


# Disruption of the ZBED6 binding site in intron 3 of *IGF2* by CRISPR/Cas9 leads to enhanced muscle development in Liang Guang Small Spotted pigs

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**Abstract** Insulin-like growth factor 2 (*IGF2*) plays an important role in the development of the foetus and in post-natal growth and development. A SNP within intron 3 of porcine *IGF2* disrupts a binding site for the repressor, zinc finger BED-type containing 6 (ZBED6), leading to up-regulation of *IGF2* in skeletal muscle and major effects on muscle growth, heart size, and fat deposition. This favourable mutation is common in Western commercial pig populations, but

is not present in most indigenous Chinese pig breeds. Here, we described the efficient disruption of the ZBED6 binding site motif in intron 3 of *IGF2* by CRISPR/Cas9 in porcine embryonic fibroblasts (PEFs) from the indigenous Chinese pig breed, Liang Guang Small Spotted pig. Disruption of the binding motif led to a drastic up-regulation of *IGF2* expression in PEFs and enhanced myogenic potential and cell proliferation of PEFs. *IGF2*-edited pigs were then generated using somatic cell nuclear transfer. Enhanced muscle development was evident in one pig with biallelic deletion of the ZBED6 binding site motif, implying that the release of ZBED6 repression has a major effect on porcine muscle development. Our study confirmed the important effect of a mutation in the ZBED6 binding site motif on *IGF2* expression

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and myogenesis, thus providing the basis for breeding a new line of Liang Guang Small Spotted pigs with improved lean meat percentage, a trait of great commercial value to pig producers.

**Keywords** IGF2 · CRISPR/Cas9 · Muscle development · SCNT · Pig

## Introduction

Insulin-like growth factor 2 (IGF2) is a maternally imprinted growth factor that promotes skeletal muscle growth by binding to the IGF1 receptor (IGF1R) and activating downstream signalling pathways to regulate cell proliferation, differentiation, and apoptosis (Florini et al. 1996). The G3072A substitution in intron 3 of *IGF2* has been identified as the causal factor of the imprinted quantitative trait locus (QTL) for porcine muscle growth, subcutaneous fat deposition, and heart size (Jeon et al. 1999; Nezer et al. 1999; Van Laere et al. 2003). This G > A substitution turns the evolutionarily conserved motif, GCTCG, into GCTCA, thus abrogating the binding site for the transcriptional repressor, zinc finger BED domain-containing protein 6 (ZBED6) (Markljung et al. 2009). This leads to a threefold up-regulation of IGF2 in skeletal muscle, a 3–4% increase in meat production, and a 20% reduction in backfat thickness (Braunschweig et al. 2004; Estelle et al. 2005; Jungerius et al. 2004). The *IGF2* QTL alleles have been denoted Q and q, for high and low muscle growth, respectively. The favorable Q allele has increased in frequency due to intensive selection in growth and carcass traits, and is close to fixation in Western commercial pig populations widely used for meat production. In contrast, most indigenous Chinese pig breeds have not been subjected to intensive selection for growth and carcass traits and therefore, the population frequency of the q allele reaches 100% (Yang et al. 2006). A recent study showed the porcine *IGF2* G3072A mutation can remove the inhibition of ZBED6 and increase the expression of IGF2, leading to enhanced growth of skeletal muscle and internal organs in a knock-in mouse model (Younis et al. 2018). Therefore, it is interesting to test whether disruption of the ZBED6 binding site is able to improve muscle mass in a Chinese indigenous pig, as

observed in the knock-in mouse model. Genome-editing technology, especially the CRISPR/Cas9 system, has developed rapidly in recent years and has been used for various genomic modifications, in both basic and applicational studies (Adli 2018). Here we used the CRISPR/Cas9 system to edit the binding motif of ZBED6 in intron 3 of *IGF2* in porcine embryonic fibroblasts (PEFs) from the Chinese indigenous pig breed, Liang Guang Small Spotted pig, and evaluate its effect on IGF2 expression and myogenesis, and finally the production of gene-edited pigs through somatic cell nuclear transfer (SCNT) technology and embryo transfer.

