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The epitranscriptome: tools to study, manipulate, and exploit RNA modifications

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Commentary to 'Existence of diverse modifications in 16–28 nt small RNAs' by Lan *et al.*, *Chemistry*, 2018 and 'Precise RNA editing by recruiting endogenous ADARs with antisense oligonucleotides' in *Nature Biotechnology*, 2019.

In 2017, the ESC working group on Cellular Biology of the Heart published a position paper in *Cardiovascular Research*, providing important guidelines for 'omic' research in the post-genomic era.[1] The authors provide a comprehensive overview of techniques to obtain both epigenomic and transcriptomic data, including advantages and limitations of each technique. Furthermore, the working group offers recommendations for dealing with the large datasets generated from both epigenomic and transcriptomic analyses. I have no doubt that these recommendations have proven to be useful to researchers, and reviewers, worldwide who venture into the post-genomic era. However, I would plea for an addition to the position paper, which includes yet another, important, 'ome', namely the 'epitranscriptome'.

RNA transcripts are not finished products. From mRNAs, we know about splicing, capping, and polyadenylation, but RNAs are also subject to modifications at single-nucleotide level. Such post-transcriptional RNA nucleotide modifications (here abbreviated as R-PTMs) are likely as various and as abundant as post-translational protein modifications (commonly abbreviated as PTMs). Currently, more than 150 different R-PTMs have been identified, occurring in organisms ranging from archaea and bacteria, to eukaryotes.[2] Although some R-PTMs were discovered already as early as in the 1950s (pseudouridine was discovered in 1951[3]), most of these modifications remain understudied even today. However, as we are discovering more about the potential regulatory roles of R-PTMs, there is an increasing attention to what has now been named the epitranscriptome.

For many forms of R-PTMs, we still have limited understanding of their molecular function, let alone about their biological role. For other R-PTMs however, their potential function is more obvious. An important example of such an R-PTM is adenosine-to-inosine (A-to-I) editing. In A-to-I editing, RNA adenosine residues are deaminated to inosines by adenosine deaminases acting on RNA (ADARs). Inosine closely resembles guanosine and indeed, rather than binding uracil, inosine binds cytosine in Watson–Crick base-pairing. This is crucial, as it means that A-to-I editing allows for specific changes the genetic code of mRNAs, potentially leading to the production of functionally different proteins. However, A-to-I editing has also been shown to direct (alternative) splicing of pre-mRNAs and, as described below, A-to-I editing can redirect microRNA target gene selection under pathological conditions (*Figure 1A*).[4,5] With regards to cardiovascular disease, Stellos *et al.*[6] demonstrated in 2016 that A-to-I editing of the Cathepsin S mRNA by ADAR1 plays an important role in atherosclerosis. In a more recent study, Jain *et al.*[7] showed that editing of the Filamin A mRNA also plays a crucial regulatory role in vascular homeostasis.

Modifying protein function without having to edit the genome would offer several crucial therapeutic benefits. By editing transcripts rather than the genome itself, effects on protein function become temporary, and thus reversible and regulatable, rather than permanent and absolute. Transcriptome editing could be a suitable means of therapy for many common diseases, including non-hereditary disorders, in contrast to genome editing, which appears mostly suitable for very specific, mostly rare, genetic disorders. The paper by Tobias Merkle *et al.*[8] in *Nature Biotechnology* describes a novel method to induce transcriptome editing in a highly specific manner.

Merkle *et al.*'s chosen tactic is to recruit endogenous ADAR enzymes to specific targets sites in the transcriptome using guide RNAs, which consist of a site-specific antisense oligonucleotide (ASO) domain and an ADAR-recruiting domain (*Figure 1C*). The technique is named RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing).[8] RESTORE is an improvement on previously published editing methods, which all relied on ectopic (over-)expression of ADARs, leading to massive off-target editing events. With RESTORE, the authors could demonstrate minimal off-target editing events. Furthermore, all off-target events that were observed, depended on reverse sequence homology with the guide RNA, indicating that potential off-target events induced by RESTORE-based strategies could be predicted and potentially prevented by optimized guide RNA design.[8] The authors further show that the intended editing events could effectively be induced (with up to ~90% efficiency) in both human cell lines and primary human cells.

