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High-Throughput AAV Barcode Screening Identifies the Optimal Serotype for Efficient Transduction and CRISPR/Cas9 Gene Editing in Mouse Embryos

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Abstract

Adeno-associated virus (AAV) has emerged as a useful vehicle for delivering gene editing reagents in early embryos. However, the relative efficiency of different AAV serotypes in zygote transduction has not been systematically quantified. Here, we present the high-throughput, barcode-based in vitro screen of AAV serotype tropism in mouse zygotes using next-generation sequencing technology. We pooled 12 naturally existing AAV serotypes (AAV1–AAV12) and exposed mouse zygotes to the mixed library. Deep sequencing of embryos revealed that AAV6 achieved the highest transduction abundance among all serotypes, providing quantitative confirmation of its superior tropism in zygotes. Following the screening, AAV6 was utilized to deliver CRISPR-Cas9 elements into zygotes and observed efficient genome editing at the target locus in all embryos. Moreover, the infection of AAV6 in zygotes did not appreciably compromise embryo development or viability relative to controls, in agreement with prior observations that AAV transduction is well-tolerated by preimplantation embryos. In summary, our study provides a comprehensive, high-throughput validation of AAV serotype infectivity in mouse zygotes.

Keywords: AAV tropism; Barcode; NGS; Zygote

1. Introduction

The conventional method for embryo gene transfer – pronuclear microinjection – has developed over 30 years ago and requires expensive micromanipulation equipment and specialized expertise[1]. Electroporation-based methods have been developed to introduce CRISPR components into embryos either ex vivo or even in situ, which obviates needle injections but still demands specific equipment and careful optimization[2]. Alternative viral-mediated strategy uses integrative lentiviral vectors to transduce early embryos, while lentiviruses are unable to transduce pre-implantation embryos unless they are injected into the perivitelline space or the zona pellucida is removed prior to infection[3]. Furthermore, lentiviruses pose risks of insertional mutagenesis[4]. AAV is a non-pathogenic, single-stranded DNA virus that can infect dividing or non-dividing cells without integrating into the host genome. AAV has been used as a promising vector for embryonic gene delivery in recent years[5].

Recombinant AAV (rAAV) is a simple, efficient, and safe method to deliver gene cassettes to embryos without extensive manipulation of the zygote. Importantly, certain AAV serotypes are capable of diffusing through the protective zona pellucida that surrounds mammalian oocytes and zygotes[6]. Initial studies in mouse zygotes indicated that natural AAV serotypes 1 and 6 can efficiently transduce early embryos in vitro[7,8]. These pioneering works established that AAV can serve as a “Trojan horse” to carry genetic payloads into fertilized eggs without micromanipulation, greatly simplifying the creation of gene-modified

embryos. Previous study demonstrates a method termed CRISPR-READI that combines CRISPR-Cas9 ribonucleoprotein (RNP) electroporation with AAV donor infection to achieve efficient knock-in mutations in mice[9]. AAV-mediated embryo editing has since been extended to many other species[10].

In this study, we examined the power of DNA barcode technology to study the performance of 12 natural AAV variants assembled into an AAV Testing Kit to evaluate on zygotes. The results demonstrate the power of this approach and validate the NGS-based testing protocol as a powerful tool could be used to screen a large number of AAV variants and selection of the top AAV candidates for individual testing in relevant models. Here, we explore the possibility of using recombinant adeno-associated viruses as vehicles for transducing intact mouse zygotes to drive embryonic gene editing and delivery. Our experiments indicate that multiple rAAV serotypes can permeate the zona pellucida and transduce intact mouse embryos at several pre-implantation stages independently of the mouse zygotes.

2. Materials and Methods

2.1 Production of Barcode AAV Plasmid Library

The generation of the plasmid library was conducted in accordance with previously established protocols[11]. Each barcode was uniquely paired with one of the 12 AAV capsid variants. To ensure construct integrity, an additional quality-control step was included to confirm each barcode sequence. Specifically, a ~300 bp fragment containing the barcode was amplified using KOD polymerase.

2.2 AAV Production and Purification

AAV particles were generated using a previously described method[12]. Briefly, HEK 293T cells (ATCC) were transfected with AAV plasmids using polyethylenimine and incubated for 72 hours. Viral particles were collected from both the culture medium and the cells at 120 hours post-transfection. Particles in the medium were precipitated with 40% polyethylene glycol (Sigma, 89510-1KG-F) in 500 mM NaCl and then combined with the cell pellet. The pellet was resuspended in lysis buffer (500 mM NaCl, 40 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$) containing 100 U/mL salt-activated nuclease (Arcticzymes) and incubated at 37 °C for 30 minutes. The lysate was clarified by centrifugation (2,000 × g) and purified via a discontinuous iodixanol gradient (15%, 25%, 40%, 60%; Sigma, D1556). The viral fraction was concentrated with Amicon filters (EMD, UFC910024) and resuspended in sterile PBS. Viral titers were then determined by qPCR quantification of DNase I-resistant genomes, using a linearized plasmid as the standard.

