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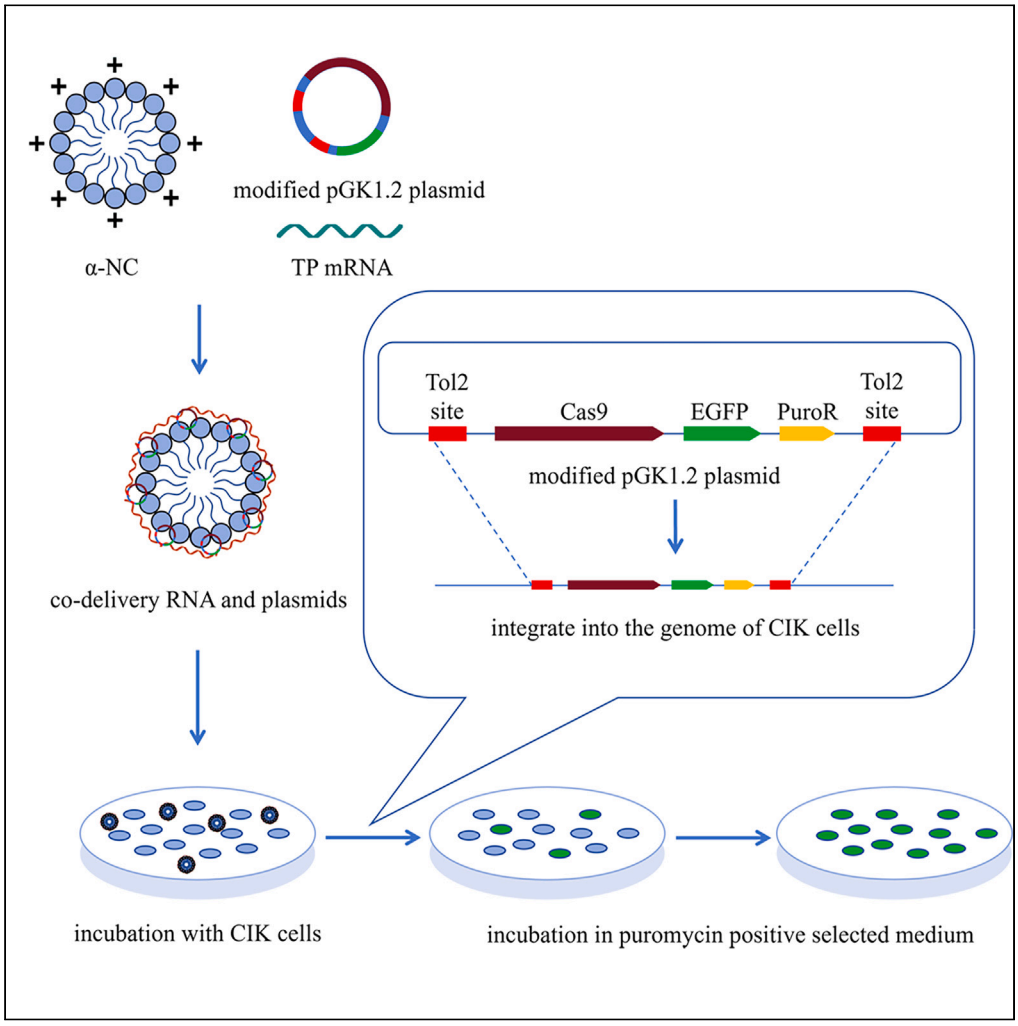

Ran Zhao, Yan Zhang, Qi Wang, ..., Ying-Jie Chen, Kai-Kuo Wang, Jiong-Tang Li

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Highlights

α-lactalbumin-based nanocarrier (α-NC) can efficiently load plasmid DNA and mRNA

α-NC was stable in cell culture medium with low cytotoxicity

α-NC had higher transfection efficiency than common liposomal reagents in CIK cell

We generated a genetically modified CIK cell line by using α-NC delivery system

Zhao et al., iScience 27, 110480
August 16, 2024
https://doi.org/10.1016/j.isci.2024.110480

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Generation of transgenic fish cell line with α-lactalbumin nanocarriers co-delivering Tol2 transposase mRNA and plasmids

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SUMMARY

Fish cells, such as grass carp (*Ctenopharyngodon idella*) kidney (CIK) cells, are harder to transfect than mammalian cells. There is a need for an efficient gene delivery system for fish cells. Here, we used CIK cell line as a model to develop a strategy to enhance RNA and plasmid DNA transfection efficiency using a nanocarrier generated from α-lactalbumin (α-NC). α-NC absorbed nucleic acid cargo efficiently and exhibited low cytotoxicity. Plasmid transfection was more efficient with α-NC than with liposomal transfection reagents. We used α-NC to co-transfect Tol2 transposase mRNA and a plasmid containing Cas9 and GFP, generating a stable transgenic CIK cell line. Genome and RNA sequencing revealed that the Cas9 and GFP fragments were successfully inserted into the genome of CIK cells and efficiently transcribed. In this study, we established an efficient transfection system for fish cells using α-NC, simplifying the process of generating stable transgenic fish cell lines.

INTRODUCTION

Generally, transfection can be divided into two types: virus-mediated transfection and nonviral gene delivery vectors. In virus-mediated transfection, also referred to as transduction, viruses serve as vectors carrying genes into eukaryotic cells.[1] This method requires steps such as constructing vectors, packaging, and purifying viruses. Although virus-mediated transfection is often used for cell types that are difficult to transfect, such as salmonid fish cells,[2] the long treatment time, complex steps, and high cost limit its practical use.[3] Compared with virus-mediated transfection, gene delivery via nonviral vectors made of lipids,[4] polymers,[5] dendrimers,[6] and peptides[7] has the advantages of being safe, simple, and convenient, allowing for its widespread use.[8] Among the various gene transfer techniques, methods relying on calcium ions (Ca^{2+}) and liposomal complexes are commonly used for the production of nonviral vectors in mammalian cells.[9–11] Unlike those in mammalian cells, low transfection efficiency in fish cells seriously hinders research and their potential applications. This is due to their special physiological characteristics and required incubation conditions.[12,13] Various studies have shown that calcium chloride and liposome-mediated transfection methods have a lower efficiency in fish cells.[14,15] Electroporation methods generally lead to a high amount of cell death.[13] Therefore, it is necessary to generate an efficient gene delivery system for fish cells.

Nanoparticles with sizes ranging from 10 to 1,000 nm have been widely used as carriers to delivery nucleic acids to mammalian cells, showing promise in nonviral vector gene delivery applications.[16–19] Nucleic acids are poorly permeable through the cellular membrane owing to their negative charge.[20] However, positive charged nanoparticals, like ionizable lipids, polymeric nanocarriers, and liposomes, are capable of carrying negatively charged nucleic acids through electrostatic interactions.[21–23] Due to their ultra-fine sizes, nanoparticles can easily deliver genetic material including DNA, RNA, and small interfering RNA (siRNA) to target cells or tissues to regulate gene expression.[24,25] Compared with other carriers, nanocarriers have a higher transport efficiency across the cell membrane.[26,27] In addition, they have little impact on cell growth and metabolism.[28–30] Hydrolyzed α-lactalbumin can further undergo self-assembly to form fibrous or tubular nanostructures.[31,32] It has been used to deliver genetic material, nutrients, and drugs.[29,33,34]

To achieve the stable expression of exogenous genes in cells, it is not only necessary to deliver the foreign gene into the cell but also to insert the exogenous sequence into the host cell genome. The Tol2 transposon element was discovered in Japanese medaka fish (*Oryzias latipes*), where an ~4.7 kb DNA insertion in the tyrosinase resulted in an albino phenotype. The insertion had the structural properties of a DNA-based transposable element.[35] Tol2 has a large cargo capacity, meaning that it can deliver a total 200 kb of DNA in several cell types and around 10 kb of DNA without compromising transposition efficiency.[36–39] It can enable sustained transgene expression following gene

