Efficient generation of FVII gene knockout mice using CRISPR/Cas9 nuclease and truncated guided RNAs

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We investigated the effects of 5′-end truncated CRISPR RNA-guided Cas9 nuclease (tru-RGN, 17/18 nucleotides) on genome editing capability in NIH/3T3 cells, and its efficiencies on generating Factor VII (FVII) gene-knockout (KO) mice. In cultured cells, RGNs on-target editing activity had been varied when gRNAs was truncated, higher at Site Two (tF7–2 vs. F7–2, 49.5 vs. 30.1%) while lower in other two sites (Site One, tF7–1 vs.F7–1, 12.1 vs. 23.6%; Site Three, tF7–3 vs.F7–3, 7.7 vs.10.9%) (P < 0.05). Out of 15 predicted off–target sites, tru-RGNs showed significantly decreased frequencies at 5 sites. By microinjecting tru-RGN RNAs into zygotes, FVII KO mice were generated with higher efficiency at Site Two (80.1 vs. 35.8%) and Site One (55.0 vs 3.7%) (P < 0.05), but not at Site three (39.4 vs 27.8%) (P > 0.05) when compared with standard RGN controls. Knockout FVII mice demonstrated a delayed prothrombin time and decreased plasma FVII expression. Our study first demonstrates that truncated gRNAs to 18 complementary nucleotides and Cas9 nucleases, can effectively generate FVII gene KO mice with a significantly higher efficiency in a site-dependent manner. In addition, the off-target frequency was much lower in KO mice than in cell lines via RGN expression vector-mediated genome editing.

Clustered, regularly interspaced, short palindromic repeat (CRISPR) RNA-guided nucleases (RGNs) can robustly induce genome editing 1–3. Repair of RGN-induced double-stranded breaks by nonhomologous end-joining or homology repair, introduces insertion or deletion mutations (indels) or specific sequence alterations 1. The Streptococcus pyogenes Cas9 nuclease (Cas9) cleaves the intervening spacer sequence directed by a single guide RNA (gRNA) with a 20 nucleotides (nt) target complementarity region, at its 5′ end. However, the frequency and magnitude of undesired off-target mutagenesis can be induced at the sites of sequence, similar to the on-target site2,5.

Several strategies to improve specificity of the Cas9 system have been reported, such as the paired Cas9 nickase approach, in which two gRNAs target adjacent sites on opposite DNA strands, and each recruit a Cas9 nickase that nicks DNA instead of cutting both strands. This method can reduce off-target modifications at sites induced by single gRNA-guided Cas96–8. Nevertheless, off-target mutations are still observed, and an additional gRNA could introduce new potential sites of off-target mutations. Each single gRNA can independently nick DNA at off-target sites, causing unwanted genome-wide mutations. The dimeric CRISPR RNA-guided FokI nuclease (RFN) strategy can reduce off-target mutations to undetectable levels, but it also reduces indel frequencies on targets9–11. Paired Cas9 nickase and RFNs need to be designed appropriately and oriented with their paired gRNAs, which may differ throughout the target gene, and present technical challenges in multiplex applications12.

Recent reports demonstrated that truncated gRNAs (tru-gRNAs) improved Cas9 nuclease specificity in U2OS, GFP and FT-HEK293 cells, by shortening the gRNAs to 17/18nt12,13. They found that 5′-end nucleotides are not required for standard gRNA (std-gRNAs, 20nt) activity, and compensate for mismatches at unwanted positions along with the gRNA target DNA interface, as shorter gRNAs are more sensitive to mismatches and therefore exhibit higher specificity. This hypothesis is proven in cultured cells, and it is not sure whether tru-RGNs can

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result in a high efficiency in generating gene knockout (KO) in animal models. Here, we investigated the activity and specificity of tru-RGNs in inducing coagulation factor VII (FVII) gene mutations in murine cells and in generating KO mice by zygote RNA microinjection.

**Results**

**Tru-RGNs induce genome editing in murine cells.** Tru-RGNs (Site One, tF7–1, 18 nt; Site Two, tF7–2, 18 nt; and Site Three, tF7–3, 17 nt) target three different sites of at the exon 2 of FVII gene (Supplementary Fig. S1A,B), and corresponding std-RGNs (F7–1, 43–63; F7–2, 46–66; and F7–3, 67–87, all 20 nt, in the exon 2) (Table 1) expression vectors were constructed as controls. Colony efficiency was determined as 0.43–0.53% among three tru-RGN and three std-RGN plasmids (P > 0.05) (Supplementary Fig. S1C). To determine efficiency and specificity of RGN-mediated genome mutations, tru-RGN and std-RGN plasmids were transfected into murine NIH/3T3 cells, and on-target mutations in the FVII gene were determined by the T7EI assay (Fig. 1A) and confirmed by sequencing. Genomic mutations were detected in cell population transfected with different RGN plasmids (Table 1). Tru-RGNs of Site Two tF7–2 yielded the highest editing frequencies in all vectors, and significantly higher than std-RGNs of F7–2 (49.5 vs. 30.1%). However, both Tru-RGNs of Site One tF7–1 (12.1 vs. 23.6%) and Site Three tF7–3 (7.7 vs. 10.9%) exhibited reduced editing activities compared to their corresponding std-RGNs, respectively (P < 0.05) (Table 1).

