Targeting Noncoding RNAs in Disease: Challenges and Opportunities
Webinar
4 September 2013

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Slide 1

Tianna Hicklin: Hello and welcome to today's *Science*/AAAS technology webinar on Targeting Noncoding RNAs in Disease. I'm Tianna Hicklin, the assistant editor for *Science*'s custom publishing office.

In this webinar, we'll be discussing the use of noncoding RNAs as therapeutic targets in human disease. Noncoding RNAs serve a wide range of functions in cellular and developmental processes and are therefore likely involved in the development and pathophysiology of many diseases. Noncoding RNAs hold the promise for new drug discovery and offers several advantages over traditional protein based targets. Thanks to the effective inhibition of micro RNAs in vivo, scientists have already made groundbreaking discoveries about the contribution of short regulating RNAs in human diseases in areas such as cancer, heart disease, and diabetes.

Slide 2

We have with us today an expert panel who will be discussing the unique opportunities and challenges that come along with targeting functional RNA in vivo. Our panelists will present data from some of their current research about noncoding RNAs in disease as well as address questions from our live audience. It is my pleasure to introduce Dr. David Corey from the Department of Pharmacology at the University of Texas Southwestern Medical Campus in Dallas, Texas; Dr. Stefanie Dimmeler from the Institute of Cardiovascular Regeneration at Goethe-University in Frankfurt, Germany; Dr. Jan-Wilhelm Kornfeld from the Department of Mouse Genetics and Metabolism, University of Cologne in Germany. Thank you all for joining us today. We're happy to have you.

Before we get started, I'd like to share some important information for our online viewers. Please note that you can resize or hide any of the windows in your viewing console and the icons at the bottom of

the console allow you to control what you see. Click on these to read speaker bios, to find additional information about technologies related to today's discussion, or to download a PDF version of the slides.

We'll begin today's webinar with a presentation from each of our speakers and end with a Q&A session during which our panelists will address the questions that are submitted by our online audience. If you're joining us live now, please submit your questions at any time by typing them into the box at the bottom left of your viewing console and clicking the submit button. If you can't see this box, click the red Q&A icon at the bottom of the screen. Please be aware that concise and broadly applicable questions are the most likely to be put to the panel and whenever possible, please direct your questions to a specific panelist.

Following today's webinar, you are invited to participate in a live online chat with representatives from our sponsor Exiqon who will be available to answer additional questions that we didn't have time to cover during the webinar. Please stay tuned and we'll provide you with a live chat link at the end of the hour.

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Finally, thank you to Exiqon for sponsoring today's webinar.

Slide 3

It is now my pleasure to introduce today's first speaker, Dr. David Corey. Dr. Corey received his undergraduate degree in chemistry from Harvard University and his Ph.D. from the University of California, Berkeley. He completed his postdoctoral training in the Department of Pharmaceutical Chemistry at the University of California at San Francisco. He has been in the Department of Pharmacology at the University of Texas Southwestern Medical Campus in Dallas since he first joined in 1992 and he has held his current position as full professor since 2003. Dr. Corey's group is interested in antigene oligonucleotides, antisense oligonucleotides, nucleic acids, RNAi, and telomerase. Welcome and thank you for being here today, Dr. Corey.

Dr. David Corey: Thank you, Tianna. I have two goals for my presentation today. The first is to give a brief introduction to the concept of using nucleic acids as dugs. The second is to show you how far the boundaries of using nucleic acids to affect gene expression can be pushed.

Slide 4

So you're all aware that most drugs bind proteins and that's a traditional and very successful strategy. However, you're probably also aware that the development of new small molecule therapeutics usually takes years and it's becoming more and more difficult.

Slide 5

As a result, there's a search for new methods to develop drugs and one of those is to develop nucleic acids that can bind to RNA and affect gene expression. The advantages of this approach are that one can identify an active oligomer, a lead compound very quickly in weeks rather than years. In addition, the medicinal chemistry and pharmacology of all of these nucleic acids is similar regardless of what kind of target they're going after and that's going to speed the drug development process. Finally, by affecting gene expression, one has the ability to treat almost any disease so it's a single class of molecule that has a very broad potential to be applied.

Slide 6
[0:05:41]

Okay. So the two main strategies for using nucleic acid to affect gene expression are to use single cell stranded oligonucleotides to bind directly to an RNA target and block their action. The other method would be to use double stranded RNA. Double stranded RNA then goes through the RNA silencing process. That machinery helps it to find a messenger RNA target and efficiently inhibit gene expression.

Slide 7

Okay. So what kind of cellular RNAs can be targeted by nucleic acids? Well they could be the RNA domains of ribonucleoproteins and the classic example of that is telomerase. One could also target messenger RNA. You could block translation or you could affect splicing so for example upregulate an isoform that might be useful in treating a disease. Today, however, we're going to focus mainly on targeting noncoding RNAs and one of those noncoding RNAs is microRNAs and by blocking the microRNA you can affect its action. In my talk specifically, I'm going to discuss targeting long noncoding RNAs, which can be used to either up or down regulate gene transcription. Okay.

Slide 8

So this area of using nucleic acids as drugs, they seem novel but it's something that needs to be taken seriously especially with news that's coming out this year. Earlier this year Kynamro, an antisense oligonucleotide that targets Ap-B1 messenger RNA, was approved by the food and drug administration. This is a systemically administered oligonucleotide that's been shown to reduce LDL cholesterol. So it's the strongest proof to date that synthetic oligonucleotides can be made on the scale that's large enough to be used as drugs and be administered to patients and get through the FDA approval process. So there are many other trials under way right now and while again I'm sure many of you have been aware that nucleic acids as drugs has been through many ups and downs over the years, it appears to becoming a more reliable drug development technology.

Slide 9

So that concludes the background to using nucleic acids as drugs. Now I'd like to move on to showing you just how far the boundaries of regulation can be pushed by discussing or work on using noncoding RNAs to regulate transcription of an operon within the eicosanoid signaling pathway.

Slide 10

Okay. So you're probably all used to thinking about genes as a messenger RNA starting at a transcription start site and ending at the end of a 3' untranslated region. However genomic programs have shown us that messenger RNAs are only one type of transcription species that messenger RNAs are often overlapped by long RNAs at both their 3' and the 5' termini as well as within the gene providing a new realm of potential targets for addressing gene expression.

