Optimizing CRISPR/Cas9 technology for precise correction of the Fgfr3-G374R mutation in achondroplasia in mice

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CRISPR/Cas9 is a powerful technology widely used for genome editing, with the potential to be used for correcting a wide variety of deleterious disease-causing mutations. However, the technique tends to generate more indels (insertions and deletions) than precise modifications at the target sites, which might not resolve the mutation and could instead exacerbate the initial genetic disruption. We sought to develop an improved protocol for CRISPR/Cas9 that would correct mutations without unintended consequences. As a case study, we focused on achondroplasia, a common genetic form of dwarfism defined by missense mutation in the Fgfr3 gene that results in glycin to arginine substitution at position 374 in mice in fibroblast growth factor receptor 3 (Fgfr3-G374R), which corresponds to G380R in humans. First, we designed a GFP reporter system that can evaluate the cutting efficiency and specificity of single guide RNAs (sgRNAs). Using the sgRNA selected based on our GFP reporter system, we conducted targeted therapy of achondroplasia in mice. We found that we achieved higher frequency of precise correction of the Fgfr3-G374R mutation using Cas9 protein rather than Cas9 mRNA. We further demonstrated that targeting oligos of 100 and 200 nucleotides precisely corrected the mutation at equal efficiency. We showed that our strategy completely suppressed phenotypes of achondroplasia and whole genome sequencing detected no off-target effects. These data indicate that improved protocols can enable the precise CRISPR/Cas9-mediated correction of individual mutations with high fidelity.
logy-directed repair (HDR) based on an exogenously supplied single strand donor DNA without inducing nonspecific off-targeting events. The resulting mice showed normal body size, were fertile, and able to transmit the corrected allele to their progeny. Thus, our study provides proof for using the CRISPR/Cas9 system to correct this dominant genetic disease.

**Results**

**Evaluation of genome editing efficiency and specificity of sgRNAs against Fgfr3-G374R by sgRNA testers**

Three overlapping sgRNAs of 20 nt were designed to spanning the Gly to Arg mutation in Fgfr3 gene (Fig. S1A), the top 10 predicted off-target locus with NGG PAM (protospacer adjacent motif) sequence were listed for further analysis (Fig. S1, B–D). The highest homology for these sgRNAs is WT Fgfr3 that contains 1 mismatched base at the “Gly to Arg” mutation site. This is followed by 1 mismatches of the Cmmn2 gene to sgRNA1; 2 mismatches of noncoding region locus Chr2: 10643681 to sgRNA2, and 2 mismatches of Extl3 genes to sgRNA3, respectively. Increasing mismatches to these sgRNAs up to 4 bases in some other genes of varying homology are shown in Fig. S1, B–D.

We then tested the cutting efficiency of the sgRNAs using a GFP reporter plasmid (pCAG-EGx-xFP-BbsI), which carries tandemly duplicated defective GFPs with a mutation in their 3’ and 5’, respectively. A dual BbsI cutting site was also introduced between two mutated GFPs for cloning of sgDNA + NGG to be tested (Fig. 1A). For each sgRNA, we cloned the mutant form and the corresponding WT form, which carries one base mismatch with the mutant form, into the tester construct (Fig. 1B) to generate 3 pairs of tester reporters. Then, each of the reporter alone or together with their corresponding CRISPR/Cas9 construct were transfected into 293 cells to test their cutting efficiency and specificity.

Our data indicated that transfection of these testers alone did not generate obvious GFP signaling, which is consistent with the fact that tandem duplicated GFPs are defective in the pCAG-EGx-xFP-BbsI construct. On the other hand, co-transfection of all three pairs of testers with their corresponding sgRNA-CRISPR/Cas9 (PX330-sgRNA) constructs generated GFP positive cells. This is because the mutant GFPs can be repaired due to homologous recombination stimulated by CRISPR/Cas9-mediated double strand break at the target site, generating GFP+ cells (Fig. 1C). The GFP expression level should reflect the cutting efficiency of sgRNA-guided CRISPR/Cas9 to the targeted sequence. The data indicated that sgRNA1 had the highest cutting effect for the Fgfr3 mutant sequence, however, it also cut the corresponding WT sequence (one base mismatch to the MT sequence) with high efficiency. sgRNA3 cut much more efficiently than sgRNA1, however, sgRNA3 also cut the WT sequence and generated obvious GFP positive signaling (Fig. S2, A–C). Thus, sgRNA2 had moderate efficiency for cutting the Fgfr3 mutant allele among the three sgRNAs tested, whereas no cutting at its corresponding WT allele (Fig. 1, C and D), reflecting its high specificity to the mutant allele. Because avoiding the off-target effect is most crucial, we selected sgRNA2 for our subsequent in vivo experiments.

