Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp

Zhaomin Zhong1,2,*, Pengfei Niu1,2,*, Mingyong Wang1,2, Guodong Huang1,2, Shuhao Xu1,2, Yi Sun1,2, Xiaona Xu1,2, Yi Hou2, Xiaowen Sun3, Yi Sun1,2, Yi Hou2, Xiaowen Sun3, Yilin Yan1,2,4 & Han Wang1,2

The common carp (Cyprinus carpio) as one of the most important aquaculture fishes produces over 3 million metric tones annually, approximately 10% the annual production of the all farmed freshwater fish worldwide. However, the tetraploidy genome and long generation-time of the common carp have made its breeding and genetic studies extremely difficult. Here, TALEN and CRISPR-Cas9, two versatile genome-editing tools, are employed to target common carp bone-related genes sp7, runx2, bmp2a, spp1, opg, and muscle suppressor gene mstn. TALEN were shown to induce mutations in the target coding sites of sp7, runx2, spp1 and mstn. With CRISPR-Cas9, the two common carp sp7 genes, sp7a and sp7b, were mutated individually, all resulting in severe bone defects; while mstnba mutated fish have grown significantly more muscle cells. We also employed CRISPR-Cas9 to generate double mutant fish of sp7a;mstnba with high efficiencies in a single step. These results demonstrate that both TALEN and CRISPR-Cas9 are highly efficient tools for modifying the common carp genome, and open avenues for facilitating common carp genetic studies and breeding.
pathway to result in indel mutations in targeted genes of human cells and many species including rat, mouse, zebrafish, cow and plants as well as in numerous fields such as basic research, clinical treatment, agriculture and animal husbandry. Moreover, it appears that CRISPR-Cas9 has advantages of ease to carry out, high efficiencies and low cost over TALEN, and particularly CRISPR-Cas9 allows for mutagenizing multiple genes in the stem cells or zygotes, and generating bialleic mutants with clear phenotypes in the F0 generation for studying gene functions without crossing animals for several generations. However, both methods have not been applied in common carp to date.

Here, we have employed both TALEN and CRISPR-Cas9 techniques to modify genes involved in bone formation such as sp7, a zinc-finger-containing transcription factor expressed in osteoblasts, which can activate pre-osteoblast cells to differentiate into mature osteoblast cells and osteocytes. In addition, we have used both techniques to modify other genes involved in bone formation including runx2 (runt-related transcription factor 2), an essential transcription factor to regulate osteoblast differentiation and bone development; spp1 (secreted phosphoprotein 1), a late osteoblast specific marker involved in bone mineralization; opg (osteoprotein), a bone-protecting molecule participated in impairing the osteoclast formation; and bmp2 (encoding for bone morphogenetic protein 2), promoting expression of runx2 and sp7 and then inducing expression of spp1, osteocalcin and other osteogenic genes. We also have used both systems to modify mstn, a member of the transforming growth factor-β superfamily and a negative regulator of the skeletal muscle growth, which knockout mice displayed 2 to 3 fold increase in both myofiber size and number of muscular cells compared to the wild-type littermates. Our results showed that mstnba-CRISPR and sp7a-CRISPR mutated common carps display defects in muscle or bone. We also generated double mutants of mstnba/sp7a in common carp with high efficiencies. Together, these results demonstrate that both TALEN and CRISPR-Cas9 systems are effective genome-editing tools for common carp genetic studies and breeding.

Results

Design of TALEN and CRISPR-Cas9 target sites. We designed TALENs and CRISPR-Cas9/gRNAs targeting genes involved in bone and muscle development. In the bone formation pathway, runx2, spp1 and mstn are mid-stream genes, and sp7 is down-stream genes. In addition, opg acts to inhibit osteoclast formation; mstn is involved in muscle formation. Among the selected genes, sp7a, runx2 and mstnba were edited using both TALEN and CRISPR-Cas9 systems to examine efficiencies of these two methods.

The cDNA sequences of zebrafish orthologs in ENSEMBL or NCBI were used to interrogate the common carp genome database (http://www.carpbase.org/login.php). Common carp DNA sequences selected were then blasted against the common carp protein database (http://www.carpbase.org/login.php) to predict the exon-intron structure. In addition, the selected target sites were blasted back against the common carp genome with reciprocal top blast hit for the target regions to minimize off-target effects.

One pair of TALENs each for runx2, sp7a, mstnba and spp1a was designed. Since the spacer length is very important to mutation efficiencies, 3 pairs of the TALENs designed for runx2a, sp7a and mstnba were longer than 22 bp and 1 pair of TALENs for spp1a was shorter than 22 bp for comparison. In addition, there were restriction endonuclease sites located in the spacers of runx2 and mstnba (Supplementary Figs S1 and S2). For CRISPR-Cas9 targeting, six genes including sp7a, sp7b, mstnba, runx2, opg and bmp2ab were selected. Most of the gRNAs were designed to target the first exon of the corresponding genes, except for runx2 and sp7b, whose gRNAs target exon 3 and exon 2, respectively (Supplementary Figs S4 and S7). All the target sites include 20-bp sequence and PAM sequence (protospacer adjacent motif NGG). The targeted regions for all these genes have restriction endonucleases sites located either near the PAM sequence or on the PAM for subsequent genotyping (Supplementary Figs S4–S7).

Evaluate mutagenesis efficiencies of TALEN and CRISPR-Cas9 of common carp genes using zebrafish embryos. Phylogenetic analysis showed that common carp have two sp7 genes and four mstn genes, while zebrafish have only one sp7 gene and two mstn genes (Fig. 1A,B), consistent with tetraploidy nature of common carp genome. RT-PCR analysis showed that mstnba is expressed highly in all the tissues/organs including the maw, liver, gill, eye, heart, brain, gut, testis and muscle, while the other three mstn genes are expressed only in some of these tissues/organs (Fig. 1C–E). We therefore selected mstnba in the following experiments.

Because of the ease of zebrafish embryos to work with, we first used them to evaluate mutagenesis efficiencies of TALENs and CRISPR-Cas9/gRNAs of common carp (Fig. 2A). Specifically, we examined common carp runx2 and sp7a TALENs and common carp mstnba and sp7a CRISPR-Cas9/gRNAs in zebrafish embryos. For TALEN, 250 ng/ul capped mRNAs of each arm plus 50 ng/ul purified plasmids carrying the common carp genomic DNA fragments containing the runx2- or sp7a-targeted sites were microinjected to one-cell stage zebrafish embryos, respectively. No toxicity was observed for zebrafish embryos at these concentrations of TALENs and purified plasmids (Supplementary Table S1). The common carp DNA fragments containing runx2- or sp7a-targeted sites were PCR amplified from the microinjected zebrafish embryos at 24 hpf. Sequencing analyses revealed that TALENs induced a 6-bp insertion mutation in runx2-targeted site and a 16-bp deletion in sp7a-targeted sites (Supplementary Table S2).