Slide 11

So what might these long noncoding RNAs do? Well undoubtedly, many of these long noncoding RNAs are simply noise. Now this remains a controversial point of just how many of these noncoding RNAs are. Most are likely noise but some might be very important and classic examples of the important ones include XIST and HOTAIR. These are genes that are known to regulate x chromosome inactivation or transcriptional multigene regulation programs. They've been reported to act in either cis or trans so they clearly can be important but the molecular details are usually obscure. It's not clear how is recognition specific site is achieved. Moreover, the analysis that one usually sees in the literature is dependent on highly

interpreted data, not the primary results or detailed biochemistry that can let you build a picture of how these are actually working.

So one might think that with RNA that RNAi factors that are so successful in regulating messenger RNA might be involved. But today they haven't really been strongly implicated in mammalian cells. So this was an area that we wanted to investigate.

Slide 12
[0:10:05]

So could there be a role for RNAi proteins? Okay. Well we know that microRNAs are in the nucleus. While it's still controversial in some sectors, we also know that RNAi factors like argonaute 2 are in the nucleus. We also know that noncoding RNAs are in the nucleus. So the players are all there but the rules might be different and what exactly they might be doing hasn't really been known yet.

Slide 13

So since 2005, my laboratory has been investigating these issues. The hypothesis that we've built up over that time is that these RNAi factors can interact with small RNAs to form what are essentially ribonucleoprotein complexes that can act to control either gene transcription or gene splicing. The RNA domain protects the RNA and promotes binding to the target. The RNA domain directs specificity to a particular RNA target inside the cell, for example a long noncoding RNA.

Slide 14

So in about 2010 working with my colleague Bethany Janowski, we decided to go very deeply into an important physiology pathway in this case the eicosanoid production pathway and the enzyme cyclooxygenase-2 and PLA2G4a.

Slide 15

With the hypothesis that it might be possible for RNA networks to coordinate the expression of these two genes.

Slide 16

So, we began by asking whether or not there was noncoding expression at the COX-2 promoter. So we characterized this expression by RNA sequencing by quantitative PCI and 5' RACE and we discovered that there were transcripts overlapping the COX- promoter in both the antisense and the sense direction. So these

provide the basics noncoding RNA raw material that might allow recognition to control gene expression of COX-2 messenger RNA.

Slide 17

Okay we then asked are there any endogenous microRNAs in the nucleus that might have the potential for regulating COX-2 by targeting the promoter? So we did this by doing computational prediction and we found that there were a substantial number of microRNAs with complementarity to the COX-2 promoter. We also did small RNA sequencing to identify microRNAs in the nucleus and microRNAs that were both in the nucleus and complementary to the COX-2 promoter became candidates for regulating COX-2.

Slide 18

The most promising of these microRNAs was microRNA-589. It had strong complementarity to two adjacent sequences within the COX-2 promoter. So that resembles how micro-RNAs recognize typically 3' untranslated regions. So it became our prime candidate for investigating for potential regulation of COX-2 expression through regulating its transcription by binding a noncoding RNA.

Slide 19

So to investigate this, we used a microRNA inhibitor. These inhibitors are small locked nucleic acids. You'll be hearing more about them in the other talks. They bind to microRNA-589 and reduce its activity. When we add these inhibitors into cells as you can see from the figure, COX-2 expression goes down. This is consistent with a microRNA binding to the noncoding RNA and activating COX-2 expression.

Slide 20

We also did the converse expression by adding microRNA-589 into cells. When we add microRNA-589 either as an exact mimic of microRNA-589 or its two complementary strands, we get activation of COX-2 expression at both the protein and the RNA level again consistent with the microRNA acting as an activator of cyclooxygenase-2 expression.

Slide 21

Okay. So we've shown transcriptional activation, what might the mechanism be? Because we've been using microRNAs as mimics and microRNAs use RNAi factors like argonaute 2, we hypothesized that argonaute 2 might be involved.

Slide 22 to slide 23

So what is argonaute 2? Well it's the catalytic engine for RNAi. When you have a fully complimentary RNA, argonaute 2 will escort it to a messenger RNA target and cause the degradation of that target. In the case of long noncoding RNAs, the hypothesis that we had built over the years was that the small RNA recruits argonaute to a noncoding RNA in proximity to the promoter where the argonaute RNA complex access a transcription factor and activates gene expression. So that was the hypothesis that we were testing here.

Slide 24

So to test it, we looked at what would happen you depleted argonaute 2 from cells. So as you can see when you add a small RNA complementary to the COX-2 promoter, you get robust activation of COX-2 expression. However, when you add a small RNA that depletes argonaute 2 first and then add that same RNA that targets, the COX-2 promoter activation is much less. So this and other information suggest that this activation is dependent on RNAi factors.

[0:15:51]

All right. So I've shown you activation by microRNAs, can we get even better activation if we target a fully complementary small RNA to the COX-2 promoter? So in this case rather than using microRNA-589, we tested a variety of microRNAs that were fully complementary and you might expect that by being fully complementary, they would bind better and possibly be more active.

Slide 25 to Slide 26

Okay. And this is something that we observed. We found that several of these small RNAs that targeted the COX-2 promoter and were fully complementary were highly active. The best one was this one RNA12. That spans between -12 and +7 around the transcriptional start site for COX-2. When we monitor RNA levels, the activation is about 20-fold.

So it's always important when you're introducing RNAs into cells to convince yourself that what you have is nontarget effect at the RNA that you planned the target and not an off target effect that's through some other mechanism. This is just one of the experiments that we did to help demonstrate that activation was on target. We introduced mismatches into either strands of the double stranded RNA and as few as one mismatch in the seed sequence of the guide strand of the double stranded RNA was enough to abolish gene

activation. So this and other evidence suggest that what we have is indeed on-target gene activation.

Slide 27

All right. So that activation led us to then think about what might be happening to adjacent genes and at that point, we discovered that the adjacent gene for COX-2 was in fact PLA2G4A. That's probably no coincidence because PLA2GF4 makes the substrate for COX-2.