2.3 Mouse Embryo Collection

Six-week-old female C57BL/6 and ICR mice (Institute of Laboratory Animal Sciences, JSJ, China) were maintained under a 12-hour light/dark cycle with ad libitum food and water. All procedures were approved by the Institutional Animal Care and Use Committee of Fudan University (Approval No. 2024JS075).

Mice were injected intraperitoneally with 5 IU Pregnant Mare Serum Gonadotropin (PMSG) to synchronize estrus; 44–48 hours later they received 5 IU human chorionic gonadotropin (hCG) (both in 0.1 mL volume) to induce superovulation. Each superovulated female was paired with a male overnight, and the presence of a vaginal plug the next morning confirmed mating. Approximately 18 hours after hCG administration, females were euthanized by cervical dislocation, the abdomen was sterilized with 75% ethanol, and an incision was made to collect the oviducts. The oviducts were transferred to pre-warmed M2 medium, and the ampullae were gently nicked with a syringe to release cumulus–oocyte complexes (COCs). COCs were treated with 0.1% hyaluronidase (~3 min) to remove cumulus cells, yielding denuded zygotes. Fertilized zygotes (identified by an extruded polar body) were selected and washed three times in equilibrated KSOM (Millipore, MR-020P-5F). These zygotes were then cultured in 15 μL drops of KSOM (30 embryos per drop) under mineral oil at 37 °C (5% CO_2, 5% O_2). After incubation, embryos were rinsed once in M2 and transferred to fresh KSOM for continued culture. Zygotes were cultured for 4–5 days to reach the blastocyst stage.

2.4 Mouse Embryo AAV Infections

For AAV library screening, each 50 Zygotes were placed into 50 μL microdroplets of KSOM medium containing 5×10^{10} GCs AAV vectors. For AAV transduction efficiency experiment, each 10 Zygotes were placed into 10-15 μL microdroplets of KSOM medium containing 1×10^{10} GCs AAV vectors. These droplets were overlaid with a layer of mineral oil in 35 mm culture dishes and maintained at 37 °C in an

incubator set to 5% CO_2 and 5% O_2. Following the incubation period, embryos were gently washed in M2 medium to eliminate residual viral particles and subsequently moved into fresh KSOM medium for continued in vitro culture. The embryos were maintained under standard conditions for 4 to 5 days, allowing progression to the blastocyst stage.

2.5 RNA Sequencing of Mouse Embryo Transduced with AAV

For RNA Sequencing of Mouse Embryos Transduced with AAV, blastocysts were harvested 5-6 days post-infection, and total RNA was extracted utilizing the QuickExtract RNA Extraction Solution (Epicenter). cDNA synthesis was conducted using the Takara cDNA Synthesis Kit. The barcode regions were subsequently PCR-amplified (TOYOBO) from each cDNA sample, and the resulting amplicons were purified using a QIAquick Gel Extraction Kit (Qiagen, 28706) in accordance with the manufacturer's guidelines. Purified PCR products, ranging from 10 to 30 ng, were then submitted for next-generation sequencing (NGS).

2.6 sgRNA expression plasmid construction

To construct the sgRNA expression plasmid, the Tyr-targeting sgRNA[13] was cloned into the SaCas9 backbone vector (Addgene #61591) according to Zhang's laboratory protocol[14]. The plasmid was linearized by BsaI (NEB) and purified by agarose gel extraction (TIANGEN). The annealed sgRNA oligonucleotides were ligated into the BsaI-digested plasmid using T4 DNA Ligase (NEB). The ligation product was then transformed into DH5α competent E. coli.

2.7 Genome editing and deep sequencing analysis of indels for endogenous sites

Five to six days after zygotic exposure to the AAV vector, blastocyst-stage embryos were isolated for analysis. Genomic DNA was extracted using the QuickExtract solution (Epicenter). To measure genome editing outcomes, the targeted genomic region was subjected to nested PCR in two sequential reactions, during which Illumina adapter sequences were incorporated. The amplified DNA fragments (approximately 200–300 bp) were isolated via gel purification and prepared for high-throughput sequencing to quantify indel formation at the intended loci.

2.8 Statistics

Statistical analyses conducted in this study were based on a minimum of n = 3 biologically independent experiments and were calculated using either an unpaired or paired two-tailed Student's t-test via the R package dplyr. Comprehensive details regarding samples and experimental replicates are provided in the figure legends.