For mstnba-CRISPR-Cas9, 25, 50 and 100 ng/ul gRNAs and 300 ng/ul capped Cas9 RNA plus 100 ng/ul purified common carp plasmid containing the targeted site were microinjected into one-cell zebrafish embryos. No toxicity was observed for zebrafish embryos at these concentrations of Cas9/gRNAs and purified plasmids (Supplementary Table S1). Then PCR products amplified from the microinjected zebrafish embryos at 24 hpf were digested with the Sty1 enzyme (Fig. 2A,B). ImageJ analysis revealed that mutation frequencies average approximately 19% (Fig. 2C). Sequencing analysis identified a 6-bp deletion mutation (Fig. 2D). For sp7a-CRISPR-Cas9
injected zebrafish embryos, digestion of the PCR product with the Hinfl enzyme estimated that the mutation frequency was approximately 2.5% (Supplementary Table S2). Taken together, these results demonstrate that TALEN or CRISPR-Cas9-induced common carp mutagenesis activities can be conveniently evaluated in zebrafish embryos50.

Mutagenesis efficiencies of TALEN and CRISPR-Cas9 in common carp embryos. TALEN pairs each for runx2, sp7a, mstnba and spp1a were microinjected into the one-cell common carp embryos at a concentration of 250 ng/ul per arm, respectively (Fig. 3A,B). DNA fragments harboring targeted sites were PCR amplified from approximately 20 embryos at 72 hpf from each injected group, and digested with T7E1 enzyme. ImageJ analysis revealed that mutagenesis frequencies are 15.2% for runx2, 36.8% for sp7a, 29.1% for mstnba and 81.5% for spp1a (Fig. 3C and Supplementary Table S3). Digestion of the PCR products containing the runx2-targeting site or the mstnba-targeting site with Hinfl or XbaI also estimated that mutation efficiencies are 1.23% for runx2, and 13.2% for mstnba (Supplementary Figs S1, S2 and Supplementary Table S3). Alternatively, these PCR-amplified fragments were cloned into the PMD-19T vector, respectively. DNA sequencing analysis showed that mutation efficiencies were 5% (1 out of the 20 single clones) for runx2, 20% (4 out of the 20 clones) for sp7a, 27.3% (3 out of the 11 clones) for mstnba and 75% (3 out of the 4 clones) for spp1a (Fig. 3D; Supplementary Figs S1–S3 and Supplementary Table S3), consistent with the enzymatic analyses. For TALEN-induced mutation efficiencies in common carp, the shorter the spacer lengths lead to the higher mutation efficiencies (Supplementary Table S4), as reported in other species previously49.

To examine the CRISPR-Cas9-induced mutagenesis efficiencies, Cas9 mRNAs plus the six individual gRNAs for corresponding genes sp7a, sp7b, mstnba, runx2, opga and bmp2ab were co-microinjected into one-cell common carp embryos, respectively (Fig. 2A). For sp7a-CRISPR, enzymatic digestion and sequencing analyses showed that 18 out of the 20 clones tested harbor mutations (Fig. 4A–C), which were reconfirmed with T7E1 enzyme analysis (Supplementary Table S5). For mstnba-CRISPR, the DNA fragment containing the targeted site was PCR amplified from 72 hpf embryos, and digested with the Styl enzyme. The results showed that the mutation efficiency was more than 70% (Fig. 4D,E), consistent with T7E1 enzymatic digestion and DNA sequencing analyses (Fig. 4F and Supplementary Table S5). In addition, runx2-, sp7b- and opga-CRISPR-Cas9 also produced gene mutations with efficient efficiencies more than 50%, but bmp2ab-CRISPR had relatively low mutation efficiency
of approximately 40% in carp embryos (Supplementary Figs S4–S7). The results of all targeted genes of common carp in F0 generation are shown in Supplementary Table S5.