Slide 28

So PLA2G4A is both the nearest functionally related gene for COX-2 as well as nearest spatially located gene. So we asked could these genes be transcriptionally linked.

Slide 29

So what we observed was that microRNA-589 was able to increase the expression of PLA2G4 in the same way that it increased the expression of COX-2.

Slide 30

When we add in that locked nucleic acid inhibitor that you remember inhibited COX-2 expression, it also inhibits PLA2G4A expression.

Slide 31

Okay. So how could we be getting this linked expression of two genes that are almost 150,000 bases apart? Well it's becoming more and more apparent that the human genome is not just organized in a linear form but it forms three dimensional interactions and the way to investigate whether these three dimensional interactions occurs is with the technique called chromosome confirmation capture in which you first crosslink DNA then digest it with restriction enzyme. You add ligase so pieces of DNA that might have been spatially close together now have the ability to ligate together and you can detect those points of ligation by quantitative PCR.

Slide 32

So we did this and the bottom line is that we detected a connection between the PLA2G4A promoter and the COX-2 promoter using a couple of different methods of 3C analysis showing that the model that you see at the bottom is operating. Now you actually have the COX-2 promoter and the PLA2G4A promoter in relatively close contact.

Slide 33

Okay this provides a pathway for introducing a signal between the two promoters. So what the body of data that we have is, which is in a paper that's just been published in nucleic acid research and is online right now, is that you have long noncoding RNAs expressed at the COX-2 promoter. No noncoding RNAs are expressed at the PLA2G4A promoter.

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When you add in a small RNA that is complementary to the COX-2 promoter or you have microRNA-589 you have that RNA in conflict with argonaute 2 and other RNA factors binding to the noncoding RNA in cis at the promoter that affects transcription factor binding at the promoter and causes up regulation of gene expression.

Slide 34

So our conclusions: We showed that RNA can induce COX-2 expression. We showed that the long noncoding RNA is a scaffold for sequence-specific recognition of RNA-protein complexes. So think about them like RNA protein transcription factors. In work that I didn't get into but which is mechanistically interesting and you can find out more about it in the paper, that long noncoding RNA, it can be cleaved by the double stranded RNA. But that cleavage is not necessary for activation. It's the binding step that's important, not any subsequent cleavage step. Finally, we've shown that RNA can organize a novel multi-gene inflammatory response pathway that had not been observed previously.

Slide 35

Finally, I'd like to thank all of this work. Most of it was done and led by Bethany Janowski and with the able assistance of Masayuki Matsui, Yungjun Chu, Keith Gagnon, and Huiying Zhang in my laboratory. So finally, I'd like to thank you for your attention and later on, I'd be happy to answer any questions. Thank you.

Slide 36

Tianna Hicklin: Thank you, Dr. Corey. Our second speaker today is Dr. Stefanie Dimmeler. Dr. Dimmeler received her undergraduate, graduate, and Ph.D. degrees from the University of Konstanz in Germany, followed by fellowships in experimental surgery at the University of Cologne and molecular cardiology at the University of Frankfurt. She has been professor of experimental medicine at the University of Frankfurt since 2001 and director of the Institute of Cardiovascular Regeneration at the Center for Molecular Medicine since 2008. Her

group studies the basic mechanisms underlying cardiovascular disease and vessel growth with the aim of developing new cellular and pharmacological therapies for improving the treatment of cardiovascular disease. Her group also focuses on epigenetic mechanisms that control cardiovascular repair, specifically the function of histone modifying enzymes and noncoding RNAs. Welcome and thank you for joining us today, Dr. Dimmeler.

Slide 37 to Slide 38

Dr. Stefanie Dimmeler

Thank you very much for the introduction. It's my pleasure to discuss with you the role of microRNAs in cardiovascular disease and the first, I'd like to remind you that despite the improvement in therapies, which really significantly extends the lives of patients with coronary artery disease, there are still a significant number of patients dying. Namely, roughly 1/3 of people are dying because of cardiovascular disease including myocardial disease as well vascular disease.

MicroRNAs may play a very important role in the heart. This has been shown. It might be attractive therapeutic targets. Several studies have identified specific microRNAs controlling cell death, fibrosis, potentially cardiomyocyte proliferation or cardiac reprogramming both might be important for regeneration of the heart. These are particularly focusing on the regulation of androgenesis, this means that the growth and migration of vessels and also androgenesis is critically regulated by microRNAs.

I will provide to you now one example microRNA-92a, which might be a target to improved neovascularization and thereby augment the recovery of the acute myocardial infarction, which is due to the limitation of blood flow in the heart.

Slide 40

We sort of studied microRNAs in endothelial cells to discover this microRNA-92a, which is belonging to the microRNA 1792 class is expressed in endothelial cells and if you further over express this microRNA, we showed that this microRNA can block androgen sprouting in vessel formation.

On the bottom, you can see two in vitro assays on the left-hand side when overexpressing microRNA-92a to abolish sprouting and tube formation and on the right-hand side you can see that even in a model organism zebrafish, over expression of a precursor of microRNA-92a induced a vascular patterning defect. But of course as

said in cardiology you want to enhance androgenesis rather than to inhibit androgenesis and therefore we went on to use inhibitors for these microRNAs.

[0:25:02]
Slide 41

There are two possibilities to inhibit microRNAs, which we tested, one of which are antagomirs. It's a cholesterol linked antisense nucleotide, which are stabilized. On the right-hand side, you can see in this cartoon a second way, which is based on locked nucleic acid molecule, which both antagomirs and the LNA antimirs efficiently block microRNA-92a expression in the heart as you can see in the bottom graph below. You may note that using LNA based antimirs even lower concentration as low as 0.05 mg/kg was very sufficient in blocking microRNA-92a expression entirely in the heart. Interestingly, these LNA based antimirs also has been safely used for clinical trials to testing that the chemistry will be usable and efficient even in human.

Slide 42

We studied these antagomirs and antimirs in the mouse models and as you can see here in the bar graph in the middle of this slide in comparison to the sham treated control animals, the PBS or antagomir controlled treated mice which where were exposed to a myocardium infarction showed a significant reduction in contractility. On the right-hand side, you can see antagomir-92a treated mice, which showed an improvement of contractility and you can see the effect of the antagomir treatment even if you just look at the apex of the heart.

