Efficient generation of B2m-null pigs via injection of zygote with TALENs

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Donor major histocompatibility complex class I (MHC I) molecules are the main targets of the host immune response after organ allotransplantation. Whether and how MHC I-deficiency of pig donor tissues affects rejection after xenotransplantation has not been assessed. Beta2-microglobulin (B2M) is indispensable for the assembly of MHC I receptors and therefore provides an effective target to disrupt cell surface MHC I expression. Here, we report the one-step generation of mutant pigs with targeted disruptions in B2m by injection of porcine zygotes with B2m exon 2-specific TALENs. After germline transmission of mutant B2m alleles, we obtained F1 pigs with biallelic B2m frameshift mutations. F1 pigs lacked detectable B2M expression in tissues derived from the three germ layers, and their lymphocytes were devoid of MHC I surface receptors. Skin grafts from B2M deficient pigs exhibited remarkably prolonged survival on xenogeneic wounds compared to tissues of non-mutant littermates. Mutant founder pigs with bi-allelic disruption in B2m and B2M deficient F1 offspring did not display visible abnormalities, suggesting that pigs are tolerant to B2M deficiency. In summary, we show the efficient generation of pigs with germline mutations in B2m, and demonstrate a beneficial effect of donor MHC I-deficiency on xenotransplantation.

Pigs are considered promising candidate donors for xenotransplantation because they share many anatomical and physiological features with humans, and have a large litter size and a relatively short gestational period. Their greater phylogenetic distance to human versus other potential donor organisms such as non-human primates may be associated with lower risk for cross-species disease transmission1. Rejection of the graft by immune-mediated mechanisms represents one of the main barriers to successful xenotransplantation. Various strategies have been pursued to genetically modify pigs to reduce immune incompatibility and to prevent host versus donor immune rejection after xenotransplantation. Approaches include pigs with transgenic expression of human proteins regulating the activation of complement, T cells, NK cells or other components of the immune system2–8, and pigs lacking a functional glycoprotein alpha-galactosyltransferase 1 (GGTA1) gene, which encodes the enzyme responsible for the expression of the immunogenic alpha 1,3-Gal epitope in non-primate species9,10.

Major histocompatibility complex class I (MHC I) peptides are cell surface molecules encoded by a large family of highly polymorphic genes. MHC receptors play a major role in the immune system of all vertebrates, and are the main target of the early alloimmune response after organ transplantation. Pig MHC I molecules have been classified into two groups11–13. The first group, termed swine leukocyte antigen 1a (SLA-1a), encompasses highly polymorphic and widely expressed proteins that are commonly recognized as the functional orthologues of the human classical MHC I antigens (HLA-A, HLA-B, and HLA-C). Proteins of the second group, SLA-1b, have been classified as non-classical MHC I antigens because these molecules exhibit limited polymorphism and restricted...
expression patterns, and their orthology or functional homology to human non-classical MHC I antigens, such as HLA-E, HLA-F and HLA-G, remains to be established.

Human CD8+ positive cytolytic cell subpopulations as well as NK cell subpopulations have been shown to directly recognize SLA-1a molecules, leading to the lysis of target cells. However, the relevance of porcine MHC I molecules in xenotransplantation is not fully understood and needs to be further explored by transplantation experiments with MHC I-deficient donor pig tissues. Porcine MHC I is a heterotrimeric complex consisting of a heavy α-chain, a light β-chain termed β2-microglobulin (B2M), and short peptides. The α-chain is highly polymorphic and is encoded by several genes that exist in multiple allelic variants. In contrast, the B2M molecule, which is indispensable to MHC I assembly on the cell surface, is non-polymorphic and encoded by a single gene. Therefore, B2m provides a simple and effective target to disrupt porcine MHC I expression on the cell surface.