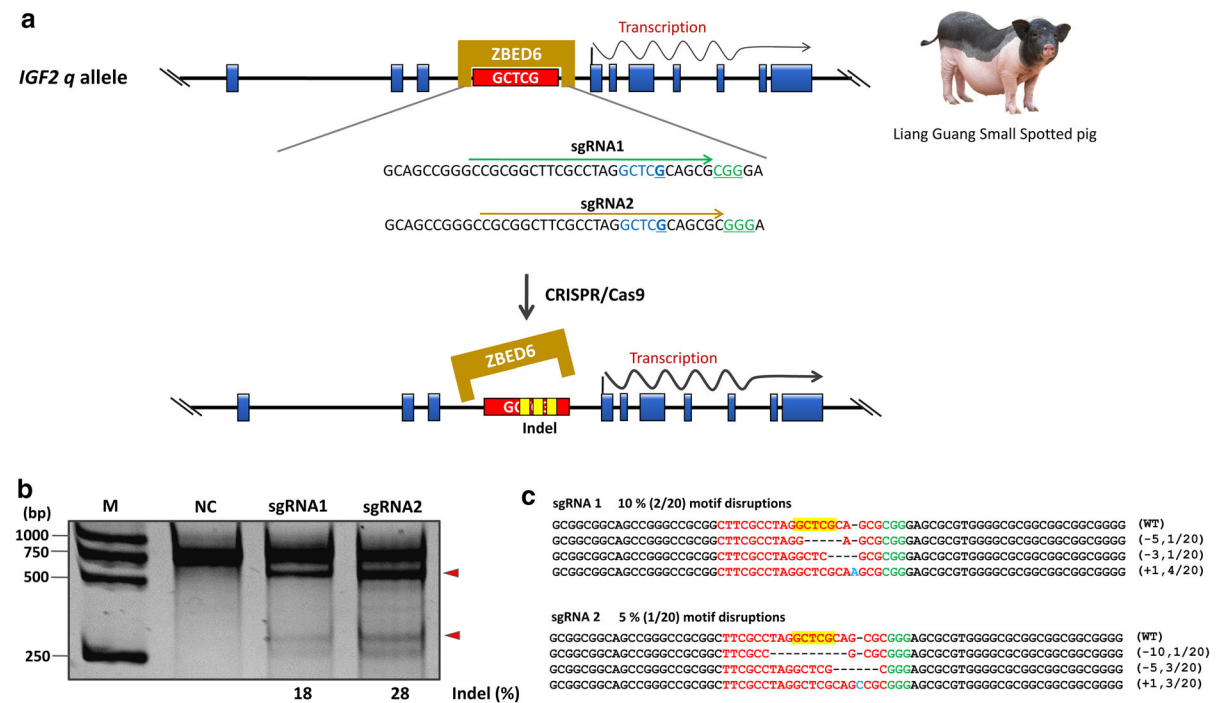
## Materials and methods

### Cell isolation and culture

PEFs were isolated from the embryos of Liang Guang Small Spotted pigs. Briefly, under a sterile environment, 35 d-old embryos were separated from pregnant sows. The back tissue of the embryos was then cut into pieces of 1 mm<sup>3</sup>, washed twice with phosphate-buffered saline (PBS) (Corning, USA), and transferred into 10-cm dishes filled with Dulbecco's modified Eagle medium (DMEM) (Corning, USA) containing L-glutamine and 1 g/L of D-glucose, supplemented with 20% fetal bovine serum (FBS) (PAN, Germany), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Sigma, USA). Visible embryonic fibroblasts migrating out from the tissue pieces after approximately 72 h of culture were harvested.

### sgRNA design and vector construction

Guide sequences for two sgRNAs targeting the ZBED6 binding motif in intron 3 of porcine *IGF2* (Fig. 1a) were selected using the open-source tool, CRISPR DE-SIGN (<http://crispr.mit.edu/>). To evaluate the targeting activities of the designed sgRNAs, synthesized oligos of each sgRNA guide sequence were cloned downstream of the human U6 promoter through *Bbs* I restriction sites in plas-mid pX330 (plasmid #42230, Addgene), as previously described (Ran et al. 2013). Target sequences for each sgRNA were introduced into the surrogate reporter RGS that encodes a monomeric mRFP-eGFP fusion protein, as described in our previous report (He et al. 2016). To



**Fig. 1** Evaluation of targeting activities of sgRNAs. **a** Schematic diagram of how targeting the ZBED6 binding site motif within intron 3 of the *IGF2* gene by CRISPR/Cas9 is anticipated to release the suppression of ZBED6, resulting in enhanced the expression of *IGF2* in Liang Guang Small Spotted pigs. Arrows indicate the guide segment of the sgRNAs. NGG nucleotide protospacer adjacent motif (PAM) sequences are shown in green. The ZBED6 binding site motif is shown in blue and the G3072A mutation site in intron 3 of *IGF2* is underlined. **b** Frequencies of mutations induced by sgRNA/Cas9, as determined by T7E1 assay. The numbers at the bottom of the

gel indicate mutation percentages, estimated from band intensities using Image J. Red arrow heads indicate the expected positions of DNA bands cleaved by mismatch-sensitive T7E1. **c** DNA sequences of the wild-type (WT) and mutant clones, with sgRNA recognition sites shown in red and the protospacer adjacent motif (PAM) sequence in green. The ZBED6 binding site motif is shown in the yellow box. Dashes and lower-case blue letters indicate deleted and inserted bases, respectively. The number of occurrences is shown in parentheses. Mutation frequencies were obtained by dividing the number of mutant clones by the total number of clones. (Color figure online)

enrich PEFs targeted by CRISPR/Cas9, synthesized oligos of each sgRNA guide sequence were cloned downstream of the human U6 promoter through *Bbs* I restriction sites in plas-mid pX458 (plasmid #48138, Addgene), as previously described (Ran et al. 2013). sgRNA sequences are listed in Table S1.