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https://doi.org/10.1016/j.isci.2024.110480

delivery and be used in the generation of transgenic cells and animals.[40] The Tol2 transposable element has been shown to create insertions in the zebrafish genome very efficiently, meaning that it is widely used in applications involving transgenic fish.[41,42]

In order to create an efficient gene delivery system for fish cells, we used α-lactalbumin to generate a nanocarrier able to absorb and deliver the target plasmid and Tol2 transposase mRNA to grass carp (*Ctenopharyngodon idella*) kidney (CIK) cells. The cell line, isolated from grass carp reovirus (GCRV)-infected tissue, is a widely used cell line for studying the cell biology, gene function, and immunology of grass carp and other cyprinoids.[43–45] We analyzed its cytotoxicity, plasmid and RNA absorption rates, as well as cellular uptake efficiency. We also evaluated the plasmid transfection efficiency using our system compared to that of two common liposomal transfection reagents. Finally, we generated a stable transgenic CIK cell line using an α-lactalbumin nanocarrier (α-NC) along with a plasmid encoding Cas9 protein and green fluorescent protein (GFP) (containing an insertion sequence with a length of 8,500 bp), demonstrating the efficient transfection of long sequence using our method. We proved that this method can be used as an efficient gene delivery system in fish cells.

RESULTS

Characterization and stability of α-NC-pDNA/mRNA complexes

A schematic illustration of the assembly of an α-lactalbumin-based nanocarrier DNA/RNA co-delivery system designed for cell transfection is shown in Figure S1. First, amphiphilic α-lactalbumin peptides were obtained after partial hydrolysis by *Bacillus licheniformis* protease (BLP) at specific sites of Glu and Asp34,46, then they were assembled into an α-lactalbumin-based nanosphere (α-NS) via ultrasonic oscillation. Subsequently, the maleimide cross-linked α-NS and poly-L-arginine (PLA) acted as bridging agents to form an α-lactalbumin-based nanocarrier (α-NC). Finally, negatively charged plasmid DNA (pDNA) or mRNA were loaded onto the surface of the positively charged α-NC by electrostatic interactions, forming either α-NC-pDNA or α-NC-mRNA complexes.

We measured the zeta potential and particle size of the α-NC complexes using a laser particle size analyzer. Following hydrolysis, assembly, and cross-linking, the initially negative charge (−21.76 mV) became positive charge (14.21 mV) (Figure 1A; Table S1). The average particle diameter and polydispersity index (PDI), which are the quality indications of size distribution, determine the stability and efficacy of nanoparticles.[46] As shown in Figure 1B, the average particle diameter of α-NC increased from 26.18 nm (PDI: 0.015) to 39.25 (PDI: 0.019) nm compared to α-NS. After being mixed with plasmids and mRNAs separately, the particle size of α-NC-pDNA was 67.59 ± 9.52 nm (PDI: 0.162) and that of α-NC-mRNA was 59.71 ± 10.92 nm (PDI: 0.112). Both were larger than the size of α-NC, indicating that the plasmids and mRNA were successfully loaded in α-NC. The morphologies of α-NS, α-NC, α-NC-pDNA, and α-NC-mRNA were confirmed using transmission electron microscopy (TEM) (Figure 2).

To verify the stability of α-NC as applied in cell transfection as a co-delivery nanocarrier, we detected the zeta potential and size distribution of α-NC, α-NC-pDNA, and α-NC-mRNA versus time in CIK cell culture medium at 29°C. As shown in Figures 3A and 3B, there were nearly no changes for at least 7 days. This indicated that α-NC was a stable nanocarrier system for cell transfection.

Low cytotoxicity and high loading efficiency of α-NC

In vitro cytotoxicity analyses of α-NC compared to commercial transfection reagents were evaluated using a Cell Counting Kit-8 (CCK-8) assay after 24 h of incubation with CIK cells. As shown in Figure 4A, the cell viabilities were >80% when α-NC was used at a concentration of 125 μg/mL. This indicates that the α-NC had no impact on the survival and growth of CIK cells at the selected concentration. However, Lipofectamine 2000 exhibited higher cytotoxicity at the required concentration for delivering the same dose of plasmid DNA or mRNA. CIK cell proliferation was observed from 0 h to 72 h at four different concentrations (40, 125, 500, and 1,000 μg/mL) (Figure S2). α-NC at the two highest concentrations led to cell death. Therefore, the highest tested concentration that did not lead to cell death (125 μg/mL) was selected for use in transfection.

The loading efficiency of α-NC with mRNA was measured via gel fluorescence intensity following the resolution of the complex in agarose gel electrophoresis (Figure 4B). There was no visible RNA band when the concentration ratio of α-NC to mRNA was increased to 100:1 (the N/P ratio was approximately 4:1). This indicates that mRNAs are efficiently loaded in α-NC when the weight ratios of α-NC to mRNA are over 100:1.

Efficient intracellular uptake of α-NC in CIK cells

Understanding how nanoparticles interact with cells and how these interactions influence their cellular uptake is essential to exploring the biomedical applications of nanocarriers, particularly for gene delivery. To confirm the ability of α-NC to transfect cells, we performed *in vitro* cellular uptake studies using fluorescence-activated cell sorting (FACS) analysis in CIK cells. The CIK cells were incubated with 125 μg/mL fluorescein isothiocyanate (FITC)-labeled α-NC for 3, 6, 12, and 24 h. Quantitative FACS analysis of the internalized fluorescence-associated cells showed that the intracellular uptake efficiency of α-NC increased from 39.1% (3 h) to 99.06% (24 h) (Figure 5A). These results have proven that α-NC can be efficiently internalized by CIK cells.

To observe CIK cells endocytosis of α-NC, fluorescently labeled α-NC was employed and imaging was performed using confocal laser scanning microscopy (CLSM). We co-loaded liposoluble Cy3 and Tol2 transposon (TP) mRNA into α-NC (α-NC/Cy3-TP mRNA) and then incubated the complexes with CIK cells for 1, 3, 6, 12, and 24 h. Subsequent CLSM imaging after TP mRNA signal labeled by fluorescein amidite (FAM) *in situ* hybridization showed that the cells incubated with α-NC/Cy3-TP mRNA exhibited strong internal red and green fluorescence signals (Figures 5B and S3). The RNA signals (FAM, green fluorescence) were first detected in the cytoplasm of CIK cells and then detected in the nucleus after 12 h of incubation (Figure 5B). These results suggest that α-NC was able to successfully deliver RNA to CIK cells.

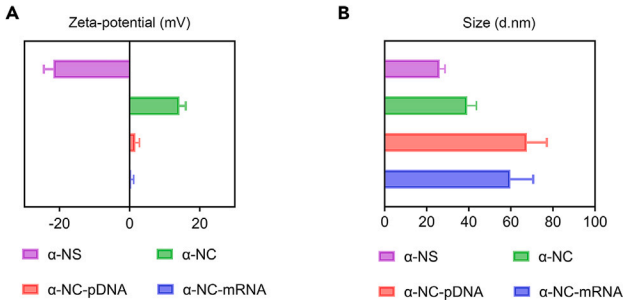


Figure 1. Characterization of the α-NC-pDNA/mRNA complexes in CIK cell culture medium

Average zeta potential (A) and size distribution (B) of the α-NCs with or without loaded plasmid DNA or mRNA. The results are reported as the mean ± standard deviation, N = 5.

