

Repair of the *TGFBI* gene in human corneal keratocytes derived from a granular corneal dystrophy patient via CRISPR/Cas9-induced homology-directed repair

Yukako Taketani (1), Kohdai Kitamoto (1), Toshihiro Sakisaka (1), Mikiko Kimakura (1), Tetsuya Toyono (1), Satoru Yamagami (2), Shiro Amano (3), Masahiko Kuroda (4), Tara Moore (5, 6), Tomohiko Usui (1)\*, Yasuo Ouchi (7)\*

1. Department of Ophthalmology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
2. Nihon University Itabashi Hospital, Tokyo, Japan
3. Inoue Eye Hospital, Tokyo, Japan
4. Department of Molecular Pathology, Tokyo Medical University, Tokyo, Japan
5. Biomedical Sciences Research Institute, Centre for Molecular Biosciences, Ulster University, Northern Ireland
6. Avellino Labs, Menlo Park, CA, USA
7. Department of Mucosal Immunology, School of Medicine, Chiba University, Chiba, Japan

\*Corresponding authors: [tomohiko-tky@umin.ac.jp](mailto:tomohiko-tky@umin.ac.jp) and [o-yasuo@chiba-u.jp](mailto:o-yasuo@chiba-u.jp)

Keywords: CRISPR/Cas 9, TGFBI, Corneal dystrophy

## **Abstract**

Granular corneal dystrophy (GCD) is an autosomal dominant hereditary disease in which multiple discrete and irregularly shaped granular opacities are deposited in the corneal stroma. GCD is caused by a point mutation in the *transforming growth factor- $\beta$ -induced (TGFB1)* gene, located on chromosome 5q31. Here, we report the first successful application of CRISPR-Cas9-mediated genome editing for the correction of a *TGFB1* mutation in GCD patient-derived primary corneal keratocytes via homology-directed repair (HDR). To correct genetic defects in GCD patient cells, we designed a disease-specific guide RNA (gRNA) targeting the R124H mutation of *TGFB1*, which causes GCD type 2 (GCD2, Avellino corneal dystrophy). An R124H mutation in primary human corneal keratocytes derived from a GCD2 patient was corrected by delivering a CRISPR plasmid expressing Cas9/gRNA and a single-stranded oligodeoxynucleotide HDR donor template *in vitro*. The gene correction efficiency was 20.6% in heterozygous cells and 41.3% in homozygous cells. No off-target effects were detected. These results reveal a new therapeutic strategy for GCD2; this method may also be applicable to other hereditary corneal diseases.

## 1 **Introduction**

2 Granular corneal dystrophy (GCD) is a bilateral, progressive, genetic, and  
3 non-inflammatory disease characterised by multiple granular deposits in the corneal stroma.  
4 Using the IC3D classification<sup>1</sup>, GCD has three subtypes, all of which are classified as  
5 Category 1, i.e., causal point mutations have been identified in the *transforming growth*  
6 *factor-beta-induced (TGFB1)* gene, located on chromosome 5q31<sup>2</sup>. TGFB1, also called  
7 keratoepithelin or Big-h3, is 68-kDa protein found in the extracellular matrix of human tissues.  
8 It is particularly abundant in the cornea.

9 There are two clinical types of GCD, GCD1 and GCD2. Although originally described  
10 in a family from the Italian region of Avellino, the R124H mutation associated with GCD2 is  
11 occurs in unrelated individuals in all populations studied and is the most common type in  
12 Asia, including Japan<sup>3,4</sup>. In GCD2, discrete grey-white granular deposits (hyaline) with  
13 snowflake, star, or disk shapes are detected in the corneal stroma at an early age<sup>5</sup> and  
14 amyloid deposits are observed in elder patients in deeper stroma<sup>6</sup>. GCD2 has a diffuse  
15 anterior stromal haze between the typical granular opacity. The haze may be caused by  
16 amyloid deposits, which are thought to be similar to Gelatinous drop-like CD, instead of the  
17 linear opacity seen in the early stage of lattice dystrophy<sup>6,7</sup>. Compared to heterozygous  
18 patients, homozygous patients may have an onset under 10 years old, and demonstrate a  
19 more rapid progression. These progressive corneal opacities cause a loss of visual acuity.

