ASOs: AN EMERGING THERAPEUTIC FOR COVID-19 AND FUTURE PANDEMICS

BY MIRIAM GOODWIN,
HANNAH VAN MULLEM,
LUYANG ZHANG
ANDREW DELANEY

INTRODUCTION

Dr. Anders M. Näär is a Professor of Metabolic Biology in the Department of Nutritional Sciences & Toxicology at the University of California, Berkeley. He received a B.S. degree in Biochemistry/Biotechnology from the University of Lund, Sweden, and a Ph.D. in Molecular Pathology with M. Geoff Rosenfeld at UC San Diego/HHMI. His research focuses on microRNA and transcriptional regulation. This interview focuses on potential ASO-based therapeutics that the Näär Lab is currently investigating for use against metabolic diseases and muscular dystrophy, as well as an intranasal SARS-CoV-2 therapeutic.

BSJ: Professor Näär, your work is mainly centered around microRNA (miRNA) and regulatory mechanisms involved in metabolism. What drew you to researching miRNAs specifically?

AN: My research has centered around gene regulation, such as transcriptional control mechanisms, ever since I was a graduate student at UC San Diego years ago. As a postdoc here at UC Berkeley, I studied transcription factors called sterol regulatory element binding proteins, or SREBPs.

We serendipitously discovered that miRNAs reside in the introns of the genes that encode SREBPs. Initially, the presence of these miRNAs baffled us, so we started trying to understand the role of miRNAs miR-33 A and B, which are inside of two SREPBs. We realized that the miRNAs and host genes are a single unit that work to control lipids such as cholesterol, triglycerides, and fatty acids. I found this fascinating. We found that the miRNA-33 A and B genes actually contribute to cardiovascular disease by virtue of shutting down the synthesis of genes that are protective against cardiovascular disease. One of these genes is called ABCA-1. This gene expresses a cholesterol efflux pump, which protects us from atherosclerosis. This is done by preventing the macrophages that live in arteries from becoming lipid-laden from taking up excess oxidized LDL cholesterol. When the miRNA prevents the pump from being expressed, you are at risk of developing atherosclerosis. This discovery was our first foray into studying miRNAs.

Based on that example of a very key pathophysiological role of a miRNA, we decided to investigate whether there are other miRNAs that contribute to coding metabolic diseases. We took an unbiased approach by using genome-wide association data from about 190,000 people and collaborated with human geneticists to look for single nucleotide polymorphisms (SNPs) within this data. Some common variants in the human genome that were linked to the incidence of normal cholesterol levels affected miRNAs. We found 69 miRNAs that other studies had completely overlooked because everyone else was looking at genes that were either directly affected by or neighboring these SNPs. However, we found that miRNAs can be pretty far away — sometimes hundreds of kilobases away — from the genetic variant and still be linked functionally to SNPs involved in the regulation of cholesterol.

BSJ: Part of your research focuses specifically on miR-128-1. Could you explain how you used antisense oligonucleotides (ASOs) to mitigate metabolic diseases associated with this miRNA?

AN: Specifically, miR-128-1 is the primary miRNA that regulates cholesterol. In our studies, we used a mouse model that is prone to atherosclerosis and has cholesterol homeostasis like in humans. We showed that injected ASOs will act like a molecular Velcro — it binds in a Watson-Crick base pair manner to the miRNA and prevents it from binding its target. By doing this, you can actually decrease cholesterol by 35% and triglycerides by 25%, which is quite dramatic. This led to us thinking that we could use ASO oligonucleotides (ASOs) to gum up miRNAs that contribute to disease. Through this technique, we realized then that miR-128-1, given its continued presence in the human population despite its contribution to disease, must reside inside of a genomic locus that is heavily positively selected.

BSJ: In the case of that positive selection, could you explain what it means for miRNA-128 to work as a "thrifty genetic element"? How has its positive selection become a maladaptation in the modern world?

