Correction

GENETICS

Correction for "Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases," by Janet Hauschild, Bjoern Petersen, Yolanda Santiago, Anna-Lisa Queisser, Joseph W. Carnwath, Andrea Lucas-Hahn, Lei Zhang, Xiangdong Meng, Philip D. Gregory, Reinhard Schwinzer, Gregory J. Cost, and Heiner Niemann, which appeared in issue 29, July 19, 2011, of *Proc Natl Acad Sci USA* (108:12013–12017; first published July 5, 2011; 10.1073/pnas.1106422108).

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Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases

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Zinc-finger nucleases (ZFNs) are powerful tools for producing gene knockouts (KOs) with high efficiency. Whereas ZFN-mediated gene disruption has been demonstrated in laboratory animals such as mice, rats, and fruit flies, ZFNs have not been used to disrupt an endogenous gene in any large domestic species. Here we used ZFNs to induce a biallelic knockout of the porcine α1,3-galactosyltransferase (*GGTA1*) gene. Primary porcine fibroblasts were treated with ZFNs designed against the region coding for the catalytic core of *GGTA1*, resulting in biallelic knockout of ∼1% of ZFN-treated cells. A galactose (Gal) epitope counter-selected population of these cells was used in somatic cell nuclear transfer (SCNT). Of the resulting six fetuses, all completely lacked Gal epitopes and were phenotypically indistinguishable from the starting donor cell population, illustrating that ZFN-mediated genetic modification did not interfere with the cloning process. Neither off-target cleavage events nor integration of the ZFN-coding plasmid was detected. The *GGTA1*-KO phenotype was confirmed by a complement lysis assay that demonstrated protection of *GGTA1*-KO fibroblasts relative to wild-type cells. Cells from *GGTA1*-KO fetuses and pooled, transfected cells were used to produce live offspring via SCNT. This study reports the production of cloned pigs carrying a biallelic ZFN-induced knockout of an endogenous gene. These findings open a unique avenue toward the creation of gene KO pigs, which could benefit both agriculture and biomedicine.

fetal fibroblasts | xenotransplantation | off target sites | complement mediated lysis assay

The knockout of an endogenous gene is an extremely valuable tool for analyzing gene function and for generating new animal models that mimic human genetic disease (1). In the realm of xenotransplantation, knockout of several immune-related genes has been shown to improve long-term survival of xenografts (2). However, traditional gene disruption techniques such as homologous recombination (HR) are very inefficient and time consuming due to a low over-all disruption frequency and the requirement for a long drug selection step. Moreover, the production of KO animals by HR is associated with the permanent introduction of an antibiotic cassette to select for the very rare (10^{-6}–10^{-5}) (3, 4) KO events. Until recently, gene KO technology in mammals has thus been restricted exclusively to mice, where germ line-competent embryonic stem cells are available.

Gene knockout has been achieved in fruit flies, zebrafish, and rats by direct injection of embryos with DNA or mRNA coding for zinc-finger nucleases (5–10). In these experiments, the frequency of targeting a single allele ranged from 7 to 46%, with approximately one-third of mutations creating single-step biallelic knockouts. Zinc-finger nuclease (ZFN)-mediated biallelic knockout of an endogenous gene in large animals (including pigs) has not yet been reported.

Pigs are a preferred species for creating new large-animal models of human diseases and for producing functional xenografts because they share important features of physiology, anatomy, and lifespan with humans (11). For example, $CFTR^{-/-}$ piglets made via conventional KO technology and cloning showed similar clinical and pathological symptoms as human neonates with cystic fibrosis (1). In contrast, knockout of the *CFTR* gene in mice was not associated with a phenotype akin to cystic fibrosis in humans (12). It is therefore desirable to improve the process of pig gene knockout to hasten development of models for human diseases that are poorly recapitulated in rodents.