Transcription activator-like effector nucleases (TALENs) are versatile genomic editing tools that have been successfully used in different species, including pigs. More recently, clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR-associated 9 systems (CRISPR/Cas9) have been developed that can mediate effective genome editing in a range of species. CRISPR/Cas9 systems can be readily modified and synthesized and allow for multiplex editing, whereas TALENs offer higher sequence specificity and are associated with a lower frequency of off-target effects. Here, we used TALEN technology to obtain a targeted disruption of pig B2m. Pigs harboring frameshift mutations in B2m were efficiently generated via cytoplasmic injection of zygotes with TALEN mRNA. Following germline transmission of mutant alleles, we obtained B2m null-mutant pigs, which were devoid of B2M in tissues derived from all three germ layers and lacked MHC I assembly on the cell surface of lymphocytes. These MHC I-deficient pigs can be used as donors to investigate the biological effects on xenotransplantation, and also provide a non-rodent model to better explore how MHC I receptors regulate immunity in various species.

Results and Discussion

Design and validation of TALENs targeting B2m. To disrupt B2M expression in pig, we designed a pair of TALEN molecules that target exon 2 of pig B2m (Fig. 1a). These B2m-TALENs did not have any predicted off-target sites (OTs) in the pig genome according to UCSC In-Silico PCR software (http://genome.ucsc.edu/cgi-bin/hgPcr). To assess B2m-TALEN-affected early stage development in pig embryos, we injected mRNAs encoding the TALEN pair (10 ng/mL each) into the cytoplasm of parthenogenetically activated (PA) pig embryos at the one-cell stage, followed by culture of embryos in vitro to the blastocyst stage. Injected PA embryos exhibited similar rates of cleavage and development to the blastocyst stage as control (untreated) PA embryos (Table 1). The slightly higher rate of blastocyst formation in microinjected embryos may be related to an increased influx of Ca2+ during micromanipulation, and is consistent with our previous observations of preimplantation development in Cas9/sgRNA-injected pig PA embryos.

To assess for TALEN activity at the B2m target locus, we isolated genomic DNA from individual embryos and employed the T7 endonuclease I (T7EN1) cleavage assay that detects heteroduplex DNA in PCR products amplified across the target site. T7EN1 cleavage bands were identified in 5 of 6 PA embryos (Fig. 1b,c). Sequencing of PCR products from the 5 embryos with T7EN1 cleavage bands identified indels and overlapping peaks in sequencing chromatographs (Fig. 1d,e; Table 2), indicating that the B2m-TALENs cleaved the target site. These data demonstrate that TALENs targeting pig B2m had no adverse effects on pig embryo development in vitro, and exhibited target-specific nuclease activity.

One-step generation of B2m knockout pigs via injection of zygotes with TALEN mRNAs. We chose to pursue the B2m knockout pig model in the Chinese Bama minipig breed. The Bama minipig is widely used as a large animal model for biomedical research, has a relatively high degree of genetic stability, and is relatively higher inbred. A total of 118 fertilized 1-cell stage embryos were recovered from the oviducts of mated sows, followed by cytoplasmic microinjection of B2m TALEN mRNAs (10 ng/mL each) and transfer of injected zygotes into eight estrus-synchronized recipient sows. Three recipients became pregnant, and one aborted. The remaining 2 recipient sows delivered 7 full-term piglets (Fig. 2a, Table 3). We isolated genomic DNA from ear punch tissue from the 7 piglets and amplified the genomic region surrounding the B2m TALEN target site by PCR. In all samples, a single band was obtained (Fig. 2b), and T7EN1 cleavage bands were detected in PCR samples of 6 of the 7 animals (Fig. 2c). Sequencing of the PCR products revealed indel mutations in the individuals with T7EN1 cleavage bands as expected (Fig. 2d, Fig. S1). Of the 6 mutant founder pigs, bi-allelic frameshift mutations were detected in 1 founder (#T5), mono-allelic null mutations in 2 founders (#T4 and #T6), and mosaicism with at least 3 different genotypes in 3 founders (#T1, #T2 and #T3) (Fig. 2e, Table 4). Interestingly, no in-frame mutation was detected in any of the founders (Fig. 2e, Fig. S1 and Table 4), suggesting a potential bias towards double strand break (DSB)-induced mutations at this site.