More muscular cells in mstnba mutant common carps. To generate sp7a-CRISPR and mstnba-CRISPR targeted common carp fish, more than 200 embryos were microinjected for each group. Most of the microinjected and uninjected control fish were put into the ponds when they were 30 days old. In addition, 20 carps each for wild type, sp7a-CRISPR and mstnba-CRISPR were raised separately in the laboratory under the same condition. All the phenotypes were examined from these 20 carps for each group. PCR products amplified with DNAs extracted from caudal fins of one-month-old common carps were digested with restriction enzyme StyI for mstnba-CRISPR. Results showed mstnba-CRISPR had high mutation efficiency in the somatic cells of one-month-old carp, averaging at 56.6% for the 20 carps, similar to the sequencing results in carp embryos (Fig. 5A).
The body weight and body length of these microinjected (all the 20 carps in the group including 3# in Fig. 5A with no detectable mutations) and uninjected control common carps were measured at one mpf (month post-fertilization), two mpf and three mpf. Unsurprisingly, mstnba-CRISPR common carps grew heavier than uninjected controls in all three time points and longer than uninjected controls in 1 mpf and 3 mpf (Fig. 5B,C). Body weights (Fig. 5B) were significantly increased at 1 mpf (P < 0.03), 2 mpf (P = 0.005) and 3 mpf (P < 0.049) in mstnba-CRISPR carp compared with wild types. Body lengths (Fig. 5C) were also significantly increased in mstnba-CRISPR carp compared with wild types at 1 mpf (P = 0.011) and 3 mpf (P = 0.047). We selected 7 mstnba-CRISPR carp with more than 90% mutation efficiency as the high-rate subgroup, 7 mstnba-CRISPR carp with 10–90% mutated efficiencies as the middle-rate subgroup and 6 mstnba-CRISPR carp with less than 10% mutation efficiency as the low-rate subgroup. Body weights were significantly increased (P = 0.007) in high-rate subgroup compared with wild types, and in high-rate subgroup (P = 0.049) compared with low-rate subgroup, implicating that the body weight exhibits a phenotype-genotype correlation, i.e., the higher the mutation rate (the high-rate subgroup), the heavier the fish compared with the low-rate subgroup (Fig. 5D). We also examined the level of phosphorylated Smad2, which is an essential intracellular transducer for the TGF-β signaling pathway51, in dorsal muscles of five-month-old mstnba-CRISPR and wild-type common carps with high mutation rate by Western blotting. Results showed approximately 25% reduced phosphorylated Smad2 in mstnba-CRISPR common carps (P = 0.024). (Fig. 5E,F), implicating that CRISPR-Cas9-induced mutations
Figure 4. CRISPR-Cas9-induced mutagenesis efficiencies of common carp genes evaluated in common carp embryos. (A) Schematic of gRNA-Cas9 targeting the exon of common carp sp7a. The restriction enzyme HinfI site in the targeted fragment is underlined. The PAM sequence is highlighted in blue. E: exon. (B) Enzymatic digestion analysis of Cas9-mediated cleavage at sp7a in carp embryos digested with HinfI. The lane 1, marker; lanes 2–4, injected groups at concentrations of 100 pg, 150 pg and 200 pg of sp7a gRNA (below the blue line), respectively; lane 5, wild-type control with digestion (below the green line); lane 6, undigested wild-type control. Uncut (256 bp) and cut (185 bp and 96 bp) bands are indicated. WT, wild type; M, marker. Mutation efficiencies are 93.5% for lane 2, 99.1% for lane 3 and 92.8% for lane 4. (C) Indels in the CRISPR-Cas9 sp7a targeted site, revealed by sequencing analysis. The gRNA-targeting sequence is labeled in green. (D) Diagram of the Cas9-gRNA-targeted site in common carp mstnba. The restriction enzyme StyI site is underlined. E: exons. (E) Enzymatic digestion analysis of Cas9-mediated cleavage at mstnba in carp embryos. The mstnba-targeted fragment was PCR amplified and digested with StyI. Lane 1, marker; lanes 2–4 (below the blue line), injected groups at concentrations of 25, 50, 100 pg of mstnba gRNA, respectively; lane 5, wild-type control with digestion (below the green line); lane 6, wild-type control without digestion. Uncut (271 bp) and cut (165 bp and 96 bp) bands are indicated. WT, wild type; M, marker. Mutation efficiencies are 80.7% for lane 2, 81.9% for lane 3 and 76.8% for lane 4. (F) Indels in the CRISPR-Cas9-targeted mstnba site revealed by sequencing. Insertions are labeled in red. Electrophoretic results were cropped from the original images shown in Supplementary Fig. S13F,G.
Figure 5. More muscular cells in CRISPR-Cas9-targeted mstnba common carps. (A) Enzymatic digestion of CRISPR-Cas9-mediated cleavage at mstnba. PCR products were digested with StyI. Lane 1, marker; lanes 2–12, digestion of PCR products of 11 carps with StyI; lane 13, wild-type without digestion. Uncut (271 bp) and cut (165 bp and 95 bp) bands are indicated. Each “#” represents a single fish, and estimated mutagenesis efficiencies are indicated at the bottom. Body weight (B) and body length (C) of one-month-old, two-month-old and three-month-old mstnba-CRISPR mutant (red) and wild-type (blue) carps. Values are means ± SEM (n = 20). (D) Comparisons of body weights in different genotypes of mstnba-CRISPR F0 at 1 mpf. High-rate represents higher than 90% of mutation efficiencies in (A) middle-rate 10–90%, and low-rate 0–10%. Each black dot represents one sample. Values are means ± SD (n = 6 or 7). (E) Western blotting analysis of phosphorylated Smad2. Proteins were obtained from dorsal muscles of mstnba-CRISPR (2#, 6#, 10#) and wild-type (3 samples) carps at 5 mpf. (F) Quantification of Western blotting results with ImageJ. Values are means ± SD. n = 3. (G,H) Representative images of muscle cells of three-month-old wild-type (G) and 7#
mstnba mutant carps (H), shown by H&E staining. Scale bar: 0.1 mm. Numbers of muscle fibers (I) and the average area of the muscle fibers (K) of mstnba mutant and wild-type carps, quantified H&E staining images (see G,H) with ImageJ. Approximately 24 stained images each were quantified. (J) Distribution analysis of the fiber area. Values are means ± SEM. (L) Quantitative RT-PCR analysis of myoda, myf5a and myogenina in mstnba-CRISPR (red) and wild-type (blue) carps. RNAs were extracted from dorsal muscles of wild-type (3 samples) and mstnba-CRISPR carps (2#, 6#, 10#) at 5 mpf. Values are means ± SD. n = 3. (M) Mutations in mstnba CRISPR-Cas9 7# carp, revealed by sequencing analysis. The targeted sequence is in green, and the PAM sequences are in blue. Electrophoretic and Western blotting results were cropped from the original images shown in Supplementary Fig. S13L,L. Two-tailed Student’s t-test or one-way ANOVA with LSD were conducted, *P < 0.05, **P < 0.01 ***P < 0.001, and N.S., no significant change.

Severe bone defects in sp7a mutant common carps. PCR products amplified with DNAs extracted from caudal fins of one-month-old common carps were digested with restriction enzyme HinfI for sp7a-CRISPR. Results showed that 16 out of the 18 sp7a-CRISPR injected carp were mutated, suggesting that almost all the somatic cells of the fin were mutated in the sp7a target site of common carp (Fig. 6A).

In contrast to mstnba-CRISPR mutants, sp7a-CRISPR mutants grew lighter and smaller than un.injected wild-type controls in all three time points (P < 0.001) (Fig. 6B,C). sp7a-CRISPR common carps exhibit conspicuous bone defects including opercula and maxilla insufficiency and bending back such as 3# sp7a-CRISPR carp in Fig. 6A (Fig. 6D,E). Alizarin Red staining of 3# sp7a-CRISPR carp showed that the irregular hemal spines and deformed centrums, and shorter inter-muscular bones than uninjected wild-type control (Fig. 6F–I). Sequencing analysis of the fin-clipped DNA of 3# sp7a-CRISPR carp showed that all the seven single clones were mutated (Fig. 6J). We selected eight carps including 1# to 06#, 9# and 10# in Fig. 6A, which all showed nearly 100% mutated rate, for Alizarin Red staining. Results showed four out of the eight carps display the crinkled neural spines and deformed centrums, and three out of the eight fishes display curved hemal spines and maxilla insufficiency (Supplementary Figs S8 and S9). Micro-CT analysis showed that both sp7a-CRISPR (11#, 12# and 13#, in Fig. 6L is 11#) and sp7b-CRISPR (1#, 2#, 3#, in Fig. 6M is 1#) showed smaller bone size compared with control group. sp7a-CRISPR carps showed more obvious bone defects than sp7b-CRISPR. The craniofacial bones and centrum bones of these sp7 mutated carps develop slowly than those of control groups (Fig. 6K–M). Further, the bone volume (BV), bone surface (BS), bone volume (BV)/tissue volume (TV) and bone surface (BS)/tissue volume (TV) in centrum bones are statistically significantly reduced in sp7a-CRISPR (P < 0.05) and sp7b-CRISPR (P < 0.05) compared with wild types (Fig. 6N). The bone volume (BV) and bone surface (BS) of craniofacial bones of sp7a-CRISPR carps are also significantly reduced (P < 0.05). However, neither the bone volume (BV)/tissue volume (TV) and bone surface (BS)/tissue volume (TV) of craniofacial bones of sp7a-CRISPR carps (P > 0.2), nor the bone volume (BV), bone surface (BS), bone volume (BV)/tissue volume (TV) and bone surface (BS)/tissue volume (TV) in craniofacial bones of sp7b-CRISPR carps are significantly reduced (P > 0.1) (Fig. 6N). In addition, sp7a-CRISPR carps display small-sized and irregular-shaped scales and fewer pharyngeal teeth compared with wild types (Supplementary Figs S10 and S11). Together, these results indicate highly efficient CRISPR-Cas9 may generate bialleic mutations resulting in obvious phenotypes in F0-generation common carp.

