Molecular Therapy
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Commentary

Trans-amplifying RNA hitting new grounds: Gene regulation by microRNA

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In the recent past, the research team of Sahin and Beissert has engineered an alphavirus-based *trans*-amplifying RNA (taRNA) split-vector system of self-amplifying RNA (saRNA).[1,2] Similar to the original saRNA vectors, taRNA can elicit specific antigen immunogenicity at significantly lower doses compared to conventional mRNA. The removal of redundant sequences has further enhanced *trans*-replicon levels, demonstrating that immunization with only 50 ng of influenza virus hemagglutinin taRNA was sufficient to elicit neutralizing antibody responses and protected mice against challenges with lethal doses of influenza virus. In a recent issue of *Molecular Therapy Nucleic Acids*, the research team of Sahin and Beissert has further expanded the taRNA technology to deliver functional microRNA (miRNA) to target cells and demonstrated its potential application for gene regulation.[3]

RNAi applications, not the least miRNAs, have been proven potent for the degradation of mRNA and regulation of gene expression,[4] which can be utilized for therapeutic approaches.[5] Although lentivirus vectors have been employed for the efficient delivery of miRNAs, the risk of insertional mutagenesis[6,7] has triggered the utilization of alternative viral vectors, such as single-stranded RNA alphavirus vectors.[8] In this context, the second-generation Venezuelan equine encephalitis (VEE) virus-based taRNA vector[2] has been engineered, where an artificial miRNA was introduced into the 3′ UTR of a protein-coding *trans*-replicon.[3]

In the latest application of taRNA, the replicase from the Trinidad donkey (TRD) VEE strain was used due to its lower cytotoxicity compared to the replicase of Semliki Forest virus strains.[9] The TRD-VEE replicase was introduced into a non-replicating mRNA (nrRNA-REPL) vector, and the commercial lentivirus artificial miRNA expression/reporter cassette comprising the emerald green fluorescent protein (emGFP) and an optimized pre-miR-155 containing an artificial miRNA targeting LacZ mRNA (miR-LacZ) were incorporated into the short *trans*-replicon vector.[10] Studies in BHK-21 cells stably expressing β-galactosidase (β-gal) showed a high transduction rate of 90% of emGFP, and the taRNA-miR-LacZ significantly reduced LacZ transcript levels by 70% and β-gal protein levels by 50%. However, the cell viability was not affected. Although the LacZ silencing was less efficient compared to similar lentiviral vectors, the taRNA-miR specifically suppressed the expression of the target protein.

Furthermore, Sahin and Beissert have replaced the miR-LacZ with two artificial miRNAs targeting firefly luciferase (Luc) in BHK-21 cells stably expressing Luc. Using a replication-deficient mutant (inactive-REPL), no Luc suppression was detected. In contrast, the TRD-REPL reduced Luc expression by 50%–60%, and co-transfection with a hyperactive replicase construct (hyper-REPL) showed superior silencing of 80%. In preparation for *in vivo* studies and particularly clinical trials, liposomal nanoparticle formulations of taRNA-miR provided similar transgene expression levels and target silencing in transfected cells as achieved by electroporation of naked taRNA-miR.

Moreover, taRNA-based downregulation by artificial miRNA targeting human p53 was demonstratedat both the transcriptional and protein levels in primary human dermal fibroblasts (HDFs), showing up to 80% knockdown efficiency for several days. An interesting part of the study addressed the functional delivery of miRNA gene clusters. In this context, five hairpin miRNAs (miRNA-302a, miR-302b, miR-302c, miR-302d, and miR-367), highly expressed in human embryonic stem cells and inducing pluripotent stem cells, were introduced into the taRNA-miR vector. Co-transfection of taRNA-miR-302/367 did not compromise the transient expression in HDFs but the DAZAP2 and transforming growth factor βR2 target genes were significantly suppressed. However, although more than 10-fold higher transgene levels were achieved for synthetic miRNA-based expression, the gene silencing was only 30% higher compared to taRNA-miR-302/367. Therefore, the taRNA-miR platform can efficiently deliver a natural polycistronic miRNA gene and should be suitable for future gene therapy applications.

The extension of the taRNA platform to provide simultaneous delivery and functional expression of protein-coding sequences and miRNA will certainly have major future implications for therapeutic interventions in the area of gene therapy but also for superior vaccine development. Although the processing of pre-miRNA by Drosha generally occurs in the cell nucleus,[11] miRNAs introduced into RNA viruses can be processed in the cytoplasm.[12] Here, the successful processing has been confirmed by efficient target mRNA suppression mediated by taRNA-miR vectors. Moreover, the gene silencing did not quantitatively affect the transgene expression, as demonstrated by the simultaneous protein expression and processing of mature miRNA. It was also shown that the kinetics of taRNA-miR-mediated targeted gene suppression and the accumulation of mature artificial miRNA correlated with the kinetics of the taRNA-miR replication. The application of a hyperactive replicase also

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confirmed that enhanced taRNA-miR transcription generated a stronger target knockdown, which lasted for several days. However, as the RNA processing is less efficient in the cytoplasm compared to the nucleus, it requires RNA amplification, which places taRNA-based systems into a favorable position compared to nrRNA-REPL.

In the context of polycistronic miRNA clusters, the miR-367 processing was most efficient, which has been demonstrated previously for Sendai virus vector-based delivery.[13] Therefore, one of the critical issues for the future development of the taRNA-based system is the identification of more efficient miRNA backbones for taRNA-miR. Another issue relates to the improvement of the efficacy of the mRNA processing by the engineering of pre-miRNA hairpin cleavage sites, which can have a positive effect on pre-miRNA processing by Drosha.[14] Alternatively, overexpression of Drosha or Argonaute-2 might contribute to pre-miRNA processing.[15]

In the context of future development of the taRNA-miR platform, it is important to identify potential off-target effects and any miRNA sequences in the vector backbone. Although the potential inhibition of taRNA expression by innate immune responses has been addressed by co-transfection of non-replicating RNA expressing the vaccinia virus immune evasion proteins E3 and B18R,[16] it might be favorable to instead engineer immune-modulating artificial miRNAs. This approach would be more appropriate for clinical applications. Implementing the modifications described above should allow superior co-delivery of therapeutic proteins and miRNAs, which will further expand the application range of the taRNA system and make it an important player in future medical use.

DECLARATION OF INTERESTS

The author declares no competing interests.

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