Higher transfection efficiency with α-NC than common liposomal reagents

We compared our transfection system with commercially available liposomal transfection reagents including Lipofectamine 3000 (Lipo 3000), FugeneHD (Fugene HD), Lipofectamine 2000 (Lipo 2000), and Fugene6 (Fugene 6). The transfection efficiency of each reagent was determined by measuring GFP-positive expression in transfected CIK cells. Quantified using flow cytometry, the CIK cells transfected using α-NC only had relatively lower ratio of GFP expression (7.15% ± 0.3%) compared to cell transfected using Lipo 3000 (9.23% ± 0.2%) after 24 h (Tables 1 and S2). The α-NC-treated cells demonstrated significantly higher GFP expression ratios (32.78% ± 0.38%, p value = 0.0006, 2.72×10^{-7}, 1.29×10^{-7}, and 3.72×10^{-8}) than Lipo 3000, Fugene HD, Fugene 6, and Lipo 2000 transfection reagent after 72 h (Figures 6A and 6B). This indicated that α-NC was able to deliver plasmids more efficiently to CIK cells than all four transfection reagents we used in this study.

Generation of transgenic CIK cell lines expressing Cas9 proteins using α-NC

Previous results have indicated that stable, genetic modification of fish cells could only be achieved at very low frequencies.[13] To determine whether transposon vectors can be used to increase the efficiency of CIK cells stable transfection, we carried out a series of transfections using the α-NC system. CIK cells were co-transfected with both Tol2 mRNA and modified pGK1.2 vectors (Figure S4) using α-NC. GFP-positive transfected cells were first observed at 3 days post transfection (Figure 7A). The transfected cells were grown in M199 medium with puromycin over the course of 56 days and cultured for eight generations post transfection. Nearly all cells were GPF positive after eight generations of culturing, which indicated that the co-transfection with Tol2 mRNA and plasmid resulted in a stable transfection (Figure 7B). The high expression levels of Cas9 and GFP in the CIK-Cas9 line, as determined by Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) values, demonstrated the insertion of the exogenous genes into the host genome (Figure 7B). We did not detect the expressions of Cas9 and GFP in the CIK line. The expression level of GFP was higher than Cas9, possibly because the activity of the CMV promoter and the enhancer of GFP is higher than the U6 promoter of Cas9.

We further analyzed the changes in the cell proliferation and gene expression of the CIK-Cas9 cell line to evaluate the impact of our exogenous gene stable expression method on cell physiology and gene function. The stable expression of exogenous genes in CIK-Cas9 cells did not significantly affect cell proliferation (0.5 h, p value = 0.55; 1 h, p value = 0.41; 2 h, p value = 0.32; 3 h, p value = 0.15) (Figure 7C). In each replicate, over 94.32% of cleaned RNA sequencing reads could be aligned to the grass carp genome (Table S3).

RNA sequencing of CIK and CIK-Cas9 cells was used to analyze the impact of the exogenous Cas9 expression on endogenous gene expression of CIK cells. In the annotated 31,348 grass carp genes (including protein-coding genes and non-coding genes), 7,204 genes

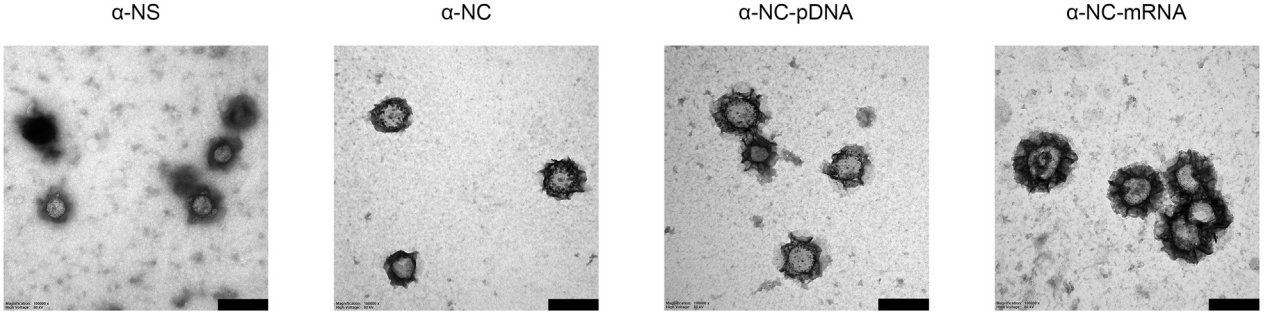


Figure 2. TEM images of α-lactalbumin-based nanocarriers with and without plasmid DNA or mRNA loading

Scale bar: 100 nm.

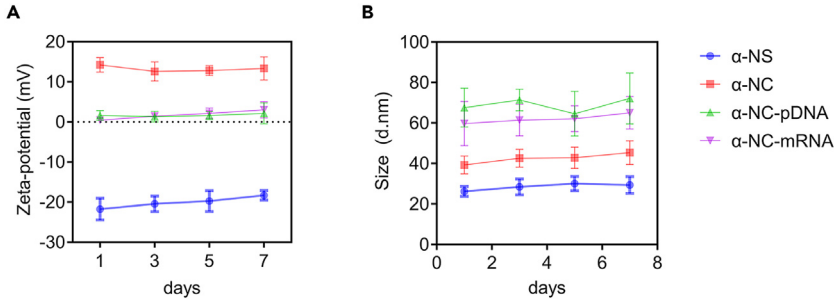
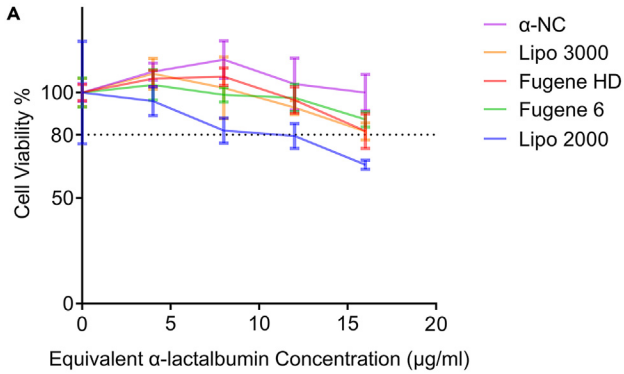


Figure 3. The stability of α-NCs loading with and without plasmid DNA (α-NC-pDNA) or mRNA (α-NC-mRNA)

Average zeta potential (A) and size (B) of α-NC, α-NC-pDNA, and α-NC-mRNA in CIK cell culture medium over 7 days. The results are reported as the mean ± standard deviation, $n = 3$.

were not expressed in either CIK-Cas9 or CIK cells, and 20,664 genes had expression signals in both cell types (Figure 7D). However, a total of 2,202 and 1,270 genes were specifically expressed in CIK-Cas9 cells and CIK cells, respectively. In comparison to CIK cells, the CIK-Cas9 cells exhibited 525 (3.91%) upregulated genes and 116 (0.86%) downregulated genes (Figure 7E).

To determine whether exogenous plasmids were integrated into the genome of CIK cells, we performed genome sequencing on CIK-Cas9 cells. Through mapping to the grass carp genome, we identified a total of 32 modified pGK1.2 plasmid integration sites in the genome of CIK-Cas9 cells (Table S4). Considering that Tol2 transposase has specific target sequence preferences and insertion site selection bias,[47,48] we further analyzed the characteristics of 150 bp DNA sequences upstream and downstream of modified pGK1.2 plasmid insertion sites in CIK-Cas9 cell genome using the RepeatMask program.[49] The results showed that 24 out of 33 insertion site sequences had features of interspersed repeats sequence (Table S4). This result indicated that the integration of exogenous plasmids in our study used the transposase



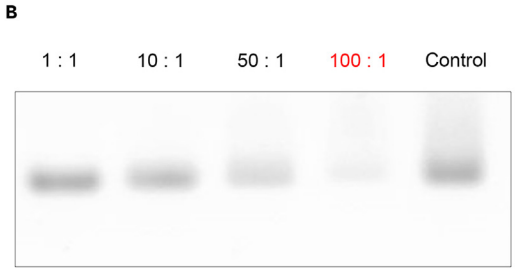


Figure 4. α-NC cytotoxicity and loading efficiency assay

(A) CCK-8 assay showing *in vitro* cytotoxicity profiles of α-NC, Lipo3000, Fugene HD, Fugene 6, and Lipo 2000 in CIK cells at the indicated concentrations after 24 h of incubation. The results are reported as the mean ± standard deviation ($n = 5$).