It usually takes 3 to 4 years for common carp to grow sexually mature. To examine the possibility of germine transmission, DNAs were extracted from the testis of 5# mstnba-CRISPR carp that has nearly 100% mutation rate based upon analysis of fin-clipped DNAs. Sequencing data show 10 out of the 10 single clones (100%) were mutated (Supplementary Fig. S12), whereby implicating that these mutations are likely transmitted to the subsequent generation.

Multiplex gene-editing in a single common carp. Common carp is a tetraploidy species with 100 chromosomes and the high DNA content. The ability to modify duplicated genes or multiple genes of common carp is important for its molecular genetic studies and breeding. To address this issue, sp7a-CRISPR and mstnba-CRISPR were co-microinjected into common carp embryos. PCR products amplified with sp7a and mstnba primers and DNAs extracted from caudal fins of one-month-old injected and uninjected control carps were digested with HinfI or StyI, respectively. The results showed that 16 out of the 21 carps had both gene mutations. Mutation efficiencies was 63.4% for sp7a gene, and 60.1% for mstnba gene, and among them, eight carps had more than 90% efficiencies in these two genes (Fig. 7A,B). These results indicated that double-gene-editing are feasible in common carp with CRISPR-Cas9 in a single step.
Due to the tetraploidy genome of common carp, it is extremely difficult to obtain the homozygous mutants using the traditional methods such as ENU, TILLING, and retroviral insertion. However, using TALEN and CRISPR-Cas9 methods we have successfully targeted carp genes of interest and observed the specific phenotypes in F0 generation. We used TALEN to target four genes, \textit{sp7a}, \textit{runx2}, \textit{mstnba} and \textit{spp1a} and CRISPR-Cas9 to target six genes, \textit{sp7a}, \textit{sp7b}, \textit{runx2}, \textit{mstnba}, \textit{opga} and \textit{bmp2ab} with high efficiencies. With TALEN, we have achieved higher mutagenesis efficiencies with average of 31.83% in common carp than previously reported 3.0% to 12.4% in zebrafish \cite{12}. We also observed that the shorter the spacer length results in the higher mutagenesis in common carp with TALEN, consistent with previous studies \cite{49}. The highest efficiency of common carp TALEN is \textit{spp1a}-TALEN that has the shortest spacer length of 15 bp.

**Figure 6.** Severe bone defects in CRISPR-Cas9-targeted \textit{sp7a} common carps. (A) Enzymatic digestion of CRISPR-Cas9-mediated cleavage at common carp \textit{sp7a}. PCR products were digested with HinfI. Lane 1, marker; lanes 2–12, digestion of PCR products form 11 injected carps with HinfI; lane 13, wild-type without digestion. Uncut (256 bp) and cut (185 bp and 71 bp) bands are indicated. Electrophoretic results were cropped from the original images shown in Supplementary Fig. S13H. Body weights (B) and lengths (C) were significantly decreased at 1 mpf ($P < 0.01$), 2 mpf ($P < 0.001$) and 3 mpf ($P < 0.001$) in \textit{sp7a}-CRISPR carp (green) compared with wild types (blue). Values are means ± SEM. $n = 20$ per sampling stage. Images of one-month-old wild-type (D) and \textit{sp7a} CRISPR-Cas9-induced mutant 3# (E) carps. Red arrow indicates the deformed bones. Scale bar, 2 mm. Alizarin Red staining of vertebra bones for one-month-old wild-type (F) and \textit{sp7a}-CRISPR-Cas9 mutant 3# (G) carps. Blue arrowheads indicate intermuscular bones, orange arrowheads hemal spine bones and yellow arrowheads the centrums region. Scale bar, 0.2 mm. (H) Average length of inter-muscular bones in wild-type (blue, three carps) and \textit{sp7a}-CRISPR (green, eight carps from Supplementary Fig. S8). All the 32 inter-muscular bones in each carp were quantified with ImageJ. Values are means ± SD. (I) Silhouettes scaled to the same final magnification to illustrate the shape of hemal spine bones. a-h are different hemal spine bones from wild types (a–d) and \textit{sp7a} CRISPR-Cas9 3# (e–h) in (F,G). (J) Mutations in \textit{sp7a} CRISPR-Cas9-3# carp, revealed by sequencing analysis. The gRNA-targeted sequence is in green, and the PAM sequences are in blue. Micro-CT images of two-month-old wild-type control (K), \textit{sp7a} CRISPR-Cas9-induced 11# mutant (L) and \textit{sp7b} CRISPR-Cas9-induced mutant (M) carps. (N) BV (bone volume), BS (bone surface), BV (bone volume)/TV (tissue volume) and BS (bone surface)/TV (tissue volume) of cranioficial bones (up panels) and centrum bones (bottom panels) in \textit{sp7a}-CRISPR (green), \textit{sp7b}-CRISPR (yellow) and WT control (blue), calculated from the Micro-CT analysis. $n = 3$. Values are means ± SD. Two-tailed Student's $t$-test or one-way ANOVA with LSD were conducted, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and N.S., no significant difference.

**Discussion**

Due to the tetraploidy genome of common carp, it is extremely difficult to obtain the homozygous mutants using the traditional methods such as ENU, TILLING, and retroviral insertion. However, using TALEN and CRISPR-Cas9 methods we have successfully targeted carp genes of interest and observed the specific phenotypes in F0 generation. We used TALEN to target four genes, \textit{sp7a}, \textit{runx2}, \textit{mstnba} and \textit{spp1a} and CRISPR-Cas9 to target six genes, \textit{sp7a}, \textit{sp7b}, \textit{runx2}, \textit{mstnba}, \textit{opga} and \textit{bmp2ab} with high efficiencies. With TALEN, we have achieved higher mutagenesis efficiencies with average of 31.83% in common carp than previously reported 3.0% to 12.4% in zebrafish \cite{12}. We also observed that the shorter the spacer length results in the higher mutagenesis in common carp with TALEN, consistent with previous studies \cite{49}. The highest efficiency of common carp TALEN is \textit{spp1a}-TALEN that has the shortest spacer length of 15 bp.
Even though TALEN and CRISPR-targeted F0 fish were genotypically complex, the highly induced mutagenesis frequencies of somatic mutations still can result in obvious phenotypes in F0 founders, as reported in mice and zebrafish. Hence CRISPR-Cas9 can have the similar effect like Morpholinos to alter the gene functions in common carp. It is applicable to the genes that function in later development or in adulthood such as mature osteoblast genes, circadian clock genes, and ageing genes.