20 To avoid visual impairment in GCD2, phototherapeutic keratectomy (PTK) is a major  
21 treatment option. However, multiple opacities usually recur within several years<sup>8</sup>. Compared  
22 with PTK, corneal clarity can be retained for longer durations using keratoplasty, but opacity  
23 eventually occurs via the gradual invasion of host corneal cells, especially in homozygous  
24 patients<sup>9</sup>. Thus, the development of a radical treatment is needed. GCD2 is typically  
25 associated with an R124H (histidine replacing arginine) point mutation in the *TGFBI* gene;  
26 accordingly, a gene therapy approach may be effective.

27 CRISPR/Cas9 (clustered, regularly interspaced short palindromic repeats  
28 (CRISPR)/CRISPR-associated protein)-mediated genome editing has been increasingly  
29 applied to repair mutated genome sequences<sup>10</sup>. This versatile tool for genome engineering  
30 enables the induction of site-specific double-strand breaks (DSBs) using guide RNAs  
31 (gRNAs)<sup>11-15</sup>. DSBs can be repaired by two major pathways, non-homologous end joining  
32 and homology-directed repair (HDR). In the presence of exogenous donor DNA as a repair  
33 template, DSBs can be repaired precisely via the HDR pathway. This technique is useful for  
34 codon replacements or reporter insertions<sup>16,17</sup>. For small genetic modifications, such as point  
35 mutations, the application of single-stranded oligodeoxynucleotides (ssODNs) as HDR  
36 templates shows higher editing efficiency than that of plasmid donors<sup>18</sup>. Here, we report the  
37 first CRISPR-mediated HDR using cultured corneal keratocytes derived from an R124H  
38 GCD2 patient. The results of this study have important clinical implications given the lack of

39 effective treatment options for GCD2.

40

## 41 **Results**

### 42 *Gene targeting strategy and construction for CRISPR/Cas9-mediated HDR of an R124H* 43 *mutation*

44 To develop an efficient strategy to repair the genetic mutation in GCD using  
45 CRISPR/Cas9, we used human cultured corneal keratocytes derived from an R124H GCD2  
46 patient as a model system. The TGFBI R124H mutant keratocytes have a monoallelic point  
47 mutation at Arg124 (GCA→ACA) in Exon 4 of *TGFBI* (Figure 1a). To repair mutant R124H  
48 cells, we designed an R124H mutation-specific gRNA based on a public algorithm (Figure  
49 1b). Then, the designed gRNAs were computationally evaluated for potential off-target  
50 effects using the E-CRISP algorithm. The gRNA with the lowest off-target risk was selected  
51 for subsequent analyses.

52 For the HDR repair template, we synthesized a 100-nucleotide (nt) donor repair template  
53 ssODN with a novel BsiWI restriction site (Figure 1b). The substitutions ensured that the  
54 sequence of the wild-type donor template was resistant to CRISPR/Cas9 cleavage by the  
55 R124H mutation-specific gRNA, and the BsiWI restriction site allowed the tracking of HDR  
56 by restriction fragment length polymorphism (RFLP) (Figure 1b). A pair of annealed oligos  
57 encoding a target sequence of R124H mutation-specific gRNA was cloned into the px458

58 vector, which enabled bicistronic expression of *Streptococcus pyogenes* Cas9 (spCas9) and  
59 green fluorescence protein (GFP) (Figure 1c).

60

#### 61 *CRISPR/Cas9-mediated HDR of an R124H mutation in human corneal keratinocytes*

62 The CRISPR plasmid expressing spCas9/gRNA was co-transfected into primary R124H  
63 mutant human corneal keratinocytes with the ssODN as a donor template. After 7 days,  
64 single GFP-expressing cells were harvested, added to individual wells of a 96-well plate, and  
65 clonally expanded. Then, the presence of a novel BsiWI restriction site was examined by  
66 RFLP-based genotyping. Genomic PCR products for wild-type alleles were not cleaved by  
67 BsiWI (Figure 2a). However, genomic PCR products for several transfected colonies were  
68 cleaved by BsiWI, suggesting target site alterations by HDR (Figure 2a). We also confirmed  
69 the genomic sequences of the PCR products (Figure 2b).