**Targeted correction of dwarf phenotype associated with Fgfr3-G374R mutation in mice**

We used the mouse model of dominant achondroplasia caused by a defined Gly to Arg mutation in exon 10 of the Fgfr3 gene [21]. As achondroplasia mice grow poorly throughout development and have reduced fertility, a Loxp-neo-Loxp (lnl) cassette was inserted into intron 10 to block the expression of the mutant allele (Fig. 2A). Heterozygous mice for the mutation (Fgfr3G1120A–/lnl) were normal, whereas homozygous mice (Fgfr3G1120A–/lnl/G1120A–/lnl) survived to adulthood and were fertile, although exhibited skeleton abnormalities similar to Fgfr3G374R– mice generated by gene targeting [22]. Deletion of the lnl cassette from mutant mice by breeding with mice carrying the Ella-Cre transgene will allow the expression of Fgfr3G1120A allele, generating mice with a dwarf phenotype (Fig. 2A). Our data indicated that the cross between homozygous male Fgfr3G1120A–/lnl mice with homozygous female Ella-Cre mice could generate dwarf phenotypes in all offspring (Fgfr3G1120A–/lnl/Ella-Cre, or Fgfr3G1120A–), thus the fertilized zygotes from this breeding were collected for pronuclear injection.

Cas9 mRNA or protein, sgRNA2 RNA, and single strand donor WT DNA were co-injected into the pronuclear of Fgfr3G1120A–/lnl zygotes, which were then implanted into pseudo-pregnant female mice for further development. Of a total of 63 live pups born, 42 mice were phenotypically normal (Table 1), suggesting the mutant allele was either disrupted or corrected.

We injected Cas9 mRNA at two concentrations, 200 and 5 ng/µl, which generated 13 and 15 pups, respectively. Of 13 pups generated by injection of 200 ng/µl mRNA, 8 (61.6%) where morphologically normal (Table 1). In contrast, none of the 15 mice generated by injection of 5 ng/µl of mRNA were normal, despite the increased concentrations of sgRNA and single strand DNA oligo (ssDO) by 2.5- and 2-fold, respectively, to increase genomic cutting and homologous recombination at the target site. This observation indicates that Cas9 mRNA at 5 ng/µl was too low to achieve effective genomic editing to the Fgfr3G1120A allele. Of note, 34 of 35 (97.1%) pups generated by injection of the Cas9 protein exhibited normal phenotype and 9 of them carry precision correction of the point mutation. Collectively, these data suggest that injection of Cas9 protein has a much higher efficiency in genome editing, although more conclusive data may be provided in future studies.