The use of porcine organs for pig-to-human transplantation is considered the most promising solution to overcome the growing shortage of human grafts for allotransplantation. Unfortunately, the α1,3-galactosyltransferase gene encodes for the enzyme responsible for the generation of Gal epitopes on the cell surface of all porcine tissues. Gal epitopes are the major antigen in a pig-to-primate transplantation, resulting in a hyperacute rejection (HAR) response mediated by preformed antibody binding (13). In a major milestone toward long-term survival of porcine xenografts, the gene for the α1,3-galactosyltransferase was successfully knocked out with conventional gene-targeting technology (14–17). The use of kidneys and hearts from *GGTA1*-KO pigs in a pig-to-baboon xenotransplantation model led to significantly improved organ survival (18, 19).

Here we demonstrate that, compared with traditional knockout technology, ZFN-mediated gene targeting dramatically increases the frequency of biallelic disruption of the porcine α1,3-galactosyltransferase gene. ZFNs were designed to target the DNA coding for the catalytic domain of the α1,3-galactosyltransferase enzyme and were transfected into primary pig fibroblasts. We show that these ZFN-treated cells can be successfully used in somatic cell nuclear transfer and result in liveborn *GGTA1*-null piglets. The ZFN-induced *GGTA1*-KO mediates similar protection against human complement-mediated lysis as *GGTA1*-KO cells previously derived by conventional HR.

Results

Design of ZFNs and Creation of *GGTA1*-KO Cells. ZFNs were designed to target the portion of *GGTA1* coding for the catalytic region of the enzyme (exon 9). The most active ZFN pair cleaved 8% of *GGTA1* alleles when assayed in transformed pig cells at 37 °C and 34% of alleles when assayed at 30 °C (Fig. 1) (20). As about one-third of the total Surveyor nuclease signal typically occurs in cells modified on both alleles, this frequency likely corresponds to ∼2.5% and ∼11% *GGTA1*-null cells, respectively. When transfected into primary pig fibroblasts suitable for somatic cell nuclear transfer (SCNT), FACS analyses of isolectin B4-FITC–stained cells revealed that 1% of the ZFN-treated cells were completely Gal^- (Fig. 2). ZFN-treated cells were subjected to counterselection with the lectin and the remaining cells assayed for *GGTA1* gene disruption and Gal expression. The Surveyor

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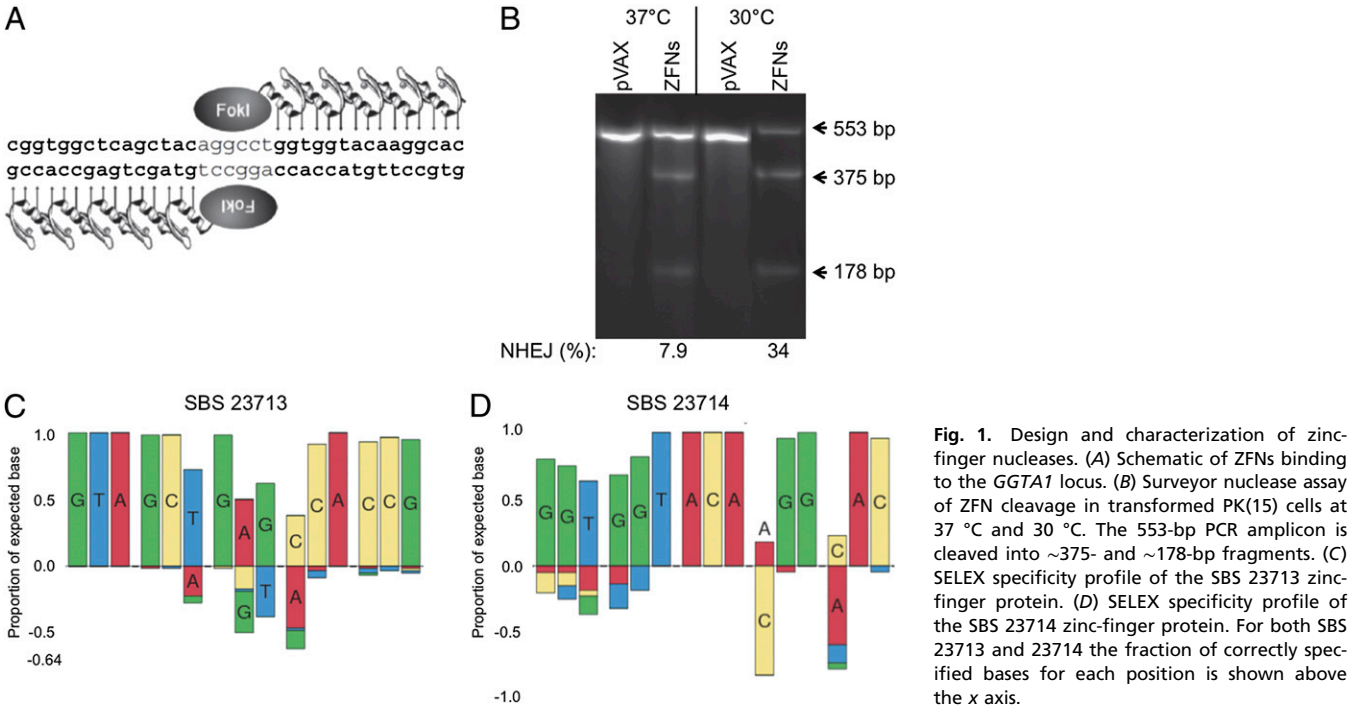