Although sp7a-targeted site with CRISPR-Cas9 has a nearly 100% mutation efficiency in embryos and somatic cells, its mutation types are different among the different cells (Fig. 6I). CRISPR-Cas9-induced mutations are complex in F0 founder carps. First, these mutations could be mosaic, meaning that the same mutations may be located in different tissues or organs, and in some cases the targeted tissues or organs may not carry the mutations and thus display no expected phenotypes. Second, several different types of mutations could be located in different tissues or organs, for example, the mutated sequences of mstnba, some causing truncated peptides and the other resulting in only deletion or insertion of a few amino acids, are different among different types of cells, as shown in Fig. 5M and Supplementary Fig. S12. Therefore the phenotypes of the body weight vary in the same group of carps (Fig. 5D). The complex nature of CRISPR-Cas9-induced mutations is also responsible for variations of Alizarin Red staining of sp7a-CRISPR F0 carps (Supplementary Figs S8 and S9) and only 25% reduced levels of phosphorylated Smad2 (Fig. 5E,F). Another aspect of the complexity of phenotypes observed in F0 founder carps is that there are multiple copies of genes under study, for instance, four mstn genes in common carp, and usually only one gene is mutated (Fig. 1B). A possible way to obtain a consistent phenotype in the F0 founder carp is to target the important functional domains such as the Z-F (Zinc-Finger) motif of sp7 with high mutation rate, which would induce biallelic mutations.

Sp7/Osterix plays an essential role in differentiation and maturation of osteoblasts and formation of osteocytes but not in cartilage development in mice and medaka. We observed that body weight is lighter in sp7a-CRISPR mutated F0 carps than wild-type control, maybe because the delayed and abnormal osteoblast development; some mutant carps have curved spines, the other mutant carps have abnormal craniofacial bones (Fig. 6D–I and Supplementary Figs S8 and S9). Further, micro-CT analysis showed the bone volume (BV), bone surface (BS), bone volume/tissue volume (BV/TV) and bone surface/tissue volume (BS/TV) are significantly reduced in sp7a-CRISPR carps (Fig. 6N). Together, these results strongly indicate that the observed phenotypes were resulted from the disruption of sp7a gene, consistent with previous studies. In contrast to the mouse model died after birth, sp7 mutant carps can survive at least 3 months, providing a unique opportunity for investigating roles of Sp7 in bone development. In particular, the inter-muscular bones are shorter in sp7a-CRISPR carps in comparison with wild types (Fig. 6H) but their number are not changed, demonstrating that Sp7 plays a role in common carp inter-muscular bone development. Even though sp7a-CRISPR mutated carps are not good aquaculture lines due to the abnormal bones and lighter body weight, they are certainly useful for finding carps without or with fewer inter-muscular bones and investigating teleost osteoblast formation.

We have used the CRISPR-Cas9 method to generate mutant carps for multiple genes with high efficiencies. Many phenotypes are derived from mutations in multiple genes rather than a single gene. There are a lot of genes with multiple copies in common carp due to additional round of genome duplication. Hence mutagenizing double genes or multiple genes with high efficiencies would facilitate generation of multiple-gene-mutated carps.
In addition, we can mutagenize multiple copies simultaneously in a single carp and study their functions. Future work will use single gRNA to modify multiple homologous genes as described previously in *Xenopus Laevis*.

For a large aquatic species such as common carp, it is important to generate mutants with high germline transmission efficiencies to reduce the cultivation time, pool space and labor force. Many studies have been focused on increasing the targeting efficiency. In this study, we have achieved mutagenesis efficiencies up to 75% for TALEN and up to 100% for CRISPR-Cas9, not only in embryos and juveniles examined, but also in the testis. Our examination of the testis indicated that almost all the testis cells including spermatogonia or spermatocytes are biallelically mutated, implicating that these CRISPR-Cas9-induced mutations are likely transmitted to the subsequent generation. A study of the F₀ monkey placenta showed that the germline cells likely would be modified by CRISPR-Cas9. Zebrafish studies showed that 87.3% mutated efficiency for *gol* in *F₀* somatic cells and 52.7% mutated efficiency for *ft* in *F₀* somatic cells with CRISPR-Cas9 both led to 100% germline transmission rate. Another zebrafish study showed that 12.5% mutated rate for *tnikb* in *F₀* somatic cells with TALEN led to 33.3% germline transmission rate. Similar to these previous studies, we should be able to obtain germline-transmitted carp lines with as high as 100% of somatic mutation rate in TALEN- and CRISPR-Cas9-targeted genes.

In summary, our studies demonstrate that TALEN and CRISPR-Cas9 technologies are effective tools for genetics studies in common carp, which will greatly promote genetic engineering in aquaculture and have the potentials for improving their qualities and economic value in the future. To our knowledge, this is the first report on targeted disruption of endogenous genes in common carp using TALEN and CRISPR-Cas9.

**Methods**

All animal care and experiments were performed in accordance with the institutional ethical guidelines for animal experiments, and all fish experimental procedures were approved by the Soochow University Committee on Animal Use and Care.

**Design of TALEN and CRISPR target sites.** The TALEN sites were selected by “TAL Effector Nucleotide Targeter 2.0” to target on the first exon of most genes. An enzyme site in the spacer was selected if it is available for genotyping. Blasting the target sequences against the whole genome DNA database (http://www.carpbase.org/login.phpweb address) of common carp was performed to avoid the off-target sites.

The gRNAs for CRISPR-Cas9 were designed by “seqbuilder” software according to the 5′-GGNNNNNNNNNNNNGG-3′ roles. The first two G are necessary for the T7 RNA polymerase and the end NGG is the PAM. The minimal number of nucleotides (N) is 19 bp depending on the sequence for each gene. A restrictive enzyme near the PAM was also selected if it is available.

**Construction of TALEN and synthesis of Cas9 and gRNAs.** The two arms of TALEN were constructed using the “Unit Assembly” method. Simply, customized TALE repeats were inserted into the 5′-end of the backbone vector (pMD-19T) by double-digesting with either NheI and HindIII or SpeI and HindIII. The final pMD-TALE repeats were confirmed by sequencing with the M13 primer. Then, the DNA fragments digested with NheI and SpeI, were ligated into the TALEN expression vectors-PCS2-PEAS and PCS2-PERR. The final pMD-TALE repeats were confirmed by sequencing with the M13 primer. Then, the DNA fragments digested with NheI and SpeI, were ligated into the TALEN expression vectors-PCS2-PEAS and PCS2-PERR. The final constructs were linearized with NotI and used as templates for TALEN mRNA synthesis with SP6 mMESSAGE mMACHINE Kit (Ambion).