(B) The efficiency of Tol2 mRNA binding to α-NC was examined via agarose gel electrophoresis. 1–4, 1 h after 100 μg/mL Tol2 mRNA was incubated with α-NC at concentrations of 0.1, 1, 5, and 10 mg/mL. 5, 100 μg/mL Tol2 mRNA without α-NC.

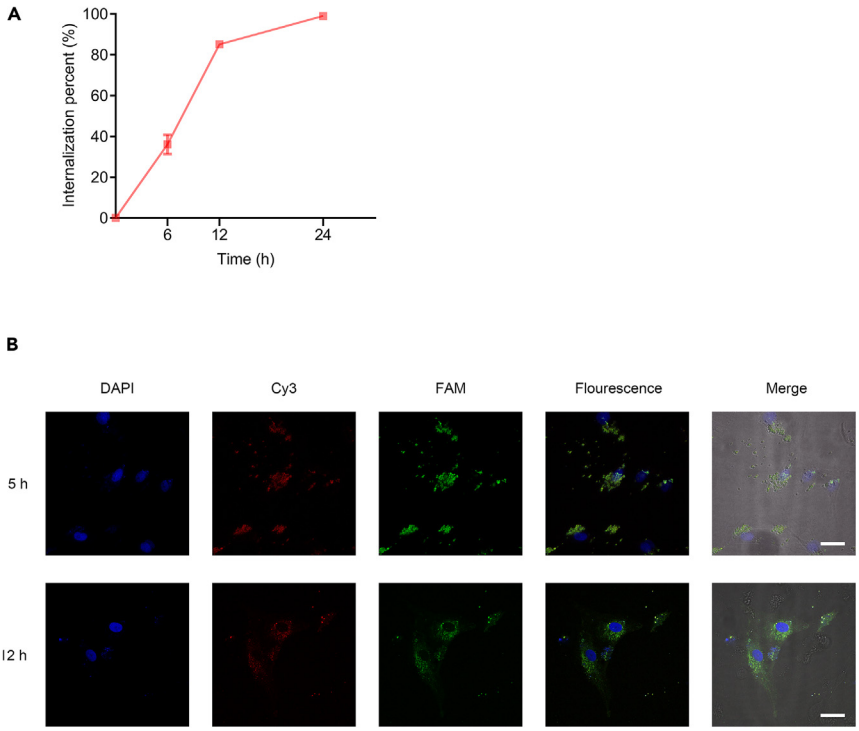


Figure 5. Intracellular uptake of α-NC in CIK cells

(A) Time-dependent cellular uptake of FITC-labeled α-NC in CIK cells analyzed by FACS. The results are reported as the mean ± standard deviation (n = 3).

(B) CLSM images of CIK cells treated with 125 μg/mL α-NC/Cy3-RNA/FAM for 5 and 12 h. Red, Cy3-labeled α-NC; green, FAM-labeled RNA; blue, nuclei. Scale bar: 20 μm.

system rather than random integration of the entire linearized plasmid. Ultimately, we successfully generated a stable, genetically modified CIK cell line by co-transfecting Tol2 mRNA and Cas9-carrying plasmids using an α-NC delivery system.

DISCUSSION

Nucleic acid transfection is a common method used to study the function of specific genes in a variety of cell models. The implementation of transfection methods has become increasingly widespread in experiments involving gene function, the regulation of gene expression, mutation analysis, and protein production. Therefore, cell transfection methods that result in a high transfection efficiency and have very few harmful effects on cell biology are needed.

In general, the transfection efficiency of vectors is associated with cell type and culture medium conditions. Applying transfection methods to fish cells is still very challenging. Most transfection reagents require a serum-free environment for optimal transfection efficiency.[50] However, a serum-free environment is highly toxic to the CIK cells used in our study, resulting in low cell survival rates. Compared with mammalian cells, the culturing environment of fish cells is maintained at a lower temperature, resulting in slower cell metabolism and reduced transfection efficiency. Given the importance of fish cells in gene function research and the genetic analysis of economic traits, it is necessary to invent a simple and efficient transfection method for fish cells, providing a more powerful tool for fish gene function research.

In this study, an α-lactalbumin-based nanocarrier system was designed for the delivery of nucleic acids and stable gene insertion in CIK cells. In our previous work, we found that α-lactalbumin can self-assemble into spherical nanoparticles and characterize internal lipophilicity

Table 1. Transfection efficiency of α-NC compared to commercial reagents

Post-transfection	α-NC	Lipo 3000	Fugene HD	Fugene 6	Lipo 2000
24 h	7.15% ± 0.3%	9.23% ± 0.2%	6.63% ± 0.12%	4.33% ± 0.12%	1.50% ± 0.07%
48 h	27.04% ± 0.47%	25.64% ± 1.44%	12.76% ± 0.73%	6.85% ± 0.17%	2.37% ± 0.06%
72 h	32.78% ± 0.38%	24.71% ± 1.35%	16.78% ± 0.14%	12.85% ± 0.17%	4.70% ± 0.18%

Proportion of fluorescent cells detected by flow cytometry. The results are reported as the mean ± standard deviation, n = 3.