On the upper picture you see a control heart, a very thin apex while in the antagomir 92a treated mice there was a significant preservation of the myocardial wall and this may also underline one of the functional effects you have seen with the antagomir 92a treatment in addition to the neovascularization response which we have seen. The treatment was very efficient. On the lower panel, you can see that antagomir 92a treatment even after three weeks of treatment was very efficiently suppressing microRNA expression in the heart.

We further went on to study the mechanism of and the summary of these studies shown in the left-hand side, you can appreciate of course that as all microRNAs, microRNA-92a has a lot of targets. We

have studied some of these targets including the demonstration of integrin alpha5 which is an antiapoptotic molecule in endothelial cells which subsequently regulates the endothelial nitric oxide synthase as well as SIRT1 might be targets of this microRNA, which constitutes the beneficial effects we are seeing, which all of them are upregulated if you block microRNA-92a the antagomir treatment.

Slide 43

Of course, these are mouse models and maybe far away from the clinic so to translate these findings further, we went on to study whether these inhibitors might used for ischemia reperfusion models in pigs. Here you can really mimic the clinical situation and this study was done in collaboration with Rabea Hinkel, Christian Kupatt from Munich.

Slide 44

But the details of the protocol are given here and we treated these pigs with these LNA based microRNA-92a inhibitors. We treated three groups of pigs, one of which received this molecule intravenously and the second and third groups there we used catheter based approaches to deliver the antimir either antegrade or retrograde meaning either in the artery or in the venous system in the heart. The idea was that we might get a more efficient knockdown in the heart and reduce the systemic effects on microRNA-92a because this micron is ubiquitously expressed and this may mean having additional effects in unwanted effects systemically.

On the bottom, you can see the knockdown efficacy of these LNA treated animals and obviously in the infarct zone, which are shown on the right-hand side, if you compare the two different control groups PBS and LNA control groups in black, all the four treatment groups given in blue showed the significant suppression of miR-92a expression. As you can also see, intravenous infusion was a little bit less efficient. This is the dark blue while the light blue bar is the local administration or even further enhancing the concentration on the right-hand side really substantially inhibited microRNA-92a expression in this pig heart cell.

Slide 45

What is the functional consequence? You can see on the next slide on the left-hand side, the global function and if the bar is up it means a worsening of heart function. It can be seen with the two control pigs. These are the dark, the black bars. While in the blue bars

indicating treated pigs, there is a significant improvement in global function. Again, intravenous infusion was a little bit less efficient compared to the local administration and a similar pattern was seen when we looked on regional function. On the right-hand side, here the basal activation means it has an increase in the regional function, which is a positive signal in all the two bars indicating treated groups. And if you tazed the pigs which is a stress response, you can even further see that the control pigs get worse while the treated pigs particularly local administration with the LNA led to a significant improvement in regional function.

Slide 46
[0:30:45]

What is the mechanism? Of course, we looked at neovascularization because this was the startup of the study. On the right panel, you can see in these images that the LNA treatment increased capillary density. It's also shown in the graph where the three right bars show the local treated, local administrated LNA-92a treated pigs showing a higher capillary density in the border zone. Also, one of the targets antigen alpha 5 was significantly increased as shown on the right-hand side.

Slide 47

In summary, I hope the slides are moving. In summary, it is shown that microRNA-92a inhibition has a beneficial effect on the recovery of the acute myocardial infarction. I haven't had time to show you all the data but clearly there's an evidence that neovascularization and perfusion is increased by a specific effect on the vasculature but also we have seen that cardiomyocytes survival was significantly increased and the point is now of course to decipher the mechanism underlying this inhibitory effect. It's a bit difficult because microRNA-92a is expressed as I mentioned in almost all cell types and if you block it with pharmacological inhibition, you may block microRNA-92a in all the different cells where it may have different functions. But we now use genetic strategies to decipher the role of microRNA 92a and some preliminary data are shown on the next slide.

Slide 48

On the upper panel, you can show that the microRNA-92a knockout mouse there's the complete blockade of 92a expression on the left-hand side. On the right-hand side, you can see the functional activity of the heart, the black box representing wild type mice showing a worsening of function and increase in wall motion core index while

the knockout mice show the preservation of function. So similar to what I've shown you before with the antimir treatment.

In contrast if you do a cardiac depletion of microRNA-92a only in cardiomyocytes, of course the knockdown is a bit less efficient and also the beneficial effects on the right-hand side that the function is not entirely preserved. So again the control black box there's an increase in wall motion's core indicating worsening of function. There's also a slight worsening of function if you knockout microRNA-92a specifically in cardiomyocytes suggesting that also other cell types may be affected by the antimir treatment, which constitutes to the beneficial effect we are seeing.

Slide 49

In the very last slide, I want to bring to your attention some recent study, which we did to study microRNAs in cardiovascular aging. As you may know, the cardiovascular system is tremendously affected by age and age is one of the most important risk factor for cardiovascular disease.

Slide 50

The cartoon on the right, on the left panel you can see that cardiovascular aging is disturbing the vascular wall and also leads into an increase in cardiomyocyte hypertrophy, cardiomyocyte cell death.

Slide 51

Several microRNAs have been published to be involved in this aging process leading to the observation of these vascular aging phenotypes and we have done one study, which is based on screen where we have identified age regulated microRNAs in the heart.

This is shown on the next slide. You can see here the hypothesis was that aging induces microRNAs or regulates microRNAs in the heart that lead to a cardiac aged phenotype and on the bottom you can see the expression profile of microRNAs. You can see several microRNAs being upregulated in old mice hearts compared to young mice hearts and the red dots represent these highly upregulated microRNAs and among them was one family the microRNA-34a family, which was significantly increased.

It was not only seen in mice but also in human hearts. On the right-hand side, you can see a correlation of age with an increased

expression of microRNA-34a in human hearts. What is the function? I'll just show you a few examples.

[0:35:08]
Slide 52

Here are data from knockout mice which we received by Heiko Hermeking's group from Munich and you can see on the top panel a comparison of knockout mice on the right-hand side and wild type mice on the left-hand side. You can significantly see a significant change, a better transfection, a better heart function in the knockout mice during aging in comparison to the wild type mice, which showed a decline in ejection fraction during age.