70 The sequence of wild-type cells had CGC, specifying arginine, at the 124<sup>th</sup> amino acid,  
71 and R124H mutant cells had CAC at this position. Neither is expected to be cleaved by  
72 BsiWI; however, gene-edited cells have CGT, which is expected to be cut at CGTAC. In an  
73 RFLP assay, we detected cells with heterozygous and homozygous editing, as shown in  
74 Figure 2c.

75

#### 76 *Efficiency of Cas9-mediated genome editing of the TGFBI R124H mutant gene*

77 To examine the editing efficiency of the R124H mutant *TGFBI* gene, genomic  
78 DNA was extracted from the clonally expanded cells in 96-well plates and examined by  
79 RFLP-based genotyping. Owing to the low growth rate and viability of flow cytometry-sorted  
80 primary keratinocytes, not all cells were sufficiently expanded by single-cell cloning in  
81 96-well plates. Cell growth and gene editing efficiency are summarised in Figure 2c.  
82 Thirty-eight out of 192 clones were sufficiently expanded and examined by RFLP. Among all  
83 examined clones, 20.6% exhibited monoallelic *TGFBI* correction and 41.3% showed biallelic  
84 correction. Accordingly, 62% showed clear *TGFBI* R124H allele correction derived from the  
85 HDR template.

86

#### 87 *Analysis of off-target cleavage by R124H mutation-specific gRNA*

88 To evaluate off-target effects mediated by the gRNA, a T7 endonuclease (T7EN1)  
89 cleavage assay was used to assess off-target cleavage. Since we rigorously designed and  
90 selected a *TGFBI*-specific gRNA to reduce the risk of off-target effects, only 3 potential  
91 off-target sites were found for the gRNA (Figure 3a, 3b). We could not find any potential  
92 off-target sites (OTS) with mismatches of less than 3 nt. The 3 potential OTS had  
93 mismatches of more than 4 nt with the *TGFBI* gRNA (Figure 3b). In the T7EN1 cleavage  
94 assay, we did not detect any off-target effects at the 3 OTS (Figure 3c).

95

96 **Discussion**

97 Current therapeutic modalities for GCD, i.e. PTK and keratoplasty, are invasive and are  
98 associated with frequent recurrence. The correction of *TGFBI* mutations in the local cornea  
99 may be a radical treatment for GCD patients, minimizing progression and the recurrence of  
100 corneal opacities. In this study, we successfully repaired point mutations in R124H mutant  
101 cells using CRISPR/Cas9 and HDR *in vitro*, without detectable off-target effects.

102 The CRISPR/Cas 9 system is an efficient tool for genome engineering and disease  
103 treatment. Kaminski et al. successfully eliminated HIV genomes in human T cells *ex vivo*<sup>19</sup>;  
104 they reported a low editing efficiency in primary culture, even using a lentivirus delivery  
105 system<sup>19</sup>. Similarly, the low transfection and growth efficiency in this study (Figure 2c) may  
106 be attributed to the use of primary culture cells. However, in general, plasmid transfection  
107 may be safer than viral transfection *in vivo*. Fortunately, despite the low growth rate, our  
108 results reveal that the efficiency of CRISPR/Cas9 in gene correction was higher compared  
109 with those of previous studies<sup>15</sup>. It is reported that the efficacy of HDR is generally not high,  
110 however, the efficiency of HDR using asymmetric donor DNA is much higher (maximum  
111 60%) than that of conventional HDR<sup>20</sup>. In this study, the efficacy of HDR using ssODN was  
112 greater than 60%. The reason for the high efficiency of HDR in our study is unclear, but may  
113 be explained by the unique characteristics of the DNA repair ability of corneal epithelial cells.  
114 Previously, Mallet et al.<sup>21</sup> demonstrated that DNA damage in human corneal epithelial cells



115 by ultraviolet radiation could be repaired faster than that in epidermal keratinocytes. This  
116 suggests that there are corneal-specific mechanisms in DSB repair. This issue should be  
117 evaluated in future studies.

