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COMMENTARY

Manipulating ribozyme enlightens large fragment delivery: Sunshine of RNA repair to gene editing

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Received: January 21, 2025; Accepted: May 20, 2025; Published Online: May 27, 2025; https://doi.org/10.59717/j.xinn-life.2025.100140

Citation: Lu L., Yuan L. and Huang Y. (2025). Manipulating ribozyme enlightens large fragment delivery: Sunshine of RNA repair to gene editing. *The Innovation Life* **3**:100140.

In a recent study published in *Science*, Lindley et al. describe a method called *StitchR*, which utilizes the unique catalytic activity of ribozymes to activate the trans-splicing mechanism of mRNA through precise cleavage.[1] It enables the seamless joining and translation of two independent mRNAs in eukaryotic cells, providing a new RNA repair- based strategy for gene therapy.

Ribozymes are widely found in nature and have unique catalytic activities that specifically recognize and cleave RNA substrates to produce specific RNA ends.[2] This property provides a natural tool base for RNA-based manipulation techniques, and the *StitchR* capitalizes on this property of ribozymes by activating mRNA trans ligation by the ribozyme. *StitchR* can precisely cleave mRNA fragments to produce the appropriate ends, which in turn facilitates the trans-joining of these fragments, enabling the re-editing and reconstruction of mRNA sequences.

Macromolecular gene delivery is the process of delivering therapeutically useful nucleic acid macromolecules, such as DNA or RNA, into cells. These nucleic acid molecules usually encode information that can correct disease-related genetic defects, regulate cellular function, or generate an immune response. Many serious genetic diseases are caused by mutations or deletions in large genes. For instance, treatment of these genetic diseases might require delivery of intact genes into patients' cells to restore normal gene function.

In gene delivery, viral vectors are one of the commonly used means, but their immunogenicity is a significant problem; liposomal delivery, although relatively low in immunogenicity, may still cause some immune-related problems, especially in the case of repeated use or high dosage, and both are unable to achieve large gene delivery. Ribozymes, on the other hand, are mainly composed of RNA, which has a relatively simple structure and is less immunogenic. To this end, Lindley's team designed a series of experiments to validate their innovative *StitchR* method, which successfully demonstrated the delivery of large proteins based on ribozymes, overcome the traditional challenges brought by AAV packaging limitations, dramatically increases gene delivery capacity. They designed and optimized the innovative *StitchR* method, successfully achieving the delivery of large molecular proteins. They first constructed an expression plasmid with non-overlapping N-terminal and C-terminal GFP reporter gene fragments (Figure 1A), and then successfully achieved trans-splicing and translation of mRNA in HEK 293T and COS7 cells, producing green fluorescence. Subsequently, they confirmed the connection at the predicted ribozyme cleavage sites using RT-PCR and Sanger sequencing technologies. Through the action of ribozymes, they found that only when both vectors were co-transfected could the full-length GFP protein be expressed, a process they named *StitchR*. To optimize the activity of *StitchR*, they systematically screened and combined optimized ribozyme sequences from different families such as Twister, HDV, and HH. By mixing pairing Nt-Luc and Ct-Luc vectors, they quantified the impact of various sequence modifications on seamless cleavage at the 5' and 3' positions. The versions of *StitchR* differ in ribozyme combinations, sequence optimization, and intron usage (Figure 1A). After continuous optimization, the resulting *StitchR* 4.0 showed a 911-fold enhancement in expression efficiency compared to *StitchR* 1.0, and its activity was validated in a variety of cell lines and demonstrated that the activity of *StitchR* was dependent on catalytic cleavage by the nuclease, providing more effective tools for applications such as gene therapy.

After in vitro verification of the *StitchR* method, Lindley's team explored its application in in vivo gene therapy. They targeted muscular dystrophy caused