**High frequency of targeted correction of the Fgfr3G1120A point mutation by the injection of Cas9 protein in mice**

The correction of achondroplasia phenotype suggests that the Fgfr3G1120A allele in these mice was edited (either through targeted disruption or point mutation correction). Because the G to A transition at position 1120 (leading to G374R mutation) generates a SfcI restriction site (CTACAG) (Fig. 2B), thus change at the genomic level at this mutation site can be monitored by SfcI digestion. Therefore, to provide a quick analysis of the status of the mutation site, we used a pair of PCR primers flanking the G1120A mutation site to amplify a 374-bp fragment followed by restriction digestion with SfcI, which should cut this fragment into 176 and 198 bp if the CTACAG site is intact, whereas the loss of this site should render the SfcI resistance.
Of 13 samples isolated from phenotype-corrected mice generated by the injection of Cas9 mRNA, 8 were SfcI resistant (pup 1 in Fig. 2, B and C), indicating the loss of cutting site; and 5 were sensitive (pups 2 and 3, Fig. 2, B and C), suggesting the G1120A mutation was not corrected. To understand the reason why these animals were normal while keeping the mutation, we conducted Sanger’s sequencing and the data indicated that pup 1 carried a 13-bp deletion, including the SfcI site, pup 2 had a deletion of “G,” and pup 3 had an insertion of “T,” respectively, in front of the SfcI site (Fig. 2D). In all three cases, the Fgfr3 G1120A allele was effectively mutated due to the frameshift caused by indels, leading to the loss of the mutant phenotype.
In 34 DNA samples isolated from injection of the Cas9 protein, 25 (73.5%) carried indels that disrupted the Fgfr3G374R allele and the animal appeared normal. In the remaining 9 mice (26.5%), the “A” was replaced by G (pups 5 and 6, Fig. 2, C–E) directed by ssDO template (Correction), all of which destroyed the SfcI site. These data indicate that injection of the Cas9 protein elicits high genome editing efficiency with a significant fraction generated by precise correction. Similar high efficiency was observed when a loxP site was introduced into the EGFP locus through homologous recombination (Fig. 3).

Targeting single strand DNA oligos of 100 and 200 nt corrected Fgfr3G1120A mutation at equal efficiency in the achondroplasia mice

The correction of the point mutation is primarily mediated by targeting ssDOs, which provide a template for repairing the double strand break created by the cutting of CRISPR/Cas9. The length of the ssDO used in the above experiment is 200 nt.

Next, we investigated whether supplying a shorter ssDO would still maintain comparable efficiency of HDR-mediated precise genome repair. Our data indicated that the injection of a ssDO of 101 nt yielded a phenotype correction efficiency of 97.5% (40/41), which is comparable with 97.1% (34/35) obtained by using the ssDO of 200 nt. DNA sequencing revealed that 31 (77.5%) mice carried indels, whereas the remaining 9 (22.5%) mice had their G to A mutation precisely corrected (Table 2).

Taken together, our data suggest that the Cas9 protein have higher efficiency to promote HDR-based genetic repairing, which was directed by exogenous oligos. 101 nt homologous ssDO keep the same efficiency for HDR compared with 200-nt ssDO. CRISPR/Cas9-mediated correction or disruption of Fgfr3G374R mutation completely suppresses phenotypes of achondroplasia mice

The most significant features of achondroplasia mice include reduced body weight, dome-shaped head, short stature, reduced...
lengths of long bone, most promptly in femurs and humerus (Fig. 4). We carefully examined all 18 mice that carried the corrected point mutation allele using these features of achondroplasia, as measured by bone length and body weight, and the data indicated that all these were absent in the phenotype-corrected mice and they were indistinguishable with WT mice (Fig. 4, A–D). One major concern of the achondroplasia therapy is whether the dwarf phenotype can be inherited to the pups. We therefore mated them with WT mice and then analyzed the pups. Progeny obtained from corrected \( \text{Fgfr3} \) point mutation mice were all normal. DNA sequencing using primer against the mutant allele showed that the pups carried the repaired \( \text{Fgfr3} \) allele originating from their parents, indicating that the corrected allele could be successfully transmitted to the next generation through the germline.

The remaining 56 (25 from using of 200 nt ssDO and 31 from using 101-nt ssDO) mice that carry non-homologous end joining-mediated insertions or deletions were also phenotypically normal. Analysis of the nucleotide sequences of the \( \text{Fgfr3} \) locus revealed that the mutant \( \text{Fgfr3}^{\text{G374R}} \) allele was disrupted by indels at or near the G1120A mutation site in all these mice, whereas the remaining WT \( \text{Fgfr3} \) allele is intact. This is consistent with our previous observation that mice heterozygous for the \( \text{Fgfr3} \) knockout mutation is phenotypically normal (22).