Fig. 1. Design and characterization of zinc-finger nucleases. (*A*) Schematic of ZFNs binding to the *GGTA1* locus. (*B*) Surveyor nuclease assay of ZFN cleavage in transformed PK(15) cells at 37 °C and 30 °C. The 553-bp PCR amplicon is cleaved into ~375- and ~178-bp fragments. (*C*) SELEX specificity profile of the SBS 23713 zinc-finger protein. (*D*) SELEX specificity profile of the SBS 23714 zinc-finger protein. For both SBS 23713 and 23714 the fraction of correctly specified bases for each position is shown above the *x* axis.

nuclease assay of a *GGTA1* PCR product showed a large enrichment of ZFN-induced mutations in the targeted region; FACS analysis revealed that these cells were 99% Gal$^-$cells.

Somatic Cell Nuclear Transfer and Cloned Transgenic Piglet Production. This pool of Gal$^-$ cells was used as a source of donor nuclei for SCNT. To obtain a preliminary assay of *GGTA1* gene knockout and cloning, the first pregnancy was killed at day 25 and six morphologically normal fetuses were harvested (an additional three fetuses were degraded and not analyzable) for initial analysis of the *GGTA1* gene and to obtain an additional pure population of KO cells for further cloning experiments. As these fetuses were found to be *GGTA1*-null (see below), cells from fetuses C1F2, C1F5, and C1F6 were used as nuclei donors in a second round of SCNT, from which four pregnancies were obtained. Finally, a fifth pregnancy was established by using a pool of primary transfected and selected cells (Table 1). In total, five of nine established pregnancies went to term, yielding nine live Gal$^-$ piglets, with birth weights ranging between 475 and 1,400 g (Fig. 3). Recloning of fetus C1F5 led to birth of two piglets. The smaller one (RC1F2) was killed 1 h after birth and the bigger one (RC1F1) was killed due to asphyxia about 48 h after birth. One piglet obtained by recloning of C1F6 (RC2F1) died 3 d after birth. One cloning experiment resulted into two live piglets, of which the smaller one (C2F2) was killed after birth; the bigger one (C2F1) developed normally. The recloning of C1F2 cells resulted in two stillborn and four live piglets (800–1,400 g body weight) of which two are still alive.

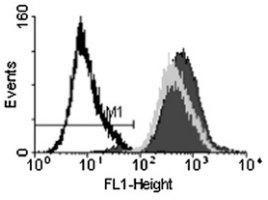

Fig. 2. 1.7% of the transfected cells (E38, not used for selection; dark gray) are located in the area where Gal$^-$ cells (black line) are located (Gal$^+$ cells, light gray line).