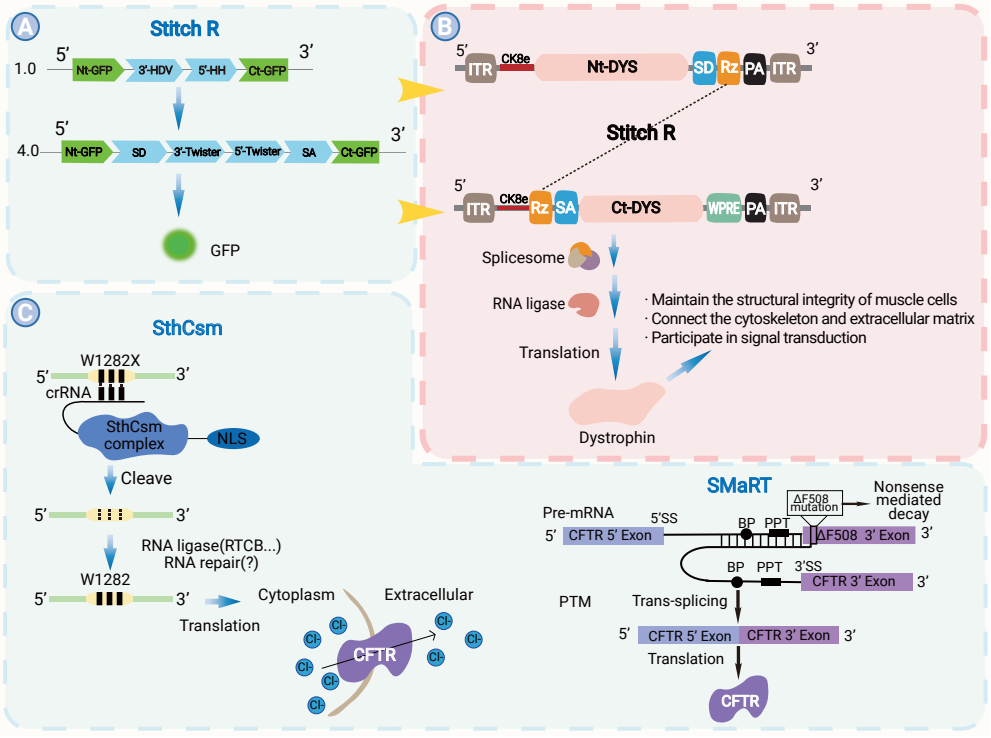

Figure 1. Involvement of RNA repair mechanism in gene editing. (A) The construction and optimization of *StitchR*. Firstly, the screening and optimization of ribozyme combinations were carried out. Through experiments, the most effective Twister ribozyme at the 3' end and the "RzB" HH ribozyme at the 5' end for cutting were determined, which significantly enhanced the activity of *StitchR*. Secondly, split intron was added to the GFP and luciferase *StitchR* reporter genes, further increasing gene expression. In addition, continuous improvements and refinements were made to the *StitchR* system to enhance its efficiency and stability in different application scenarios. These optimization measures have enabled *StitchR* to have broader application prospects in gene editing and therapy fields. (B) Utilizing RNA repair mechanism for large gene delivery. *StitchR* system to express full-length Dysferlin (DYSF) in Dysferlin-KO mice. In gene delivery, by using the RNA repair-related pathways or processes existing in cells to process and repair RNA molecules related to large genes, the effective delivery and expression of large genes can be achieved. Lindley's team, in the Dysferlin gene knockout mouse model, through the RNA repair mechanism and the *StitchR* system, enabled the mice to express the complete Dysferlin protein, thus alleviating Duchenne muscular dystrophy. (C) Utilizing RNA repair mechanism for gene correction: SMaRT and SthCsm to CFTR. Through the pre - trans - splicing molecule or SthCsm-crRNA complex, it recognizes and binds to the mutation site of CFTR pre - mRNA, and then utilizes the intracellular trans - splicing or repair mechanism to repair or correct the mutation, restoring the normal function of CFTR.

by the lack of large functional proteins and used *StitchR* to construct a dual adeno-associated virus (AAV) vector system for splitting large genes (Figure 1B). In the study, the *StitchR* vector was injected into Dysferlin gene-knockout mice and Dystrophin gene-knockout mice. It was found that in Dysferlin gene-knockout mice, the *StitchR* vector enabled Dysferlin expression in muscles and hearts similar to that of wild-type mice. Also, *StitchR* technology successfully expresses large therapeutic proteins Dysferlin and mini-dystrophin in vivo, ameliorates muscle pathology and improves disease phenotype. This demonstrated that *StitchR* has a unique advantage, being able to produce only full-length target proteins.

In addition to its application in large gene delivery, *StitchR* technology has shown unique advantages in the field of prime editing. The researchers developed the *StitchR* - PE system for expressing the plasmid editor PEmax and related parts. Compared with the conventional single-ORF-expressed PEmax and the previously reported split-intron pairs, *StitchR* - PE showed outstanding performance in terms of gene editing activity, reaching 81.2% of the activity of the single-ORF-expressed PEmax, and twice the activity of the split-intron pairs. Moreover, the editing activity of this system was obviously inhibited without the addition of trimethoprim (TMP), but significantly enhanced with the addition of 1 mM TMP, which realized the chemical regulation of the editing process and provided a new strategy for the precise application of gene editing technology in therapy.