AN: Essentially, miR-128 allows you to store fat more efficiently, making it a thrifty genetic element. In ancient times, having more fat storage was probably beneficial when there was food scarcity. However, at least in the Western world, we generally no longer encounter famines, so now this locus is actually linked to obesity and Type 2 diabetes. This likely explains the increased propensity for stored fats driven by this miRNA, which is a maladaptation. We proved that this is the case in mouse models: we fed the mice a high-fat diet, which led to obesity, but if we also injected ASO targeting the miRNA, there was a decrease in fat storage by about 5%. We are also seeing beneficial effects on glucose homeostasis, insulin tolerance, and other diabetes-related phenotypes. Fatty liver disease is another

Graphical Abstract

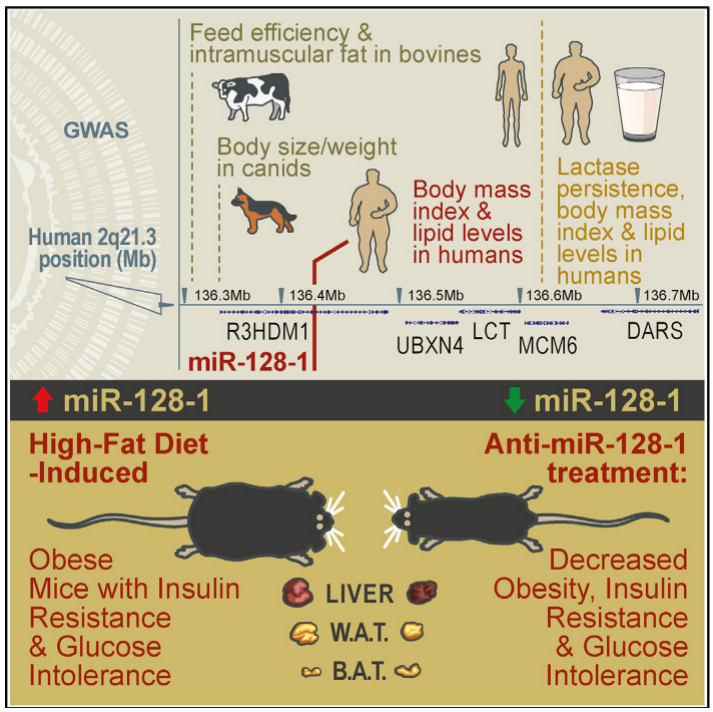

Figure 1: Correlation between miR-128-1 and metabolic disorders. *miR-128-1 was found from genome-wide association studies (GWAS) and is in a metabolically significant locus that is indicated with BMI and lipid levels in humans. Decreasing miR-128-1 with an anti-miR-128 ASO decreases obesity, insulin resistance, and glucose intolerance in mice.*

metabolic disease influenced by miR-128, where lipid accumulation in the liver leads to inflammation, fibrosis, and eventually cirrhosis, hepatocellular carcinoma, and a disease called nonalcoholic steatohepatitis (NASH). We have found that targeting miR-128 alleviates all phenotypes of NASH, which is also something we are interested in pursuing therapeutically.

BSJ: You have also found that miRNA-128-1 has a link to Duchenne muscular dystrophy (DMD). Do you see a therapeutic approach targeting muscular dystrophy-associated miRNAs with ASOs?

AN: Absolutely! People who suffer from DMD get very weak muscles, and eventually, many of them die from a lack of breathing or heart failure. There was a paper that came out from Louis Kunkel, the researcher who discovered the genetic cause of DMD. He found a gene called dystrophin that causes aberrations when it gets mutated, both in the muscles of DMD patients and the animal models he developed. Nearly all therapeutics today for DMD are aimed at restoring dystrophin. Surprisingly, they found a golden retriever in Brazil who had a complete loss of dystrophin but did not suffer from muscle dystrophy. While trying to understand why this dog was healthy without dystrophin, they genetically identified a gene called Jagged1. Jagged1 is a Notch ligand that signals between cells. The researchers suspected that the upregulation of Jagged1 causes increased regeneration, which compensates for the loss of dystrophin. I discovered miR-128-1, which regulates Jagged1, so I reached out about collaboration. When we collaborated, we injected our ASO into his zebrafish model, and about 30% of the fish were completely cured of the muscular dystrophy phenotype, and the rest were partially rescued, so we thought this is really cool.