**Tru-RGNs significantly induce genome editing in mice in a site-dependent manner.** To determine the editing efficiency of tru-RGNs in generating gene-modified mice, tru-gRNAs and Cas9 mRNA were co-injected into pronuclear stage zygotes to produce FVII KO mice. Std-gRNAs (50 ng/μL) and Cas9 mRNA (100 ng/μL) were injected into zygotes as controls. Ninety-eight, 75, and 120 embryos were injected, and 38, 20, and 20 newborns were born from the tru-RGN groups (tF7–1, tF7–2, and tF7–3, respectively). From the std-RGN groups (F7–1, F7–2, and F7–3), 78, 110, and 80 embryos were injected, and 18, 24, and 6 newborns were obtained, respectively (Table 2). Mutagenesis of the newborns was detected by T7EI assays (Fig. 2A) and confirmed by sequencing. In order to distinguish the monoallelic and biallelic mutations, T7EI assays were implemented by mixing equal amount of PCR amplicons over newborns and wild type mice. The results of generating KO mice clearly indicated the site specific and dependent mutations in newborns mediated by tru-RGNs compared to

| ID gRNAs | Target sites (5′-3′)* | Length (nt) | Sites in exon 2 | Mutation frequency (%) Mean ± SEM |
|----------|------------------------|-------------|-----------------|----------------------------------|
| F7–1     | AAGGCGTGCCAACTCCTCC TGG 20 43–63 | 23.6 ± 0.5a |
| tF7–1    | GGCGTGCCAACTCCTCC TGG 18 45–63 | 12.1 ± 0.2b |
| F7–2     | GCCTGCCAACTCCTCC TGG AGG 20 46–66 | 30.1 ± 0.9a |
| tF7–2    | GTGCCAACTCCTCCTGG AGG 18 48–66 | 49.5 ± 1.0b |
| F7–3     | GAGCGTTGGCCGGCTCTCCTCC TGG 20 67–87 | 10.9 ± 1.3a |
| tF7–3    | GCTTTGGCCGGCTCTCCTCC TGG 17 70–87 | 7.7 ± 0.9b |

Table 1. Mutation frequencies of the FVII gene in transfected cells induced by tru-RGNs and std-RGNs.

*a,b values with the different numbers within the same column showed significant differences (P < 0.05). The statistical comparison was performed in pair between std-RGNs and tru-RGNs, that was F7–1 vs. tF7–1, F7–2 vs. tF7–2, F7–3 vs. tF7–3 (n = 3). *Either TGG or AGG at 3′-end of each gRNA was shown as protospacer adjacent motif (PAM) necessary for gRNA recognition. The target sites were counted from the first nucleotide of exon 2 in FVII (GenBank Accession No. U66079, as shown in Fig. 1S A).
Results show that RGNs editing activity varied to a certain extent when 5'-end complementary nucleotides of gRNAs were truncated, with an increase at one target site (Site Two) and a decrease at other sites (Site One and Site Three). The indel mutations mediated by tF7–1 tru-RGNs was much higher when compared to std-RGNs (55.0 vs. 3.7%, P < 0.05). Similarly at Site Two, tF7–2 tru-RGNs induced the highest percentage of indel mutations (80.1%) in all three target sites, which is significantly higher than its F7–2 std-RGNs controls (35.8%) (P < 0.05). At Site Three, both F7–3 tru-RGNs and F7–3 std-RGNs induced similar percentage of mutations (39.4 vs. 27.8%, P > 0.05). In tru-RGNs groups of tF7–1, tF7–2, and tF7–3 KO mice, 1, 15 and 8 mice contained monoallelic mutations, whereas 18, 0 and 0 mice carried biallelic mutations, respectively. The std-RGNs groups of F7–1, F7–2, and F7–3 resulted in 1, 2 and 2 mice with monoallelic mutations, and 0, 6 and 0 mice with biallelic mutations, respectively (Table 2).

We further analyzed and confirmed the modified target sites by DNA sequencing and found that the mutations mainly included deletions, insertions, nucleotide transition and transversion (Fig. 3).