Analysis of Cloned Fetuses and Piglets. FACS analysis of cells from the six cloned fetuses (C1F1–F6), two cloned piglets (C2F1 and C2F2), and all recloned piglets (RC1F1, RC1F2, RC2F1, RC4F1–3, and RC5F1–3) revealed an absence of Gal epitopes in all clones (Fig. S1), illustrating that ZFN-mediated genetic modification did not interfere with the cloning process. DNA analysis of the α1,3-galactosyltransferase gene of the cloned fetuses showed a heterozygous band pattern for fetuses C1F1, C1F2, C1F3, and C1F5, whereas fetuses C1F4 and C1F6 gave a homozygous cleavage pattern (Fig. S2*A*). Analysis of piglets cloned from primary transfected cells revealed that C2F1 carried a heterozygous and C2F2 a homozygous mutation (Fig. S2*B*). DNA sequencing of the ZFN target region in *GGTA1* in all cloned fetuses and piglets confirmed mutation of the gene (Fig. 4). All recloned piglets (RC1F1, RC1F2, RC2F1, RC4F1–3, and RC5F1–3) were found to have the same mutations as their donor nuclei.

To ensure that ZFN plasmid DNA did not integrate into the pig genome, we developed an assay for plasmid integration on the basis of PCR amplification of the FokI nuclease domain. Integration of the ZFN plasmid was not detected in DNA from any fetal cell or piglet (Fig. S3). Furthermore, we determined the specificity profiles of the zinc-finger proteins and performed a computational scan of the pig genome to identify the 10 most likely off-target sites (OTS) of ZFN cleavage. Assay of these 10 loci by Surveyor nuclease showed no cleavage products, indicating that no unwanted mutations had occurred at these genomic sites (Fig. S4, showing the results of six analyses).

Susceptibility of *GGTA1*-Null Fetal Fibroblasts to Antibody/Complement-Mediated Lysis. ^{51}Cr-release assays were performed to study whether absence of Gal epitopes on ZFN-*GGTA1*-KO fibroblasts results in protection of the cells from lysis by human antibody/complement. As expected, control fibroblasts (wild type) were readily destroyed by increasing concentrations of human antibody/complement (Fig. 5). Some variability in specific lysis was found among the six tested ZFN-*GGTA1*-KO fibroblast lines, even between lines that originated from the same cell clone (C1F1, C1F3,

Table 1. Somatic cloning and recloning results using ZFN modified transgenic porcine cells with number of transferred embryos, pregnant sows, born piglets, and cloning efficiency

Cloning date	Recipient	Donor cells	No. of transferred reconstructed embryos	Pregnant sows	Stillborn	Liveborn	Cloning efficiency, %	Piglet number and birth weight
6/23/10	331/25	E41	95	Killed on day 30			6.3	C1F1–F6
2/2/11	G77	C1F1	98	Killed on day 30			2.0	RC3F1+2
6/23/10	331/29	E41	90	Resorbed	—	—	0	
8/11/10	299/45	C1F2	96	Resorbed	—	—	0	
8/18/10	293/47	C1F5	98	Delivered	0	2	2.0	RC1F1 (1,200 g), RC1F2 (475 g)
	178/100	C1F5	100	Resorbed	—	—	0	
8/25/10	322/44	C1F5	100	—	—	—	0	
9/1/10	K357	C1F2	90	—	—	—	0	
	359/59	C1F2	81	—	—	—	0	
9/8/10	K356	C1F6	85	Delivered	0	1	1.2	RC2F1 (800 g)
	335/23	C1F6	79	Resorbed	—	—	0	
9/9/10	328/88	E45	90	—	—	—	0	
	358	E45	92	Delivered	0	2	2.2	C2F1 (850 g), C2F2 (475 g)
12/15/10	291/59	C1F2	90	Delivered	2	1	3.3	RC5F1–F3 (400–800 g)
	319/38	C1F2	85	Delivered	0	3	3.3	RC4F1–F3 (800–1,400 g)
Total	*n* = 15		1,369	11/15 = 73.3% pregnancy rate	2 (18%)	9 (81%)	In average: 1.4	