3. Results

3.1 Barcode AAV Library Generation

To systematically evaluate AAV tropism in mouse embryos, we assembled a multiplexed AAV library compromising 12 natural serotypes (AAV1–AAV12). All vectors were engineered to carry an identical reporter cassette (driving enhanced green fluorescent protein, EGFP) and a unique 12-nucleotide DNA barcode in the 3′ untranslated region, similar to previously described AAV barcode libraries[15]. Instead of laboriously microinjecting each embryo, we adopted a simplified approach by incubating zona-intact zygotes overnight in virus-containing medium, allowing AAV entry through the zona pellucida via natural binding and uptake. After a defined exposure period (overnight incubation), we washed the embryos to remove excess virus and cultured them to the blastocyst stage. Total RNA was extracted from pooled embryos, and high-throughput sequencing was performed to quantify barcode abundance, thereby indicating the relative transduction efficiency of each AAV serotype (Figure 1A).

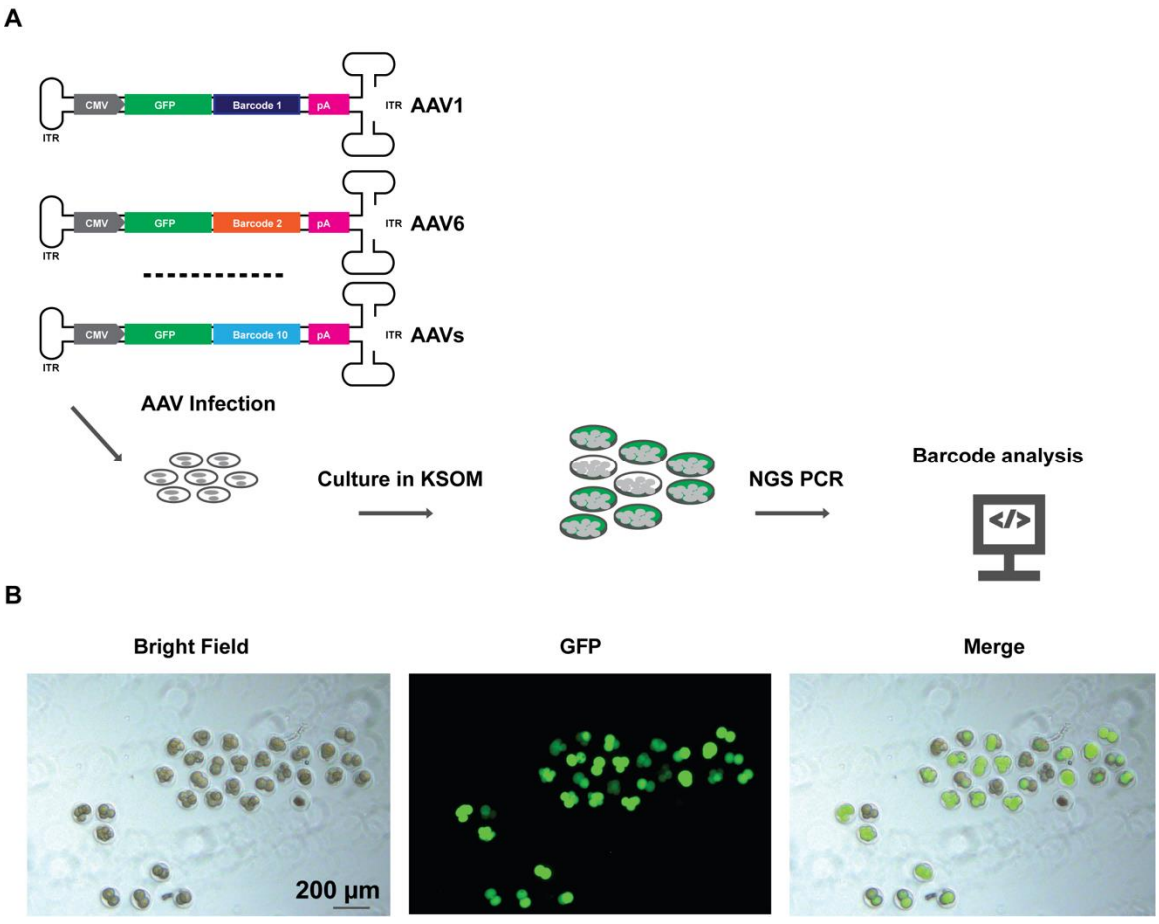

Figure 1. Overview of the AAV barcode screening strategy. (**A**) Schematic of the AAV-barcode assay for serotype tropism. A panel of 12 rAAV variants (serotypes 1–12) was constructed, each carrying an enhanced GFP reporter gene driven by a constitutive promoter and a unique 12-bp barcode sequence inserted in the 3′ UTR of the GFP transcript (immediately downstream of the stop codon). The individual barcoded AAVs were mixed to create a library, which was then used to infect mouse zygotes ex vivo (at the late one-cell stage). After a 24-hour incubation allowing viral entry and initial gene expression, we harvested embryos for molecular analysis. RNA was extracted from pools of embryos and PCR-amplified across the barcode region, followed by NGS to count the representation of each barcode. (**B**) Representative fluorescence microscopy images of mouse zygotes infected with mixed barcode AAV vectors. Scale bar: 200 µm.