**Off-target mutagenesis induced by tru-RGNs in transfected cells and mutant mice.** Potential off-target sites were predicted using the MIT Design Tool. Five off-target sites with the highest homology and affinity to each tr-uRNA at each site were subjected to mutation analysis with T7EI assays. In NIH/3T3 cells, off-target mutation frequencies induced by tru-RGNs at most predicted sites had been decreased in a different degree compared to std-RGNs (Table 3). As a result, at a total of 15 predicted off-target sites, the editing frequencies at 5 sites were decreased significantly (5/15, OT1–5, OT2–1, OT2–5, OT3–4 and OT3–5) (P < 0.05) while off-target frequencies decreased but without statistical differences at 4 sites (4/15, OT1–1, OT1–2, OT1–4 and OT3–3) (Fig. 1B). There were 5 sites without off-target mutations (5/15, OT1–3, OT2–2, OT2–3, OT2–4 and OT3–1). However, only one off-target mutation at OT3–2 site mediated by tru-RGNs of tF7–3 had a significantly increased frequency when compared to std-RGNs control (22.0 vs. 3.7%, P < 0.05) (Table 3). Six mice generated with tru-RGNs of tF7–3 carried off-target mutations at one predicted site (OT3–2, Fig. 2B; Supplementary Table S2), with its off-target ratio of 29.3% in mutant mice versus newborns (n = 20). In other sites, both tru-RGNs and std-RGNs did not generate off-target mutations in FVII KO mice (Table 3).

**Phenotype changes in tru-RGN-mutagenized mice.** Mice in tru-RGN group with biallelic mutations died in few days after birth (Table 2), while mice with monoallelic mutations were all alive but suffering deformation in limb in various degree of severity. Similar symptoms of limb deformation and newborn death were observed in std-RGNs groups. It was reported that homozygous FVII+/− neonates which were generated by homologous recombination, suffered of fatal-abdominal bleeding, and all died within 24 d30. In tru-RGN-modified mice (H68, H69, H78 and O68), the plasma phenotype of prothrombin time (PT) was significantly prolonged compared to WT mice (P < 0.05) (Fig. 2C). The FVII protein expression in plasma of these four mice was further determined by Western blot, with its level decreased to about 1/2 in wild type plasma in all PT-prolonged KO mice (Fig. 2D). Deducd amino acid sequences of mutant locus from these heterozygous FVII+/− mice (H68, H69, H78 and O68), indicated the frame shift of translation at the sites of mutations, and all showed the pre-mature termination of protein translation (Supplementary Table S1). We further mated H68, H69, H78 and O68 with wild-type mice (FVII−/−), Our study has successfully demonstrated, for the first time, that gene KO mice can be generated using tru-RGNs microinjection to fertilized eggs, at the highest efficiency of 80.1%. Fu et al.15 reported that the truncated guide RNAs significantly improves CRISPR-Cas9 nuclease specificity in human cell lines. It was also reported that Std-RGNs can mediate genome modification in one-celled embryos and generate KO mice14–17. In our study, we found that the ratios of KO mice in newborns using tru-gRNAs, at Site One and Site Two are significantly higher than std-gRNAs, which reveals that tru-gRNAs are more efficient in generating gene KO mice by RNA embryo microinjection.

We have compared the genome editing frequencies of tru-RGNs and std-RGNs in mouse NIH/3T3 cells. Results show that RGNs editing activity varied to a certain extent when 5'-end complementary nucleotides of gRNAs were truncated, with an increase at one target site (Site Two) and a decrease at other sites (Site One and

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**Table 2. Generating FVII KO mice with gRNA and Cas9 mRNA co-injection.**

| ID gRNAs | No. injected embryos | No. embryos transferred | No. of Replicates | No. recipients | No. newborns | Total No. mutant mice | No. with one allele (Alive) | No. with two alleles (Deceased) | Mutant mice / total mice tested (%) (Mean ± SEM) |
|----------|----------------------|-------------------------|------------------|----------------|-------------|----------------------|-----------------------------|---------------------------------|---------------------------------|
| F7–1     | 78                   | 71                      | 3                | 3              | 18          | 1                    | 1 (1)                       | 0                               | 3.7 ± 3.7a                      |
| tF7–1    | 98                   | 98                      | 3                | 3              | 38          | 19                   | 1 (1)                       | 18 (18)                        | 55.0 ± 5.0b                     |
| F7–2     | 110                  | 106                     | 3                | 4              | 24          | 8                    | 2 (2)                       | 6 (6)                           | 35.8 ± 5.8a                     |
| tF7–2    | 75                   | 67                      | 3                | 3              | 20          | 15                   | 15 (15)                     | 0                               | 80.1 ± 7.4c                     |
| F7–3     | 80                   | 77                      | 3                | 3              | 6           | 2                    | 2 (2)                       | 0                               | 27.8 ± 14.7c                    |
| tF7–3    | 120                  | 115                     | 3                | 4              | 20          | 8                    | 8 (8)                       | 0                               | 39.4 ± 6.1c                     |