118 CRISPR/Cas9 itself has some probability of causing off-target mutations<sup>22,23</sup>.  
119 CRISPR RNA-guide endonucleases tolerate single and double mismatches in their  
120 sequences at the gRNA interface in bacterial cells<sup>22</sup> and human cells<sup>23</sup>. Wu et al.<sup>24</sup> reported  
121 that only 2 out of 12 samples had off-target mutations when they co-injected Cas9 mRNA  
122 and a single gRNA into mouse zygotes with dominant mutations in *Crygc* that cause  
123 cataracts. Additionally, off-target mutations were detected at 1 of 10 potential OTS in the two  
124 samples. Thus, although off-target effects are an important safety issue for clinical use, they  
125 can be greatly reduced by a cautious gRNA sequence design<sup>25,26</sup>. Moreover, according to  
126 previous studies, gRNA does not cleave nonspecific targets with mismatches of 3 nt or  
127 more<sup>23,27</sup>. In our study, based on these findings, we made highly specific gRNAs using an  
128 off-target prediction tool. A T7 Endonuclease 1 cleavage assay was performed to examine  
129 off-targets effects, but the three predicted OTS were not detected in any sample (Figure 3c).

130 In ocular tissues, several reports have demonstrated successful gene editing using  
131 the CRISPR/Cas9 system<sup>28-31</sup>. Wu et al. corrected a genetic disease in mice that show  
132 early-onset cataracts using non-homologous end joining and HDR<sup>24</sup>. The gene correction  
133 was conducted at the embryonic stage and cataracts occurred in 10 out of 12 mice. Wang et

134 al.<sup>32</sup> and Bakondi et al.<sup>30</sup> successfully edited retinal genes by electroporation, and Hung et  
135 al.<sup>28</sup> also successfully edited retinal genes using a virus delivery system. In the cornea,  
136 Courtney et al. reported the effectiveness of DNA cleavage by CRISPR/Cas9 for the  
137 treatment of cornea dystrophy caused by a KRT12 mutation<sup>29</sup>. To our knowledge, this study  
138 is the first to demonstrate *in vitro* gene correction in mutant human primary corneal cells  
139 using CRISPR/Cas9 and HDR. The cornea is an excellent tissue for the application of  
140 genome editing therapy owing to its accessibility and high amenability to naked plasmid  
141 DNA transfection via intrastromal injection<sup>33</sup>. Thus, gene editing is a radical GCD treatment  
142 and the *in vivo* application of this system is ideal for clinical settings in which conventional  
143 treatments are limited. In the future, it is necessary to develop safer and more efficient  
144 methods to modify local corneal genes *in vivo*.

145 In conclusion, we used CRISPR-Cas9-mediated HDR to correct the R124H mutation.  
146 Our data suggest that the approach is highly specific, with no observed off-target effects.  
147 Given the lack of effective treatment options for GCD2, this gene editing system is a  
148 potentially radical treatment for TGFBI-related corneal dystrophy and can be used to protect  
149 corneal opacities. The *in vivo* application of this system is an important future challenge.

150

151

152 **Methods**

153 **Cell culture**

154 Primary human corneal keratocytes of a GCD2 patient with a heterozygous TGFBI  
155 mutation (R124H) were isolated from a surgical specimen during deep anterior lamellar  
156 keratoplasty. Ethics approval for this work was obtained from the Institutional Review Board  
157 of the Inoue Eye Hospital and informed consent was obtained from the patient. All tissues  
158 were provided from Inoue Eye Hospital and no tissues were procured from prisoners. All the  
159 experimental methods were carried out in accordance with the guidelines verified and  
160 approved by the Ethics Committee of The University of Tokyo.

161 The cell culture method was described previously<sup>34-36</sup>. Briefly, the corneal epithelium  
162 was removed from the stroma of the surgical specimen by scraping with a razor blade. A  
163 stromal button was incubated overnight at 37°C in basal medium, i.e. DMEM/F12 medium  
164 supplemented with B27 (Invitrogen, Carlsbad, CA, USA) containing 0.02% collagenase  
165 (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the digested tissue and cells were  
166 dispersed by pipetting and centrifuged at 800 × g for 5 min. After removing the supernatant,  
167 the keratocytes were resuspended in 1.0 mL of basal culture medium and seeded in culture  
168 dishes. The medium was changed every 2 days until the cells reached confluence.  
169 Second-passage cells were used in the subsequent transfection and other assays.