**Transfection and sorting**

PEFs were harvested, counted, and  $1 \times 10^6$  cells were resuspended in 100  $\mu$ L of buffer R (Invitrogen, USA), that contains 5  $\mu$ g of RGS reporter plasmid and 5  $\mu$ g of each pX330-IGF2-sgRNA plasmid. The mixture was then transfected by electroporation at 1650 V for 10 ms in 3 pulses, using the Neon Transfection System (Invitrogen, USA) and seeded in 6-well plates with

2 mL of preheated culture medium. Forty-eight hours after transfection, cells were dissociated with trypsin (Sigma, USA) at 37  $^{\circ}$ C for 4 min and resuspended in PBS (Gibco, USA). They were then analyzed and collected by fluorescence-activated cell sorting (FACS) using an Aria II Cell Sorter (BD Biosciences, USA). DsRed and EGFP-positive cells were sorted into 1.5 mL centrifuge tubes and then centrifuged for isolation of genomic DNA for T7 endonuclease I (T7E1) assays. Similar conditions were used for the transfection of pX458-IGF2-sgRNA1 plasmids. Cells with the top 15% and the top 5% of EGFP intensity were sorted for the isolation of genomic DNA for T7E1 assays.

## T7E1 assays

Genomic DNA samples were extracted from sorted cell populations using a DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The targeted sites were amplified using a 2 × Phanta Max Master Mix (Vazyme, China) with the primer pairs listed in Table S2 and purified with a gel extraction kit (Omega, USA). Five hundred nanograms of purified PCR product was denatured and annealed in NEBuffer 2 using a thermocycler (Bio-Rad, USA). Samples were then digested with T7E1 (NEB, USA) for 30 min at 37 °C and separated by 10% native polyacrylamide gel electrophoresis (native-PAGE). Mutation frequencies were calculated based on band intensities using Image J software (Kim et al. 2011). PCR products were then cloned into a pMD-18 vector (Takara, Japan) and sequenced to confirm the mutation efficiency. Primers used for PCR are listed in Table S2.

## RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from sorted EGFP-positive cells using TRIzol Reagent (Invitrogen) and was reverse transcribed using a cDNA synthesis kit (TransGen Biotech, China), according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed with ChamQ SYBR qPCR Master Mix (Vazyme) using a Roche Light Cycler 480 (Roche, Switzerland). The expression levels of *IGF2*; myogenic factors, including myogenic differentiation 1 (*MYOD1*), myogenin (*MYOG*), and desmin (*DES*); and proliferation and apoptosis related genes, including cyclin D1 (*CCND1*), *BCL2*, and *BCLXL*, were measured. Target gene expression levels were normalized to the housekeeping gene, beta actin, using the  $2^{-\Delta\Delta Ct}$  method. The primers used for qPCR are listed in Table S3.

## Protein extraction and western blotting

Total protein was extracted from sorted EGFP-positive cells using RIPA lysis buffer (GenStar, China) supplemented with PMSF (GenStar). Proteins were separated on 10% SDS-PAGE gels and were then transferred to a PVDF membrane at 18 V for 40 min. Targeted proteins on the membranes were detected by

incubation with primary anti-beta-actin (#ab8227; Abcam, UK), anti-MYOD1 (#ab16148, Abcam), anti-IGF2 (#sc-5622; Santa Cruz Biotechnology, USA), and anti-desmin (#sc-14026, Santa Cruz Biotechnology) antibodies, followed by incubation with a secondary antibody conjugated with horseradish peroxidase (HRP). The fluorescence signal was detected based on the catalysis of an electrochemiluminescence (ECL) reagent (Pierce Biotechnology, USA) by HRP.

## EdU assay

Cell proliferation was assessed with an EdU assay, using the Cell-Light™ EdU Apollo 567 in vitro flow cytometry kit (RiboBio, China), according to the manufacturer's instructions. Briefly, cells were exposed to 50 mM of EdU for 2 h, washed with PBS, fixed with 4% paraformaldehyde, and then permeabilized with 0.5% Triton X-100. After washing three times with PBS, cells were incubated in Apollo reaction solution for 1 h and counterstained with Hoechst 33342 for 30 min. The ratio of EdU<sup>+</sup> positive cells then was calculated under a fluorescence microscope.