We also defined targets of these microRNA and identified a new target, which is called PNUTS. PNUT is an interesting protein because it's associated with telomere, it's associated with TRF2 and if you see on the left-hand side on the bottom, there is a reduction of PNUTS protein expression seen in aged hearts which is very dramatically.

We then looked on the function of PNUTS and showed that PNUTS regulates both telomere dysfunction as well as the DNA damage response pathway. On the left-hand side, you can see Lenti-mock transduced cardiomyocytes treated with stressors inducing an augmentation of DNA damage response pathways while the Lenti-PNUT transduced cardiomyocytes given in the black box showed a protection against DNA damage response.

On the very right side, you can see the telomere effects. These individual dots represent individual telomere and you see on the middle panel the Lenti-PNUT overexpression does not affect the longest telomere. So it's not working like telomerase. We suspect increasing telomere length wall but it's the physical effect occurrence of short telomeres in comparison to the mock-transduced cells. This was specifically attributed to the binding to ATF2, which is shown on the very right panel if you treat the lenti-PNUTS mutant which *[Indiscernible]* to binding activity, this effect is gone suggesting that as you can see in the cartoon above that PNUTS regulate telomere dysfunction and DNA damage response pathways involved in regulating cardiac function.

Slide 53

In the final slide, you can see that if the overexpressed is PNUT, even in vivo in a myocardial infarction model, we got a significant effect meaning the preservation of cardiac function. If you overexpress

PNUTs and together our data showed that acute myocardial infarction, I haven't had time to show you but also aging, induces microRNA 34a, which affects the protein PNUTS and of course also other targets like 31. But PNUTs is showed to have a predominant role, which regulates telomere dysfunction in DNA damage response pathways leading to apoptosis and cell death and finally contributes to contractile dysfunction.

Slide 54

I would like to thank the funding agencies supporting our work as well as particularly the people who did the studies.

Slide 55

The study was started with Angie Bonauer who worked on microRNA-92a in endothelial cells and Ariane Fischer, Dannie Penzkofer now contributed to the pig studies and the knockout mice together with Rabea Hinkel, Christian Kupatt of Munich, and Eva Van Rooij from Miragen provided the LNA inhibitors for the pig study. The aging heart was led by Reinier Boon and was done in collaboration with Heiko Hermeking whom I already mentioned and Hugo Katus as well as Andreas Zeiher who supported all of this study. Thank you very much for your attention.

Slide 56

Tianna Hicklin: Thank you Dr. Dimmeler. And a quick reminder to our live audience please submit your questions at any time by typing them into the textbox at the bottom of the screen. If you don't see the box, click the red Q&A icon and it should appear.

Slide 57

Our third and final speaker for today is Dr. Jan-Wilhelm Kornfeld. Dr. Kornfeld was originally trained in cancer biology and immunology and completed his Ph.D. studies (at the Ludwig-Boltzmann-Institute for Cancer Research in Vienna, Austria. He became interested in the molecular basis of obesity-associated liver cancer and remained at the LBICR to pursue his postdoctoral training. Now Dr. Kornfeld is an EMBO postdoctoral fellow at the Institute for Genetics at the University of Cologne. His research focuses on the study of energy and glucose homeostasis and the role of noncoding RNAs in insulin resistance and type 2 diabetes and understanding the role of functional RNAs in the regulation of brown adipose tissue function. Welcome Dr. Kornfeld, and thank you for being here with us today.

Dr. Jan-Wilhelm Kornfeld: Thank you, Tianna, for that kind introduction and thanks for giving me the chance to present our past and current research activities.

Slide 58

So what I would like to start with or what I would like to talk about today is give an example about how the in vivo inhibition of a small noncoding RNA, a microRNA, can be harnessed to combat complex diseases in vivo, in this case obesity associated insulin resistance which precedes diabetes.

[0:40:21]

On this sketch, I would like to start out with a small summary on maybe how our concept of the genome changed within the years. So what I still learned in graduate school is that the flow of genomic information basically is unidirectional, which means that genes which are encoded as DNA serve as template for the transcription into messenger RNAs which then are translated into proteins.

Slide 59

So this so-called central dogma of molecular biology received some first wrinkles in the '70s when it was shown that mobile DNA elements either directly as DNA transposons or indirectly via messenger RNA precursor so-called retrotransposons can also change the genomic information not unidirectionally. Furthermore, it has been shown that proteins can also change the information content of other proteins, which is the famous prions.

Slide 60

It has been shown also in the '70s that there is a small set of noncoding RNAs, which serve mostly infrastructural purposes. So for example ribosomal RNAs, TRNAs, small nucleolar RNAs or H19 and Xist which are implicated in the regulation of the edge of two locus or in each chromosomal inactivation.

Slide 61

But I think mostly due to the advance of new sequencing techniques summarized in the term next generation sequencing, we have been witnessing really an explosion of the zoo of noncoding RNAs. So as presented by my speakers before for example, small noncoding RNAs called microRNAs and lately long noncoding RNAs.

Slide 62

So what I just summarized here are some features of the noncoding transcriptome because I think this is really important for the talk. So

what has been found out by large consortia like the Ncode and the Phantom Consortium has been that most or the majority of the genome basically is transcribed and at least for the murin situation, we can think of that we have more than 30,000 noncoding RNAs encoded in the murin genome. Furthermore, I think it's important to stress that as shown by Genomewide Association Studies, it was found that most disease variants actually are located outside of protein coding genes. Furthermore, noncoding RNAs exhibit a rather tissue specific expression pattern when compared to messenger RNAs. A finding which I personally find really intriguing is the fact that the number of noncoding RNAs per species scales with eukaryotic complexity, which means we have more or less the same amount of protein coding genes as a worm or a fly but we have much more noncoding RNAs. What has been shown also is that the expression of noncoding RNAs is regulated in complex diseases like type 2 diabetes.

Slide 63

So on the first part of my talk, I would basically give you a hands-on example on the role of a microRNA in the regulation of hepatic glucose homeostasis.