std-RGNs controls. At Site One, the percentage of mice which carried FVII indel mutations mediated by tF7–1 tru-RGNs was much higher when compared to std-RGNs F7–1 (55.0 vs. 3.7%, P < 0.05). Similarly at Site Two, tF7–2 tru-RGNs induced the highest percentage of indel mutations (80.1%) in all three target sites, which is significantly higher than its F7–2 std-RGNs controls (35.8%) (P < 0.05). At Site Three, both F7–3 tru-RGNs and F7–3 std-RGNs induced similar percentage of mutations (39.4 vs. 27.8%, P > 0.05). In tru-RGNs groups of tF7–1, tF7–2, and tF7–3 KO mice, 1, 15 and 8 mice contained monoallelic mutations, whereas 18, 0 and 0 mice carried biallelic mutations, respectively. The std-RGNs groups of F7–1, F7–2, and F7–3 resulted in 1, 2 and 2 mice with monoallelic mutations, and 0, 6 and 0 mice with biallelic mutations, respectively (Table 2).

We further analyzed and confirmed the modified target sites by DNA sequencing and found that the mutations mainly included deletions, insertions, nucleotide transition and transversion (Fig. 3).
Site Three). These results support previous findings in human cells\textsuperscript{13,18}. The genome mutation frequencies induced by RGNs are site-dependent, when using both tru-gRNAs and std-gRNAs. In most cases, the ratio of off-target mutations mediated by tru-RGNs in cell lines were decreased, but varied in a certain degree, with a significant decrease at 5 sites. This conforms that magnitudes of sensitivity to mismatches are site-dependent. Fu et al.\textsuperscript{13} reported that tru-gRNAs can dramatically decrease undesired mutagenesis at off-target sites in human U2OS, EGFP and FT-HEK293 cells, dependent upon cell lines and targeting sites. They also suggested that lengthening the 5′ end of gRNA reduces on-target editing efficiency, as 5′ nucleotides might not be necessary for full gRNA activity. Thus, shortening the gRNA can potentially decrease off-target effects 5000-fold or more in human cells\textsuperscript{13}. A subsequent study further showed that both the number of off-target sites and mutation frequencies are decreased using tru-RGNs as detected by GUIDE-Seq\textsuperscript{19}. In contrast, we did not observe such large magnitude of editing improvement in mouse NIH/3T3 at three designated sites when gRNA complementary nucleotides are

**Figure 2. Analysis of genomic mutations in mice.** (A) PCR amplicons of target sites in murine FVII were subjected to T7E I assays. The amplicons from mutant mice (a) and from mutant mice mixed with wild type (WT) amplicons (b, 1:1) were digested to identify biallelic mutations. Amplicons from WT mice served as negative controls (c). (B) Amplicons of off-target site (OT3–2) from FVII mutant mouse were subjected to T7E I digestion (a). The amplicons were mixed with WT amplicons (1:1) and digested to identify biallelic mutations (b). Amplicons from WT mice were served as negative controls. The target and off-target sites were subsequently confirmed by either PCR-sequencing or TA cloning-sequencing. (C) Detection of plasma prothrombin time (PT) in FVII mutant mice. a, b values with the different numbers within the same table column were indicative of significant differences (P < 0.05). Plasma from four WT mice served as controls. (D) Western blot analysis of FVII expression in plasma of mutant mice. Total plasma proteins were separated by SDS-PAGE, and FVII expression was detected by Western blot. The samples H78, H69, H68, and O68 showed a decreased FVII expression to an extent lower than its 1/2 WT level. FVII proteins in Western blot were indicated out by arrow. (E) Founder mice (H78, H69, H68, and O68) were breed by mating with wild-type mice, and the rates of germline transmission were recorded. a values within the same column showed no significant differences (P > 0.05).
truncated from 20 nt to 17 or 18 nt. Off-target mutations are screened out in the same predicated off-target sites, while at 9 out of 15 off-target sites, the respective mutation frequencies are not significantly different between tru-RGNs and std-RGNs. Moreover, off-target mutation frequency at one off-target site (OT3–2) is significantly increased by tru-gRNAs instead of std-gRNAs (22.0 vs. 3.7%). Similar results are observed in other previous studies in which increased off-target mutation frequencies resulted from tru-RGN use (tru-RGNs: 6.88% vs. std-RGNs: 3.88%) in U2OS.EGFP cells13.