The Cas9 mRNA and gRNAs were synthesized as described previously with modifications. Briefly, the Cas9 mRNA was synthesized by in vitro transcription using T7 mMESSAGE mMACHINE Kit (Ambion). The DNA templates of gRNA were generated by PCR with a pair of primers (Supplementary Table S6), and then purified by phenol and chloroform. gRNAs were in vitro transcribed with SP6 Riboprobe Systems (Promega), and purified with lithium chloride and ethanol precipitation.

**Zebrafish maintenance and microinjection.** Zebrafish (*Danio rerio*) are maintained at 28.5 °C under 14 h light:10 h dark (14:10 h LD) cycles at the Soochow University Zebrafish Research Facility. To test the targeting efficiency of TALEN pairs and the CRISPR-Cas9 system, 50–100 pg purified plasmid containing gene specific DNA fragment of carp plus 250 pg TALEN mRNAs of each arm or 300 pg Cas9 mRNA plus 25–100 pg carp gRNAs were microinjected into one cell-stage zebrafish embryos. Genomic DNAs extracted from injected embryos at 24 hpf (hours postfertilization) were used as templates for PCR.

**Artificial insemination of common carp.** The brood stocks of common carp were obtained from The Heilongjiang Fisheries Research Center, Harbin, Heilongjiang, China, and are maintained at the New Era Fish Breeding Facility of Xiangcheng, Suzhou, Jiangsu, China. Well-developed 3-year-old brood fish with the average body weight of 2 kg of adult carp were selected. Female fish were injected with 5 ug/kg luteinizing hormone-releasing hormone A2 (LHRH-A2, Ningbo secondary Hormone Company, China) and 1 mg/kg domperidone (DOM, Ningbo secondary Hormone Company, China) together dissolved in 0.68% physiological saline in a total volume of 2 ml, while male fish received only half of this dosage. Male and female fishes were kept separately in circular breeding ponds with flowing water stimulation. Then common carp eggs were manually stripped into a dry centrifuge tube, while milts were manually stripped from the males into a centrifuge tube with 10 ml Hank’s solution (0.4 g KCl, 8 g NaCl, 0.35 g NaHCO3, 0.09 g NaH2PO4·7H2O, 0.1 g MgSO4·7H2O, 0.1 g MgCl2·6H2O, 0.06 g KH2PO4, 0.14 g CaCl2 and 1 g glucose in 1 L sterile deionized water) for in vitro fertilization.

**Microinjection and maintenance for the embryos of common carp.** Microinjection began at 20 min after fertilization. Short but sharp needles were pulled by the Micropipette Puller (P-1000, Sutter Instrument) using the thin wall borosilicate tubing with filament (Catalog Number: BF120-94-10; Outside Diameter: 1.20 mm;
Inside Diameter: 0.94 mm; Overall Length: 10 cm; Sutter Instrument), and the program of pulling the needle was Heat: 750; Pull: 80; Vel: 55; Time: 250; Pressure: 500; and Ramp: 765.

For the injection of the TALEN arms, 250 pg TALEN mRNAs of each arm were co-microinjected into one-cell carp embryos; For CRISPR-Cas9, 300 pg Cas9 mRNA and 25–200 pg gRNA were co-injected into the embryos. Microinjected embryos were grown at room temperature and collected for DNA extraction at 48–72 hpf.

**Common carp larval feeding and maintenance.** Four dpf-12 dpf larvae were fed with paramecia; then fed with brine shrimps until 25 dpf. 20–90 dpf juveniles were fed with the eel powder for two days, and then were transferred into pools. Those fish to be examined were taken to the laboratory. Each fish was raised in a 3-L tank at 22 °C under 14 h light:10 h dark (14:10 h LD) cycles and fed with brine shrimp and eel powder. The food was fed equivalently in all the groups.

**Mutagenesis efficiency analyses in injected embryos.** Restriction digestion assay. TALEN- and CRISPR-Cas9/gRNA-induced mutation efficiencies were examined by the restriction digestion assay as described previously. PCR products were digested with corresponding restriction endonuclease enzymes for 2 h at 37 °C. The intensities of cleaved and uncleaved bands were quantified with the ImageJ software.

**T7 endonuclease I (T7EI) assay.** 300 ng of purified PCR products were denatured and slowly re-annealed to facilitate heteroduplex formation. The re-annealing procedure includes a 5-min denaturing step at 95 °C, followed by cooling to 85 °C at −2 °C/sec and further to 25 °C at −0.1 °C/sec. The re-annealed amplicon was then digested with 10 U of T7EI endonuclease I (New England Biolabs) at 37 °C for 90 min, and digested amplicon was electrophoresed on a 2% agarose gel. Band intensities were quantified with the ImageJ software.

**DNA sequencing.** For DNA sequencing, DNA fragments containing the targeted sites were PCR amplified with DNAs extracted from caudal fins of 1-month old carps, and cloned into the PMD-19T vector. Single clone was picked up, verified by PCR and sequenced by Sangong sequencing (GENEWIZ, Inc.).

**RNA isolation, RT-PCR and quantitative real-time PCR analyses.** Total RNAs were extracted from the maw, liver, gill, eye, heart, brain, gut, testis and muscle of 3-month-old common carp fishes with Trizol (Invitrogen, CA, USA), and reverse transcribed into cDNAs with Superscript III Reverse Transcriptase (Invitrogen, CA, USA), respectively. RT-PCRs were carried out with the following thermal profile for 38 cycles. Quantitative real-time PCR (qRT-PCR) was performed in an ABI StepOnePlus instrument with the SYBR green detection system (Invitrogen) using a software (Version: 1.10.11.0), as described previously. PCR products were digested with corresponding restriction endonuclease enzymes for 2 h at 37 °C. The intensities of cleaved and uncleaved bands were quantified with the ImageJ software.

**Alizarin red staining.** Mineralization of calcium deposits was assessed by Alizarin Red S (Sigma) staining conducted as previously described. Three-month-old carps from mutant and control groups were anesthetized with 0.03% Tricaine (Sigma, USA). The stained matrix was examined using a Leica stereomicroscope and was photographed using an Olympus 35-mm camera (Olympus, Tokyo, Japan).

**H&E (Hematoxylin and eosin) staining.** Three-month-old carps from mutant and control groups were anesthetized with 0.03% Tricaine (Sigma, USA). Subsequently, carp dorsal muscles were dissected out and fixed in 4% paraformaldehyde for 4 hr at room temperature for 24 hrs, and then sectioned and stained with hematoxylin/eosin as described previously. Dorsal muscles of carp pelvic fins were counted. Cell number was calculated as the number of fibers per cross-sectional muscle area. The muscle fiber numbers and area were determined with the ImageJ program. The muscle numbers were calculated by the “Cell Counter” features of ImageJ.