Slide 64

So just to put things into perspective, so the amount which is spent to counteracting obesity alone in the US, which is $147B, is enough or almost enough to what is needed to end hunger worldwide. This sum is manifold the amount which would be needed to basically halt the spreading of most – of the biggest killers in the developing world which is malaria and HIV. So there's a huge economic interest in understanding the principles underlying the pathogenesis of obesity-associated comorbidities.

Slide 65 to Slide 66

So we basically asked the question so are noncoding RNAs involved in the development of obesity associated insulin resistance. As a start, we concentrated on small noncoding RNAs called microRNAs and doing this, we basically got hold on mouse models of obesity and insulin resistance.

Slide 67

As you can see on the left part, mice which were fed a calorie-rich high fat diet and on the other hand mice which harbor the diabetes mutation of the leptin receptor, which is a genetic model for obesity. Using whole immunome screenings, we could reveal that the

expression of microRNA-802 is coordinately upregulated in both mouse models. Furthermore, in collaboration with scientists at the University Clinic of Hamburg, we could show that the expression of human microRNA-802 which is conserved to the mouse microRNA also correlates with human body mass index. So we probably have the same function of microRNA-802 in mice and men.

[0:45:50]
Slide 68

So to get insight into the in vivo role of microRNA-802, we generated a transgenic mouse model where the gavage of doxycycline basically leads to the expression of mature microRNA-802. Using this and using insulin and glucose tolerance tests, we could show that the overexpression of microRNA 802 leads to a deterioration of insulin sensitivity as shown in the lower left part as well as a deterioration of glucose clearance as shown on the lower right part.

Slide 69

In the next step, we asked whether the in vivo inhibition of microRNA-802 would be able to improve the detrimental effect of diet-induced obesity. For this purpose, we used locked nucleic acids, which have been presented by my previous speaker, to silence the expression of microRNA-802 by administration via intravenously. So what you can appreciate in the lower upper left part is that basically injection of anti-microRNA-802 LNAs twice leads to a profound reduction of microRNA expression in liver and kidney. In the upper right part, you can see that administration of microRNA-802 locked nucleic acids leads to an amelioration of the metabolic states as shown here by fasting insulin levels. Furthermore, the loss of microRNA-802 predominantly in the liver and the kidney led to a reinstatement of insulin sensitivity as well as glucose tolerance shown in the lower part.

Slide 70

So then of course we were interested in the molecular mechanisms underlying this amelioration of the metabolic state by microRNA-802 loss. For this, we performed luciferase assays and we could show that the diabetes risk gene hepatocyte nuclear factor 1b or HNF1b constitutes one target of microRNA posttranscriptional gene silencing. This was also exemplified by the fact that lots of microRNA-802 using locked nucleic acids led to an increase of HNF1 beta protein levels.

Slide 71

As no study before has addressed the role of hepatic HNF1 beta, we generated adenoviruses, which encode either for short hairpin RNA targeting HNF1 beta or controlled adenoviruses. These were injected into mice and as you can appreciate on the left part is that the silencing of hepatic HNF1 beta leads to hyperglycemia so increased glucose levels in the random fed and in the fasted state. Furthermore, deletion of HNF1 beta leads to a deterioration of glucose metabolism shown on the right part via glucose tolerance test.

Slide 72

So basically, summarizing our finding, what we propose is that in the liver a microRNA-802 HNF1 beta access regulates glucose homeostasis. In this case, the expression of microRNA-802 leads to the silencing of HNF1 beta and the reduction of HNF1 beta protein levels, a finding which can be shown in diabetic mice as well. HNF1 beta itself in turn inhibits the expression of cytokine signaling negative regulators namely SOCS1 and SOCS3 as well as the reduction of gluconeogenic master regulators like PCK and G6P.

Slide 73

Now in the obese state, we have the condition of an excessive microRNA-802 tone. This overactive microRNA-802 expression leads to a further decrease of HNF1 beta, which on one hand leads to increased levels of SOCS1 and SOCS3, which directly impinge on the insulin receptor signal transduction.

On the other hand, increased microRNA-802 levels are coupled to a decreased HNF1 beta tone, which leads to a consecutive activation and upregulation of PCK and G6P, which leads to hyperglycemia and finally insulin resistance in these mice.

[0:50:09]
Slide 74

So this is to summarize our finding. What I try to convince you about is that the expression of hepatic microRNA-802 is upregulated in obese mice and humans. Furthermore, the overexpression of microRNA-802 impairs glucose tolerance. The loss of microRNA 802 alleviates detrimental aspects of diet-induced obesity like insulin resistance and glucose intolerance. Furthermore, the diabetes risk gene HNF1 beta constitutes one effector target of microRNA 802. We were the first to show that the loss of hepatic HNF1 beta leads to glucose intolerance and an excess of microRNA-802 HNF1 beta tone causes hyperglycemia. Of course, the ultimate dream would be that

the silencing of microRNA-802 is a novel avenue for combatting diabetes also in the human situation.

Slide 75

So on the second part of my talk, I would like to talk briefly about the role of microRNAs in brown adipose tissue. So what is brown adipose tissue?

Slide 76

Brown adipose tissue's role is in contrast to white adipose tissue the generation of heat. So the brown adipose tissue intrinsic uncoupling protein 1 leads to heat generation via dissipation of the mitochondrial protein gradient. Brown adipose tissue is not only found in human and infants but has also been found recently to be present in adult humans. Interestingly now, the activity of brown adipose tissue correlates with obesity and age and the dream of course is that if you could reactive this decreased BAT activity in age and obese individuals that this might constitute a very powerful antiobesity regimen.

Slide 77

So we asked the question are microRNAs implicated in the homeostasis of brown adipose tissue?

Slide 78

So as a starting point, we generated a new genetic tool, a CRE driver specific for brown adipose tissue. So in these mice, the expression of the CRE recombinase is driven by the brown adipose intrinsic UCP1 promoter. These mice we crossed with mice heterozygous for the Dicer1 allele or flox-Dicer1 allele and we ended up with mice in which one allele of Dicer, which is the canonical downstream processing node of microRNA maturation, is deleted. What you can see on the right side is that the expression of most mature microRNAs is compromised in these mutant mice.

