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MEETING REPORT

NON-CODING RNAs AND CANCER

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SUMMARY

The American Association of Cancer Research (AACR) Conference on Non-Coding RNAs and Cancer was introduced by a keynote address from David Baltimore (California Institute of Technology), and included sessions on topics such as long non-coding RNAs (lncRNAs), biology and regulation in development of microRNA (miRNA), RNA in cancer and disease, and RNA therapeutics. Approximately 120 scientists from industry and academia attended the meeting. Non-coding RNAs are transcribed from a large percentage of the genome. Until recently, their existence and their potential to possess important functions have been unappreciated. However, miRNAs are now recognized as important regulatory agents, rivaling proteins in their importance for normal physiology and disease. The function and therapeutic impact of lncRNAs are less well known, but early investigations have implicated them in many important processes. The field of oligonucleotide therapeutics is also advancing, with steady progress being made with small interfering RNAs (siRNAs) and antisense oligonucleotides. The intersection of non-coding RNA biology and oligonucleotide therapy may provide opportunities for translating basic advances into promising therapeutic programs. Non-coding RNAs and cancer is an emerging area in which rapid progress is occurring. Although many questions remain unanswered, this meeting highlighted that non-coding RNAs have the potential to have a major impact on our understanding of basic biology and therapeutic development over the next decade.

Key words: Non-coding RNAs – miRNA – RNA therapeutics – Cancer – Obesity

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THE BIOLOGY OF lncRNA

Jeannie Lee (Massachusetts General Hospital) discussed the spreading of X-chromosome inactivation via a hierarchy of defined polycomb stations. Xist RNA is a 17-kB long non-coding RNA (lncRNA) transcribed within the X-chromosome inactivation center. lncRNAs are known to be involved in promoting histone modification and epigenetic silencing on the silent X-chromosome, and, by analogy with better-characterized silencing mechanisms in yeast, it was hypothesized that RNA-mediated modification in mammalian cells might involve the polycomb protein PRC2. RNA immunoprecipitation (RIP) was used to address this hypothesis. Using an antibody to PRC2 and PCR of the immunoprecipitate, the presence of Xist RNA was detected. However, key questions remained unanswered, including whether Xist acted in the *trans* or *cis* conformation. The dogma was that Xist was *cis* acting; however, when Xist was expressed from a transgene, Xist expression from the endogenous inactive X-chromosome was squelched within 24 hours. Deletional analysis identified a squelching domain within the RNA: a repetitive sequence termed "repeat F". Repeat F appeared to be an RNA localization motif, and was found to bind the YY1 protein. Mutagenesis of repeat F eliminated the binding of YY1 protein and squelching. These data suggest that YY1 is a Xist receptor and that repeat F is the nucleation center for Xist binding to YY1.

Dr. Lee also addressed how differences arise between the active and inactive X-chromosome. YY1 binds to the inactive X-chromosome

only. Xist binds YY1 through both the F repeat and another repeat, the C repeat. The X-chromosome is active as long as the antisense transcript at the Xist locus is made. During X inactivation, the antisense transcript disappears. It was then explained how silencing could spread throughout the inactive X-chromosome. The use of allele-specific ChIP sequencing to examine how polycomb protein location changes over time was discussed. Polycomb can first be detected at the X inactivation center. It then localizes to over 100 positions, which are characterized by CpG islands or 5′ gene ends. Next, protein localization spreads to approximately 3,000 non-canonical sites. It is not clear why localization to the non-canonical sites occurs, as they lack polycomb response elements or conserved motifs.

