugs that could shift the miRNA expression profile of a cancer cell toward that of a normal tissue (35). Recent findings have shown that restoring the global miRNA expression (miRNAome) could have a therapeutic effect (36, 37). By modulating multiple miRNAs simultaneously, such a miRNAome modifying approach may be much more effective for therapy than strategies that aim to regulate a single miRNA. Reconstitution of downregulated miRNAs offers the theoretical edge of correcting the malignant defect by inducing small changes in miRNA gene dosage to a homeostatic level achieving phenotypic alterations that counteract malignant transformation. Few studies of fluoroquinolones have demonstrated a significant growth inhibition of some tumor cells including translational cell carcinoma of bladder, colorectal carcinoma and prostate cancer cells (38-40). Enoxacin belongs to this family of synthetic antibacterial compounds based on a fluoroquinolone skeleton (41). Enoxacin has been used to treat bacterial infections ranging from gonorrhea to urinary tract infections (42). Clinically, side effects have been minimal in adults (42). This small molecule also enhances RNAi induced by either shRNAs or siRNA duplexes (37). A new “miRNAome-based” strategy to restore global miRNAs expression has been suggested (36). The small molecule enoxacin enhances RNAi and promotes processing by binding to TRBP (36, 37). After being administered to human cancer cells lines and systemically delivered to xenograft mouse models it as restored downregulated miRNAs to a more “normal-like” miRNA expression pattern and tumor growth has been inhibited with great efficiency (36). Most importantly, the drug showed no effect on normal cells and was not associated with toxicity in the mouse models (37).

Using MiRNAs to Sensitize Tumors to Chemotherapy

The design of cancer chemotherapy has become increasingly sophisticated, but there is still no cancer treatment that is 100% effective against disseminated cancer. Chemotherapy resistance occurs when cancers that have been responding to a therapy suddenly begin to grow. Acquired resistance to chemotherapy is a major obstacle to successful cancer treatment. The identification of new approaches that could circumvent this problem would be key to sensitize resistant cells to commonly used cancer therapies. Because miRNAs target a multitude of mRNAs involved in different signaling pathways that are often

impaired in cancer, the potential of using miRNAs or antagomirs to make the tumors responsive to chemotherapy is of great promise. However this is still a growing field therefore *in vivo* data is not abundant. Nonetheless, recent *in vitro* experiments have shed light in what can become the new era of tumor sensitization drugs. One of the most common reasons for acquisition of resistance to a broad range of anticancer drugs is expression of transporters that detect and eject anticancer drugs from cells.

The miRNA miR-9 as been described in a recent report to be a negative regulator of SOX2, which induces indirectly ABCC3 and ABCC6 transporters (43). Therefore when SOX2 is overexpressed, cancer cells are able to excrete drugs to the extracellular environment becoming resistant (43). The overexpression of mir-9 in a glioma stem cell line (chemotherapy-resistant) resulted in reduced ABC transporter expression and resulted in increased drug retention (43). This is hopeful data in reducing cancer cells drug efflux through manipulation of miRNAs.

Another example is the resistance to tamoxifen, a widely used oestrogen receptor modulator. These tumors repress miRNAs miR-15 and miR-16 and are able to restore the anti-apoptotic BCL-2 expression (44). In this regard, re-expression of miR-15 and miR-16 decreased BCL-2 expression and cells become sensitized to tamoxifen. The same results proved true for miR-342 which forced overexpression sensitized cells to tamoxifen-induced apoptosis (45).

5 - Fluorouracil (5-FU) is being used as a chemotherapy agent against cancer for more than 40 years. It acts mainly as a thymidylate synthase inhibitor blocking the synthesis of thymidine; therefore rapid dividing cancer cells undergo cell death (46). A recent miRNA profiling report as unraveled high miR-21 levels as being correlated with poor treatment outcome in colon cancer due to the direct downregulation by miR-21 of genes of the mismatch repair machinery of the cells (47). The same results stand true for hepatocellular carcinoma and pancreatic cancer (48, 49). In this regard, antagomir against miR-21 was able to sensitize cultured cells to 5-FU treatment suggesting this miRNA therapy as a promising path for tumors resistant to 5-FU that overexpress miR-21 (49).

Off-Target Effects

Nonspecific side effects are as important as effectiveness and duration of miRNA expression or inhibition. Each miRNA can target up to 200 transcripts directly or indirectly, and multiple miRNAs can target a given gene. Therefore the potential regulatory network afforded by miRNAs is extremely complex and the therapeutic outcome of a miRNA therapy will directly depend on the number of targets and the affinities for each one of those targets that are expressed in that specific cellular context. Also delivery to the appropriate cell type or tissue is an important aspect of effective miRNA mimicry to prevent unwanted side effects.

Some investigators have argued that miRNA mimics or inhibitors are specific enough to distinguish between similar miRNAs (50, 51). However, cross-reactivity between miRNAs of similar sequence is likely to be unavoidable at high doses of antagonists or agonists. Another possible side effect is that high expression of miRNA mimics may interfere with

endogenous miRNA action by saturating the cellular machinery for miRNA processing or action. This may result in a change in expression of other miRNAs, leading to a deleterious effect in the cells. Indeed, a fatal side effect as a result of saturation of miRNA pathways has been reported (52). To minimize undesirable side effects, the expression or knockdown of a miRNA should be improved so that it is more accurate and controllable. An alternate approach to improving specificity is to target the pre-miRNAs with antisense or siRNA strategies.

Despite of all the progress that has been made in the new era of miRNA therapeutics there is still a significant gap between basic research on miRNAs and clinical application. Extensive preclinical and translational research is required to increase the efficacy and minimize the side effects of miRNAs-based therapy.

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Figure 1.

MiRNAs are transcribed mainly by RNA polymerase II into an immature form of about 80 nt in length called primary microRNAs (pri-miRNA; ref. 3). The stem loop structure of the pri-miRNA is recognized in the nucleus by Drosha and its partner DGCR8 and it is further processed to the precursor miRNA (pre-miRNA; ref. 3). The hairpin shaped pre-miRNA is then transported from the nucleus to the cytoplasm by exportin-5 (XPO5) where it is loaded by the Dicer-TRBP complex and cleaved into a double-stranded miRNA in a process known as dicing (3). After strand separation the mature miRNA, in combination with Argonaute proteins, form the RNA-induced silencing complex (RISC; ref. 4). The expression of the target mRNAs is silenced by miRNAs on the RISC complex, either by mRNA cleavage or by translational repression (4).