**Correction of the \( \text{Fgfr3} \)-\( \text{G374R} \) related achondroplasia**

The CRISPR/Cas9 system sometimes suffers an off-target effect by cutting at sites with shared homology (15–17). To investigate this, we conducted whole-genome DNA sequencing for 7 mice delivered by one surrogate mother, including 3 mice

![Figure 3. Knock-in a loxP site into GFP transgene through pronuclear injection of CRISPR/Cas9 system.](image)
with targeted mutation correction, 3 mice with indels (these 6 mice are phenotypically normal), and 1 mouse with uncorrected G1120A mutation (achondroplasia phenotype) revealed by SfcI digestion. As shown in Fig. 5, A and B, #1083 still contains the G to A mutation, whereas showing the dwarf phenotype. #1081, #1084, and #1089 only contained WT allele, suggesting the G1120A mutation was corrected. The remaining 3 mice, #1082, #1085, and #1086, exhibited mixed ratios of WT allele and indels. In the #1082 and #1086 mice, the WT allele was presented at 60 and 42%, respectively, which is considered the normal range of heterozygosity. Meanwhile, the #1082 mouse also contained a deletion of 17 bp and an insertion of a T, presented at 16 and 23%, respectively, suggesting this animal carries these two types of mutations in different portions of cells, presumably due to two distinct indels at 2- or 4-cell staged embryos. A similar situation was observed in #1086, which also carried insertion of a C and a T in different cells besides the WT allele. Mouse #1085 contained WT allele at 81.4% and an allele of 27 bp deletion at 18.6%, suggesting a precise correction of G1120A, and a deletion of 27 bp occurred in different cells at early 2-cell or 4-cell staged embryos. All these events (precision correction and indels) are mediated by CRISPR/Cas9 and effectively disrupted the Fgfr3G1120A mutation, resulting in the correction of achondroplasia phenotype of the mutant mice.

Next, we examined the entire Fgfr3 locus spanning 15.4 kb and detected no alterations. We then studied all homologous loci (Fig. 1C) for sgRNA2 with 1–4 mismatched nucleotides and detected no indels generated. These analyses indicate that the G1120A mutation was corrected specifically without obvious off-targeting events.

Discussion

CRISPR/Cas9 is a powerful tool for genome editing that has been used in multiple organisms (1, 2, 4). However, CRISPR/Cas9, at its current stands, also suffers some limitations. For example, the CRISPR/Cas9 system frequently suffers an off-target effect by cutting at sites with shared homology (15–17). Furthermore, due to its high efficiency cutting at targeting sites, CRISPR/Cas9 preferably generates more indels than precise modifications, casting difficulty to generate the desired mutations. Using Fgfr3G374R achondroplasia mice as a model system, we have addressed some of the potential problems. Our optimized experiment conditions have achieved high specificity of targeted correction of achondroplasia with several navel features.

Unintended binding, modification, and cleavage of nucleic acids, so called the off-target effect, is a major challenge to the CRISPR/Cas9 system (3). To overcome this problem, many sgRNA designing tools have been developed to increase specificity of sgRNAs at the silicon level (23, 24). A number of methods have also been employed to avoid off-target cutting or identify undesired mutations, such as using single-based editors correct base-pairing to avoid inducing a dsDNA break, or replace Cas9 with Cas13a to target RNA editing (3). In our study, the off-target effect still occurred even if CRISPR design software was used to design our sgRNAs. Because of the newly designed GFP tester, we found two of three sgRNAs could cut the WT Fgfr3 sequence with a single mismatched base, whereas the other sgRNA cuts only at the MT Fgfr3 sequence. We used this sgRNA for further genome editing in mice and it indeed did not cut the corresponding WT Fgfr3 sequence with single mismatched base.
More recently, there are some discussions about whether or not the CRISPR/Cas9 system could generate unexpected mutations \textit{in vivo} (25–29). To investigate this, we conducted whole genome sequencing, and detected no off-target events in the region near the targeting site, the entire \textit{Fgfr3} locus, and in all predicted potential off-target sites, which share varying degrees of homology with the target sequence. Altogether, we demonstrated that by using our optimized sgRNA, the CRISPR/Cas9-mediated targeted correction of the \textit{Fgfr3} \textit{G374R} point mutation completely suppresses phenotypes of achondroplasia mice with high fidelity. This result demonstrates in principle the importance of using such a tester system to avoid potential off-target effects prior to the experiment. For example, if sgRNA 1 or 3 was used in our experiment, it might have generated off-target events. The GFP tester is user friendly, and reliable for providing functional evaluation of sgRNAs for their specificity and efficiency. From the long run, highly suspected sequences for off-target predicted by software could also be tested first to avoid any potential problems.

