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MicroRNAs: Regulatory Function and Potential for Gene Therapy

When Victor Ambros, Rosalind Lee, and Rhonda Feinbaum published the first finding of a microRNA, lin-4, in 1993 (Bartel 281), it spawned a cascade of research on these new biological molecules. In less than two decades, groups have found and studied the functions of hundreds of different microRNAs, and artificial microRNAs like siRNAs have been developed for transfection and further study. As researchers begin to appreciate the growing importance of microRNAs for *in vivo* gene regulation, this knowledge has naturally led to the interest in microRNAs as either the targets or the means of gene therapy for cancers. This paper will describe the microRNAs' regulatory function, particularly in relation to cancer, and it will describe the ongoing proposals and obstacles for microRNA gene therapy.

MicroRNAs, shortened as miRNAs, are small, noncoding RNAs about 22 nucleotides in length. miRNAs are different from normal messenger RNAs because they function as transcriptional regulatory molecules which control the messenger RNAs and thus the expression of genes. Generally, they are coded from the introns of other known genes, but some have their own promoter. According to Bartel, the miRNA genes code for a miRNA precursor, pri-miRNA, which forms a long hairpin loop. Endonuclease Drosha cleaves this precursor in the nucleus, which is then exported to the cytoplasm where Dicer cuts it into its mature form, and a helicase makes the miRNA single-stranded (287). This mature microRNA functions as the regulatory molecule which binds to the messenger RNA, usually at the 3' UTR, and either cleaves the mRNA and destroys it, or binds to the mRNA and prevents the ribosomes from translating it. In this way, the miRNA controls gene expression at the translational level. The

importance of miRNAs is shown in the strong conservation of genes coding for them within closely related organisms, as well as the fatal phenotypic effects of not having them. Mice lacking the Dicer endonuclease selectively in the heart muscle cells, for example, died within weeks of birth from cardiac failure (Zhao 129), likely due to a loss of mature miRNAs to regulate cardiac muscle function. miRNAs are at least superficially important because their loss is fatal.

More specifically, miRNAs are crucial in proper gene expression regulation throughout the body. Their influence throughout the body can be seen in the wide range of functions that they control. According to Stefanie Sassen, papers have been produced demonstrating the importance of miRNA in the regulation of diverse biological processes such as cell division and cell death (8). A specific example is in the immune system, where Bradley S. Cobb and his group suggest that Dicer produces miRNAs that activate and mature T regulatory cells in the thymus (9). Yunhe Xu and his group also showed that artificially transfected siRNA control 1 had a similar effect, inducing differentiation of adipocyte cells (4). miRNA seems to play an important role in differentiation and in maintaining the cell lineage of a committed cell. Finally, miRNAs have been shown to be important in the maintenance of stem cell lines in the body. According to Sassen, Bernstein showed that with the removal of the Dicer1 gene, no viable stem cell lines could be grown, and the mouse died before birth (8). Since cancer cells behave much like stem cells, a disruption of the miRNA regulatory pathway can easily lead to cancer due to a loss of proper cell lineage and quiescence. Clearly, for a set of regulators that affects the body so universally, any disruption of this regulation has drastic and even carcinogenic consequences.

The fatal effect of the loss of miRNAs is shown specifically with respect to the onset of cancer. Research groups have shown that certain miRNAs act as tumor suppressors for

oncogenes. Mayr, Hemann, and Bartel, for example, showed that let-7 miRNA is a suppressor for oncogene Hmga2, and disruption of let-7 results in tumorigenesis (1576). Hmga2, or High Mobility Group A2, allows for anchorage independent growth and mobility of tumor cells. Since miRNAs recognize their target mRNAs by the specific base pairing of the miRNA to the mRNA at the 3' UTR, certain mutations of either the target site or the miRNA itself prevent proper binding of the miRNA to the mRNA. When let-7 or Hmga2 is mutated, the luciferase assay with the cloned UTR of Hmga2 can produce luciferin because there is no translational repression. Another example is miR-15a and miR-16-1, which, according to George A. Calin, are tumor suppressors because they induce apoptosis of cancer cells (15). The locus where miR-15 and miR-16 genes are located is a hot spot for chromosomal translocations, resulting in mutated microRNAs which cannot regulate apoptosis and, when cancer cells grow, cannot be stopped from continual proliferation. Leukemia patients, for example, generally show decreased levels of miR-15 and miR-16. The mechanism of miR-15 and -16's activity is likely in repressing multiple transcripts which signal the cell division cycle. miRNAs, then, can be important preventers of cancer.