Moreno-Mateos et al.20 found that the most efficient alternatives to canonical gRNAs are gRNAs shorter at the 5′ end by 1–2 nt or gRNAs of canonical length but with one mismatch in the 5′ GG region. The gene editing activity of alternative gRNAs has decreased according to GG sequence variants in order. We have designed truncated gRNAs with two or three nucleotides deleted, at Site Three where tru-gRNA is 17 nt, where target mutation efficiency on cells is significantly decreased, and there is no increase of mutation efficiency in newborn mice. This implies that truncation of gRNA to 17 nt might be considered the limit for gRNA activity. Off target mutations in FVII mutant mice are observed in 17 nt gRNA (OT3–2) at 29.3% (Supplementary Table S2). It was reported that shortening canonical gRNAs at 5′- by 4 nt to 16 nt tru-gRNA, has significantly reduced on targeting efficiency13.

At Site One and Site Two where gRNAs are truncated to 18 nt, and GC ratio in gRNAs were 66.7% (tF7–1) and 61.1% (tF7–2), the on target mutation efficiencies of cells and animals are different. Compared with std-gRNA, tF7–1 gRNA has induced a decreased mutations in cultured cells, but has significantly increased the efficiency.

Figure 3. DNA sequences of FVII gene mutations induced by RGNs in mice. The guide RNA sequences were labeled as underlines in wildtype. Mutations of nucleotide (nt) transition, transversion, and insertion were marked in grey highlighted, nucleotide deletions were shown as “−”.

| Founder | Target sequences (5′–3′) | Indels |
|---------|--------------------------|--------|
| RGNs F7–1 | | |
| WT | AAGGCGTGCACCTTGGAGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | |
| 068 | AAGGCG--------------------TTGAGGAGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ25 nt |
| RGNs tf7–1 | | |
| WT | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | |
| I18 | TTGGCTGCTGTTTGGAGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | C-T, C-G |
| RGNs F7–2 | | |
| WT | CTTGACGAGGAGGAGTTGGGCGTCTGAGGAAGAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | |
| I31 | CTTGACGAGGAGGAGTTGGGCGTCTGAGGAAGAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | C-T |
| I34 | CTTGACGAGGAGGAGTTGGGCGTCTGAGGAAGAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | C-T, T-G |
| RGNs tf7–2 | | |
| WT | AAGGCGTGCACCTTGGAGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | |
| H66 | AAGGCG--------------------TTGAGGAGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ30 nt |
| H67 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ33 nt |
| H68 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ7 nt |
| H69 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ16 nt |
| H71 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ25 nt |
| H72 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ27 nt |
| H73 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ2 nt |
| H74 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | +1 nt, C-T |
| H75 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ9 nt |
| H76 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | +13 nt |
| H78 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ17 nt |
| H79 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ26 nt |
| H82 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ36 nt |
| H83 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ11 nt |
| H84 | AAGGCGTGCACCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ18 nt |
| RGNs F7–3 | | |
| WT | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | |
| I66 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ24 nt |
| I68 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ22 nt |
| RGNs tf7–3 | | |
| WT | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | |
| H54 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | G-C, C-G, T-A |
| H59 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | +1 nt |
| H60 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ4 nt |
| H61 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | C-T |
| H62 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ26 nt |
| H63 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ24 nt |
| H64 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ28 nt |
| H85 | CTTGAGGGAGACTTTGGCCGCTCCTGAGGAAGAGGAGTTGGGCGTCTGAGGAAGAGAGAGCTGCA | Δ23 nt |
of generating mutant mice. On the other hand, tf7–2 has increased mutations in both cells and animals. This suggests that the editing capacity of tru-RGN is site-dependent. It is recommended that, prior to generating KO animals, gRNAs should be first tested in cell lines to screen out the best construct from several designed gRNAs. This approach can save time and reduce the use of animal resource in animal studies. Although we have taken precautions in designing gRNAs, it is often necessary to remove one or more nucleotides when the gRNA-encoding oligomer is cloned into an RGN expression plasmid at BbsI or BbsI restriction sites21,22, or into a T7 promoter reading frame that requires a 5′-GG region for start of RNA transcription23.

Table 3. Comparison of off target mutation in both mouse cell lines and mice induced by tru-RGNs and std-RGNs. a,b values with the different numbers within the same row showed significant differences (P < 0.05). The frequencies of mutation at predicated off-target (OT) sites were compared separately in either cell lines or newborn mice between each tru-gRNA and std-gRNA group. At each OT site, the different nucleotides mismatch to the targeted sequence were marked in grey highlighted. The mutations were detected by T7E1 assay together with PCR-sequencing or TA cloning-sequencing.