SMALL RNA-MEDIATED ACTIVATION AND SILENCING OF TRANSCRIPTION

Transcriptional activation or silencing by small RNAs was discussed by David Corey (University of Texas Southwestern). Published data were described regarding the discovery of transcriptional silencing by small RNAs complementary to sequences adjacent to gene promoters, and the concept that small RNAs can function like protein transcription factors was introduced. Protein transcription factors can inhibit transcription in one genetic context and activate it in another, so it was hypothesized that RNA might also be able to activate expression. This hypothesis was validated by the observation of activation in a cell line with low target gene expression. It was pointed out that the mechanism did not involve recognition of DNA, but that the small RNAs bind to nascent non-coding transcripts at gene promoters. Binding requires protein argonaute-2, a central protein in the RNAi machinery. Nascent transcripts also overlap 3′ gene ends, and it was hypothesized that they might also be targets for modulatory RNAs. A 3′ sense RNA overlapping the progesterone receptor was identified and it was shown that it was a target for small RNAs that modulate gene expression. It was then reported that the chromosomal locus encoding progesterone receptor loops juxtaposes the 3′ and 5′ termini of the gene, providing a short path for transducing a signal from 3′ interactions. Further work, conducted in collaboration with Bethany Janowski (University of Texas Southwestern), was also presented. Mechanistic information was gained from prior studies to understand RNA modulation of a gene involved in cancer. This was used to discover non-coding transcripts that overlapped the promoter of *COX2*, and identified small RNAs capable of activating *COX2* gene expression by 25-fold. It was also found that the expression of *PLA2G4*, an adjacent gene, was activated. Analysis showed that the *COX2* and *PLA2G4* genes were also spatially close, providing an explanation for the shared activation program.

piRNAs

Small RNA pathways in germline cells were discussed by Alexei Aravin (California Institute of Technology). Piwi proteins are essential for gametogenesis and their absence in flies is known to cause male sterility. These proteins provide a general mechanism for controlling transposons in flies. Piwi acts to generate piwi-interacting RNAs (piRNAs). Unlike microRNAs (miRNAs), which number in the hundreds, there are millions of piRNAs, and a single precursor transcript gives rise to many piRNAs, seemingly at random. The only common feature appears to be uridine at the 3′ end. lncRNAs are major sources for piRNAs and 90% of them appear to match transposon sequences. In flies, the *flamenco* mutation is known to reduce the effects of piRNAs. *Flamenco* is an insertional mutation that, rather than occurring within a protein-encoding gene, appears to break up a cluster of piRNAs and probably disrupts their promoter sequence. Efforts to construct artificial piRNAs were then discussed. When protein-encoding sequences were inserted into a piRNA cluster, the piRNAs were still observed. Furthermore, piRNAs were still observed when all uridines were removed. It was concluded that a genomic context is critical for piRNA formation. More research is needed to determine the specifics of piRNAs.

miR-208a – A POSSIBLE TARGET FOR OBESITY RESEARCH

Eric Olson (University of Texas Southwestern) presented results describing miRNA function in vivo, first focusing on miR-208a. This miRNA is expressed with the myosin (*MYH*) gene and is only found in cardiac muscle. Oligonucleotides containing locked nucleic acid (LNA) bases were used to inhibit the action of miR-208a in mice. These LNAs protected mice from cardiac damage and prolonged their lifespan. The mice treated to inhibit miR-208a function had reduced fat mass and reduced serum triglycerides. Experiments suggested that this phenotype was due to reduced energy expenditure and was not related to heart function. These data suggest that the heart is generating a product, possibly a peptide or small-molecule hormone, that can influence energy balance. Identification of this factor would be a major result and provide an important target for obesity research. Regarding the mechanism, MED-13, a component of the mediator complex, appeared to be a target for miR-208a. When miR-208a was inhibited, expression of MED-13 increased, and overexpression of MED-13 made animals resistant to obesity. When MED-13 was genetically knocked out, mice became obese when fed a high-fat diet and metabolic syndrome was observed. MED-13 also has several targets for another miRNA pair, miR-378 and miR-378*. Animals lacking these miRNAs develop normally but, as seen for the miR-208a knockout animals, they are resistant to a high-fat diet.

TOUGH DECOYS FOR IN VIVO RESEARCH IN MICE

RNA transcripts that contain miRNA target sequences, known as tough decoys, were described by Phillip Zamore (University of Massachusetts Medical School). When overexpressed, these decoys compete for the cellular pool of miRNA complementary to the target and deplete the pool of free miRNA. This depletion derepresses the miRNA's target genes. It was shown that the tough decoys can be introduced into an adeno-associated viral (AAV) vector and delivered to mice, and expression of the decoy can be detected for > 20 weeks. miR-122 was noted as a classical system for studying miRNA inhibition, because reduction of miR-122 leads to reduced serum cholesterol, an effect that can easily be observed. It was reported that one injection was sufficient to achieve sustained phenotypic change. The current goal is to make AAV vectors capable of inhibiting most mouse miRNAs. This tough decoy approach promises to be faster than generating knockout mice and less expensive than the use of synthetic oligonucleotide anti-miRNAs.