## Somatic cell nuclear transfer (SCNT) and embryo transfer

To generate cloned embryos, oocytes isolated from ovaries collected from an abattoir, were matured in vitro, as we have previously described (Ji et al. 2013). Matured oocytes were then selected for SCNT, following our previously reported protocol (Ji et al. 2014). Briefly, the nucleus and polar body were removed with an enucleation pipette made by pulling a glass capillary, and an EGFP-positive PEF was injected, using a transfer pipette, into the perivitelline space to form a couplet. The reconstructed couplets were fused and activated simultaneously with a single DC pulse of 120 kV/cm for 30 ms, using a BTX Electro Cell Manipulator 2001 (BTX, USA). Reconstructed embryos then were placed in PZM-5 medium and incubated at 37 °C. Cloned embryos were transferred into Large White sow recipients on day 1 after their first standing oestrus. Embryos were surgically transferred into the ampullary-isthmic junction of the oviduct of the surrogate.

## Genotyping of cloned pigs

Genomic DNA was extracted from piglet ear biopsies using the DNeasy Blood and Tissue Kit (Qiagen). *IGF2*-specific primers (Table S2) were used for PCR amplification as follows: 95 °C for 5 min; 36 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min; and finally, 72 °C for 10 min. PCR products were separated on 2% agarose gels, and subsequently, cloned into pMD18-T vectors (Takara) for Sanger sequencing.

## Results

Effective enrichment of PEFs with ZBED6 binding site in intron 3 of *IGF2* disrupted by CRISPR/Cas9

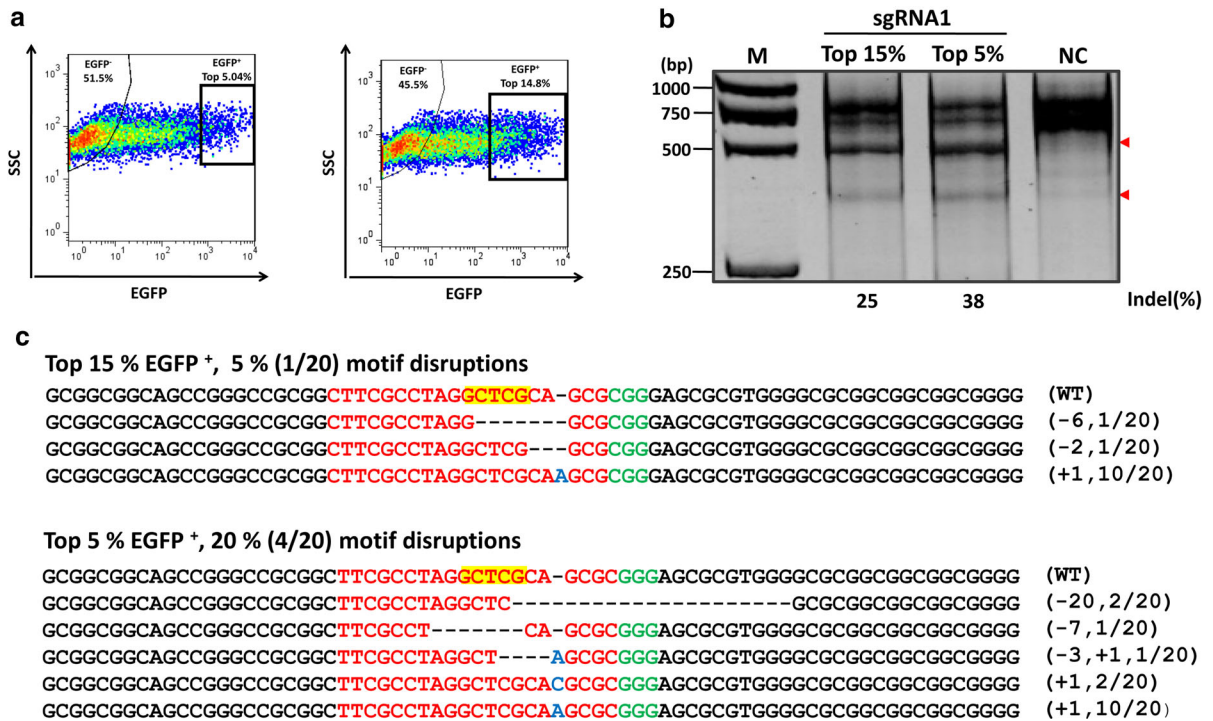
To disrupt the ZBED6-binding motif in intron 3 of *IGF2*, we designed two sgRNAs to target this motif on the *IGF2* *q* allele of Liang Guang Small Spotted pigs (Fig. 1a). The ZBED6 binding motif is within a DNA fragment that is GC-rich and hypermethylated, which could affect the targeting activities of the designed sgRNAs. Therefore, we employed an RGS reporter (He et al. 2016) to enrich PEFs with mutations induced by CRISPR/Cas9. T7E1 assays (Table S2) of genomic DNA purified from sorted EGFP- and DsRed-positive PEFs, revealed that sgRNA1 and sgRNA2 were able to induce non-homologous end joining at their target sites, with frequencies of 18% and 28%, respectively (Fig. 1b). Further clonal sequencing confirmed the targeting activities of these two sgRNAs, with indels induced by sgRNA1 and sgRNA2 reaching 30% (6/20) and 35% (7/20), respectively. The cutting site of sgRNA1 is one base closer to the ZBED6 binding motif and therefore, it seems to induce a higher frequency of on-motif disruption (10%) compared with sgRNA2 (5%, Fig. 1c). Therefore, sgRNA1 was used in subsequent experiments for obtaining PEFs with desired mutations.