| Target sites | Recognition sites | Mutation frequencies in cells (%) | Mutant mice / total mice tested (%) |
|--------------|-------------------|----------------------------------|-----------------------------------|
|              | std-NGs (20 nt)    | Tru-NGs (17/18 nt)               | Std-gRNA Mean ± SEM | Tru-gRNA Mean ± SEM | Std-gRNA Mean ± SEM | Tru-gRNA Mean ± SEM |
| FVII site 1  | AAGGGTGCCAATCTCAATCC TGG | GGGGGTGCCAATCTCAATCC TGG | 13.3 ± 0.8a | 13.1 ± 0.3* | 0* | 0* |
| OT1–1        | AAGATGGGCGGCGCCATCC TGG | GATGGGCGGCGCCATCC TGG | 6.6 ± 0.2a | 6.2 ± 0.3* | 0* | 0* |
| OT1–2        | AAGGGTGCCAATCTCAATCC TGG | GAGGGTGCCAATCTCAATCC TGG | 0* | 0* | 0* | 0* |
| OT1–3        | AAGGGTGCCAATCTCAATCC TGG | GAGGGTGCCAATCTCAATCC TGG | 21.4 ± 0.6a | 20.3 ± 1.0a | 0* | 0* |
| OT1–5        | AAGCCCTCCTAAATCTCAATCC CAG | GCCCTCCTAAATCTCAATCC CAG | 2.1 ± 0.4a | 0* | 0* | 0* |
| FVII site 2  | GCTGGCAAACTCACTCCTGG AGG | TGGCAAACTCACTCCTGG AGG | 4.4 ± 0.4a | 3.0 ± 0.1a | 0* | 0* |
| OT2–1        | GCAGGGCGAATCTCACTCACTC TGG AAG | CGAGGGCGAATCTCACTCACTC TGG AAG | 0* | 0* | 0* | 0* |
| OT2–2        | GCGCCACTCACTCACTCACTCACTCACTCACTC TGG AGG | CGCCACTCACTCACTCACTCACTCACTCACTC TGG AGG | 0* | 0* | 0* | 0* |
| OT2–3        | GTGCTCTACTCACTCACTCACTCACTCACTCACTC TGG GGG | GTGCTCTACTCACTCACTCACTCACTCACTCACTC TGG GGG | 0* | 0* | 0* | 0* |
| OT2–4        | CTGCTCCCAATTCACTCACTCACTCACTCACTCACTC TGG TAG | CTGCTCCCAATTCACTCACTCACTCACTCACTCACTC TGG TAG | 0* | 0* | 0* | 0* |
| OT2–5        | AGGTACCAGCTCACTCACTCACTCACTCACTCACTC TGG GGG | AGTACCAGCTCACTCACTCACTCACTCACTCACTC TGG GGG | 8.5 ± 0.5a | 6.0 ± 0.3a | 0* | 0* |
| FVII site 3  | GGGCGGGCTGGCCAGGCTCTC TGG | GCTGGCCAGGCTCTC TGG | 3.7 ± 0.5a | 22.0 ± 0.6a | 0* | 29.3 ± 7.1a |
| OT3–1        | AGGGCGGGCTGGCCAGGCTCTC TGG | AGGGCGGGCTGGCCAGGCTCTC TGG | 5.7 ± 0.4a | 5.2 ± 0.4a | 0* | 0* |
| OT3–2        | AGGGCGGGCTGGCCAGGCTCTC TGG | AGGGCGGGCTGGCCAGGCTCTC TGG | 6.2 ± 0.3a | 5.2 ± 0.2a | 0* | 0* |
| OT3–3        | ACAGTTGCGGGCTCTC TGG | AGTGGCGGGCTCTC TGG | 7.2 ± 0.1a | 5.6 ± 0.2a | 0* | 0* |

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Tru-RGNs can induce both biallelic and monoaallelic mutations in FVII gene. We found that all biallelic FVII deficient mice (FVII−−) died several days after birth, whereas mice with monoaallelic mutations (FVII+/−) were alive, although some of them suffered of limb deformation. FVII gene can be inactivated by disrupting the entire coding sequence for the mature FVII protein19-20. Rosen et al.30 reported that FVII-deficient (FVII−−) embryos developed normally, but 70% of the FVII−− newborn neonates suffered of fatal-abdominal bleeding, and the majority of remaining neonates died from intracranial hemorrhage before 24 days of age. At 9.5 day of embryonic development, FVII maternal–fetal transfer was undetectable. They suggested that the survival of embryos did not depend on cellular receptor tissue factor (TF)-FVII, responsible for initiating fibrin formation. Mice carrying FVII+/− genotype have demonstrated substantial phenotypic changes such as prolonged PT in plasma. In our study, targeted sites are located in exon 2 of FVII gene, and any mutations occurring in these sites significantly affect their gene functions, such as completely inactivating FVII expression (Supplementary Table S1). The deduced amino acid sequences are shown as pre-mature termination of translation. Our results of prolonged PT and lowered (half) adult plasma FVII levels in heterozygous FVII KO mice (FVII+/−), strongly support the hypothesis that FVII−− mice only express half amount of FVII protein compared to wild type mice (FVII+/+), and that FVII is biallelicly expressed in the plasm of wild type (WT) mice. FVII-KO mouse model can be utilized in cardiovascular disease research and pharmaceutical developments.