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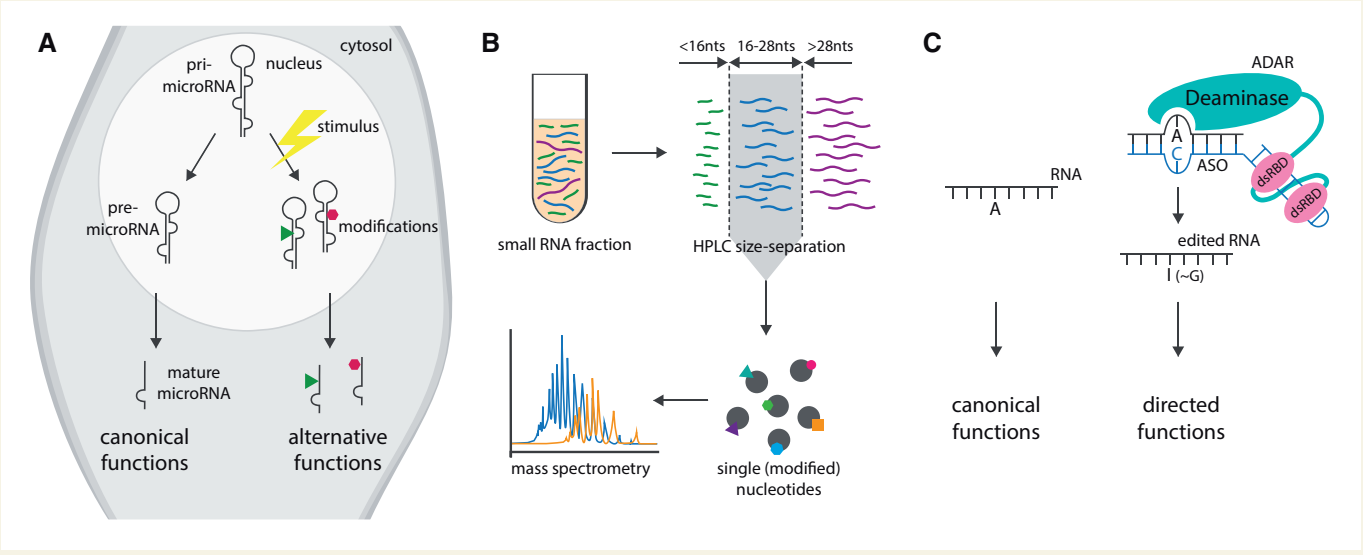

Figure 1 Schematic overview of two novel epitranscriptome tools. (*A*) RNAs, including (pri- and pre-) microRNAs, as depicted here, can undergo post-transcriptional modifications. Both my group and others have shown that such modifications are induced by cellular stress, altering the function of the affected RNAs. (*B*) To detect modifications in small RNAs, total RNA is extracted from cells and the small RNA fraction is isolated. This fraction is further separated by size using high pressure (or high performance) liquid chromatography. 16–28 nucleotide long RNAs are hydrolyzed to single (modified) nucleotides, which are identified by mass spectrometry (Adapted from Lan *et al.*[11]). (*C*) The epitranscriptome can be manipulated specifically, potentially for future therapeutic applications. An ASO consisting of a target-site-specific domain and an ADAR-recruiting domain directs the deaminase enzyme ADAR to a target site in the transcriptome, leading to site-specific A-to-I editing of the target RNA (Adapted from Merkle *et al.*[8]). Image created by Vesna Krajina.

The authors varied the degree of chemical modifications on the guide RNAs used and found that both the target-site-specific ASO domain and the ADAR-recruiting domain accept extensive chemical modifications.[8] This will facilitate their use, as it allows for optimization of both stability and cellular uptake. A further benefit of chemically modifiable oligos is of a more practical nature. As described in a commentary by Stellos *et al.*[6] in *Cardiovascular Research*, there are many challenges to the clinical translation of RNA therapeutics. The ability to use chemical modifications to match other ASOs in clinical development could facilitate development procedures, by learning from previous experience with similarly modified compounds.