Then, we tried performing the same experiment in mice, and the same thing happened. Now, we have developed a much more potent ASO, and we see about a 90% rescue of all phenotypes in mice. Thus, we think this could be a therapeutic approach for DMD. This prompted our collaboration with researchers in Germany who have developed a pig model for DMD. In this model, muscular dystrophy is incredibly severe, and most of the pigs die within a few months due to heart failure. However, we decided to treat three of them with our ASO, and it significantly ameliorated their heart function. The pathological role of this miRNA is conserved from fish to mice to pigs. Others have shown that miR-128-1 is significantly elevated in the blood and muscle of people with DMD. So, we believe it is a very strong pathological contributor to DMD. Part of the known disease mechanism is that miR-128-1 prevents regeneration, and we think that lots of different targets of this miRNA are involved. So by taking out miR-128-1, we can rescue many different phenotypes, and this is very exciting. We are about to submit these results to *Nature*.

BSJ: We also wanted to ask you about your ASO therapeutic that targets SARS-CoV-2. How has your background in researching metabolic disorders informed your perspective on working with SARS-CoV-2 therapeutics?

AN: It was not exactly our metabolic research components that prompted us to tackle SARS-CoV-2; it was more our ASO approach. We thought my lab was going to be shut down; but, the Vice Chancellor for Research allowed SARS-CoV-2 research, so I thought, "Why not try it?". SARS-CoV-2 is a single-stranded, positive-sense RNA virus that is about 30 kilobases long. miRNAs and messenger RNAs are also single stranded. So, we felt that perhaps we could use ASOs to bind functionally important portions of the viral RNA and gum it up somehow. We scanned the genome of the virus and designed about 150 different ASOs to target different parts of the viral sequence. We also targeted ACE2 receptors on the virus to see if we can block them as well. We found several ASOs that bound to different viral sequences to be extremely efficacious in human cells in preventing viral replication.

We were quite surprised that they worked. Then, more impor-

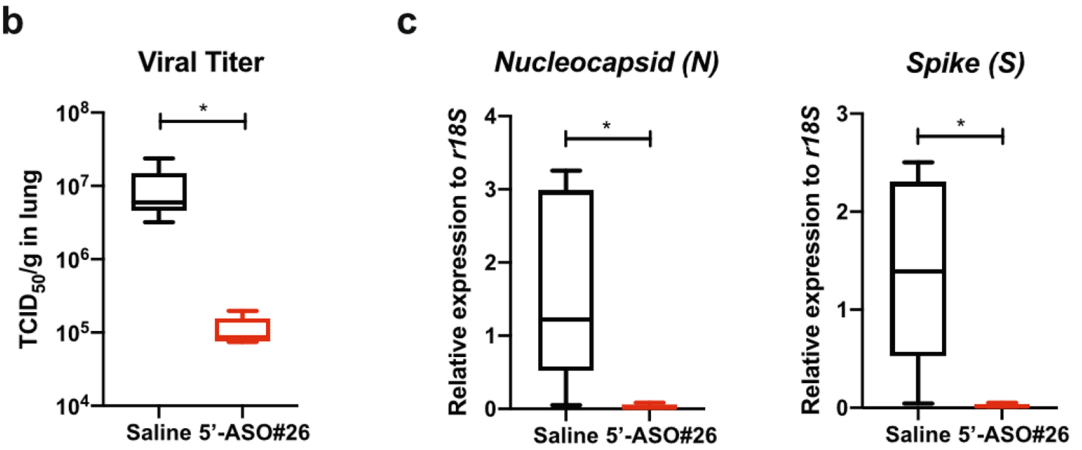

Figure 2: ASO therapeutic decreases viral pathogenesis. *Viral titer and mRNA encoding essential viral proteins are both decreased when mice were treated with 5'-ASO#26 6 hours post-infection.*

"We found several ASO oligonucleotides that bound to different viral sequences to be extremely efficacious in human cells in preventing viral replication."

tantly, it actually worked in vivo in mice and hamsters, which was quite surprising as well. Typically, we inject ASOs under the skin subcutaneously when we try to treat metabolic disease by targeting miRNAs. We tried that in mice and hamsters for SARS-CoV-2, and it didn't work at all. So I said, "Why don't we just spray it into the nose of these animals?" And it worked. We were quite shocked. Nobody's ever shown that ASOs can work in the respiratory system. That was kind of a surprise and a serendipitous discovery.