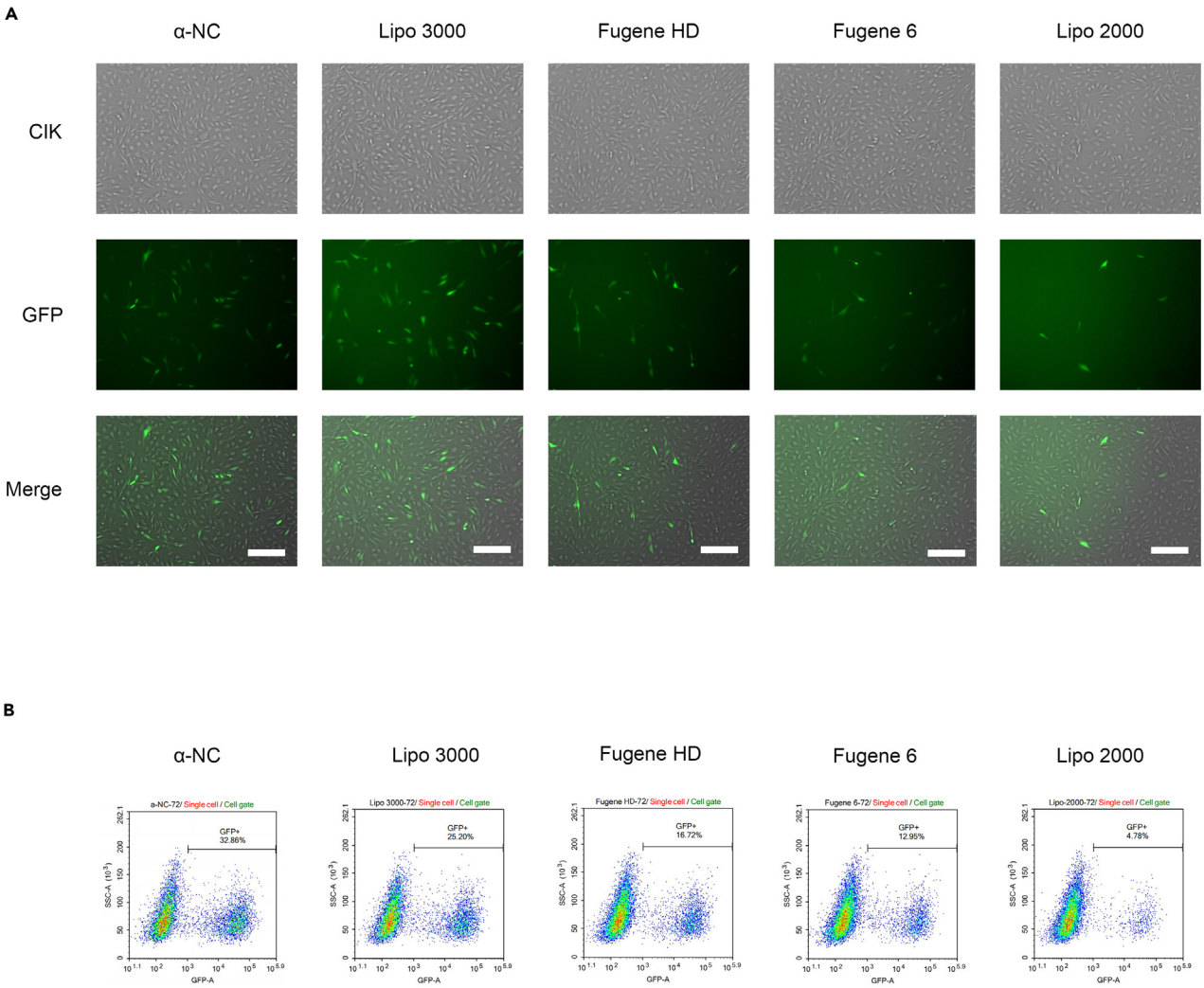


Figure 6. Transfection efficiency assays of α-NC and common liposomal reagents

(A) Visible, fluorescent, and merge fields of microphotographs of CIK cells transfected with CMV-GFP plasmid by α-NC, Lipo3000, Fugene HD, Fugene 6, and Lipo 2000 after 72 h, respectively. Scale bar: 200 μm.

(B) A GFP-positive cell assay was used to validate the transfection efficiency of α-NC, Lipo3000, Fugene HD, Fugene 6, and Lipo 2000 by flow cytometry after 72 h.

and surface hydrophilicity.[33] α-lactalbumin nanoparticles can be absorbed by mammalian cells both *in vitro* and *in vivo*, making them an ideal drug carrier.[51–53] In order to modify these nanocarriers to have a positively charged surface amenable to cellular nucleic acid uptake, poly-arginine was linked to α-lactalbumin nanoparticles, enabling the loading of negatively charged nucleic acids. Thus, α-NC was able to bind to the negatively charged membrane through ionic interactions and enter the cell through spontaneous endocytosis.[54] At the same time, we validated the stability of the particle size and zeta potential of α-NC. The *in vitro* cytotoxicity analysis in CIK cells showed that α-NC was safe for gene delivery applications.

Cellular uptake efficiency is an important factor affecting gene delivery using nanocarriers. We generated an α-NC co-loaded with liposomal Cy3 and FAM-labeled mRNA (α-NC/Cy3-mRNA/FAM) to determine the CIK cell internalization efficiency of α-NC by observing the fluorescence signals via FACS. After 12 h of co-incubation with NC/Cy3-mRNA/FAM, over 80% of CIK cells contained the α-NC fluorescence signal. This result demonstrated that α-NC can be internalized by most CIK cells within a short period of time.

Following cell internalization, nanocarriers are usually captured by lysosomes and degraded via enzymatic action in their acidic environment. This results in a reduction in or even loss of their delivery function.[55,56] Therefore, timely escape from lysosomes is crucial for the application of nanocarriers. In previous research, it was confirmed that the internalized α-NC could escape from lysosomes, which paved the way for the release of nucleic acid in the cytoplasm and nucleus.[57] Using laser confocal microscopy, the mRNA FAM signal was observed in the nucleus of CIK cells after 12 h of co-incubation with α-NC/Cy3-mRNA/FAM. This result indicated that α-NC is an ideal gene delivery system for CIK cells.

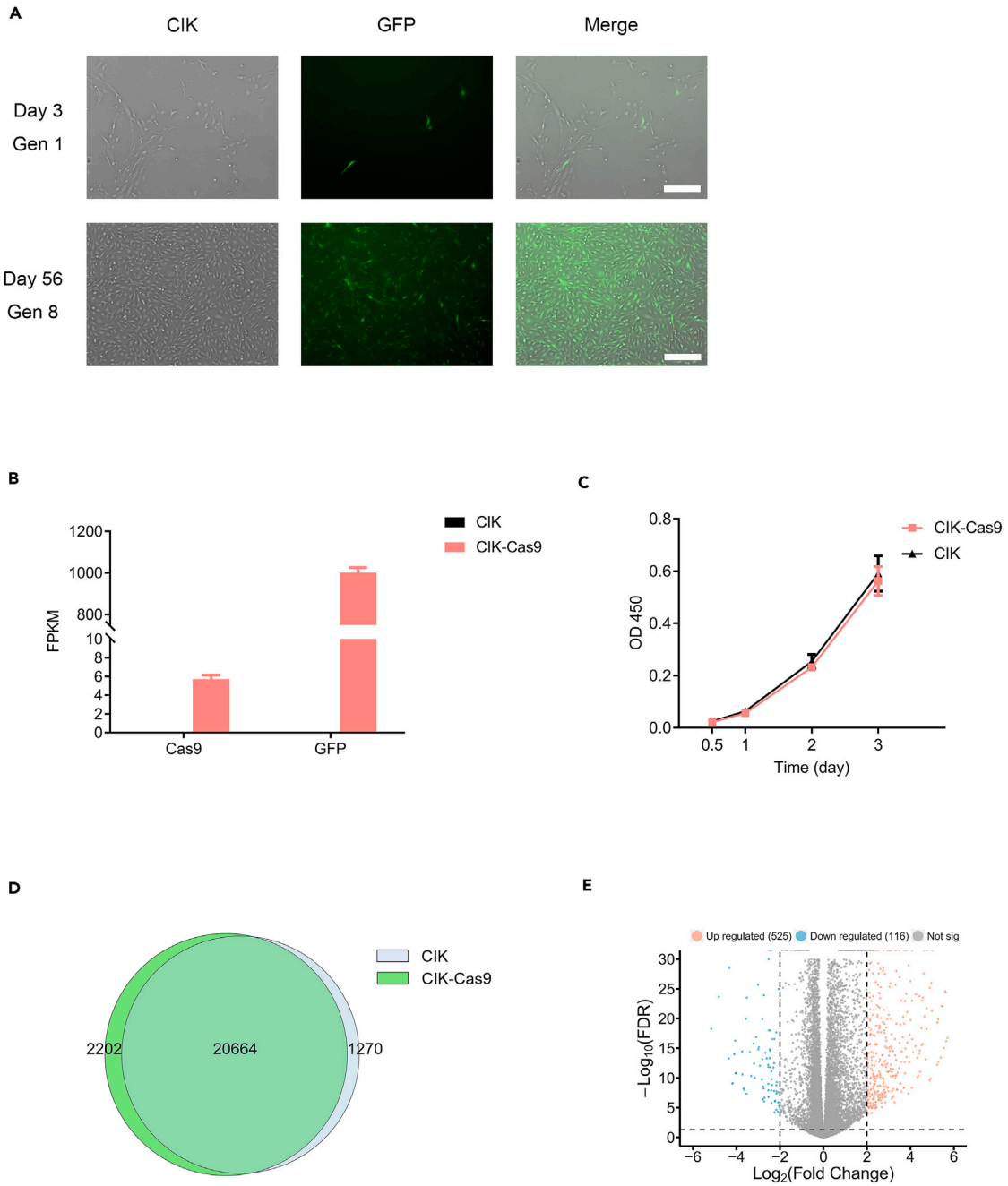


Figure 7. Generation of transgenic CIK cell lines expressing Cas9 proteins using α-NC

(A) Microphotographs of CIK cell line co-transfected with Tol2 mRNA and pGK1.2 plasmid by α-NC (CIK-Cas9) on the third day after transfection and on the 56th day in puromycin-selected M199. Scale bar: 200 μm.