and C1F5). Fibroblasts from piglet C2F1 showed an identical pattern in this assay. In all cases, there was no dose-dependent increase and even at the highest antibody/complement concentration (1:4 dilution), lysis did not exceed 10%. Compared with 40% of specific lysis found in wild-type cells, these data clearly show that elimination of Gal epitopes by ZFN-mediated *GGTA1* knockout leads to effective protection. Protection from antibody/complement-mediated lysis was also observed in control HR-derived *GGTA1*-KO fibroblasts. The lysis assays using different ZFN-*GGTA1*-KO fibroblast lines and HR-*GGTA1*-KO control cells were performed four times. In the experiment depicted in Fig. 5, lysis of some ZFN-*GGTA1*-KO cells was indeed slightly higher than in the controls. This difference, however, is within the normal range of variability of the test system. In different experiments we also observed lysis of HR-*GGTA1*-KO cells (e.g., at a 1:4 dilution of serum) that ranged from 0 to 12%.

Discussion

Here, we demonstrate the feasibility of a ZFN-mediated biallelic knockout of the porcine α1,3-galactosyltransferase gene and the successful production of cloned pigs by SCNT using ZFN-targeted cells. ZFN-mediated KO is superior to conventional targeting due to its high efficiency and its ability to achieve a biallelic KO via a single transfection. It is thought that a pig suitable for xenotransplantation will require multiple genetic modifications. Several selection cassettes would be necessary to screen for cells that carry all desired genetic modifications using traditional technology. An advantage of using ZFNs is that neither an antibiotic selection cassette nor often deleterious drug-based selection for cells with the desired genetic modification is necessary. Furthermore, de novo methylation of the selection construct can result in silencing of the locus. Use of ZFNs to produce knockout animals avoids this phenomenon.

We found no evidence for unwanted modification of the pig genome: neither integration of the ZFN plasmid nor cleavage at the 10 most-likely off-target sites were detected. Whereas this assay does not rule out off-target ZFN cleavage at other loci, this specificity profile, combined with the otherwise normal physiology of the cloned pigs makes this unlikely. Finally, the 1% frequency of biallelic KO observed in our primary cell population is more than 10,000-fold higher than the HR targeting efficiency reported previously (3).

We enriched for Gal^- cells by magnetic bead-based removal of Gal^+ cells (17). Compared with fluorescence-activated cell sorting (FACS), magnetic bead separation achieved a similar sorting efficiency but much better cell viability. After counterselection, Gal^- cells grew normally and were used as donor cells for SCNT. Six normal fetuses were obtained, a typical number for pregnancies with cloned porcine embryos. The presence of three degraded fetuses was likely due to selection in the uterus against false or incomplete nuclear reprogramming (21). On the basis of our previous extensive experience with the somatic cloning of transgenic porcine cells (2, 22, 23) we conclude that the relatively high rate of embryonic loss in this experiment is likely due to the SCNT procedure and not ZFN treatment. Pigs carrying a *GGTA1*-KO are known to suffer from low birth weights, a low cloning efficiency, and health problems (14). The birth of nine live piglets out of 9 pregnancies resulting from 15 transfers is superior to previous reports, using eight recipients, resulting in four births and three live offspring carrying a monoallelic *GGTA1*-KO (17) or 48 transfers of *GGTA1* null embryos resulting in 17 pregnancies and six born piglets (24).

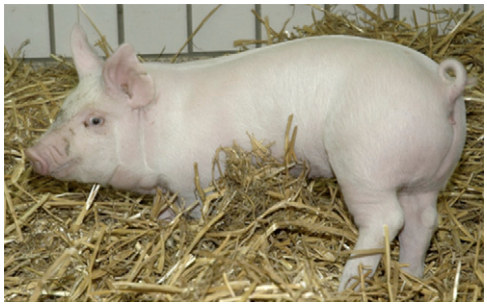

Fig. 3. Live-born piglet C2F1 (Lia), 3 wk old.