BSJ: For our readers, can you explain how ASOs work in the context of viral therapeutics and what separates ASOs from the available vaccines?

AN: So vaccines come in a variety of different "flavors" as you may know. There are attenuated viruses, which is the classic way of making the vaccine. The new kind of vaccines, messenger RNA vaccines, express a portion of the viral surface protein, the spike protein, by enclosing the RNA within a lipid bubble called a lipid nanoparticle. You have your RNA, encoding the spike protein in this lipid bubble, which is taken up by cells when injected in your arm. Your own cells can then make spike protein, which is recognized as foreign to generate an antibody response. In contrast, the ASO is simply a tiny snippet of a chemically modified DNA-like oligonucleotide that binds in a complementary manner to the viral RNA and prevents the RNA from working. In this case, what we targeted in vivo is a hairpin structure that is critical for the virus to translate protein for replication. The ASO blocks this hairpin structure from forming and makes it straight. Now, the virus cannot replicate.

BSJ: One major public health concern that comes with SARS-CoV-2 is how rapidly it mutates to allow the virus to evade vaccines. Can you explain how the ASO therapeutic you have developed is effective against emerging SARS-CoV-2 variants?

AN: The spike protein in variants of concern gets heavily mutated to evade the monoclonal antibodies against it that are produced by the vaccines. In contrast, the ASO oligonucleotide targets a highly conserved structure, the 5' leader hairpin, and of all 10 million isolates that have been sequenced, there is not a single variation in this sequence. The virus requires the hairpin to stay in this structure; otherwise, it does not "work." All the variants of concern have exactly the same leader sequence. That is why the ASO works equally well against all of them.

"All the variants of concern have exactly the same sequence. That is why the ASO works equally well against all of them."

BSJ: Because the ASO targets a structurally conserved region of viral RNA, could it be used for other RNA viruses?

AN: The particular sequence our ASO targets is conserved only in SARS-CoV-2. However, there is another structural element called the frameshift stimulation element (FSC) which we also described in the paper. We did not focus on it, but that could also

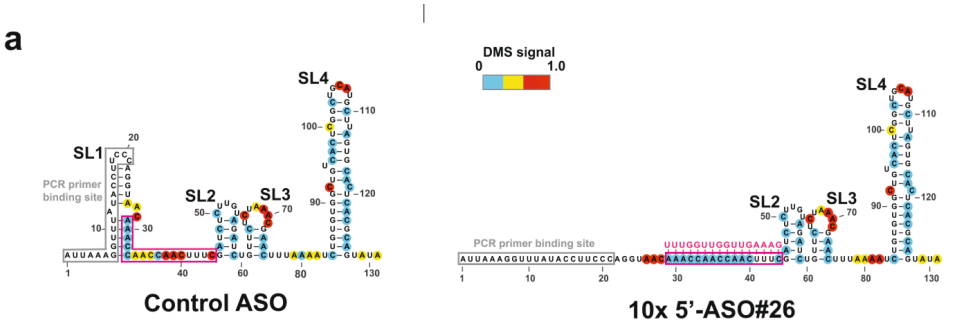

Figure 3: 5'-ASO#26 disrupts the hairpin loop in the leader sequence of the SARS-CoV-2 mRNA. *The ASO binds complementary to the part of the viral sequence which forms hairpin SL1, effectively blocking translation of viral proteins necessary for replication.*

in theory be targeted. The FSC is conserved all the way to SARS-CoV-1 and to MERS. The whole betacoronavirus family could be targeted by this other ASO. In fact, we are thinking about combining our ASO with one that targets the FSC to make a cocktail therapy that can then target any betacoronavirus today or in the future. Additionally, ASO therapeutics are very stable. You could actually stockpile these ASOs in the freezer indefinitely. They are very easy to make because they are simply oligonucleotides. We hope to deploy ASOs as a nasal spray in developing countries that do not have the refrigeration capacity that vaccines require. So, the nasal spray could be widely deployed and cheaply produced.