Germline transmission of mutant B2m alleles. The two founder pigs (#T1 and #T5, Table 4), in which frameshift, but no other types of mutations were detected and affected both alleles, indicated by the absence of a wild-type allele, did not exhibit any obvious phenotypic abnormalities and grew normally into adults, suggesting that the lack of a B2M molecule had no apparent adverse effects on the development or health of these animals. However, gene-modified founder animals produced by the injection of customized engineered endonucleases into zygotes often exhibit mosaicism for the targeted mutations. Therefore, genetically unmodified cells may be present in various tissues of the founders and functionally compensate for the B2m null mutant cells, masking potential phenotypic manifestations. To address whether pigs were truly tolerant of B2M-deficiency and whether the B2m mutations could be transmitted through the germline, we mated the female founder pig #T1, harboring three different frameshift mutations and no wild type target sequence according to ear tissue analysis, with the male founder #T6, which should produce offspring with bi-allelic B2m-null mutations. We obtained
4 piglets in the F1 generation (F1 piglets) and performed genetic analysis as before. No additional bands were detectable in PCR reactions amplifying across the \(B2m\) target area, and T7EN1 cleavage bands were detected.
inability of B2M may affect pig MHC I assembly on the cell surface in a dose-dependent manner, similar to findings type control peak, suggesting that haploid expression of B2M also reduced MHC I complex assembly. Thus, avail-

B2m deletions was shifted in intensity towards the iso-

SLA-1 peak of the sample from the pig with mono-allelic

with biallelic B2m pig and a mutant pig with mono-allelic

as a negative control, with a FITC-conjugated mouse IgG isotype antibody without known binding specificity. As

samples stained either using a FITC-conjugated mouse monoclonal antibody specific for pig MHC I (SLA-1) or,

whether the lack of B2M expression eliminated or reduced pig MHC I assembly on the cell surface, we collected

indicating that the frameshift

frameshift mutations were devoid of MHC I complexes on the cell surface. Interestingly, the

pig and a mutant pig with mono-allelic

Table 2. Summary of alleles from test embryos with B2m-TALEN-mediated modifications. Del: deletion; WT: wild type.

Table 1. Summary of test embryo microinjection of B2m-TALEN. COC: cumulus-oocyte complex.

Phenotypic analysis of B2m-null pigs. Germline transmission of mutant B2m alleles provided an opportu-
nity to verify absence of the B2M protein in various tissues and to evaluate the functional consequences of B2m null mutations on MHC I assembly and immune rejection after xenotransplantation. Using a monoclonal antibody specific for pig B2M protein, we performed immunohistochemical staining to investigate B2M expres-
sion in skin, liver, and intestine, which are derived from different germ layers. As shown in Fig. 4, B2M was not detectable in tissues from the F1 pig (#P2) with bi-allelic B2m mutations, but detected in wild-type control tissues, indicating that the frameshift B2m mutations effectively disrupted B2M protein expression. To further investigate whether the lack of B2M expression eliminated or reduced pig MHC I assembly on the cell surface, we collected peripheral lymphocytes from F1 animals and performed fluorescence-activated cell sorting (FACS) analysis of samples stained either using a FITC-conjugated mouse monoclonal antibody specific for pig MHC I (SLA-1) or, as a negative control, with a FITC-conjugated mouse IgG isotype antibody without known binding specificity. As shown in Fig. 5, the SLA-1 histogram of the sample from the F1 pig with bi-allelic null mutations of B2m completely overlapped with the isotype control peak, whereas distinct peaks were present in samples from a wild-type pig and a mutant pig with mono-allelic B2m deletions. These data indicate that lymphocytes from the mutant pig with biallelic B2m frameshift mutations were devoid of MHC I complexes on the cell surface. Interestingly, the SLA-1 peak of the sample from the pig with mono-allelic B2m deletions was shifted in intensity towards the iso-
type control peak, suggesting that haploid expression of B2M also reduced MHC I complex assembly. Thus, avail-
bility of B2M may affect pig MHC I assembly on the cell surface in a dose-dependent manner, similar to findings in B2m knockout mouse lymphocytes35. The above data demonstrated that the frameshift mutations caused by B2m-TALENs effectively disrupted B2M expression, and eliminated pig MHC I assembly on cell surface.