3.2 High-Throughput AAV Barcode Screening Identifies Optimal AAV Vehicle for Zygote Transduction

After infection of mouse zygotes with the 12-serotype AAV barcode library, deep sequencing of barcode amplicons was used to determine each variant's relative abundance in the embryo samples. NGS results revealed marked differences in transduction efficiency among the AAV serotypes. In our screening, AAV6 achieves the highest transduction with an approximately 5-fold higher efficiency than the next best serotypes (~25% of total on average), significantly above the next best serotypes AAV1 and AAV7 (Figure 2A). In contrast, several serotypes including AAV2, AAV4 and AAV11 were barely detected above background, suggesting poor or negligible zygote transduction by those capsids (Figure 2B). Certain serotypes like AAV2[16] and AAV8[17] that are efficient in some somatic tissues were surprisingly ineffective in zygotes, underscoring that AAV tropism is highly context dependent.

Overall, the barcode screening unambiguously pointed to AAV6 as one of the most capable serotypes for delivering genes to mouse embryos. Our finding aligns with prior qualitative observations by previous studies that AAV6 can robustly transduce embryos, and with a recent mini review highlighting that AAV6 was uniquely able to cross the zona pellucida and infect fertilized oocytes[5]. Thereby, we justify a focused evaluation of AAV6 in subsequent experiments.