Through previous RNA editing of trans-splicing, known as SMaRT technology, strategies for treating cystic fibrosis[3] and autosomal dominant retinitis pigmentosa[4] have been proposed, but ultimately have limited due to low splicing efficiency and immunogenicity. Lindley et al. achieved the treatment of muscular dystrophy diseases by delivering large molecular proteins, further revealing the potential of alternative splicing in gene therapy and gene editing. Many previous studies and this study focus on RNA repair. RNA plays an important role in cellular life activities, especially in protein synthesis. Meanwhile, non-coding RNAs, such as miRNAs and lncRNAs, do not directly participate in protein coding, but play a crucial role in the gene expression regulatory network. Compared with DNA, RNA exhibits unique dynamic properties. Its synthesis and degradation processes are more efficient and rapid, with a significantly faster turnover rate. RNA repair can precisely target functional abnormalities in specific RNA molecules while avoiding over-repair. The in-depth investigation of the mechanism of RNA repair and the effective utilization of RNA repair system are of great scientific significance and potential application value. In this regard, Blake et al. have made a remarkable breakthrough in their research.[5] They successfully identified a series of key enzymes closely related to RNA repair, and skillfully applied these enzymes to achieve precise repair of specific genes. They utilized the SthCsm complex to accurately identify and remove the mutation site in the CFTR gene, successfully restoring the normal expression of the CFTR protein. This innovative research result not only opens up a new potential pathway for the treatment of cystic fibrosis, but also provides a valuable paradigm and idea for gene therapy and gene editing in a broader sense, fully demonstrating the great potential of RNA repair in overcoming hereditary diseases (Figure 1C).

Among the many key molecules involved in RNA repair, RTCB, as an important enzyme with RNA ligase activity, plays a central role in the process of RNA repair. RTCB can specifically recognize the specific terminal structure of damaged RNA molecules and binds to it tightly. When RNA is damaged and there is a broken RNA strand, RTCB, like a skillful "craftsman", acts precisely on these broken ends, and by virtue of its unique ligase activity, efficiently promotes the formation of phosphodiester bonds between the broken fragments, thus realizing the reconnection and repair of the RNA strand. Through this process, RTCB effectively restores RNA integrity and function, ensuring its critical role in cellular processes such as protein synthesis and gene expression regulation. In view of the key position and unique function of RTCB in the RNA repair process[5], the complex mechanism of RTCB and its synergistic cofactors undoubtedly deserves our in-depth investigation and attention, which will help to further reveal the mysteries of RNA repair and lay a solid foundation for the development of novel therapeutic strategies and biotechnology based on RNA repair.

In conclusion, the *StitchR* technology developed by Lindley et al. is excellent in overcoming the AAV packaging capacity limitation, realizing the effective delivery of large genes, and accurately regulating the gene editing activity, which provides a strong technical support for the further development of the gene therapy field and is expected to promote the breakthrough progress in the treatment of more diseases. As an innovative tool for RNA manipulation, *StitchR* technology shows great potential application value in various fields, such as gene therapy, protein engineering, gene editing and basic research. Through the dual AAV vector strategy, it realizes the efficient expression of large therapeutic proteins in vivo, providing new hope for the treatment of diseases caused by large gene mutations. To protein engineering, *StitchR* can be used to design and express complex proteins with specific functions as well as to realize the multifunctionalization of proteins, which provides the possibility of developing new therapeutic proteins. For gene editing, it can deliver large-scale gene editing tools and realize precise gene editing, expanding the application scope of gene editing technology. In addition, *StitchR* technology contributes to the exploration of RNA processing and repair mechanisms in basic research, as well as the study of new strategies for gene expression regulation, providing new methods for the development of life science and medicine. However, the ribozyme activity may be interfered by intracellular RNA-binding proteins, and the stability needs to be further optimized.

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FUNDING AND ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (Nos. 82201594, 81502582), Construction Project of Liaoning Provincial Key Laboratory, China (2022JH13/10200026). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. We thank members in our laboratory for helpful discussion.

AUTHOR CONTRIBUTIONS

Y.H. and L.Y. provided the conception, funding support, revision and supervision. L.L. conducted the literature research and wrote the initial manuscript and visualized the figure. Y.H., L.Y., and L.L. are responsible for revision and proofreading of the manuscript. All authors have read and approved to publish the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.