sgRNA1 was then cloned into pX458, which co-expresses an EGFP reporter, and transfected into PEFs. PEFs with the top 15% and the top 5% of EGFP intensity were then sorted to evaluate whether fluorescence-assisted selection could improve the on-motif editing efficiency (Fig. 2a). T7E1 assays showed that collection of PEFs with the top 5% of

EGFP intensity was able to reach an editing efficiency of 38%, which was greater than the 25% editing efficiency found in PEFs with the top 15% of EGFP intensity (Fig. 2b). Further clonal sequencing revealed that, in PEFs with the top 5% of EGFP intensity, the frequency of ZBED6 binding motif disruption reached 20%, a great improvement from the 5% disruption of the binding motif in PEFs with the top 15% of EGFP intensity (Fig. 2c). This indicates effective enrichment of PEFs with the desired mutations in the ZBED6 binding motif, based on fluorescence selection.

Disruption of ZBED6 binding site enhances myogenic potential and proliferation ability of PEFs from Liang Guang Small Spotted pigs

*IGF2* is a maternally imprinted growth factor, with the paternal allele expressed and the maternal allele shut off. In order to deliver the edited paternal allele to progenies when we have generated the *IGF2*-edited pigs, PEFs derived from male foetuses of Liang Guang Small Spotted pigs were identified through PCR amplification of the *SRY* gene (Fig. S1 and Table S4). #2 PEFs, derived from a male foetus, was selected for subsequent experiments. PEFs are of mesodermal origin. Mesoderm forms muscles during the myogenesis process. Therefore, we examined whether *IGF2*, as a regulator involved in myogenesis, is expressed in PEFs. RT-PCR results clearly indicate that *IGF2* and *ZBED6* are expressed in PEFs (Fig. S2). Next, we checked whether disruption of the ZBED6 binding motif is able to enhance the expression of *IGF2* (Fig. 1a). #2 PEFs were transfected with pX458-*IGF2*-sgRNA1, and cells with top 5% of EGFP intensity were sorted for quantitative PCR analysis. Disruption of the ZBED6 binding motif increased *IGF2* transcription by several hundred fold. This result was confirmed by western blot analysis of *IGF2* protein expression in edited cell populations (Fig. 3a). Previous studies have shown that IGFs promote MYOD1 expression in C<sub>2</sub>C<sub>12</sub> cells (Hsu et al. 1997). MYOD1 belongs to the MRF family, which plays an important role in vertebrate skeletal muscle cell differentiation. Therefore, it is considered to be a critical marker for the detection of myogenic potential. Both qRT-PCR and western blot analysis demonstrated that MYOD1 expression was significantly up-regulated in *IGF2*-edited cell populations (Fig. 3b), implying that *IGF2*-edited PEFs have enhanced