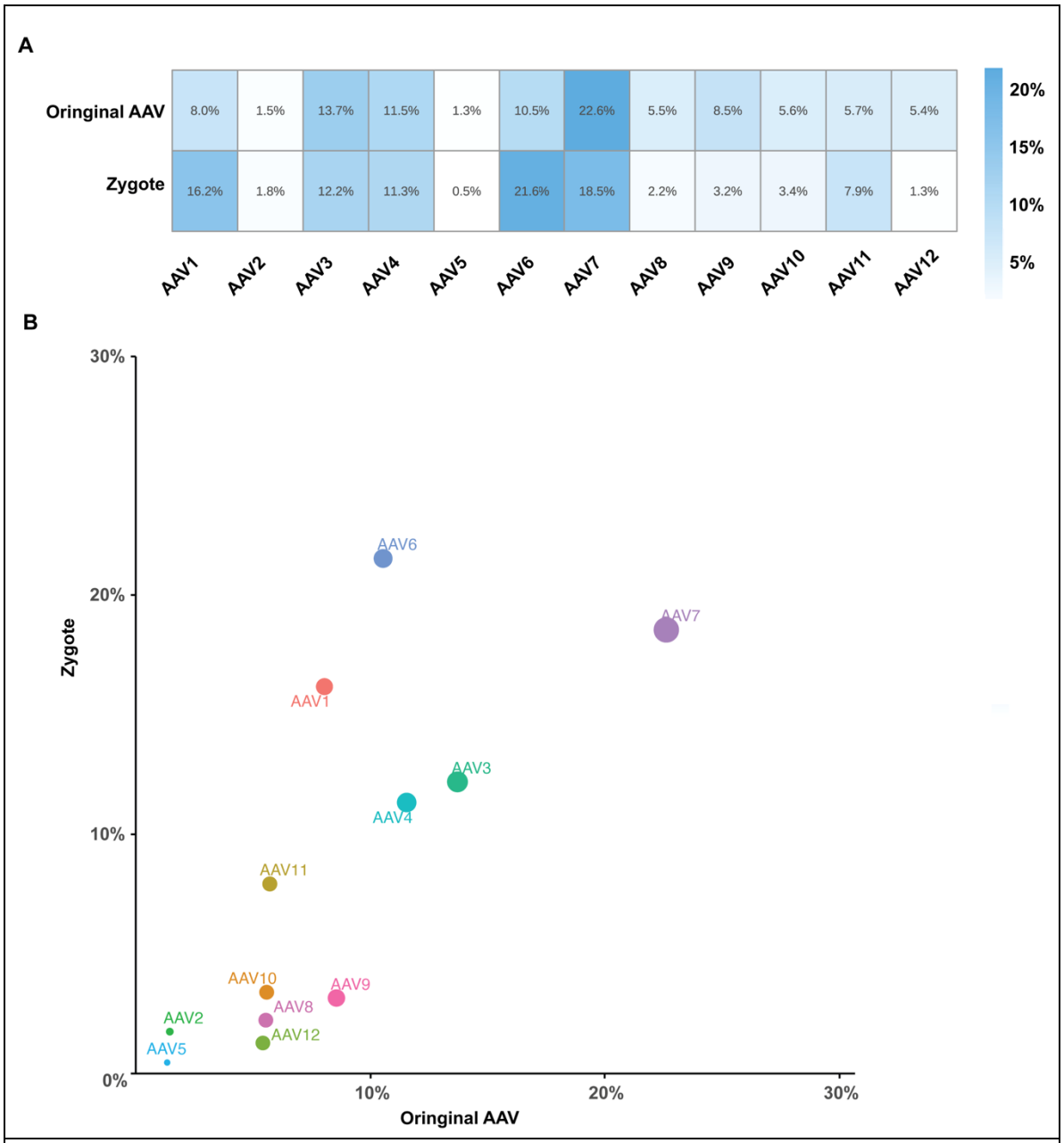

Figure 2. NGS analysis of AAV serotype tropism in mouse zygotes. (**A**) Barcode representation heatmap. Each row corresponds to an independent biological replicate (pool of 110 zygotes) infected with the 12-serotype AAV library, and each column corresponds to one of the AAV serotype 1-12. The color intensity reflects the frequency of that serotype's barcode in the NGS reads from the embryos; darker colors indicate higher transduction by that AAV variant. (**B**) Transduction efficiency of each AAV serotype, presented as a line chart. The Y-axis indicates the proportion of viral transcription equivalents in the embryos attributable to each serotype's vector (percentage of total).

3.3 AAV6 Efficiently Transduces Mouse Zygotes and Drives Reporter Expression

We next sought to validate the candidate vector in direct infection experiments and to visually confirm transgene expression in embryos. Zygotes from mice were collected and incubated with purified AAV6 particles carrying a CAG promoter-driven EGFP reporter (scAAV6-CAG-GFP). For comparison, we performed parallel infections using AAV1 and AAV5 vectors encoding the same GFP cassette, as these serotypes showed the next-best (albeit much lower) transduction in our screen. After 24 hours of culture, we examined the embryos under a fluorescence microscope to assess GFP expression. Representative images of embryos infected with AAV1, AAV5, or AAV6 at same 1×10^{10} vector genomes per culture droplet (Figure 3A). AAV6-treated embryos exhibited bright, widespread GFP fluorescence, whereas embryos exposed to AAV1 or AAV5 showed little detectable GFP signal.

To quantify the relative transduction efficiencies, we extracted total RNA from batches of AAV-infected embryos and performed reverse transcription followed by quantitative PCR (RT-qPCR) for the GFP transcript. Figure 3B summarizes the GFP mRNA levels (normalized to housekeeping gene expression) in embryos infected by AAV1, AAV5, or AAV6. Consistent with the microscopy observations, AAV6 yielded significantly higher GFP expression in zygotes – approximately a three-fold increase in GFP mRNA compared to AAV1, and similarly well above the AAV5 group (Figure 3B). These differences were statistically significant. Thus, by both qualitative imaging and quantitative molecular readouts, AAV6 demonstrates markedly superior gene delivery to embryos relative to other common serotypes. This validates the barcode screening outcome and identifies AAV6 as an optimal vector for transducing mouse zygotes. It is worth noting that AAV6's efficiency may stem from its natural capsid properties – AAV6 is known to utilize cell surface sialic acid and heparan sulfate proteoglycans for entry, which could be abundantly accessible on the zona or embryo membrane. In contrast, AAV2 (which was ineffective in zygotes) typically requires heparan plus co-receptors that might be absent in early embryos. Our results here provide a foundation for using AAV6 as a vehicle to introduce functional payloads (such as genome editors) into embryos.