170

171 **gRNA design and CRISPR-Cas9 construct**

172 Single gRNA targeting the R124H mutation site of the human *TGFBI* gene was  
173 designed using the CRIPSR design tool (publically available at <http://crispr.mit.edu/>,  
174 <http://www.e-crisp.org/E-CRISP/>). To construct the CRISPR-Cas9 plasmid targeting the  
175 human *TGFBI* gene, the complementary oligonucleotides hTGFBI gRNA-F and hTGFBI  
176 gRNA-R were phosphorylated using T4PNK (TAKARA, Kusatsu, Japan), annealed, and  
177 cloned into pSpCas9 BB-2A-GFP (PX458, plasmid #48138; Addgene, Cambridge, MA,  
178 USA) via the BbsI restriction sites. To utilize HDR to edit the human TGFBI R124H mutation,  
179 a 100-nt ssODN (hTGFBI R124H HDR ssODN) was designed to target the R124H mutation  
180 site.

181 The oligonucleotide sequences were as follows:

182 *hTGFBI* gRNA-F: 5'-CACCACTCAGCTGTACACGGACCACA -3',

183 *hTGFBI* gRNA-R: 5'-AAACTGTGGTCCGTGTACAGCTGAGT-3',

184 and *hTGFBI* R124H HDR ssDNA:

185 5'-GAGACCCTGGGAGTCGTTGGATCCACCACCACTCAGCTGTACACGGAC**CGTACGG**

186 AGAAGCTGAGGCCTGAGATGGAGGGGCCCGGCAGCTTCACCATCT-3'.

187

### 188 ***Transfection and cloning***

189 CRISPR-Cas9 constructs (2.5 µg per well) and ssODN (1 µg per well) were  
190 transfected into R124H primary cells using FuGENE (Promega, Madison, WI, USA)

191 according to the manufacturer's instructions and the cells were incubated for an additional  
192 48 h. Images were obtained by fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan).  
193 The cells expressing GFP were single-cell-sorted by FACS (Aria III, Becton-Dickinson,  
194 Franklin Lakes, NJ, USA) at 1 week after transfection. The sorted cells were then clonally  
195 expanded and analysed as described below.

196

### 197 ***Indel analysis by restriction fragment length polymorphism (RFLP)***

198 Total DNA was extracted from cells using the Nucleospin Kit (Takara Bio Inc.).  
199 Polymerase chain reaction (PCR) using specific primer sets (Forward:  
200 5'-GTTGAGTTCACGTAGACAGGC-3', Reverse: 5'-GACTCCCATTTCATCATGCCCA-3')  
201 was performed to amplify the DNA using the KOD FX Kit (KOD FX; Toyobo, Osaka, Japan)  
202 with the following temperature profile: 94°C for 2 min, followed by 40 cycles of 98°C for 10 s  
203 and 55°C for 30 s, and 72°C for 2 min. The PCR products were treated with the restriction  
204 enzyme BsiWI (New England Biolabs, Ipswich, MA, USA). One microgram of DNA was  
205 treated with 1 unit of enzyme and NE Buffer 2.1 at 55°C for 15 min. The samples were  
206 analysed by electrophoresis on a 5% polyacrylamide TBE gel.

207

### 208 ***DNA sequencing analysis***

209 The target site (exon 4 of *TGFBI*) was amplified by PCR with primers (Forward:

210 5'-GTTGAGTTCACGTAGACAGGC-3', Reverse: 5'-GACTCCCATTTCATCATGCCCA-3')

211 targeting the genomic DNA of R124H-edited cells. After the purification of PCR products, the

212 sequence of samples was analysed using a contract genome sequencing service.

213 (Eurofins Genomics Inc., Tokyo, Japan).

214

### 215 **T7 Endonuclease I cleavage assay**

216 The genome editing efficiency was investigated using a T7 endonuclease I

217 cleavage assay. Genomic regions surrounding the target sites and potential off-target sites

218 of gRNAs were amplified by PCR using Takara ExTaq (Takara Bio Inc.). Two hundred

219 nanograms of gel-purified PCR products was re-suspended in NEB Buffer 2, and a

220 hybridization reaction was performed using a thermocycler (BioRad, Hercules, CA, USA)

221 with the following settings: 95°C for 5 s, 95–85°C at –2°C/s, 85°C for 30 s, 85–25°C at

222 –0.1°C/s, 25°C for 30 s, followed by maintenance at 4°C. Five units of T7 endonuclease I

223 (New England Biolabs) were added to digest the re-annealed DNA. After 2 h of incubation at

224 37°C, DNA products were loaded on a 2% agarose gel and visualised after staining with

225 ethidium bromide. Primers are listed in Table 1.