To assess the functional consequences of the absence of MHC I complex in pig tissues on xenotransplantation,

we performed skin grafting from pigs to mice as described previously31,32. For improved graft survival, grafted

skin pieces were covered with a dressing of several layers of gauze pieces and secured with a transparent film

dressing (Fig. 6a). Skin grafts from the F1 bi-allelically mutant pig (#P2) exhibited remarkably prolonged survival

compared to those from the littermate F1 pig lacking B2m mutation (#P214) (Fig. 6b,c and d). On day 6 post graft-
ing, the majority of grafts without B2m mutation exhibited necrosis, and grafts were almost completely rejected on day 10 post grafting, demonstrating that acute immune rejection against grafts occurred (Fig. 6c). In contrast, the majority of skin grafts with bi-allelic B2m mutations exhibited no detectable necrosis on day 10 post grafting (Fig. 6b). These data suggest that donor MHC I complex deficiency may be beneficial for xenotransplantation.


| Embryo No. | Target site mutation   |
|------------|------------------------|
| #1         | overlapped mutation    |
| #2         | WT                     |
| #3         | overlapped mutation    |
| #4         | overlapped mutation    |
| #5         | del 10bp; del 16bp     |
| #6         | del 5bp; del 7bp       |

460 965 452 230 222 49.1% (113/230) 47.3% (105/222) 16.1% (37/230) 14.4% (32/222)
However, compatibility may require the additional elimination of the genes encoding the alpha heavy chain of pig MHC I, i.e., pig classical MHC I molecules (SLA-1, 2, 3), to completely prevent intracellular MHC I expression. While this manuscript was in preparation, pigs with disruption of the classical MHC I molecules (SLA-1, 2 and 3) were reported. However, the presence of porcine B2m gene in these pigs would hamper any attempted humanization of MHC I, because endogenous porcine B2M could combine with human MHC I heavy alpha chains in hybrid complexes as observed in rodent models. Furthermore, the presence of pig non-classical MHC I molecules would be immunogenic after xenotransplantation. Therefore, the combined disruption of B2M and MHC I alpha heavy chains in pigs will be required to investigate the functional consequences of pig MHC I deficiency on xenotransplantation. Such MHC I-null pigs could be used for MHC I humanization with transgene encoding the human counterparts, especially non-classical MHC I molecules such as HLA-G, HLA-E and HLA-F, which are of limited immunogenicity for the human immune system due to their limited polymorphism and inhibitory function on human NK cell activation.

**Figure 2.** Detection of B2m-TALEN-mediated modifications of B2m in founder pigs. (a) A representative photo showing 52-day-old founder pigs carrying B2m mutations. (b) PCR products of the targeted region of B2m from founder pigs co-microinjected with a pair of TALEN mRNA. Eight piglets were born and one died soon after birth. The seven alive founders were named from T1 to T7. Con denotes wild-type pig as control. (c) Detection of TALEN-mediated on-target cleavage of B2m by T7EN1 cleavage assays. All PCR products from (b) were subjected to T7EN1 cleavage assays. All the founders except T7 could be digested by T7EN1 which suggests that these founders carry B2m mutations. (d) Sequencing results of modified B2m alleles detected in founder pigs. At least 16 TA clones of the PCR products were analyzed. Sequences targeted by left and right TALEN are labeled in red and green respectively. The mutations in blue, lower case; deletions (−), N/N indicates positive colonies out of total sequenced. See also Figure S1. (e) Summary of generated B2m mutant pigs by TALENs. Founders showed more than two genotypes are considered as mosaicism.
In summary, we demonstrate the efficient generation of B2m-null pigs after germline transmission of mutant alleles that were generated by the injection of zygotes with TALENs targeting exon2 of B2m. We find that the absence of B2M protein prohibits the assembly of MHC I on the cell surface but has no detectable adverse effects on pig development and health. Furthermore, our study also showed a possible beneficial effect of donor MHC I complex deficiency on xenotransplantation success.

**Materials and Methods**

**Animals.** The animals used in this study were regularly maintained in the Laboratory Animal Center of the Third Military Medical University. All the protocols involving the use of animals were in accordance with approved guidelines of the Institutional Animal Care and Use Committee of the Third Military Medical University (Approval ID: SYXK-PLA-2007036).

**Vector constructs and in vitro transcription.** B2m-TALENs were designed using TALEN-NT software and constructed by Golden Gate methods as previously described. pCS2-PEAS and pCS2-PERR were utilized as upstream and downstream TALEN-assembling backbones, respectively, as described. The CDS region of the TALEN vectors were cut out using SpeI and NheI, and subcloned into pcDNA3.1 vector to be under the drive of T7 promoter. The TALEN-expressing plasmid was linearized using Pmel, of which the cutting site was located at the 3′-end of the FokI domain. B2m-TALEN mRNA was prepared via in vitro transcription using the linearized plasmid as the templates with T7 Ultra Kit (Ambion, Austin, TX), and further purified by RNeasy Mini Kit (Qiagen).