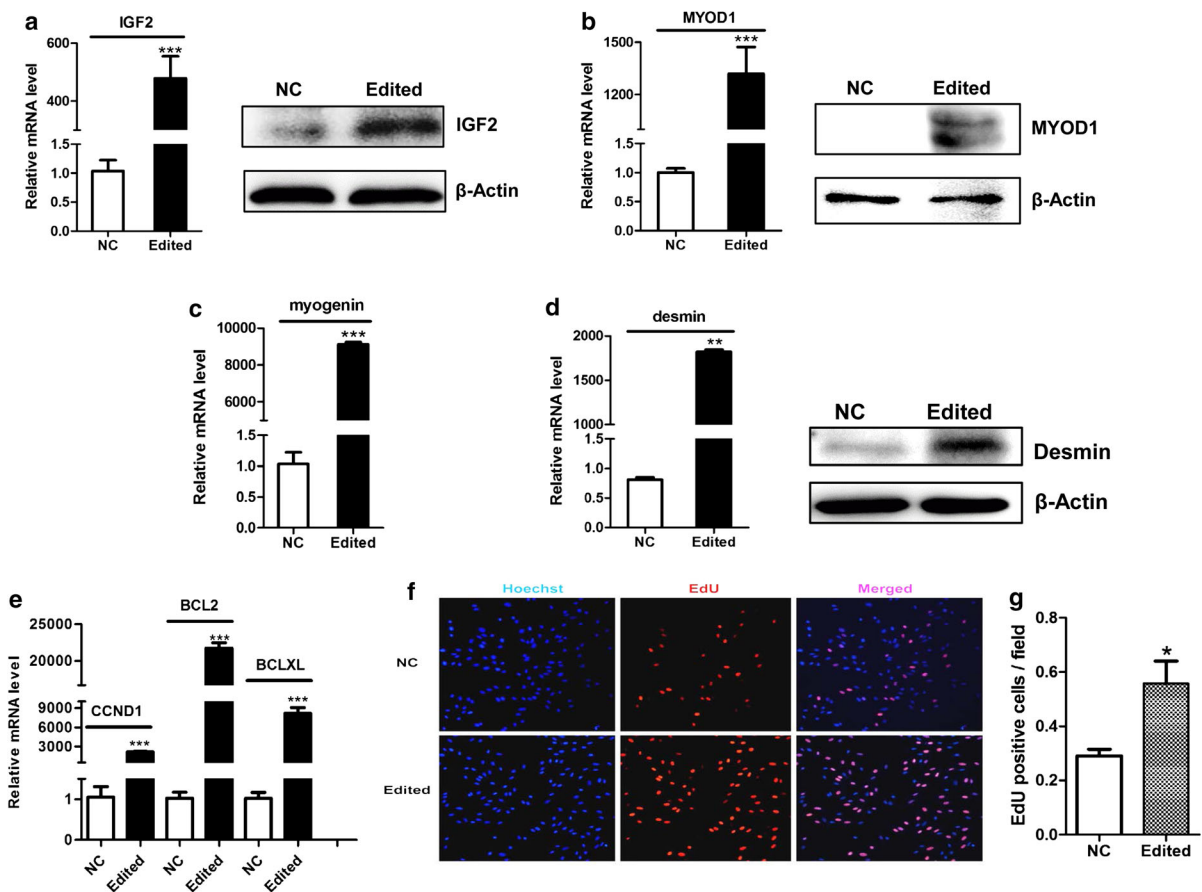


**Fig. 2** Enriching *IGF2*-edited PEFs through fluorescence-assisted selection. **a** Images of flow cytometry scatter plots showing the collection of transfected PEFs with the top 5% and top 15% EGFP fluorescence intensities. **b** The frequency of CRISPR/Cas9-induced mutations in PEFs with the top 5% and top 15% of EGFP fluorescence intensity were determined by T7E1 assay. The numbers at the bottom of the gel indicate mutation percentages estimated from band intensities using Image J. Red arrow heads indicate the expected positions of

DNA bands cleaved by mismatch-sensitive T7E1. **c** DNA sequences of the wild-type (WT) and mutant clones, with sgRNA recognition sites shown in red and the protospacer adjacent motif (PAM) sequence in green. The ZBED6 binding site motif is shown in a yellow box. Dashes and lower-case blue letters indicate deleted and inserted bases, respectively. The number of occurrences is shown in parentheses. Mutation frequencies were obtained by dividing the number of mutant clones by the total number of clones. (Color figure online)

myogenic potential. This prompted us to further study the impact of ZBED6 binding motif disruption on the expression of the myotube-formation-related factors, myogenin and desmin. As expected, the transcriptional levels of both factors were significantly enhanced, as revealed by qRT-PCR analysis. An increase in desmin protein levels was also detected by western blot analysis (Fig. 3c, d). *IGF2* also plays an important role in cell proliferation and apoptosis. Thus, we examined the impact of ZBED6 binding motif disruption on the expression of cell proliferation and apoptosis related factors, including cyclinD1 and the anti-apoptosis factors, *BCL2* and *BCLXL*. qPCR results indicated that these marker genes were all significantly up-regulated in *IGF2*-edited PEFs (Fig. 3e), thus implying that disruption of the ZBED6 binding motif inhibited cell apoptosis and promoted

cell proliferation. Therefore, we further characterized cell proliferative ability by EdU assay. The ratio of EdU-positive cells to EdU-negative cells in *IGF2*-edited PEFs was nearly twice the ratio in non-edited PEFs (Fig. 3f, g). These results indicated that disruption of the ZBED6 binding site enhanced the in vitro myogenic potential and proliferation ability of PEFs from Liang Guang Small Spotted pigs, which strongly implies that it could also enhance myogenesis and muscle development in vivo. This prompted us to generate *IGF2*-edited Liang Guang Small Spotted pigs using SCNT.