(B) Expression levels of Cas9 and GFP in CIK-Cas9 cell line in puromycin-selected M199 on the 56th day post-transfection, using non-transfected cells as a control. The results were reported as the mean ± standard deviation, $n = 3$.

(C) CCK-8 assay showing proliferation of CIK-Cas9 and CIK cells as a function of time. The results are reported as the mean ± standard deviation, $n = 5$.

(D) The Venn diagram of the expressed genes in CIK and CIK-Cas9 cells.

(E) The vocal map of differential expression of mRNAs in the CIK and CIK-Cas9 cells.

Although liposomal transfection reagents have a wide range of applications, they may not be efficient for all cell types. To verify the transfection efficiency of α-NC in CIK cells, we selected two commonly used transfection reagents, non-liposome Fugene 6 and cationic liposome Lipofectamine 2000, as the controls. Both Fugene 6 and Lipofectamine 2000 are well-documented transfection reagents. They can successfully transfect most types of adherent and suspension cells and are also widely used in fish cells.[12] Our research has proven that α-NC has a higher transfection efficiency than Fugene 6 and Lipofectamine 2000 in CIK cells.

Furthermore, we attempted to use the α-NC system to generate a CIK cell line which can stably express exogenous genes. The conventional method for constructing stable cell lines was to use viral vectors for infection. However, viral vectors have biosafety issues, and their infection efficiency in fish cells is lower than exhibited in mammalian cells. The Tol2 transposon element is an autonomously active transposon, containing a gene encoding a complete and functional transposase that is capable of identifying, excising, and reinserting the DNA element defined by its inverted terminal repeats (ITRs) or other elements with the same ITRs. The integration of the targeted gene by Tol2 is mediated by a "'cut-and-paste" mechanism, which does not cause any rearrangement or modification at the target site except for the creation of an eight bp duplication. The Tol2 transposon shows no preference with respect to integration sites, which means that it can theoretically translocate anywhere on a chromosome. Single-copy insertions can be achieved, and DNA inserts of fairly large sizes (as large as 13 kb) can be cloned between these sequences. This is a considerable advantage compared to Tc1/mariner-type transposons such as Sleeping Beauty, which is limited in terms of cargo size.[58] In this study, we utilized the delivery efficiency enabled by an α-NC system, co-delivering TP mRNA and donor DNA plasmids to CIK cells. The positive CIK-Cas9 cell lines were screened through a selective medium and were able to stably express exogenous Cas9 and GFP protein. There are two reasons why we construct a Cas9-expressing cell line using α-NC delivery system in our study. (1) We want to evaluate the ability of α-NC delivery system to integrate long-length sequences into the host genome. The length of the insertion sequence (8,500 bp) of modified pGK1.2 plasmid we used in this study, which contained the coding sequence of Cas9 and GPF, makes it difficult to transfect and integrate into the host cell genome. (2) Constructing a Cas9-stable expression CIK cell line provides an ideal experimental platform for subsequent research on fish gene function. Compared to simultaneously transfecting Cas9 plasmid and gRNA, only gRNA transfection is needed in this cell line, which makes gene editing more convenient.

Considering the shortcomings of conventional fish cell transfection methods in terms of efficiency and cytotoxicity, the present invention uses α-lactalbumin as a carrier and can effectively improve cell transfection efficiency by adsorbing plasmids and delivering them to CIK cells for expression. α-NC, the framework constructed in this study, can serve as an efficient CIK cell gene delivery vector with versatility, allowing for the delivery of other genes of interest or combination genes. The present invention provides a method for transfecting carp CIK cells that is simple, easy to operate, and has a higher transfection efficiency than the existing liposome-based transfection methods. It can be used as a gene introduction vector for fish cells. With more emerging research on fish and aquatic cell lines, this delivery system is expected to provide a fast and effective method for the *in vitro* verification of gene function in cultured fish cells, showing good development prospects.

In general, we report the development of a new class of α-lactalbumin-based nanocarrier (α-NC) that are uniquely suited for the co-delivery of long nucleic acids (Tol2 transposase mRNA at ~1.9 kb nucleotides and plasmid DNA that is ~10.5 kb nucleotides) and can be used to generate CIK cell lines stably expressing exogenous genes. This approach improves transfection efficiency in CIK cells over other methods and simplifies the generation of transgenic fish cell lines using the Tol2 transposon system.

Limitations of the study

In this study, a stable gene transfer system suitable for CIK cells was evaluated and optimized. α-NC system is potentially valuable in the functional gene analysis of fish cells. The use of α-NC complexes in combination with the Tol2 transposon system has proven to be an effective, rapid, and non-toxic alternative strategy for sustained transgene expression *in vitro* but not fully representing the gene transfer effect within the fish body. Further studies are needed to determine whether the utilization of α-NC adapted for the delivery of the Tol2 transposon system can be used in *in vivo* conditions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110480.

ACKNOWLEDGMENTS

This research was funded by the Agricultural Biological Breeding-2030 major project (2023ZD04054), the Central Public-interest Scientific Institution Basal Research Fund, CAFS (no. 2023TD25), and the National Freshwater Genetic Resource Centre (grant number FGRC: 18537).

AUTHOR CONTRIBUTIONS

R.Z.: conceptualization, methodology, software, validation, formal analysis, data curation, visualization, writing – origin draft, project administration, and funding acquisition. Y.Z.: methodology, formal analysis, resources, and funding acquisition. Q.W.: software. Y.-M.C.: methodology. M.-X.H.: methodology. X.-Q.S.: methodology. S.-T.Y.: methodology. Y.-J.C.: methodology. K.-K.W.: methodology. J.-T.L.: conceptualization, validation, resources, data curation, writing – review and editing, supervision, project administration, and funding acquisition. All the authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 22, 2023
Revised: April 7, 2024
Accepted: July 8, 2024
Published: July 9, 2024