**Micro computed tomography (Micro-CT).** Two-month-old common carp with approximately 3.5 cm of body length were anesthetized with 0.03% Tricaine (Sigma, USA) and gently put into 5 ml centrifuge tubes. The whole body was imaged in a SkyScan 1176 high-resolution micro-CT scanner (SkyScan, Kontich, Belgium) using a 8.8 μm voxel size. The applied X-ray voltage was 45 kV and current was 550 μA. Scans were over 180° with a 0.7° rotation step. Images were reconstructed and binarised with global thresholding using SkyScan CT Analyser software (Version: 1.10.11.0), as described. A region of interest was traced around individual whole body or head models prepared using the “Double Time Cubes” 3D reconstruction method. Cortical bone mineral density (BMD) was estimated by comparing bone density with calibration phantoms of known BMD, scanned at the same time as the head.

**Western blotting analysis.** Proteins extracted from dorsal muscles of five-month-old common carps were washed with fish water and homogenized in lysis buffer as described previously. Protein samples were separated with sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) using 5% acrylamide stacking gel followed by 10% gradient separation gel and transferred to NC membranes. After blocking with 5% nonfat milk (blocking solution) for 1 h at room temperature. The blocking solution was also used as dilution solutions for antibodies. NC membranes were incubated with primary antibody overnight at 4 °C; anti-phospho Smad2 (1:500, Millipore Corp., MA, USA), anti-Tubulin (1:1000, Cell Signaling Technology, Inc., MA, USA). The next day, membranes were washed in TBST (0.5% Tween 20) four times, each for 5 min, and then incubated for 2 h.
with goat anti-rabbit HRP-conjugated secondary antibody (1:10000; Santa Cruz Biotechnology, Inc., CA, USA) at room temperature. After washing with TBST four times, 10 min each, membranes were detected by Amersham ECL prime (GE Healthcare). Equal loading of the proteins was confirmed with an anti-Tubulin antibody.

**Statistical analysis.** Statistical analyses were performed with the unpaired, two-tailed Student's *t*-test or one-way ANOVA with post hoc LSD method. All statistical analyses were executed with SPSS 16.0 software and *P* < 0.05 was regarded as a statistically significant difference, *P* < 0.05, **P* < 0.01, ***P* < 0.001.

**References**

1. Xu, P. *et al.* Genome sequence and genetic diversity of the common carp, *Cyprinus carpio*. *Nat Genet* 46, 1212–1219 (2014).
2. Zhang, X. *et al.* A consensus linkage map provides insights on genome character and evolution in common carp (*Cyprinus carpio* L.). *Mar Biotechnol* (NY) 15, 275–312 (2013).
3. Laghari, M. Y. *et al.* QTL mapping for economically important traits of common carp (*Cyprinus carpio* L.). *J Appl Genet* 56, 65–75 (2015).
4. Xu, P. *et al.* Genomic insight into the common carp (*Cyprinus carpio* L.) genome by sequencing analysis of BAC-end sequences. *BMC Genomics* 12, 188 (2011).
5. Li, X. *et al.* Gut microbiota contributes to the growth of fast-growing transgenic common carp (*Cyprinus carpio* L.). *PloS One* 8, e64577 (2013).
6. Zhong, C. *et al.* Increased food intake in growth hormone-transgenic common carp (*Cyprinus carpio* L.) may be mediated by upregulating Agouti-related protein (AgRP). *Gen Comp Endocrinol* 192, 81–88 (2013).
7. Ma, J. *et al.* Screening and body correlation analysis of microsatellite markers related to intermuscular bone number in common carp (*Cyprinus carpio*). *Dongwuxue Yanjiu* 34, 406–410 (2013).
8. B. P. *et al.* Characterization of common carp transcriptome: sequencing, *de novo* assembly, annotation and comparative genomics. *PloS One* 7, e35152 (2012).
9. Xu, P. *et al.* Generation of the first BAC-based physical map of the common carp genome. *BMC Genomics* 12, 537 (2011).
10. Xu, J. *et al.* Genome-wide SNP discovery from transcriptome of four common carp strains. *PloS One* 7, e48140 (2012).
11. Zhang, Y. *et al.* Genetic linkage mapping and analysis of muscle fiber-related QTLs in common carp (*Cyprinus carpio* L.). *Mar Biotechnol (NY)* 13, 376–392 (2011).
12. Huang, P. *et al.* Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotechnol* 29, 699–700 (2011).
13. Hwang, W. Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 31, 227–229 (2013).
14. Wood, A. J. *et al.* Targeted genome editing across species using ZFNs and TALENS. *Science* 333, 307 (2011).
15. Pennisi, E. The CRISPR craze. *Science* 341, 833–836 (2013).
16. Sander, J. D. *et al.* Targeted gene disruption in zebrafish cells using engineered TALENs. *Nat Biotechnol* 29, 697–698 (2011).
17. Tesson, L. *et al.* Knockout rats generated by embryo microinjection of TALENs. *Nat Biotechnol* 29, 695–696 (2011).
18. Bedell, V. M. *et al.* In vivo genome editing using a high-efficiency TALEN system. *Nature* 491, 114–118 (2012).
19. Lei, Y. *et al.* Efficient targeted gene disruption in Xenopus embryos by engineered transcription activator-like effector nucleases (TALENs). *Proc Natl Acad Sci USA* 109, 17484–17489 (2012).
20. Li, T. Liu, B., Spalding, M. H., Weeks, D. P. & Yang, B. High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat Biotechnol* 30, 390–392 (2012).
21. Shan, Q. *et al.* Rapid and efficient gene modification in rice and brachypodium using TALENs. *Mol Plant* 6, 1365–1368 (2013).
22. Song, J. *et al.* Generation of RAG 1- and 2-deficient rabbits by embryo microinjection of TALENs. *Cell Res* 23, 1059–1062 (2013).
23. Li, H. *et al.* Antagonistic Roles of Dmrt1 and Foxl2 in Sex Differentiation via Estrogen Production in Tilapia as Demonstrated by TALENS. *Endocytology* 154, 4814–4825 (2013).
24. Wang, H. *et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153, 910–918 (2013).
25. Wang, Y. *et al.* The CRISPR/Cas system mediates efficient genome engineering in Bombyx mori. *Cell Res* 23, 1414–1416 (2013).
26. Carlson, D. F. *et al.* Efficient TALEN-mediated gene knockout in livestock. *Proc Natl Acad Sci USA* 109, 17382–17387 (2012).
27. Ding, Q. *et al.* A TALEN genome-editing system for generating human stem cell-based disease models. *Cell Stem Cell* 12, 238–251 (2013).
28. Auer, T. O., Duroure, K., De Cian, A., Cordet, J. P. & Del Bene, F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res* 24, 142–153 (2014).
29. Chang, N. *et al.* Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell Res* 23, 465–472 (2013).
30. Hruscha, A. *et al.* Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* 140, 4982–4987 (2013).
31. Ran, F. A. *et al.* Genome engineering using the CRISPR/Cas9 system. *Nat Protoc* 8, 2281–2308 (2013).
32. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823 (2012).
33. Jao, L. E., Wente, S. R. & Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci USA* 110, 13904–13909 (2013).
34. Yang, H. *et al.* One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154, 1370–1379 (2013).
35. Nakashima, K. *et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108, 17–29 (2002).
36. Zhang, C. *et al.* Inhibition of Wnt signaling by the osteoblast-specific transcription factor Osterix. *Proc Natl Acad Sci USA* 105, 6936–6941 (2008).
37. Zhou, X. *et al.* Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice. *Proc Natl Acad Sci USA* 107, 12919–12924 (2010).
38. Zhang, Y. W. *et al.* A RUNX2/Pebp2alpha A/Cbfal mutation displaying impaired transactivation and Smad interaction in chondrocraniopharyngioma. *Proc Natl Acad Sci USA* 97, 10549–10554 (2000).
39. Apsechner, A., Huitema, L. F., Ponsioen, B., Peterson-Maduro, J. & Schultze-Merkel, S. Zebrafish enpp1 mutants exhibit pathological mineralization, mimicking features of generalized arterial calcification of infancy (GACI) and pseudoxanthoma elasticum (PXE). *Dis Model Mech* 7, 811–822 (2014).
40. Peal, D. S., Burns, C. G., Macrea, C. A. & Milan, D. Chondroitin sulfate expression is required for cardiac atrioventricular canal formation. *Dev Dyn* 238, 3103–3110 (2009).
41. Kitamura, K. *et al.* Zebrafish scales respond differently to in vitro dynamic and static acceleration: analysis of interaction between osteoblasts and osteoclasts. *Comp Biochem Physiol A Mol Integr Physiol* 166, 74–80 (2013).
42. Crotwell, P. L., Sommervold, A. R. & Mabee, P. M. Expression of bmp2a and bmp2b in late-stage zebrafish median fin development. *Gene Expr Patterns* 5, 291–296 (2004).
43. Grobet, L. *et al.* A deletion in the bovin bovine myostatin gene causes the double-muscled phenotype in cattle. *Nat Genet* 17, 71–74 (1997).
Targeted disruption of sp7 in common carp.