Also, I should mention that ASOs can be used against other viruses as well. Right now, we are targeting the influenza virus. We have a pan influenza A emphasis that we have already shown efficacy with in cell culture, and the next step is to test it in mice. That could be a game changer for influenza. It could be used prophylactically or as a treatment for just "garden variety flu," but it could also be stockpiled for an H1N1 pandemic. The last three pandemics before the current one were actually influenza pandemics. We could prevent something like the Spanish flu that happened in 1918 with such a stockpile that is ready to deploy. So for that, I think there is a lot of excitement as a pandemic preparedness avenue.

BSJ: You have talked about how you might use a combination of ASOs to target different regions in the viral genome to make it more effective, or maybe design ASOs to target other coronaviruses. Are there any other ways that you are optimizing ASOs and improving on the technology?

AN: Can we improve ASOs? Yes, I think we can improve ASOs in multiple ways. Currently, the oligonucleotide that we deliver is in saline and is naked. Probably, we can chemically couple lipids to this ASO, causing it to enter cells even more readily. We are also working with a company to see if we can develop an oral ASO therapy. That is perceived as the holy grail —to just take a pill to cure coronaviruses. Those are the two main improvements.

BSJ: How do you envision ASOs will change the vaccination and therapeutic world in the near future?

AN: I think ASOs are a nice complement to vaccines. There are a lot of folks who are vaccine-hesitant in the US. And as I mentioned, there are areas around the planet that have not seen vaccines yet because of infrastructure challenges and a lack of funding. So, I believe there is quite a large number of people who would benefit from an ASO approach. Like I mentioned, ASOs can be used as a treatment post-infection, and perhaps it could complement Paxlovid. For example, in the clinical setting, if you are infected with SARS-CoV-2, you may take both Paxlovid and this ASO spray to prevent long COVID. That is how we envision it.

BSJ: What would the next steps look like to understanding this therapeutic before it can be made available to the public?

AN: The next steps are prescribed by the FDA. You basically have to jump through a few hurdles in animals called pharmacokinetic and pharmacodynamic studies, and then toxicology trials are run. These have to be done in two different animals. For example, rats and non-human primates. The FDA requires this type of work for safety and more of an understanding of how the ASO works before we deploy it in humans. After that, there are phase one human safety trials. We are currently in discussion with the Gates Foundation and hope that they will help us fund these next steps in bringing our ASO therapy to the public.

"There are areas around the planet that have not seen vaccines yet because of infrastructure challenges and a lack of funding. So, I believe there is quite a large number of people who would benefit from an ASO approach."

REFERENCES

1. *Figure 1*: Wang, L., Sinnott-Armstrong, N., Wagschal, A., Wark, A. R., Camporez, J. P., Perry, R. J., ... & Näär, A. M. (2020). A microRNA linking human positive selection and metabolic disorders. *Cell, 183*(3), 684-701.
2. *Figure 2*: Zhu, C., Lee, J. Y., Woo, J. Z., Xu, L., Nguyenla, X., Yamashiro, L. H., ... & Näär, A. M. (2022). An intranasal ASO therapeutic targeting SARS-CoV-2. *Nature communications, 13*(1), 1-13.
3. *Figure 3*: Zhu, C., Lee, J. Y., Woo, J. Z., Xu, L., Nguyenla, X., Yamashiro, L. H., ... & Näär, A. M. (2022). An intranasal ASO therapeutic targeting SARS-CoV-2. *Nature communications, 13*(1), 1-13.