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
α-Lactalbumin (≥85%)	XinYu Biotechnology	Catalog # 9051-29-0
N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride crystalline (EDC)	Sigma-Aldrich	Catalog # 25952-53-8
Bacillus licheniformis protease (BLP) with 15 units/mg enzyme activity	Sigma-Aldrich	Catalog # 9014-01-1
Poly-L-arginine (PLA)	Sigma-Aldrich	Catalog # 26982-20-7
Hydrophobic Cy3-SE	Fanbo Biochemicals	N/A
Liposoluble Cy3	Fanbo Biochemicals	N/A
Liposoluble FITC	Fanbo Biochemicals	N/A
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Catalog # 10099158
Medium199 Hanks' salts (M199)	Thermo Fisher Scientific	Catalog # 12350039
Opti-MEM™	Thermo Fisher Scientific	Catalog # 31985070
Trypsin-EDTA	Thermo Fisher Scientific	Catalog # 25200072
Penicillin and streptomycin (P/S)	Thermo Fisher Scientific	Catalog # 25200072
Phosphate-buffered saline (PBS)	Thermo Fisher Scientific	Catalog # 15140122
Lipofectamine™ 2000 transfection reagent	Thermo Fisher Scientific	Catalog # 11668019
Lipofectamine™ 3000 transfection reagent	Thermo Fisher Scientific	Catalog #L3000015
FuGENE® 6 transfection reagen	Promega corporation	Catalog #E2691
FuGENE® HD transfection reagen	Promega corporation	Catalog #E2311
NotI-HF®	New England Biolabs	Catalog #R3189S
4% Paraformaldehyde (PFA)	Beyotime Biotechnology	Catalog #P0099
TRIzol® Reagent	Qiagen	Catalog # 79306
Critical commercial assays		
Cell Counting Kit-8	Beyotime Biotechnology	Catalog #C0037
T7 High Yield RNA Transcription kit	Nanjing Vazyme Biotech	Catalog # TR101-02
RNA clean Kit	TIANGEN Biotech	Catalog # 4992728
TIANamp Genomic DNA Kit	TIANGEN Biotech	Catalog # 4992254
Hieff NGS® OnePot Pro DNA Library Prep Kit V2	Yeasen Biotechnology	Catalog # 12205ES24
Deposited data		
The RNA-seq data of CIK and CIK-Ca9 cells	National Center for Biotechnology Information (NCBI) Sequence Read Archive database	Accession number: PRJNA1014127
The genomic sequencing data of CIK-Cas9 cells	National Center for Biotechnology Information (NCBI) Sequence Read Archive database	Accession number: PRJNA1033438
Experimental models: Cell lines		
Ctenopharyngodon idella kidney (CIK) cell line	China Center for Type Culture Collection, Wuhan, China	Catalog #GDC0086
Oligonucleotides		
FAM-labeled TP mRNA probe: ACGAAAAGU CGGGGUUUUCUCGAUCC-FAM	Taihe Biotechnology	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
Plasmid: pGK-1.2	Huayueyang Biotechnology	N/A
Plasmid: modified pGK-1.2	Taihe Biotechnology	Catalog # GS212125
Plasmid: pCS2FA-transposase T7	Taihe Biotechnology	Catalog # GS211439
Software and algorithms		
Fastp	Chen et al.[59]	v0.19.5
HISAT2	Kim et al.[60]	v2.2.1
StringTie	Pertea et al.[61]	v2.1.2
edgeR	Robinson et al.[62]	v3.22.5
Burrows Wheeler Alignment Tool	Jo[63]	v0.7.17

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jiongtang Li (lijt@cafs.ac.cn).

Materials availability

The authors confirm that the materials are available from the lead contact, Jiongtang Li (lijt@cafs.ac.cn), upon reasonable request.

Data and code availability

- The RNA-seq data of CIK and CIK-Cas9 cells presented in this study are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database under accession number PRJNA1014127. The genomic sequencing data of CIK-Cas9 cells presented in this study are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database under accession number PRJNA1033438. All data list above are publicly available as of the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell line

Ctenopharyngodon idella kidney (CIK) cell line was purchased from the China Center for Type Culture Collection (Wuhan, China). CIK cells were cultured at 29°C in Medium 199 Hanks' salts (M199) medium supplemented with 10% FBS and 1% Penicillin-Streptomycin without CO_2.

METHOD DETAILS

Formulation of α-lactalbumin nanocarriers

The synthesis of α-lactalbumin nanocarriers (α-NC) was optimized based on a previously described method with modifications.[33] The α-lactalbumin (2.5 mg/mL) was dissolved in PBS at pH 7.5, hydrolyzed by BLP with the weight ratio of BLP to α-lactalbumin being 1:25, then incubated at 50°C for 45 min to generate α-lactalbumin peptides. α-lactalbumin nanospheres (α-NS) were self-assembled from α-lactalbumin peptides via ultrasonic vibration for 1 min. Then, the α-NS mixture solution was passed through a 0.22 μm filter and dialyzed with ultrapure water three times. The carboxyl groups of α-NS were activated by EDC with gentle rotation on a vertical mixer for 5 min; the weight ratio of α-NS to EDC was 5:1. Maleimide (Mal) was conjugated to α-NS after EDC activation at room temperature for 4 h to generate α-NS-Mal nanospheres; the weight ratio of α-NS to Mal was 1.2:1. Excess EDC and maleimide were removed using a centrifugal filter tube (10 kDa, Millipore Co., Germany) spun at 3,500 rpm for 30 min. To generate the α-NC, PLA was finally conjugated to α-NS-Mal after EDC activation via gentle rotation on a vertical mixer at room temperature for 4 h. Excess EDC and PLA were removed using a centrifugal filter tube (10 kDa, Millipore Co., Germany) at 3,500 rpm for 30 min. The α-NC was directly used in subsequent experiments or freeze dried for storage at −20°C.

Characterization of α-NC system

The morphological characteristics of α-NS, α-NC, α-NC-Plasmid, and α-NC-mRNA were observed via transmission electron microscopy (TEM) (JEM-F200, Japan). The plasmid and mRNAs used here were the pGK1.2 plasmid and Tol2 mRNA; details of their preparation are outlined in section of plasmid and Tol2 mRNA preparation. Samples were dispersed with ultrapure water at a concentration of 0.1 mg/mL (equivalent to

the α-lactalbumin concentration), placed on a 200-mesh copper grid, stained with uranyl acetate for 1 min, and then air dried at room temperature. The size distribution, size polydispersity index (PDI) and zeta-potential analyses of α-NS, α-NC, α-NC-Plasmid, and α-NC-mRNA were determined via BeNano 90 Zeta (Bettersize Inc., Liaoning, China) at room temperature. The measurements were performed in triplicate, respectively.

Stability test of α-NC system

For the stability test, 2.5 mg/mL (equivalent to the α-lactalbumin concentration) α-NC, α-NC-Plasmid and α-NC-mRNA solution were prepared in CIK cell culture medium and incubated at 29°C. Subsequently, the size distribution and zeta potential were determined via BeNano 90 Zeta (Bettersize Inc., Liaoning, China) each day for 7 consecutive days. The measurements were performed in triplicate, respectively.

Cytotoxicity analysis

Cell viability was measured using a Cell Counting Kit-8 (Beyotime Biotechnology, Shanghai, China). Prior to sample treatment, CIK cells were cultured in 96-well plates (Corning Inc., USA) at a density of 5,000 cells per well for 24 h. Afterward, the cells were incubated in fresh M199 medium containing α-NC (at concentrations of 20, 40, 80 and 125 μg/mL), Lipo 3000 (at dose of 0.05, 0.1, 0.2 and 0.3 μL/well), Fugene HD (at dose of 0.16, 0.32, 0.64 and 1 μL/well), Fugene 6 (at dose of 0.16, 0.32, 0.64 and 1 μL/well) and Lipo 2000 (at dose of 0.08, 0.16, 0.32 and 0.5 μL/well) for an additional 24 h, separately. Then, 10 μL of CCK-8 solution was added and the treatment, which continued for 1 h. The absorbance values at 450 nm were recorded using an ELx800 multi-mode microplate reader (BioTek Inc., USA).

Plasmid and Tol2 mRNA preparation

To evaluate the ability of a-NC delivery system to transfect long sequences into the fish cell, we tried to deliver the pGK1.2 plasmid carrying Cas9 protein and GFP protein (Huayueyang Biotechnology, Beijing, China) into CIK cell using our method. We modified the pGK1.2 plasmid by inserting forward and reverse Tol2 inverted terminal repeat (ITR) sequences into the upstream region of the CMV enhancer and the downstream region of the bGH polyA signal (a total length of 8,500 bp), respectively (Figure S4). The pCS2FA-transposase T7 plasmid was purchased from Taihe Biotechnology (Beijing, China). The preparation process of Tol2 mRNA was as follows: pCS2FA-transposase T7 plasmids were linearized using *NotI-HF* (New England Biolabs, MA, USA) as transcription templates. *In vitro* transcription was performed on linearized DNA templates using a T7 High Yield RNA Transcription kit (Nanjing Vazyme Biotech, Jiangsu, China). Following transcription, the mRNA product was purified using an RNA clean Kit (TIANGEN Biotech, Beijing, China). Gel electrophoresis was used to confirm for the presence of mRNA, with concentration determined via a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific inc., MA, USA).