Cloned fetuses C1F1, C1F3, and C1F5 and fetuses C1F4 and C1F6 carried the same mutations. Because independent generation of the exact same mutations on both alleles is unlikely, these fetuses probably originated from the same transfected cell clone. Homozygous mutations at *GGTA1* (such as C1F4, C1F6, and C2F2) might result from mutagenic repair of the first allele by nonhomologous end joining followed by homology-directed repair of the second allele using the imperfectly repaired first allele as a template. Alternately, the small deletion of one base may have happened by coincidence or by a biased DNA repair process at this locus. The type of mutations recovered are similar to previously observed ZFN-created alleles and include deletions and insertions (9, 10, 25–28). These small mutations (−7 bp to +4 bp) (Fig. 4) in the DNA coding for the catalytic domain of α1,3-galactosyltransferase caused a functional *GGTA1*-KO due to reading frameshift. A similar (but spontaneous) small change has been shown to give a complete knockout of the *GGTA1* gene (16).

FACS analysis confirmed that the six cloned fetuses and nine live-born piglets did not express any Gal epitopes on their cell surface. Correspondingly, all fetuses showed a significantly decreased susceptibility to complement-mediated lysis comparable to the HR-derived *GGTA1*-KO cells described previously (23, 29).

The injection of ZFN-encoding mRNA is potentially another way to obtain mutated offspring; however, this process is more suited to small animal experiments [e.g., rat (9) or zebrafish (7)] where the numbers of embryos and resulting offspring that can be studied is high and can compensate for the lower efficiency of biallelic gene disruption. Indeed, we performed one mRNA microinjection experiment, but did not obtain a KO fetus. Rather the method we have explored here combining SCNT and ZFN-mediated gene disruption appears both robust and portable because recently a ZFN-induced knockout of the eGFP transgene was achieved in primary porcine somatic cell cultures and hemizygous eGFP-KO pigs were produced (26, 30), and ZFN-targeting resulted in a KO of a single allele of the PPARγ gene (31). Our data reveal that the ZFN approach can successfully target all alleles of an endogenous porcine gene, eliminating the need for further rounds of targeting and/or breeding to homozygosity (a length proposition with large animals). It is now feasible to introduce genetic modifications into the porcine genome with high efficiency, paving the way for the establishment of a collection of porcine KO strains, similar to those made in mice. As the pig is in many ways much more closely related to humans than mice are, we anticipate that the pig will be increasingly used as a model of human disease.

In conclusion, the relatively high percentage of disruption of the two alleles (1%) demonstrates that ZFNs are active in porcine fetal fibroblasts and can be used to produce knockout pigs via SCNT. ZFNs can be engineered against a broad range of endogenous genes within a few weeks and ZFN-driven gene disruption and generation of KO pigs by cloning can be accomplished within 5 mo. Our success with this strategy enables a whole new range of transgenic technologies beneficial to both agriculture and biomedicine, ultimately leading to the generation of new therapies and drugs to treat human disease.

Fig. 4. *GGTA1* sequencing results of wild type (WT), six cloned fetuses (C1F1–6), and cloned piglets (C2F1 and C2F2). Recloned piglets carry mutations identical to the donor nuclei (C1F5 and -F6). Gray boxes indicate binding sites of ZFNs *GGTA1*-23713 and -23714. -, indicates base deletion. Base insertion is shown with the boxed arrow.