However, mRNAs are not the only RNAs that are edited. And of course, editing is only one of many potential epitranscriptomic modifications. Just like mRNAs, microRNAs are subject to post-transcriptional modifications at single-nucleotide level, abbreviated here as miR-PTMs. MicroRNAs are small endogenous RNA molecules that inhibit translation of their target mRNAs. They are crucial players in all forms of vascular remodelling and cardiovascular disease.[9] Last year, my group demonstrated that an important vasoactive microRNA, miR-487b-3p is subject to A-to-I editing in the seed sequence of the microRNA. In fact, the proportion of edited miR-487b-3p is significantly increased under ischaemia, which leads to a more active form of the microRNA, with a completely different 'targetome', that induces post-ischaemic neovascularization.[5] But the same miR-487b-3p adenosine residue that can be edited, is also subject to another miR-PTM, namely 2′O-ribose-methylation (2′OMe). 2′OMe of ribosomal RNAs and small nuclear RNAs is essential for pre-mRNA splicing and ribosomal protein synthesis and is directed by small nucleolar RNAs (snoRNAs). We demonstrated that 2′OMe of miR-487b-3p is likely snoRNA-dependent as well.[5] Although no snoRNAs have been identified so far that specifically direct microRNA-methylation, many orphan snoRNAs (snoRNAs without known RNA-targets) exist. Earlier this year, we demonstrated that a specific set of orphan snoRNAs, transcribed from the same gene locus as miR-487b, plays a regulatory role in human cardiovascular disease, in heart failure in particular.[10]

Although we know that many miR-PTMs exist and that they play regulatory roles in human (cardiovascular) physiology and pathology, miR-PTMs are even less studied than 'regular' R-PTMs, not in the last place because of a lack of reliable methods to detect and quantify modifications specifically in small RNAs. Lan *et al.*[11] present an effective pipeline to detect R-PTMs in small RNAs, based on small RNA isolation and enzymatic digestion, followed by liquid chromatography, electrospray ionization, and tandem mass spectrometry. Besides methodology to reliably detect R-PTMs in small RNAs, the authors also provide a first-ever overview of R-PTMs in mammalian small RNAs (*Figure 1B*). Lan *et al.* detected 24 different R-PTMs robustly present in human small RNAs, including both modifications described above. Even though the number of different R-PTMs identified in small RNAs was lower than in long RNAs in HEK293T cells (24 vs. 57), this study does demonstrate that R-PTMs likely play an important role in small RNA biology.[11]

The epitranscriptome is still largely a scientific *Terra Incognita*, particularly in small RNAs, but its relevance to human (cardiovascular) disease is evident. The two papers by Merkle *et al.*[8] and Lan *et al.*[11] discussed here offer exciting new methods to both study and manipulate the epitranscriptome for research and future therapeutic purposes.

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Biography: Anne Yaël Nossent after completing her postdoc at the University of Copenhagen and the University of Southern Denmark, Dr Nossent initiated an independent research line on the role of microRNAs in neovascularisation at the Leiden University Medical Center in Netherlands. She was awarded a personal Veni grant by Netherlands Organization for Scientific Research to support this line of research. In her Veni project, she demonstrated a role for the 14q32 miRNAs in post-ischemic neovascularization. Dr Nossent and colleagues also demonstrated positive effects of 14q32 miRNA inhibition on atherosclerosis and restenosis. Besides 54 miRNAs however, the 14q32 locus also encodes 3 lncRNA and 41 snoRNAs. Dr Nossent was recently awarded grants by Netherlands Heart Foundation and the Rembrandt Institute for Cardiovascular Sciences to support further research on the role of these lncRNAs and snoRNAs in cardiovascular disease. In September 2017, Dr Nossent moved to Vienna, Austria, where she continues her research on noncoding RNAs, supported by a Lise Meitner Grant from the Austrian Science Fund FWF. She now holds a dual affiliation with both the Medical University of Vienna and the Leiden University Medical Center.