44. Chisada, S. et al. Myostatin-deficient medaka exhibit a double-muscling phenotype with hyperplasia and hypertrophy, which occur sequentially during post-hatch development. Dev Biol 359, 82–94 (2011).
45. Zhu, X.-F. et al. Transcriptome analysis reveals the time of the fourth round of genome duplication in common carp (Cyprinus carpio). J Mol Evol 83, 359–369 (2016).
46. Dong, Z. et al. Heritable targeted inactivation of myostatin gene in yellow catfish (Pelteobagrus fulvidraco) using engineered zinc finger nucleases. PLoS One 6, e28897 (2011).
47. Sinha, K. M. & Zhou, X. Genetic and molecular control of osterix in skeletal formation. J Cell Biochem 114, 975–984 (2013).
48. Liu, L., Yu, X. & Tong, J. Molecular characterization of myostatin (MSTN) gene and association analysis with growth traits in the bighead carp (Aristichthys nobilis). Mol Biol Rep 39, 9211–9221 (2012).
49. Christian, M. et al. Targeted DNA double-strand breaks with TAL effector nucleases. Genetics 186, 757–761 (2010).
50. Dong, Z. et al. Heritable targeted inactivation of myostatin gene in yellow catfish (Pelteobagrus fulvidraco) using engineered zinc finger nucleases. PLoS One 6, e28897 (2011).
51. Chisada, S. et al. Myostatin-deficient medaka exhibit a double-muscling phenotype with hyperplasia and hypertrophy, which occur sequentially during post-hatch development. Dev Biol 359, 82–94 (2011).
52. Li, D. et al. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nat Biotechnol 31, 681–683 (2013).
53. Renn, J. & Winkler, C. Osterix/Sp7 regulates biomineralization of otoliths and bone in medaka (Oryzias latipes). Matrix Biol 34, 193–204 (2014).
54. Chen, S. et al. Key role for the transcriptional factor, osterix, in spine development. Spine J 14, 683–694 (2014).
55. Wang, J. T., Li, J. T., Zhang, X. F. & Sun, X. W. Transcriptome analysis reveals the time of the fourth round of genome duplication in common carp (Cyprinus carpio). BMC Genomics 13, 96 (2012).
56. Niu, Y. et al. Targeted mutagenesis of multiple and paralogous genes in Xenopus laevis using two pairs of transcription activator-like effector nucleases. Dev Growth Differ 56, 108–114 (2014).
57. Niu, Y. et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156, 836–843 (2014).
58. Hwang, W. Y. et al. Heritable and precise zebrafish genome editing using a CRISPR-Cas system. PLoS One 8, e68708 (2013).
59. Doyle, E. L. et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156, 836–843 (2014).
60. Wang, J. T., Li, J. T., Zhang, X. F. & Sun, X. W. Transcriptome analysis reveals the time of the fourth round of genome duplication in common carp (Cyprinus carpio). BMC Genomics 13, 96 (2012).
61. Walker, M. B. & Kimmel, C. B. A two-color acid-free cartilage and bone stain for zebrafish larvae. Biotechnol Histochem 82, 23–28 (2007).
62. Fuentes, E. N. et al. Transient inactivation of myostatin induces muscle hypertrophy and overcompensatory growth in zebrafish via inactivation of the SMAD signaling pathway. J Biotechnol 168, 295–302 (2013).
63. Spoorenbroek, K. M. et al. Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. Development 135, 3765–3774 (2008).
64. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30, 2725–2729 (2013).

Acknowledgements

This work was supported by the grants from National High Technology Research and Development Program of China (863 Program) (#2011AA100402-2), National Basic Research Program of China (973 Program) (# 2012CB947600), the National Natural Science Foundation of China (NSFC) (#31030062, #31271356, #81070455), the Jiangsu Distinguished Professorship Program (#SR13400111), and the Natural Science Foundation of Jiangsu Province (#BK2012052), the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions (#XY13400214), the High-Level Innovative Team of Jiangsu Province and the “333” project of Jiangsu Province (BRA2015328).

Author Contributions

H.W., Y.Y. and Z.Z. conceived and designed the experiments; Z.Z., P.N., M.W., G.H., S.X., Y.S., X.X., Y.H. and Y.Y. performed the experiments; X.S. provided materials; H.W., Z.Z., Y.Y. and P.N. analyzed the data and wrote the paper. All authors critically revised and approved the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep.

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhong, Z. et al. Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp. Sci. Rep. 6, 22953; doi: 10.1038/srep22953 (2016).