Both Cas9 mRNA and proteins have been widely used for achieving genome editing with variable frequencies (30, 31). Our data revealed significant higher frequency of CRRSPR/Cas9-mediated genome cutting at the target site and the precise correction of the \textit{G1120A} point mutation completely suppresses phenotypes of achondroplasia mice with high fidelity. This result demonstrates in principle the importance of using such a tester system to avoid potential off-target effects prior to the experiment. For example, if sgRNA 1 or 3 was used in our experiment, it might have generated off-target events. The GFP tester is user friendly, and reliable for providing functional evaluation of sgRNAs for their specificity and efficiency. From the long run, highly suspected sequences for off-target predicted by software could also be tested first to avoid any potential problems.

Conventional gene targeting by homologous recombination has high demand on the length of targeting homology to achieve ideal targeting efficiency (32). Because the CRISPR/Cas9 system generates double strand breaks at the target sites that greatly stimulates efficiency of homologous recombination, the long homologous arm of targeting constructs is less critical (32). Donor oligo or targeting constructs with less than 1000-bp homology arms are frequently used for genome editing in mouse zygotes (33). In our study, we first tested targeting ssDO with 200 nt (with around 100 nt on each side of the G to A mutation) and obtained genome editing frequency in 34/35 (97%) mice. In the 34 mice, 9 carry precise \textit{G1120A} mutation correction (26.5%). To further test the effect of the length of homology, we injected a 101-nt ssDO and obtained comparable editing frequency and mutation correction rate. These data demonstrate that the targeting oligos of 101 and 200 nt precisely corrected the mutation at similar efficiency. As oligos of 101 nt can be generated much more economically, our finding is of great significance when designing targeting ssODs.

In summary, we have demonstrated that the CRISPR/Cas9 system can be used to cure achondroplasia in mouse by directly correcting the genetic defect through homology-mediated gene edit-
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Experimental procedures

Animal model

As previously described to bypass the lethal effect of Fgfr3G1120A mutation, a lnl cassette is inserted between exons 10 and 11 to knockout the mutant Fgfr3 gene (21). Mice harboring homozygous Fgfr3G1120A-lnl/G1120A-lnl exhibit phenotypes similar to Fgfr31/1 mice (22) and are maintained to serve as stud mice. Homozygote of Ella-Cre (JAX, ME) female mice were superovulated by injection with pregnant mare’s serum gonadotropin followed by human chorionic gonadotropin, and then crossed with Fgfr3G1120A-lnl/G1120A-lnl stud mice to delete the lnl cassette and obtain Fgfr3 point mutant embryos for pronuclear injection. All animal procedures were performed under the ethical guidelines of the University of Macau (animal protocol number: UMAEC-037-2015).

sgRNA reporter plasmid construction

sgRNA reporter plasmid (pCAG-EGx-xFP-BbsI) was constructed by modifying pCAG-EGxxFP (Addgene). Briefly, a short dual BbsI cassette was amplified from plasmid PX330 (1) and inserted into the pCAG-EGxxFP digested with EcoRI to obtain pCAG-EGxxFP-BbsI (Fig. 1A). sgRNA tester sequence was designed based on the sgRNA sequence including the NGG PAM sequence, then the adapter was added for further cloning. To clone the target sequence into the reporter plasmid, paired oligos with adaptor were synthesized (Fig. 1A). Each pair of oligos was phosphorylated and annealed followed by ligation with a BbsI pre-digested reporter plasmid.

Microinjection

sgRNA(s) were designed by using Optimized CRISPR Design online tools (34), then ligation with PX330 plasmid. sgRNAs with a T7 promoter were amplified by PCR and in vitro transcribed using a MEGASHortscript T7 kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA).

The combination of these factors greatly facilitates the introduction of the desired genome modification with high fidelity. sgRNA(s) were designed by using Optimized CRISPR Design online tools (34), then ligation with PX330 plasmid. sgRNAs with a T7 promoter were amplified by PCR and in vitro transcribed using a MEGASHortscript T7 kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA). After transcription, the sgRNAs were purified with a MEGAclear kit (Thermo Fisher Scientific, MA).
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