On the other hand, there are certain miRNAs which actually act oncogenic and enhance the growth of cancer. Intuitively, they seem to work by downregulating the tumor suppressors or the expression of genes which code for tumor suppressors. According to Espinosa and Slack, miR-155, for example, seems to enhance the effects of oncogene MYC, but it also acts as an oncogene itself when overexpressed in mice (18). MYC normally codes for transcriptional factors, but when mutated, becomes oncogenic and results in a loss of transcriptional control usually of cell division. Both oncogenic MYC and miR-155 promote uncontrolled cell proliferation. Overexpression of miR-155 is found in many patients with B-cell lymphomas.

Another example is miR-21, which by itself is not oncogenic, but with the activation of oncogenes and the growth of tumors, sustains the cancer cells by turning off cell apoptosis signals (Gammell 19). The tumor cells are allowed to proliferate freely without cell death. Micro-RNAs, then, have a dynamic relationship with cancers, working either to suppress them or to benefit them.

Due to the strong connection between miRNAs and cancer, possible gene therapy treatments have focused on utilizing the current knowledge of miRNAs. The fact that certain miRNAs act as tumor suppressors provide one means of therapy by replenishing these miRNAs or supplementing currently existing ones; concurrently, the fact that certain miRNAs are oncogenic provide a potential gene therapy target. According to Rengaswami Rajaraman, cancer is often caused by oncogenes. These oncogenes are normally quiescent but are turned on by mutations (3). Meiko Takahashi's group and Christine Mayr have independently shown how mutations in the RNA transcript result in the inability of the miRNA to bind to it (Takahashi 1, Mayr 1577), and Mayr has shown that mutations on the miRNA prevent its binding ability. More importantly, Mayr showed that by introducing either the normal miRNA to a normal mRNA sequence or by introducing a mutant miRNA to match the mutated mRNA sequence, translational repression was rescued (1577). This information provides a key to miRNA gene therapy because siRNAs with the proper sequence can be injected into a patient to match the target mRNA and provide translational repression just like tumor suppressor miRNAs. For oncogenic miRNAs, oligonucleotides which bind to these miRNAs have been the key focus of treatment. Called anti-miRNA oligonucleotides or AMOs, these artificial RNAs would have sequences complementary to the carcinogenic miRNAs and thus competitively inhibit them. MiRNA gene therapy has huge potential for the future of cancer and disease treatments.

Unfortunately, miRNA therapy treatments are still in the developmental stage. According to Lars Aagaard, there are two main obstacles that must be overcome in order to use miRNA gene therapy methods, mainly, toxicity and delivery (3). As shown before, miRNAs control a vast variety of biological processes in the body, and one miRNA can have multiple target mRNAs due to sequence homology of the binding sites; therefore, in using siRNAs as tumor suppressors or in targeting an oncogenic miRNA, there will be multiple side effects which could be potentially detrimental. Since the exact functions and targets of many of the known miRNAs have not been fully analyzed yet, using miRNA gene therapy right now may be unethical without knowing the full functions of miRNAs. Furthermore, delivery of gene therapy treatments is difficult because RNA in general is more unstable than DNA and tends to degrade easier. siRNAs may also cause an immune response which causes the body to reject the foreign RNA. Transfection inefficiency is a major problem both in treatment and in study of miRNA. Gene therapy utilizing or targeting miRNAs, therefore, have a long time before clinical fruition.