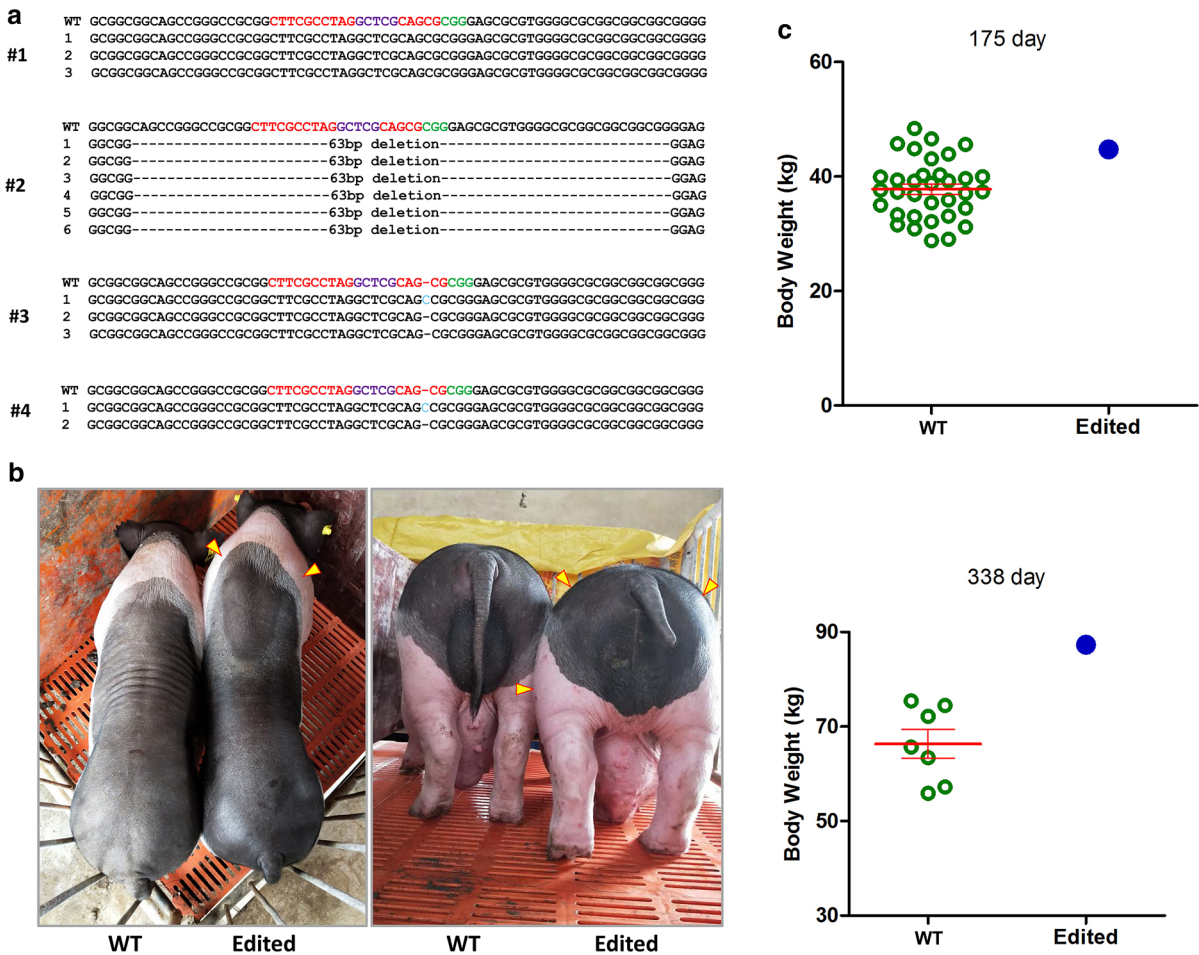
**Fig. 3** Evaluation of the myogenic potential and proliferation ability of *IGF2*-edited PEFs. **a** qRT-PCR and western blot analysis of *IGF2* expression in *IGF2*-edited PEFs. **b** qRT-PCR and western blot analysis of the expression of the myogenic marker, *MYOD1*, in *IGF2*-edited PEFs. **c** qRT-PCR analysis of the expression of the myotube-formation-related factor, myogenin, in *IGF2*-edited PEFs. **d** qRT-PCR and western blot analysis of the expression of the myotube-formation-related

factor, desmin, in *IGF2*-edited PEFs. **e** qRT-PCR analysis of the expression of apoptosis-related genes, cyclinD1, *BCL2*, and *BCLXL* in *IGF2*-edited PEFs. **f** Cell proliferation ability of *IGF2*-edited PEFs was determined by EdU assay. Cells active in DNA replication were stained with EdU, as shown by red fluorescence. **g** The percentage of EdU-positive cells was counted from the image. (Color figure online)