In conclusion, truncated gRNAs together with Cas9 mRNAs (tru-RGNs) can be effectively used to generate FVII knockout mice with a much higher efficiency than using standard RGNs, although in a site-dependent manner. RGNs editing activity can be altered to a certain extent, depending upon where truncated gRNAs to 17/18 complementary nucleotides are located at different target sites. When the geneedited KO mice are generated by RGNs microinjection into zygotes, the frequency of animals carrying off-target mutation is much lower than cells mediated by expressed RGNs and their cellular genome editing.

Methods
Animals. All protocols for animal treatment were approved by the Animal Care and Use Committees of Nanjing Normal University (NSD-2013-30). This study was also carried out in accordance with the recommendation in the Guided for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were maintained in an SPF animal facility at Nanjing Normal University and bred in IVC cages (4 mice per cage) with free access to food and water. Mice were kept under 12:12 h light: dark cycles in a room maintained at 24 ± 2°C and 50 ± 20% relative humidity. A proper anesthesia before any procedures was performed to all animals which were part of the experiments.

Construction of tru-RGN and std-RGN expression plasmids. Target sites at the second exon of the FVII gene (Supplementary Fig. S1) were analyzed by the MIT CRISPR Design Tool21. Off-target sites were identified in parallel, and gRNAs with the highest scores were chosen. Tru-gRNAs contained 17 or 18 target complementary nucleotides shortened from std-gRNAs. Oligomers encoding gRNAs were synthesized (Supplementary Table S4), annealed, and cloned into the PX459 vector (#48139, Addgene) at its BbsI site as previously described22. An artificial BbsI restriction site was generated for each vector by adding 4–5 nucleotides (Supplementary Table S3). Recombinant plasmids were sequenced and subsequently used for cell transfection.

gRNA and Cas9 mRNA in vitro transcription. For the in vitro synthesis of gRNAs, the T7 promoter was inserted upstream of gRNAs by PCR using oligomers as previously described13,14, and 5′-GG transcription start region was arbitrarily created (Supplementary Table S4). The T7-gRNAs were gel-purified for in vitro transcription. Both std-gRNAs and tru-gRNAs were transcribed in vitro with the MEGAscript ShortScript Kit (AM1354, Ambion). The PCAG-T3-hCas-pA (#48625, Addgene) plasmid was digested by NruI, and the DNA fragment encoding Cas9 was recovered by gel extraction for in vitro transcription. Cas9 mRNA was transcribed with the mMESSAGE mMACHINE® T3 Transcriptio Kit (AM1348, Ambion). All RNAs were purified with the MEGAClear Transcription Clean-Up Kit (AM1908, Ambion) and stored at −80°C until use.

Transfection of NIH/3T3 cells. Mouse embryonic fibroblast cells (NIH/3T3; ATCC, CRL-1658) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, HyClone) at 37 °C with 5% CO2. Plasmid RGN DNA at the concentration of 50 μg/μL was used to transfect cells. Cells were transfected at 70–80% confluence using electroporation (ECM2001, BTX, USA) at 300 V with 1 μs and one pulse in a 0.2-cm cuvette with electro-transfection solution (272 mM sucrose, 7 mM K2HPO4, 1 mM MgCl2). Transfected cells were cultured in medium supplemented with 2 μg/ml puromycin after replating at 24 h and addition of fresh medium after 72 h. Cells that reached 80% confluence were recovered for genomic DNA extraction, 200μl cell suspension containing 5 × 104 cells was mixed with 400μl Lysis Solution and incubate at 65°C for 5 min. The transfected cells were screened 72 h by puromycin and cultured 2 d for colony counting. Colony efficiency = No. colonies/total of transfected cells × 100%. DNA extraction was carried out using Genomic DNA Purification Kit (K0512, Thermo) according to the manufacturer’s instructions.

gRNA and Cas9 mRNA co-injection into murine zygotes and breeding. Female mice (C57BL/6), 4–6 weeks old) were used as embryo donors for superovulation. After mating, resultant fertilized embryos were collected from the oviducts and cultured in KSOM medium as previously described22,23. Microinjection was performed using an Olympus IX71 inverted microscope with the Narishige microinjection system. gRNA (50 ng/μL) and Cas9 mRNA (100 ng/μL) were mixed and injected into the cytoplasm of the zygotes with visible pronuclei in M2 medium as previously described23. The injected embryos were cultured overnight in KSOM medium at 37°C with 5% CO2. Embryos at the two-cell stage were transferred into oviducts of pseudopregnant ICR females. Tails of 3-week-old live pups were collected for extraction of genomic DNA. Approximately 1 cm long tail from
each mouse was digested with Proteinase K butter. DNA was extracted using Phenol/Chloroform and ethanol as previously described\textsuperscript{34}. Presumptive heterozygous FVII founders (FVII\textsuperscript{+/−}) were mated with wild-type mice to determine whether offspring followed the Mendelian hereditary pattern.