However, several groups have attempted to develop microRNA gene therapy on animal trials and have improved the potential for this kind of treatment in clinical use. In dealing with toxicity, dosage seems to be an important factor in the treatments. Because of transfection inefficiency, miRNA expression models are generally saturated, but this naturally brings the problem of having too much miRNA in the system. Dirk Grimm has shown that by using a minimal yet effective dosage of miRNA transfections, suppression of Hepatitis B in mice was possible for about 5 months (16), so simply modulating the amount of siRNA used is the first step to improving toxicity levels. The next step taken by research groups is modifying the AMOs themselves so that they are less toxic. By changing or adding to the AMO structure, developers can make them more cell-specific and thus reduce the number of other unrelated

transcripts being repressed. According to Espinosa, the most common modification used is LAN, or locked nucleic acid-modified oligomers. The 2' position of the AMO is linked to the 4' position by a methylene bridge to form a bicyclic structure (20). These modified AMOs provide high target specificity to the treatment because of the steric bulkiness of the ring structure that prevents a large amount of miRNA-mRNA mismatch. Also, Ted Chu has shown that miRNAs bound to nucleic acids called aptamers were integrated within 30 minutes into the injected cells (3). They are also highly specific because they bind to specific cell surface markers and are internalized by the cell. Researchers are slowly overcoming the toxic side effects of miRNA gene therapy.

Other groups have tried to tackle the other problem of miRNA gene therapy, delivery. One solution is methylating the 2' OH group of the AMO, which provides a measure of stability by reducing nuclease affinity to the AMO and increasing mRNA affinity. Stabilizing the molecule itself is one means of increasing delivery efficiency, but the vehicle of transport has also been a problem. According to Grimm, the traditional mode of transport has been encapsulating siRNAs within a lipid particle complex called Stable Nucleic Acid Lipid Particles or SNALPS (4). While this complex provides an effective means of delivering siRNAs for silencing, it has the tendency of degrading in the liver, and repression only lasts for about 11 days. While this is longer than the half-life of siRNAs by themselves, it is still not long enough for long term treatments of chronic diseases. Another option being studied is the use of viral vectors, which would not only allow higher delivery efficiency than simple siRNA transfection of SNALPS, but would also provide another means of controlling toxicity levels. Grimm suggests that since viral vectors like AAVs, lentiviruses, and adenoviruses have their own promoter, the amount of microRNA expressed and the specificity of mRNA repression could be

controlled to a high degree (5). Efficiency would also be high since viruses can infect many types of cells, while the relatively innocuous nature of the vectors themselves has minimal toxic side effects. If stem cells were infected with virus vectors ligated to siRNAs, and these stem cells were then transplanted into a patient, the stem cells would proliferate to make cells with the siRNA, and the patient would effectively treat himself after transplantation. Developers have made small breakthroughs towards improving the viability of miRNA gene therapy.

Furthermore, preliminary animal trials of miRNA gene therapy show promising results. The Omoto group showed that siRNA derivatives of HIV-1 miRNAs could be used to inhibit HIV-1 viral replication in mice (7). HIV-1 nef is a coding accessory gene coding for RNAs important in regulation of viral replication. Omoto showed that overexpression of nef-derived miR-N367 can actually repress nef transcription, so miRNAs can actually be used for treatment of diseases like HIV-1. The findings also suggest that HIV-1 produces its own miRNA for self-regulation; therefore, researchers can not only utilize the miRNAs found in the human body, they can also manipulate the miRNAs of the pathogens for disease control. Another promising study is the one by Dong Sun An, et al. His group transplanted hematopoietic cells into HIV-1 infected rhesus monkeys. The cells were previously infected with lentiviruses coding for siRNAs that targeted chemokine receptor 5, and upon transplantation, could produce lymphocytes expressing the siRNAs and effectively repressing chemokine receptor 5 (1). Afterwards, the cells were more resistant to the simian immunodeficiency virus. Chemokine receptor 5 (CCR5) is a coreceptor crucial for HIV-1 to infect cells, so it is an important target for treatment of HIV-1. Given that the transplantation was performed on rhesus monkeys, a close relative of humans, this result is very optimistic for the future of clinical treatments. It also demonstrates the effectiveness of using lentiviruses and stem cells as the means of siRNA

delivery because the monkeys have had decreased CCR5 expression for at least 14 months and no toxicity has been observed. Gene therapy which utilizes miRNA has made important steps towards improvement and practical use.

The widespread importance of microRNA regulation in biological processes and the close tie of microRNAs to cancer lend themselves to targeting and use in potentially powerful gene therapy treatments. While the future of such treatment still has obstacles to overcome, many positive steps have been taken so that clinical treatment may eventually be possible.

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