Slide 79

So what I would just like to end with is that already the heterozygosity of microRNAs in brown adipose tissue in normal diet mice as seen on the left side is sufficient to impair glucose, a proper glucose tolerance. If you put these mice now on a high fat diet as seen in the rat figure on the right side is that this phenotype is even more compromised. In the moment, we are in the brink of identifying individual microRNA isoforms which might be implicated in this phenomenon.

Slide 80

So what I would like to do is acknowledge our collaborators namely Jorg Heeren and Ludger Scheja from the University Clinic in Hamburg as well as Markus Stoffel from the ETH in Zurich as well as Silvia Cereghini from the INSERM in Paris. Furthermore, I would like to stress the supervision by my supervisor Jens Bruning as well as Cathy Baitzel was implicated in all projects shown here.

Slide 81

In the last slide, I would just basically try to visualize that I think we are really in a noncoding revolution now because I mean new RNA isoforms pop out month by month. So recently it has been shown that there are circular RNAs which serve as microRNAs sponges and I think this is a really challenging and interesting time and with this I would like to conclude and thank you very much for your attention.

Tianna Hicklin: Thank you, Dr. Kornfeld, and thank you to each of our speakers for your presentations.

Slide 82

Now we're going to move right along to answering some of our audience's questions. A reminder, following the Q&A, we will have a short presentation from our sponsor and we will then provide you with a link to our online chat room where you can ask additional questions to the sponsor's representatives. Moving right along to our first question, Dr. Dimmeler, we can start with you, what makes microRNA and noncoding RNA in general interesting therapeutic antisense targets?

[0:55:00]

Dr. Stefanie Dimmeler: Basically, there are two reasons why I would be interested. The first is that microRNAs particularly in contrast to conventional targets have very mild effects on one specific gene expression of one pathway but modulate the networks of genes in a moderate manner. So this means by blocking microRNAs or by overexpressing microRNAs, you get a change in gene expression networks which might be something very attractive for the treatment of chronic disease. Now we know that it's not only one single pathway which is disregulated but multiple things go wrong. So I think this could be something which could be very interesting particularly because microRNAs are also stress responses meaning they are changed during diseases, this may be very effective.

The second thing is that microRNAs are at least in our view are very easy to be targeted by these antimirs making it quite easy to study these microRNAs in disease models. Of course the way to the clinic is very long and there are a lot of chances also but at least the tools available look quite easy in comparison to the development of specific inhibitors for kinases for example to name an example.

Tianna Hicklin: Great. Thank you. Dr. Corey, microRNAs can potentially regulate hundreds of targets, how can microRNA inhibitors have such a specific effect as shown in the presentations?

Dr. David Corey: Well they're not necessarily going to have a specific effect. What everyone has to understand is if you're getting into drug development with these as a target, it's like drug development anything else has to be taken on a case by case basis. So when you start developing a lead compound to bind to a particular microRNA, there's a possibility that you're going to have off target effects. Effects that are not just affecting the specific pathway that you intended and that's like any other drug. So you're going to have to take it on a case by case basis and you ought to evaluate the potential for those off target effects upfront by thinking about what the biology of the target is. So basically, the answer is just keep your eyes open.

Tianna Hicklin: Great. Thank you. Dr. Kornfeld, how long does the effect of a microRNA inhibitor last in vivo?

Dr. Jan-Wilhelm Kornfeld: That is a good question. We basically stopped our experimental regimen after two weeks but we have some preliminary data that at least the loch nucleic acids which we use in our studies might have the suppression effect for up to four weeks given at the dose which we use them in.

Dr. David Corey: Yeah this is David Corey, can I chime in?

Tianna Hicklin: Sure, that would be great.

Dr. David Corey: The clinical data on other nucleic acids you know, from trials with lots of patient show that these class of agents has a surprisingly long effect once it's administered. So we could be talking about if it has to be administered by injection maybe once a month or even less frequently.

Tianna Hicklin: Great.

Dr. Stefanie Dimmeler: I fully agree. We have also seen in the mouse studies effects lasting for six weeks.

Tianna Hicklin: Okay. Dr. Dimmeler, have you observed any side effects with the antimicroRNA-92 treatment?

Dr. Stefanie Dimmeler: That's a difficult question to answer because of course we did not look yet for extensive toxicology and things you would do in a classical track testing. I can just say that we haven't seen any obvious histological defects in the mice we have treated so far and we have done studies after treatment of several months for example with the inhibitor against 92a without seeing defects in organ functions or things like this. Creatinine levels, liver enzyme levels were all okay and also in the pigs, we didn't see anything but this doesn't mean -- I think careful testing is essential of course before concluding that there is no side effect.

Tianna Hicklin: And unfortunately, we are running short on time so maybe I can just throw out one final question that each of you can perhaps comment on. What do you find is the most challenging aspects of inhibiting microRNAs in vivo?

Dr. David Corey: This is David Corey. I mean you know, I'll start. The common answer in the field would be that delivery is probably going to be the most challenging aspect when you get into the clinic. As of right now, the clinical data show that nucleic acids can be efficiently delivered into the liver. That's what the approved drug does. Local administration is good too. For other tissues, we're going to have to see that on a case by case basis.

Tianna Hicklin: Dr. Dimmeler?

Dr. Stefanie Dimmeler: I am not so worried about delivery because at least in the hearts we have seen really a nice effectivity even in pigs and large animal studies and this is also shown by others for example Eva van [Indiscernible] has a nice publication on pigs as well. So this appears not the major or my view of the major trend is that these multiple targets which are moderately modulated, it's very difficult to define the precise molecular action of these microRNA inhibitors which makes all the development of these inhibitors a bit more difficult because you want to exactly know what's happening if you add these inhibitors. We only see minor changes in a lot of different genes so it's a bit difficult to define the specific activity.

[1:00:41]

Tianna Hicklin: Dr. Kornfeld?

Dr. Jan-Wilhelm Kornfeld: In general, I don't really see that much at least in our hands, the problem with the inhibition of microRNAs. I think what one mainly has to bear in mind is that the overexpression of microRNAs may constitute a problem when you basically oversaturate the microRNA processing machinery and this is something which we saw that for example if we express microRNAs, we also got off target effects by our improper maturation of other microRNAs. So I think this is something which one would have to be worried about. But for the inhibition at least in the animal models, we didn't see that much of a problem.