**PCR amplification of on-target and off-target sites.** Transfected cell and mouse tail genomic DNA were used as template with high fidelity DNA polymerase (New England BioLabs, NEB). Target or off-target regions (500–700 bp) were amplified by PCR primers (Supplementary Table S5). Most sites were amplified successfully (95 °C, 20 s; 58 °C, 20 s; 72 °C, 40 s) after 32 cycles. PCR products were analyzed by agarose gel electrophoresis to verify amplicon size and quality.

**T7E1 assays for determining mutation frequencies.** T7E1 assays were performed as previously described\textsuperscript{31}. Briefly, 500 ng purified PCR products from transfected cell DNA were denatured and annealed to form heteroduplexes by incubating at 95 °C for 10 min and cooling to 25 °C at 5 °C/min. For mouse tail genomic DNA, 250 ng PCR products were mixed with 250 ng wild-type (WT) genomic DNA PCR products and then denatured and annealed. Hybridized PCR products were digested with T7 Endonuclease I (T7EI, M0302L, NEB) for 30 min at 37 °C. Reaction products were analyzed by agarose gel electrophoresis. On/off target mutation frequencies were calculated using the following formula as previously described\textsuperscript{32}:

\[
\text{Indel} (%) = 100 \times \left(1 - \sqrt{1 - f_{\text{cut}}} \right)
\]

\[f_{\text{cut}} = \text{amount of digested PCR fragments compared to total PCR fragments.}
\]

Further confirmation of mutations was performed by either PCR sequencing or TA cloning-sequencing described below.

**Cloning and Sanger sequencing to identify sequence modifications.** Target region amplicons were cloned into a TA-cloning vector (PCR Cloning Kit, E1202S, NEB) and transformed into 	extit{E. coli} DH5α competent cells as previously described\textsuperscript{32}. Briefly, 50 ng PCR product was added into 100 μl of ice-cold chemically competent DH5α cells. The cell mixtures were incubated on ice for 10 min, heat-shocked it at 42 °C for 30 s and returned it immediately to ice for 2 min, finally plated onto on LB plate containing 100 μg/ml ampicillin and incubated at 37 °C overnight. Plasmid DNA was isolated and sequenced by commercial sequencing company (Sangon Biotech).

**Thrombin reaction in mice.** Blood from WT mice and putatively KO mice was collected by orbital venipuncture into plastic tubes containing 1/10 volume 0.129 M buffered trisodium citrate. Plasma was recovered by centrifugation at 4 °C, 2500 × g for 15 min, transferred into plastic tubes, and stored at 4 °C for immediate use or at −80 °C for later use. Coagulant activity of plasma FVII was measured by a one-stage prothrombin time (PT)-based assay\textsuperscript{35}. Fifty microliters blood plasma were incubated at 37 °C for 3 min and immediately mixed with 100 μl pre-warmed thromboplastin reagent (Prothrombin Time Assay Kit, F007, Nanjing Jiancheng BIO). The PT was then recorded.

**Western blot analysis of FVII expression in mice.** Total plasma proteins were quantified with the BCA assay kit (GK5011, Shanghai Generay Biotech). Thirty micrograms total plasma proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was carried out with FVII-specific antibody (1:1000 rabbit anti-FVII antibody, Lannuo Biotechnologies Wuxi Inc.). Primary antibodies were visualized with goat anti-rabbit IgG-HRP secondary antibody (SC-2004, Santa Cruz Biotechnology) using an Enhanced ECL detection kit. Western blot analysis was then recorded.

**Statistical analyses.** The data on genomic editing and off-target mutation frequencies induced in cells and newborn mice by RGNs, were analyzed using the SPSS software (SPSS 18.0, IBM). Percentage data in each replicate were arcsine transformed and subjected to one-way ANOVA. Means were compared by Fisher’s least significant difference test (PLAS). Statistical significance was defined as \(P < 0.05\).
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Author Contributions
L.A. and F.D conceived and designed the experiments. L.A., Y.H. and S.C. performed most experiments with assistances from X.Z., P.L. and F.Z. Molecular analyses were performed by J.L., Y.L., Y.C. and L.Y. Data analysis was by L.A., Y.H. and L.Y. L.A. G.A.P. and F.D. prepared the manuscript and made critical discussion. All authors reviewed and revised the manuscript.

Additional Information
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