Absorption efficiency of plasmids and mRNA

The modified pGK1.2 plasmid and Tol2 transposon mRNA (TP mRNA) were mixed with 2.5 mg/mL α-NC solution at increasing weight ratios (the weight ratios of α-NC to plasmid DNA or Tol2 mRNA were 10:1, 20:1, 50:1, and 100:1), respectively, to generate α-NC-Plasmid or α-NC-mRNA. Gel shift assays were conducted on 1.5% agarose gels. The results were visualized and imaged using a standard imaging system (Bio-Rad Laboratories, CA, USA).

Cellular uptake assay

Liposoluble Cy3 (red) or FITC (green) were incubated with α-NC using gentle rotation on a vertical mixer for 4 h in the dark to generate α-NC/Cy3 or α-NC/FITC. The unabsorbed Cy3 or FITC were removed via dialysis with ultrapure water. The FAM-labeled TP mRNA probe (5′ ACGAAAAGUCGGGGUUUUCUCGAUCC 3′-FAM) was synthesized from Taihe Biotechnology (Beijing, China). The TP mRNA was incubated with α-NC/Cy3 to generate an α-NC/Cy3-TP mRNA complex (the weight ratio of α-NC to TP mRNA is 100:1). The CIK cells were grown in 24-well plates (Corning Inc., NY, USA) in M199 at 29°C without CO_2 after culturing with α-NC/Cy3-TP mRNA at 250 μg/mL (equivalent to the α-lactalbumin concentration concentration) for a predetermined time.

The cellular uptake amount of α-NC/Cy3-TP mRNA by CIK cells was observed using A1 confocal laser scanning microscopy (CLSM) (Nikon Co., Tokyo, Japan). The fluorescence intensity exhibited in each group was used to quantify cellular uptake. CLSM was performed to visualize the delivery efficiency of α-NC into the CIK cells. CIK cell monolayers were incubated with α-NC/Cy3-TP mRNA in M199 at 29°C without CO_2 for 1, 3, 5, 6, 12 and 24 h. The α-NC was removed, the cells were washed three times with PBS, and then they were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. After washing with PBS, TP mRNAs in CIK cells were hybridized by FAM-labeled probes and then DAPI was added for 15 min to mark CIK cell nuclei. Finally, the Cy3 and FAM signals delivered by α-NC in CIK cells were visualized using CLSM.

Further, the cellular uptake of FITC-labelled α-NC (α-NC-FITC) into CIK cells was quantified using FC500 fluorescence-activated cell sorting (FACS) (Beckman Coulter, Inc., CA, USA). Cells treated with α-NC-FITC for 6, 12, and 24 h, as well as unstained cells were collected in 200 μL of PBS and measured via FACS.

Transfection activity assay

The transfection activity of CMV-GFP plasmids (Huayueyang Biotechnology, Beijing, China) mediated by α-NC, Lipofectamine 2000 and Fugene 6 were investigated in CIK cells. CIK cells were seeded in 24-well plates at a density of 5×10^4 cells/well, the number of cells

were measured by using C100-SE automated cell counter (RWD Life Science Co., LTD, Guangdong, China) before being seeded an equal amount onto the culture plates. After 24 h of culturing, the M199 medium in each well was aspirated. An aliquot of 50 μL of 2.5 mg/mL α-NC suspension containing 1.25 μg CMV-GFP plasmids (α-NC-GFP) was added into 500 μL of fresh M199 medium, vortexed for 20 s, and then added into each well. Lipofectamine 3000 complex (an aliquot of 50 μL transfection mix containing 1.5 μL Lipofectamine 3000, 1 μL P3000 reagent and 1.25 μg CMV-GFP plasmids), Fugene HD complex (an aliquot of 50 μL transfection mix containing 5 μL Fugene HD and 1.25 μg CMV-GFP plasmids), Lipofectamine 2000 complex (an aliquot of 50 μL transfection mix containing 2.5 μL Lipofectamine 2000 and 1.25 μg CMV-GFP plasmids) and Fugene 6 complex (an aliquot of 50 μL transfection mix containing 5 μL Fugene 6 and 1.25 μg CMV-GFP plasmids) set as four controls. 50 μL Lipofectamine 3000 complex, Fugene HD complex and Fugene 6 complex was added into each well with 500 μL of fresh M199 medium separately. 50 μL Lipofectamine 2000 complex was added into each well with 500 μL of fresh Opit-MEM medium. The cells were incubated with each transfection reagent mix for 12 h, respectively. Next, the cells were washed twice with PBS and incubated in 0.5 mL of fresh M199 medium/well for 24, 48, and 72 h. Afterward, the cells were collected, suspended in 0.5 mL of PBS, then analyzed via FACS.

Construction of stable transgenic CIK cell line

CIK cells were plated in six-well plates 24 h prior to transfection in order to produce a monolayer. The cells were transfected at 60% confluency, using α-NC loaded with either 2.0 μg of Tol2 mRNA or 2.0 μg of modified pGK1.2 plasmid. The cells were passaged in puromycin-positive, M199 selection medium. Fluorescent cells were identified using fluorescence microscopy (Zeiss, Germany) at day 3 post-transfection in order to determine the initial rates of transfection. This analysis was repeated 3 weeks post-transfection to determine the frequency of the stable integration.

Cell proliferation assay

The proliferation of CIK cells post after transfection was assessed using a CCK-8 kit (Beyotime Biotechnology, Shanghai, China). CIK-Cas9 cells were plated in 96-well plates at a density of 100 cells per well in 100 μL of M199 medium containing 10% FBS.[64–67] Cell proliferation was measured at serial time points of 0.5, 1, 2, and 3 days. Non-transfected CIK cells were used as a negative control. The experiment was repeated five times for each sample.

RNA sequencing and differential expression analysis

To analyze the impact of the exogenous Cas9 and GFP expression on endogenous gene expression of CIK cells, we performed the RNA-sequencing for the CIK-Cas9 and CIK cells. The total RNAs of CIK-Cas9 and CIK cells were extracted using TRIzol Reagent (Qiagen, Germany). The RNA quality was determined using a 5300 Fragment Analyzer System (Agilent, CA, USA) and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific inc., MA, USA). The extracted RNAs were used to construct a sequencing library. Each library was sequenced in triplicate using an Illumina NovaSeq 6000 platform (Majorbio Bio-pharm, Shanghai, China). Clean reads were obtained using Fastp (v0.19.5)[59] and then aligned against the grass carp reference genome (NCBI accession: GCF_019924925.1) with HISAT2 (v2.2.1).[60] The expression levels of all genes were evaluated as Fragments Per Kilobase per Million mapped reads (FPKM) using StringTie (v2.1.2).[61] In CIK cells or CIK-Cas9 cells, if one gene had an FPKM value of 0 in all triplicates, there was considered to be no expression; if the value different from 0, this gene was considered to be expressed. Differential expression analysis was performed using edgeR (v3.22.5).[62] We identified significantly differentially expressed genes with the FDR-corrected P-values $\leq$0.05 and|Log2FC (fold change)|> 2.

DNA sequencing and integration site analysis

The genomic DNA of CIK-Cas9 cells was extracted using a TIANamp Genomic DNA Kit (TIANGEN Biotech, Beijing, China). The DNA library for sequencing was prepared using a Hieff NGS OnePot Pro DNA Library Prep Kit V2 (Yeasen Biotechnology, Shanghai, China). Then, the library was sequenced using an Illumina NovaSeq 6000 platform (Ma-jorbio Bio-pharm, Shanghai, China). Clean reads were obtained using Fastp (v0.19.5), then aligned with the grass carp reference genome (NCBI accession: GCF_019924925.1) and the modified pGK1.2 plasmid sequence using the Burrows Wheeler Alignment Tool (v0.7.17).[63]

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism software (GraphPad, San Diego, CA) except for the differential expression analysis. At least three independent experiments were performed, and all data were presented as the mean value ±SD. Significant statistical differences were defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.