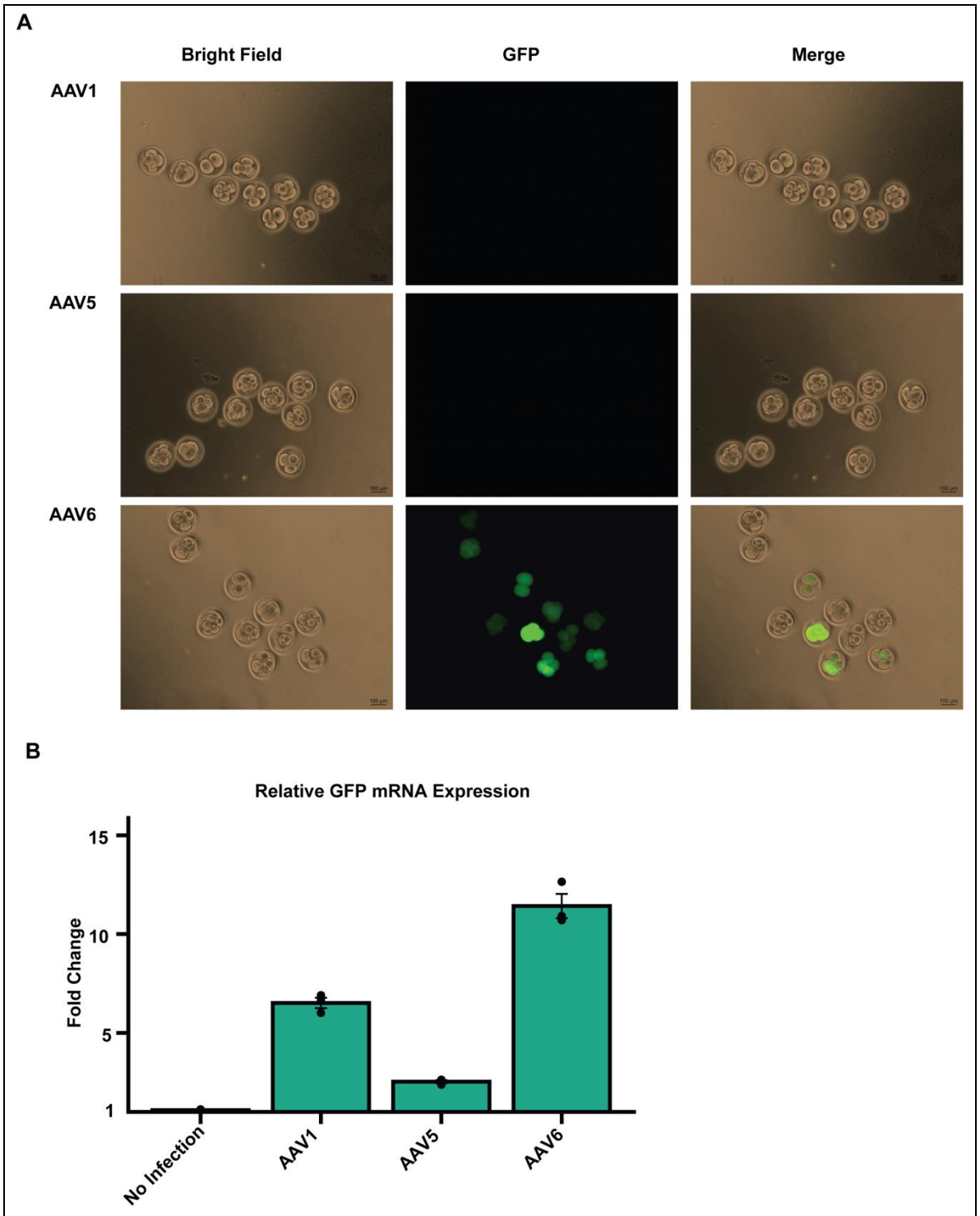

Figure 3. Comparative analysis of transduction efficiency among selected AAV serotypes. (**A**) Representative fluorescence microscopy images comparing transduction efficiency of AAV1, AAV5, and AAV6 vectors expressing GFP. AAV6-infected zygotes exhibited significantly stronger GFP fluorescence. (**B**) Quantitative RT-PCR analysis demonstrating significantly elevated GFP mRNA expression in zygotes infected with AAVs (n=3 biological replicates; $p<0.01$, Student's t-test).

3.4 AAV6-mediated delivery of CRISPR-Cas9 in zygotes achieves efficient genome editing without impeding embryo development.

We next tested whether AAV6 could effectively deliver SaCas9 genome editing tools to embryos. Zygotes were exposed to AAV6 vectors encoding the SaCas9 nuclease and a guide RNA targeting a Tyr locus (Figure 4A). Using a 1×10^{10} vector genome of AAV6 (ssAAV6-SaCas9:Tyr), all groups of AAV6-SaCas9 treated embryos implanted and gave rise to healthy blastocysts at frequencies 76.9% statistically indistinguishable from untreated controls 82.4% (Figure 4B-C), indicating that the AAV6-mediated CRISPR treatment is compatible with normal embryonic development.

In our cohort, we observed that nearly all treated embryos carried the intended mutation at the target Try locus. Moreover, every blastocyst derived from these embryos was found to harbor the edited allele (Figure 4D). This high editing success rate is consistent with reports of essentially 100% editing frequency achieved with AAV6 under optimal conditions.

Taken together, these results validate AAV6 as an efficient delivery vehicle for CRISPR genome editing in zygotes with a high editing efficiency, while being non-injurious to embryo viability. By quantitatively confirming AAV6 leading performance among AAV serotypes and functionally integrating it into an embryo editing workflow, we have established a framework for using viral vectors in the production of genome-edited embryos.