THE ROLE OF LET-7 IN THE REGULATION OF DEVELOPMENT AND CANCER

Richard Gregory (Children's Hospital Boston/Harvard Medical School) discussed miRNA regulation in stem cells and cancer. It was noted that the let-7 miRNA accumulates during development, and that increased let-7 is due to enhanced stability, not increased transcription. The protein Lin-28A was found to bind to let-7 and was shown to control its accumulation. Binding is through a loop sequence, but the sequence is not conserved, raising the question of how it is recognized. A recent structure revealed two modulatory domains connected by a flexible linker. When Lin-28A binds to let-7, terminal uridylyltransferase (TUTase) is recruited, which causes uridines to be added to the miRNA and leads to its destruction. A related protein, Lin-28B, also appears to regulate let-7, but through a TUTase-independent mechanism. Inhibition of expression of either Lin-28A or Lin-28B using small interfering RNAs (siRNAs) was found to reduce cell proliferation.

RNA THERAPEUTICS

Results of studies investigating the targeting of miRNAs with synthetic anti-miRNAs were presented by Sakari Kauppinen (Santaris Pharma). When targeted to miRNA seed sequences, short LNA oligomers take advantage of the high affinity of LNA–RNA recognition to bind tightly. An eight-base anti-miRNA targeting miR-9 reduced liver tumor growth when formulated in saline. This result suggests that short anti-miRNAs that are less than half the size of most antisense oligonucleotides currently in clinical trials may be effective lead compounds for development. The clinical progress of miravirsin, a 15-base anti-miRNA drug designed to treat hepatitis C virus (HCV) infection was then described. This anti-miRNA was designed to target miR-122, which plays a role in cholesterol metabolism. Inhibition of miR-122 reduces serum cholesterol levels, providing a sensitive output for monitoring the success of in vivo administration. miR-122 is also a host factor that binds viral protein and increases viral abundance, making it a promising target. In animal studies, dosing reduced viral load in chimpanzees, while phase I clinical studies demonstrated reduction of serum cholesterol with only minor side effects. The half-life in plasma was 30 days, suggesting that infrequent dosing would be adequate. A phase IIa study in patients showed a reduction in viral load, with some patients having no detectable virus. As with the phase I study, only minor side effects were seen. Because miR-122 is a host factor, the development of resistance may be less likely.

Claes Wahlestedt (University of Miami) described efforts to modulate gene expression by targeting lncRNAs. It was noted that there were many ways to inhibit gene expression, but relatively few strategies for enhancing gene expression. Targeting an antisense transcript that overlaps the brain-derived neurotrophic factor (BDNF) locus with antisense oligonucleotides was detailed as an example. The oligonucleotides reduced antisense transcript levels and induced a twofold increase in the levels of BDNF. Research at CURNA, a subsidiary of OPKO Health, yielded seven validated candidate oligonucleotides capable of upregulating gene expression.

The development of miRNA therapeutics for cancer was discussed by Eric Marcusson (Regulus Therapeutics), who described targeting miR-21 using methoxyethyl (MOE) oligonucleotides. MOE was chosen because it has been used in thousands of patients in clinical trials over the past decade and has a proven ability to reach targets in people. The anti-miR-21 therapeutic, which is being investigated in collaboration with Sanofi, can be delivered in vivo in a saline formulation and was tested in mouse models of hepatocellular carcinoma. All 23 untreated mice were dead after 100 days, while 10 of 13 drug-treated mice survived after 160 days. Delivery of anti-miRNA oligonucleotides to the brain to treat glioblastoma was also described. It was shown that distribution throughout the CNS after intrathecal injection was robust. Inhibition of miR-21 was shown in cells derived directly from patients. These studies provide a promising platform for developing drugs to treat an intractable disease.

DISCLOSURES

The author states no conflicts of interest.

The website for this meeting can be found at http://www.aacr.org/home/scientists/meetings-workshops/special-conferences/noncoding-rnas-and-cancer.aspx.