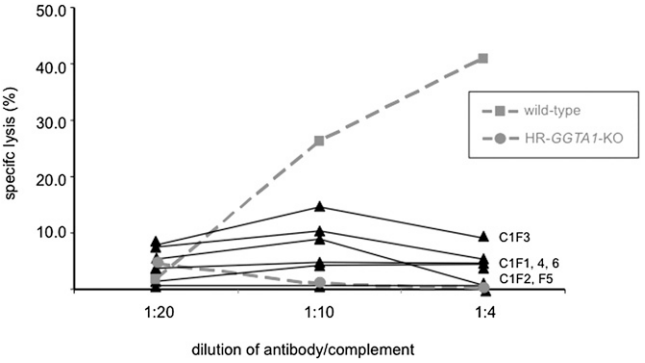

Fig. 5. Protection of ZFN-*GGTA1*-KO fibroblasts from antibody/complement-mediated lysis. ^{51}Cr-labeled fibroblasts from six cloned fetuses (C1F1–F6), from a wild-type control, and Gal-KO fibroblasts generated by conventional knockout (HR-*GGTA1*-KO) were incubated with increasing concentrations of human antibody/complement. The amount of radioactivity released into the supernatant was determined after 4 h by measuring an aliquot of 25 μL. The percentage of specific lysis was calculated as described in *Materials and Methods*. The data shown were obtained in a single experiment. Similar patterns were observed in a second and third experiment. The HR-*GGTA1*-KO sample is represented by one cell line.

Materials and Methods

Design of Zinc-Finger Nucleases. ZFNs designed to cleave a region of the *GGTA1* gene coding for the catalytic core of the enzyme were assembled as previously described using the EL/KK FokI heterodimer mutations (32, 33). ZFN cleavage was assayed in porcine PK(15) cells (American Type Culture Collection; CCL-33) by transfection of 400 ng of each individual *GGTA1* ZFN by Amaxa nucleofection with solution SF and program FF-137. Transfected cells were harvested after 2 d of growth at either 30 °C or 37 °C (20). ZFN activity was assayed by PCR of the *GGTA1* locus with primers GJC 200F (5′-CTA GAA ATC CCA GAG GTT AC-3′) and GJC 201R (5′-TCC TTG TCC TGG AGG ATT CC-3′) followed by Surveyor nuclease treatment as previously described (34). The first through fifth helices of the lead ZFNs are as follows: SBS 23713: RSDTLSE, QKATRIT, RSHNLAR, QSSDLSR, and QSGSLTR; SBS 23714: DQSNLRA, RSDHLSA, QSATRTN, TSGHLSR, and TSGHLSR.

Cell Culture and Transfection of Cells. Primary porcine fetal fibroblasts were established from a day 25 postconception fetus as previously described (2) and cultured for seven passages in Dulbecco's modified Eagle medium (DMEM) (PAA Laboratories) supplemented with 1% nonessential amino acids, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (AppliChem), 0.1 mM β-mercaptoethanol, 0.1 mM sodium pyruvate (Sigma-Aldrich), and 30% FCS (PAA Laboratories) (Batch nos. E00909-0027, E00910-0380, E00910-1556, and E00910-2368). A total of 3×10^6 cells were trypsinized (EDTA-Trypsin; PAA Laboratories), washed with PBS (Sigma), centrifuged, and finally suspended in 800 μL electroporation buffer (Bio-Rad) containing 7.5 μg DNA of each ZFN plasmid. Fetal fibroblasts were electroporated (Gene Pulser XcellTM; Bio-Rad) at 250 V/400 μF. After electroporation, cells were plated on three 25-cm^2 dishes. Transfected cells were incubated in humidified 95% air with 5% CO_2 at 37 °C for 7 d. Cells were cultured in DMEM supplemented with 30% FCS.

FACS Analysis. Seven days after electroporation, cells were analyzed by flow cytometry (FACScan; Becton Dickinson). A total of 1×10^6 cells were trypsinized, washed with PBS, and centrifuged. Cells were resuspended in 1 mL PBS and incubated with 3 μg Isolectine-B4-FITC (Enzo Life Sciences) for 5 min at 37 °C. After washing with PBS, cells were used for FACS analysis. Wild-type cells and HR-*GGTA1*-KO cells served as controls. Data were processed using WinMDI software.