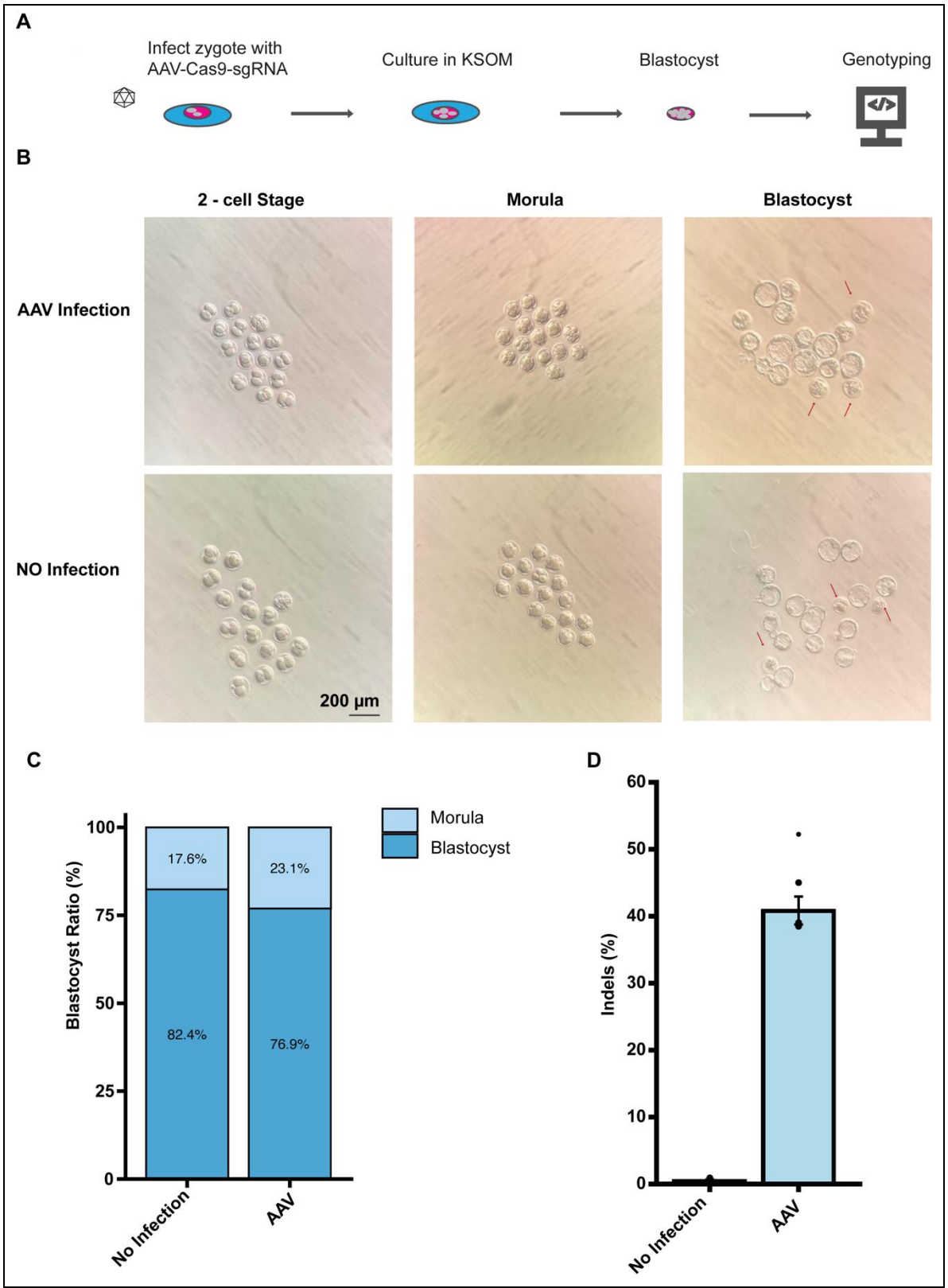

Figure 4. AAV-Cas9 efficiently induces genome editing in mouse zygotes. (A) Workflow for AAV-Cas9 genome editing in mouse zygotes. (B-C) Morphological analysis demonstrating normal embryonic development from 2-cell stage to blastocyst after infection with AAV6-SaCas9. Scale bar: 200 µm. (D)

Deep sequencing analysis quantifying the editing efficiency at the Tyr gene locus in blastocysts following AAV6-Cas9 transduction. Data are represented as the mean ± SD.

4. Discussion

Our study provides the first comprehensive, high-throughput evaluation of AAV serotype tropism in mouse zygotes, and the results carry important implications for the field of genome engineering. AAV6 emerged as the top-performing serotype for zygote transduction in our experiment, which agrees with earlier reports that highlighted AAV6's strong ability to infect early embryos[18-20]. Notably, previous studies had initially screened a collection of AAV vectors on mouse embryos and observed that serotype 6 produced the highest level of reporter expression in morulae[19,21-23.] Our work builds on and reinforces that observation by providing quantitative evidence: using a barcode sequencing approach, we showed that AAV6 outcompetes eleven other serotypes in reaching the interior of the zygote. This systematic comparison removes potential confounding differences in experimental conditions, as all serotypes were tested side-by-side in the same embryos. The confirmation of AAV6's superior tropism is therefore robust, and it cements AAV6 as the vector of choice for delivering genetic cargo to one-cell embryos.