226

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- 302

### 303 **Author Contribution Statement**

304 YT and KK did experiments and prepared all figures. YT also wrote the initial draft of the



305 manuscript. TS prepared human tissue. MK and TT supported experiments. SY, SA, MK and  
306 TM contributed to data collection and interpretation, and critically reviewed the manuscript.  
307 TU and YO designed the study, and wrote the manuscript as corresponding authors. The  
308 final version of the manuscript was approved by all authors.

309

### 310 **Acknowledgements**

311 This work was supported by a grant-in-aid for Scientific Research from the Japanese  
312 Ministry of Education, Culture, Sports, Science and Technology. We would like to thank  
313 Azusa Nakamura, assistant researcher, for her assistance with the experiments.

314

315

### 316 **Additional Information**

317 **Competing financial interests:** Dr. Tomohiko Usui has had financial support from Santen  
318 Pharmaceutical Co., Ltd., Senju Pharmaceutical Co., Ltd., and Alcon, Inc. The other authors  
319 declare no competing financial interests.

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324

### 325 **Figure Legends**

326

### 327 **Figure 1**

328 **Gene targeting strategy for CRISPR/Cas9-mediated HDR of a TGFBI R124H mutation.**

329 **(a)** Schematic diagram of the *TGFBI* mutation in GCD2 in humans. **(b)** In GCD2, the 124<sup>th</sup>  
330 protein position is histidine (H), instead of arginine (R). The recognition sight of donor  
331 single-strand DNA is also shown. **(c)** Linear structure of the plasmid transfected into R124H  
332 mutant cells. The plasmid (px 458) includes guide RNA targeting R124H mutant cells, Cas 9  
333 protein sequences, and EGFP. TGFBI, transforming growth factor  $\beta$ -induced; GCD2,  
334 granular corneal dystrophy; HDR, homology-directed repair.

335

336 **Figure 2**

337 **Correction of the mutation in TGFBI R124H mutant keratocytes using**

338 **CRISPR-mediated HDR.**

339 **(a)** Result of an RFLP analysis of edited R124H cells. *TGFBI* exon 4 was amplified by PCR,  
340 and the products were treated with the BsiWI restriction enzyme. The lane with three bands  
341 was edited heterozygously and the lane with two bands was edited homozygously. **(b)** DNA  
342 sequences of PCR products amplified from the *TGFBI* gene of wild-type cells, a  
343 heterogeneous R124H mutant, and a repaired allele by HDR after transfection of Cas9 guide  
344 RNA and ssDNA. Two peaks were observed in the sequence of the R124H heterogeneous  
345 mutant, while the base of HDR-repaired cells was corrected to T. **(c)** Editing efficiency of

346 CRISPR/Cas9-mediated HDR of an R124H mutation. RFLP, restriction fragment length  
347 polymorphism TGFBI, transforming growth factor  $\beta$ -induced; HDR, homology-directed  
348 repair.

349

350 **Figure 3**

351 **Off-target sites and T7 endonuclease cleavage assay of potential off-target loci.**

352 **(a)** Ranked list of potential off-target loci for R124H mutation-specific gRNA. Number of  
353 mismatched bases, PAM sequence, chromosomes, and target gene are indicated. **(b)**

354 Sequence alignments of R124H mutation-specific gRNA and potential off-target loci. **(c)**

355 Potential off-target sites in edited cells were amplified by PCR. After T7 endonuclease  
356 treatment, no off-targets effects were found at any site.

357 N/A: not applicable

358

359 **Table 1 Primer set used for the T7 endonuclease cleavage assay of potential**  
360 **off-target loci.**

Column1	Forward	Reverse
#1	5'-ATGTCAGAAGTCCCGCTGTG-3'	5'-TGATGGGGTCAGAGGGCATA-3'
#2	5'-GCAGCAAAGCACTCAAGAGG-3'	5'-CAAACCTTCTGCCTGGGCATC-3'
#3	5'-CTTCCTGCTCTGTGTTTAGCCA-3'	5'-ACCTCCAAGTTGAGCAGTGTC -3'

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