**TALEN efficacy test via pig parthenogenetic embryo injection.** The efficacy of B2m TALENs was tested in pig parthenogenetically activated (PA) embryos. To prepare pig PA embryos, the cumulus-oocyte complexes (COCs) were collected from slaughter house and cultured for in vitro maturation as described.

| Injected materials | Injected embryos | Transferred embryos | Recipient amount | Established pregnancy | Piglets born |
|--------------------|------------------|---------------------|------------------|-----------------------|--------------|
| 1st B2m-TALEN mRNAs (10 ng/mL each) | 40 | 40 | 2 | 0 | 0 |
| 2nd B2m-TALEN mRNAs (10 ng/mL each) | 26 | 26 | 1 | 0 | 0 |
| 3rd B2m-TALEN mRNAs (10 ng/mL each) | 59 | 57 | 2 | 2 | 5 |
| 4th B2m-TALEN mRNAs (10 ng/mL each) | 22 | 22 | 1 | 0 | 0 |
| 5th B2m-TALEN mRNAs (10 ng/mL each) | 41 | 41 | 2 | 1 | 2 |
| Total | 188 | 186 | 8 | 3 | 7 |

**Table 3. Summary of embryo microinjection with B2m-TALEN mRNAs.**

| Founder No. | Target site mutations | Sequenced colonies |
|-------------|-----------------------|--------------------|
| T1          | del 1 bp (T)          | 1                  |
|             | del 2 bp (TT)         | 11                 |
|             | del 2 bp (AC)         | 1                  |
|             | del 16 bp             | 3                  |
| T2          | del 1 bp              | 5                  |
|             | del 7 bp              | 4                  |
|             | WT                    | 6                  |
| T3          | del 1 bp              | 9                  |
|             | del 7 bp              | 3                  |
|             | del 16 bp             | 2                  |
|             | WT                    | 5                  |
| T4          | del 2 bp              | 7                  |
|             | WT                    | 14                 |
| T5          | del 11 bp             | 4                  |
|             | del 25 bp             | 13                 |
| T6          | del 1 bp (A)          | 6                  |
|             | WT                    | 16                 |
| T7          | WT                    | 20                 |

**Table 4. Summary of allele from founders with B2m-TALEN-mediated modifications.** Del: deletion; WT: wild type.
mRNAs of the designed TALEN pair were mixed and diluted to be the final concentration of 10 ng/μL each using RNase-free deionized water. The pig oocytes were freed of cumulus, and the matured oocytes with extruded polar body were selected out and subjected to cytoplasmic microinjection with diluted TALEN mRNAs as described28,37. The injected oocytes were activated by direct current electrical pulses (1.2 KV/cm, 30 μs, two times, 1 sec interval) and the activated oocytes (PA embryos) were cultured in PZM-3 media as described by Wang et al.37. The cleavage rate of PA embryos was counted at 48 h post activation and blastocysts were harvested at 144 h post activation. Pig genomic DNA was extracted from individual PA blastocysts by incubating individual embryos in lysis buffer as described28,37. Using the genomic DNAs as templates, a primer pair set (B2m-TAL-F: 5’-CGGTGAAAATCTCTGGGAC-3’; B2m-TAL-R2: 5’-GCCTGTGCTTCCCTGAGACT-3’; product size: 658bp) were used to amplify modified B2m alleles in injected embryos by PCR, and the amplification products were subjected to T7 endonuclease 1 (T7EN1, NEB) cleavage assay or Sanger sequencing after purification using gel extraction kit (Qiagen).

Production of B2m knock-out pigs via zygote injection with TALENs. The TALEN mRNAs were mixed and diluted as described above. Pig zygotes were surgically collected from the oviducts of mated sows. The collected zygotes were subjected to cytoplasmic microinjection with the diluted TALEN mRNA mixture in the same way as that for parthenogenetic embryos as described above. Shortly after injection, the injected zygotes were transferred into estrus-synchronized foster mother sows as described 28,37. Pregnancy was investigated by observing the oestrus behaviors of recipient sows at every ovation circle.