It is worth reflecting on why AAV6 is particularly effective in this context. AAV6 is a naturally occurring variant (closely related to AAV1) that can utilize both α2,3/α2,6 N-linked sialic acid and heparan sulfate proteoglycans for cell entry, broadening its host cell range[24]. The mouse zona pellucida and embryonic membranes may present glycan structures that AAV6 can bind, facilitating its uptake. In contrast, other serotypes like AAV2 (which relies heavily on heparan sulfate) or AAV5 (which binds to platelet-derived growth factor receptor) may not find their receptors readily available on zygotes or may not efficiently traverse the zona. Our finding that AAV1 had the second-best (though much lower) transduction aligns with its capsid similarity to AAV6; AAV1 and AAV6 differ by only a few amino acids, and prior studies noted that both can infect preimplantation embryos while many other serotypes lag[7]. Interestingly, a recent report by Romeo et al. demonstrated that an engineered AAV capsid (AAV-DJ, a synthetic hybrid of multiple serotypes) could also penetrate the mouse zona and transduce zygotes, but that it was associated with higher toxicity than AAV1[6]. This underscores a key point: beyond raw infectivity, a useful vector for embryo work must also be biocompatible with early development. Our data indicate that AAV6 meets this criterion well – it infects efficiently and is well tolerated by zygotes – whereas some vectors with different capsid properties might disturb embryonic physiology. We did not detect any detrimental effects of AAV6 on embryo progression to blastocyst or on offspring viability, consistent with the notion that wild-type AAV serotypes are generally low in toxicity[25-27]. There have been isolated reports of developmental perturbation with certain AAV serotypes or preparations (e.g., helper-dependent AAV2 vectors causing zygote toxicity in one study)[22], but these appear to be the exception rather than the rule. In our hands and others', AAV6 stands out as a reliable compromise of high efficacy and minimal toxicity in the embryo context[22,28].

A major innovation of this study is the application of a multiplexed barcode sequencing platform to preimplantation embryos. High-throughput approaches are increasingly used in gene therapy research to evaluate large libraries of AAV capsid variants in adult animals or cell cultures[29-32]. By adapting this strategy to embryos, we have demonstrated that one can rapidly screen many vectors for gene delivery to the germline stage. This has several advantages. First, it is resource-efficient: dozens of AAV variants can be tested in a single batch of embryos, which is particularly useful given the limited availability of zygotes and the cost of generating and purifying many viral vectors. Second, it provides a quantitative readout of relative performance, which is more informative than binary "works or doesn't work" assessments. For example, our data not only identified AAV6 as a good serotype but also quantitatively ranked others, revealing gradations in tropism that could guide secondary choices. We foresee that this barcode-based screening platform could be employed to evaluate not just natural serotypes but also engineered AAV capsids (e.g., those evolved for specific traits) for their ability to target embryos. Indeed, the development of novel AAV variants for gene therapy is a rapidly moving field[33,34], and applying our screening method could help discover or validate capsids that confer even higher infection rates or specificity for gametes/early embryos. It is interesting to note that Yoon et al. managed ~100% embryo transduction with AAV6, yet still only ~80-90% of resulting pups carried editing[7], implying some embryos were infected but not effectively edited. This could relate to timing issues; hence, capsids or methods that accelerate transgene delivery (such as self-complementary genomes or using smaller Cas nucleases) might further improve outcomes. Recent advances in Cas9 engineering, such as the creation of high-fidelity or compact

Cas9 variants[35-37], offer additional opportunities to optimize the cargo that AAV delivers to embryos. A smaller Cas9 (or a different genome editor like base editors or Cas12 variants) could fit more easily into a single AAV vector, simplifying the delivery and perhaps yielding more uniform editing. Combining such improved reagents with AAV6's delivery capability could enhance efficiency and reduce mosaicism.

5. Conclusion

In summary, AAV6 is reaffirmed as an outstanding vehicle for zygotic gene editing, and our barcode screening approach lays the groundwork for future optimization of viral vectors in developmental biology. These findings will help guide researchers in selecting and developing AAV tools for creating genetically modified animals and could inform translational efforts where safe and effective delivery of gene editors to early embryos is desired. Our results emphasize that systematic screening and validation is the key to unlocking the full potential of viral vectors in genome engineering.

Data sharing agreement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Conflicting Interests: The authors declared no potential conflicts of interest with respect to the research, author-ship, and publication of this article.

Author Contributions: TY. Wang conceived and designed the experiments. TY. Wang, YF. Tian performed zygote experiments. L Liu, JW. Chen performed in vitro experiments. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consents were obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

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