### Production and genotyping of *IGF2*-edited Liang Guang Small Spotted pigs

To generate *IGF2*-edited Liang Guang Small Spotted pigs using SCNT, #2 PEFs were transfected with pX458-*IGF2*-sgRNA1 and cells with the top 5% of EGFP intensity were sorted as donors for SCNT, to generate reconstructed embryos. A total of 4515 reconstructed embryos were then transferred to 20 surrogate pigs. Six surrogate sows became pregnant, and 34 male piglets were delivered, with 24 born alive. However, only 11 remained healthy in the first week and only 4 of these remained healthy for a biopsy after two weeks (Table S5). Genomic DNA was purified

from piglet ear biopsies and was used for PCR and clonal sequencing. The sequencing results showed that piglets #2, #3, and #4 harboured indels induced by CRISPR/Cas9 in intron 3 of *IGF2*. However, only piglet #2 showed disruption of the ZBED6 binding motif on both alleles (Fig. 4a). Unfortunately, piglets #3 and #4 died at an early stage, due to improper nursing. Thus, we were unable to determine whether modification outside the ZBED6 binding motif affects pig muscle development. However, piglet #2 remained healthy and presented with increased muscle size, especially on the rump, shoulder, and legs, at around 4 months of age (Fig. 4b). At 175 d old, this founder male pig was 18% heavier than the average



**Fig. 4** Genotyping *IGF2*-edited piglets. **a** DNA sequences of the wild-type (WT) and *IGF2*-edited piglets, with CRISPR/Cas9 recognition sites shown in red and the protospacer adjacent motif (PAM) sequence in green. Dashes and lower-case blue letters indicate deleted and inserted bases, respectively. **b** Image of *IGF2*-edited pig #2 and a WT pig, both at approximately

4 months of age. The more muscular parts of the *IGF2*-edited pig are indicated by yellow arrow heads. **c** Body weight measurements of the WT pig and *IGF2*-edited pig #2 at approximately 6 months of age (top) and approximately 11 months of age (bottom). Results for WT pigs are shown as mean ± SEM. (Color figure online)

body weight of wild-type pigs at a similar age, and at 338 days, it was 32% heavier than wild-type pigs (Fig. 4c and Table S6). This confirms that disruption of the ZBED6 binding motif had an important impact on porcine muscle development in vivo.

**Discussion**

Currently, most livestock genome editing focuses on disruption of coding regions, whereas editing regulatory sequences is reported less frequently (Tan et al. 2016). Our study strongly supports the findings of Leif

Anderson’s group, who identified ZBED6 as a special transcriptional inhibitor that binds to a conserved motif (GCTCG) in intron 3 of the porcine *IGF2* gene and inhibits its expression. They proposed that ZBED6 repression may be released by germline or somatic mutations at target sites in the binding motif (Markljung et al. 2009). Our in vitro study showed that disruption of the ZBED6 binding motif by CRISPR/Cas9 in 20% of PEFs from Liang Guang Small Spotted pigs, led to a significant up-regulation of *IGF2* expression and enhanced myogenic potential and cell proliferation. In addition, the enhanced muscle development observed in a piglet with the



ZBED6 binding motif completely deleted on both paternal and maternal alleles, showed that the release of ZBED6 repression has a major effect on muscle growth in Liang Guang Small Spotted pigs (Fig. 4b).

As the cutting site of Cas9 nuclease is several bases away from the ZBED6 binding motif, most editing events resulted in indels outside of the binding motif and precise disruption of the binding motif was difficult. Therefore, we applied fluorescence-assisted selection to enrich for precise targeting of the ZBED6 binding motif and obtained 20% on-target efficiency, as compared to 80% total editing efficiency (Fig. 2c). The marked up-regulation of IGF2 expression in PEFs with only 20% of cells presenting precise binding motif disruption suggests that the modification of the flanking sequence of the binding motif may also have an effect on the release of ZBED6 repression. This is supported by the recent findings published while our paper was under review, showing that indels induced by CRISPR/Cas9 in the region surrounding the ZBED6 binding motif have a similar effects on porcine growth as targeted disruption on the ZBED6 binding motif (Xiang et al. 2018).

As an indigenous Chinese pig breed, Liang Guang Small Spotted pigs have not been subjected to intensive selection for growth and carcass traits and the frequency of the *IGF2* q allele is fixed in the population (Yang et al. 2006). Our study clearly showed that precise modification of the q allele by disrupting the ZBED6 binding site motif is able to increase the expression of IGF2 in vitro and increase body weight and enhance muscle development in vivo. The phenotype of the single male founder pig is similar to that observed in a knock-in mouse model carrying a mutation of the porcine q allele (Younis et al. 2018). The percentage of cloned, genome-edited pigs born alive (Table S5) was in line with our expectations (unpublished data), but most pigs did not survive their early stage of life. In contrast to Western commercial breeds, the Liang Guang Small Spotted piglets are more susceptible to swine enzootic pneumonia (SEP). The piglets in this study were delivered by Large White surrogates on a farm where workers are mainly experienced at breeding Western commercial pigs. Therefore, the lack of the experience with nursing these genome-edited piglets, resulted in most of them dying of SEP at an early stage, with only one male founder pig remaining. Therefore, a more systematic analysis of the effect of ZBED6 binding

motif disruption on the expression of IGF2 in skeletal muscle, muscle fibre formation, skeletal muscle mass, meat production, internal organ size, and subcutaneous fat deposition need to be performed once a considerable progeny population of piglet #2 is established.

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