Surveyor Nuclease Assay (Mutation Detection Assay). ZFN-induced mutations were detected using the Surveyor Mutation Detection kit (Transgenomic) in

accordance with the manufacturer's protocol (35) and reported recently (34). One week after electroporation, genomic DNA from ZFN-treated and control cells was extracted. PCR was performed using *GGTA1* primers ZFN-*GGTA*-F (5′-CTA GAA ATC CCA GAG GTT AC-3′) and ZFN-*GGTA*-R (5′-TCC TTG TCC TGG AGG ATT CC-3′) under the following conditions: 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 57 °C for 45 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. DNA concentration was measured on an ethidium bromide-stained agarose gel (1%). Subsequently, sample DNA alone and equal amounts of sample DNA plus wild-type DNA were mixed, denatured, and allowed to rehybridize. Three hundred nanograms of DNA was incubated with 1 μL of Surveyor nuclease at 42 °C for 30 min. The product was separated on a polyacrylamide gel (9%) to confirm ZFN-induced mutations.

Counterselection of Gal$^-$ Cells. Gal$^-$ cells were selected by magnetic beads (Dynabeads; Invitrogen) as previously described (17). A total of 3×10^6 cells were harvested, washed, and incubated in 1 mL PBS supplemented with 5 μg biotin-conjugated Isolectine-B4 (Enzo Life Sciences) for 15 min on ice. Subsequently, cells were incubated with 600 μL streptavidin-conjugated Dynabeads for 30 min. Gal$^-$ cells were separated from Gal$^+$ cells in a magnetic field. The supernatant containing Gal$^-$ cells was transferred into a new tube. Cells were washed with PBS, centrifuged, and placed on one 96-well containing DMEM + 30% FCS.

SCNT. Somatic cell nuclear transfer was performed as described (22). Magnetic bead-selected cells served as donor cells for cloning experiments. One pregnancy was killed on day 25 of gestation to establish fetal fibroblast cell lines. Cloned fetuses were analyzed by FACS and Surveyor nuclease assay as described. Fetal cells served also as donor cells for recloning to obtain living offspring. Delivery was induced by injecting 0.006 mg dexamethasone on day 117 of gestation followed by a prostaglandin injection (Estrumate; Intervet) on day 119 of gestation. One pregnancy had to be terminated by cesarean section on day 120.

Sequencing *GGTA1* Gene of Cloned Fetuses. The PCR product of the targeted region within exon 9 of the *GGTA1* gene was subcloned into the pGEM-T Easy Vector system (Promega) in accordance with the manufacturer's protocol. For each fetus, three to six colonies were picked and sequenced using the T7prom primer.

Exclusion of Plasmid Integration into Genome. To exclude integration of the ZFN plasmid, FokI nuclease-specific PCR was performed using primers ZFN*Fok1*-F (5′-GGC ACA AGC TGA AGT ACG TG-3′) and ZFN*Fok1*-R (5′-TTG CAG TTG GTG ATG TGG TT-3′). The PCR product of 433-bp length (obtained with an annealing temperature of 60 °C) was separated on an ethidium bromide-stained agarose gel (1%).

Off-Target Sites (OTS) Analysis. The specificity of the ZFNs was assayed, the 10 most-likely sites of potential off-target ZFN cleavage were determined (36) and assayed for ZFN cleavage via the Surveyor nuclease assay (for PCR primers, Table S1). A *GGTA1* amplicon from one cloned fetus served as positive control for Surveyor nuclease activity.

Assay for Complement-Mediated Lysis of Porcine Fibroblasts ^{51}Cr-release assays were performed to study the susceptibility of *GGTA*1-null cells and controls to lysis by human antibody/complement. Porcine fibroblasts (1×10^6) were labeled with 100 μCi of sodium [^{51}Cr]-chromate (GE Healthcare; 1 Ci = 37 GBq) and plated at 1×10^4 cells per well in microtiter plates. After 18 h, dead fibroblasts were removed by "dumping." Cells were incubated with increasing concentrations (1:20, 1:10, and 1:4 dilution) of pooled complement-preserved normal human serum (Dunn Labortechnik). After 4 h, 25 μL of the cell supernatant was removed and the amount of radioactivity was measured in a Microbeta scintillation counter (Wallac). The mean cpm obtained in triplicate cultures was used for all calculations. The spontaneous release of ^{51}Cr was determined by incubating the target cells with medium alone, whereas the maximum release was determined by incubating in 2% Triton X-100. The specific lysis was calculated as follows: % specific lysis = (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release) × 100.

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