Tianna Hicklin: Well unfortunately, we have come to the end of the Q&A portion of our webinar and we'll now have a short presentation from Dr. Neils Frandsen from Exiqon who will talk about their available technology for inhibiting noncoding RNAs. Welcome, Dr. Frandsen.

Slide 83

Dr. Neils Frandsen: Thank you. In this presentation, I will give you a brief overview of Exiqon's LNA based products for function analysis of coding and noncoding RNA.

Slide 84

Exiqon's combined scientific expertise in RNA analysis with our proprietary LNA technology offering state of the art products for RNA research. In addition, we offer advanced bioinformatics tools and a wealth of supporting material and scientific advice. Join us after this short presentation for a live discussion of your function analysis questions.

Slide 85

Exiqon products are based on proprietary LNA technology. LNA, locked nucleic acid, is a nucleotide analogue that has the unique property of greatly increasing the strength of base pairing. LNA therefore enable us to make short LNA DNA mixmer oligonucleotides with dramatically increased affinity for complimentary DNA and RNA strands.

Slide 86

Exiqon offers highly potent antigens tools for the analysis of RNA function. In addition to increasing target affinity, LNA also allows us

to design the mode of action of our antisense agents. Gapmers with large gaps between LNAs catalyze RNase H-dependent knockdown of RNA with efficacy similar to siRNA. In contrast, anti-sense oligos with short gaps between LNAs do not provoke RNA degradation. This is useful for the design of microRNA inhibitors or a series of blockers that mask ribonucleoprotein binding sites on RNA modules.

Slide 87

We've developed a range of products for microRNA function analysis including two types of predesigned high affinity microRNA inhibitors for in vitro experiments. Regular LNA modified inhibitors with normal backbone and power inhibitors with a phosphorothioate backbone, which radically increases the resistance enzymatic degradation making power inhibitors the most potent on the market.

Slide 88

Some microRNAs fall in families of co-expressed and highly similar family members predicably with redundant functions. We have therefore developed special inhibitors for the knockdown of an entire family of microRNAs.

Slide 89

We also provide inhibitor libraries in 96-well plates convenient for high throughput screening of microRNA function.

Slide 90

Sometimes it is of interest to investigate the significance of a specific microRNA-messenger RNA interaction. Target site blockers are custom designed to mask a specific microRNA binding site on a messenger RNA of interest. By competing effectively with the microRNA, it will stabilize the messenger RNA and enhance its translation.

Slide 91

Finally, we offer custom-designed LNA inhibitors for the study of microRNA function in live animals. These are short oligonucleotides with a phosphorothioate modified backbone optimized for in vivo efficacy and to minimize problems of specificity.

Slide 92

Our in vivo inhibitors have sparked a revolution in microRNA research by enabling discovery of important and surprising microRNA functions that could not have been deduced from in vitro

experiments. Today's presentations by Dr. Kornfeld and Dr. Dimmeler are testament to this along with more than 20 publications in the last four years. The table shows that microRNA inhibition is observed in a wide range of organs including the heart where antisense activity generally have been difficult to achieve.

[1:05:02]

A recent publication of promising results from unlimited human phase 2 trial with an LNA and microRNA inhibitor highlights the two drug-like properties of these compounds. Could you please advance the slides? Okay.

Slide 93

LNA gapmers are short, single stranded oligonucleotides specifically designed to catalyze RNase H-dependent degradation of the RNA target. We've developed and empirically derived a design algorithm that incorporates more than 20 different design parameters. Gapmers are highly potent, as efficient or better than siRNA, and are very attractive alternatives for a number of reasons: Gapmers have fewer and different off-target effects; as RNA H is present in the nucleus. Gapmers are highly effective [Indiscernible] RNAs that are not easily addressed with siRNAs. Finally, as I will show you on the next slide, our gapmers display potent activity in live animals upon administration. Next slide please.

Slide 94

This graph shows the very first result of Reinier Boon from Dr. Stefanie Dimmeler's group. Ten gapmers designed the knock down of malat 1 highly expressed nuclear long noncoding RNA with screen in vitro. Three gapmers were chosen for testing in mice. As yet, no effort had been made to optimize the dose machine. Almost complete knockdown is achieved and delivered as measured by QPCR and around 60% knockdown is observed in muscle and heart where gapmers traditionally have failed. So just as our in vivo inhibitors have done for microRNA research, we feel confident that LNA gapmers liberate the discovery of long noncoding RNA function in animals. With that I will conclude my presentation and invite you to join our live chat, next slide please, a live chat session with myself and my colleagues where you're welcome to ask any questions you may have about RNA's function in analysis. Thank you.

Tianna Hicklin: Thank you, Dr. Frandsen. And this concludes our webinar for today.

Slide 95

I'd like to thank each of our wonderful panelists once again for being with us today and for the great talks and discussion they've provided, Dr. David Corey from the University of Texas Southwestern Medical Campus, Dr. Stefanie Dimmeler from Goethe University, and Dr. Jan-Wilhelm Kornfeld from the University of Cologne. Thank you to all of our online viewers for your great questions. I'm sorry we didn't have the time to get to them all today but please feel free to move over to the online chat room where you can continue the conversation. Simply click on the link that should now be up on your slide viewer.

Slide 96

And this audio webinar will be available to listen to again on demand within the next 48 hours. We encourage you to share your thoughts about the webinar by sending an email to the address now shown in your slide viewer webinar@aaas.org.

Slide 97

For more resources related to today's discussion, please visit the URL now listed at the bottom of the slide and please be on the lookout for more webinars from *Science*, which you can find by visiting webinar.sciencemag.org. Thank you again to all of our panelists and to Exiqon for generously sponsoring today's educational seminar. Thank you to our online audience for taking the time to be with us today and once again if you'd like to join us with the live chat, you still can.

Slide 98

I've placed the URL up in your webinar viewer now. Thank you for listening and I hope you'll join us again for the live chat and for *Science* in the next *Science*/AAAS Technology Webinar.

[1:08:32] **End of Audio**