T7EN1 cleavage assay and sequencing. Pig tissue samples were digested in lysis buffer (0.4 M NaCl, 2 mM EDTA, 1% SDS, 10 mM Tris–HCl, and 100 mg/ml Proteinase K) overnight. Genomic DNAs were extracted from the lyses after treatment with phenol–chloroform and recovered via alcohol precipitation. For performing T7EN1 cleavage assay, the DNA fragment covering the target site was amplified from the genomic DNAs as templates, a primer pair set (ssB2m-TAL-F/R, of which the sequences were: 5’-GGGAGCTCATTGTGGCCTAGACT-3’ and 5’-CTCTCGAGAAGGTGCTACTAGACG-3’ reverse), respectively, and the product size was 565bp. After

Figure 3. Genetic analysis of the four offspring piglets of F1 generation derived from the founder T#1(♀) and #T6(♂). (a) PCR amplification of the region around target site in B2m using the primers: B2m-TAL-F: 5’-CGGTGAAAATCTCTGGGAC-3’; B2m-TAL-R: 5’-GCCTGTGCTTCCCTGAGACT-3’; product size: 658bp; M: DNA marker DL1000; WT: wild-type pig genomic DNA; P2, P215, P216 and P214 indicate the piglet #P2, piglet #P215, piglet #P216 and piglet #P214, respectively; Cont: wild type pig as control. (b) All the PCR products from F1 piglets subjected to T7EN1 cleavage assay. (c) Sequencing results of the modified B2m alleles in the four piglets of F1 offspring; the sequences targeted by left and right TALEN are labeled in red and green respectively. The mutations in blue, lower case; deletions (−). N/N indicates the positive colonies out of total sequenced.
purification with a PCR cleanup kit (Axygen, AP-PCR-50), the purified PCR product was denatured and re-annealed in NEBuffer 2 (NEB) using a thermocycler. Thereafter, the re-annealed PCR products were digested with T7EN1 (NEB, M0302L) for 30 min at 37 °C and separated on a 2.5% agarose gel. The PCR products exhibiting additional band(s) after T7EN1 cleavage assay were sub-cloned into T vector (Takara, D103A). For each sample, the no less than 16 colonies were randomly picked up and sequenced using M13F primer.

Immunohistochemical assay. The freshly collected tissues were fixed in 4% paraformaldehyde solution overnight, washed with PBS for 3–4 times and stored in 70% alcohol at 4 °C before use. The fixed tissues were embodied in paraffin and 5-μm sections were conventionally prepared. The sections were subjected to immunohistochemical staining using the monoclonal antibody of murine origin specific for porcine B2M protein (LifeSpan, Cat.: LS-1858) and HRP-conjugated rabbit anti-mouse IgG Fc antibody (PIERCE, USA) as the primary and secondary antibody, respectively. After stained with DAB kit (Zhongshan Biotech, China), the sections were examined and photographed under microscope (Olympus, Japan).

Fluorescence-activated cell sorting (FACS) assay. Pig whole blood sample (~50 μL) was placed into Eppendorf tube containing 1 mL of PBS with 2 mM EDTA, and then subjected to centrifuge at 1200 rpm, room temperature for 5 min. After centrifuge, the blood cells were re-suspended with 5 mL of PBS. 1 mL of ACK solution was added into the sample and mixed thoroughly to lyse the red blood cells. The lysis process of red blood cells was repeated twice, and then the remaining blood cells were stained with 50 μL of FACS buffer (PBS with 2% (v/v) fetal bovine serum) containing 1:200 diluted FITC-conjugated SLA Class 1 antibody (GenTex, Cat.: GTX43353) or FITC-conjugated IgG1 isotype control antibody (eBioscience, Cat. No.: 8011-4714) at 4 °C for 30 min. Thereafter, 1 mL of FACS buffer was added and the sample subjected to centrifuge at 1400 rpm for 5 min.

Figure 4. Immunohistochemical analysis of B2m expression in the F1 piglet #P2 with bi-allelic mutation. The antibody specific porcine B2M molecule (LifeSpan, LS-B1858) was used to detect B2m expression in skin, liver and intestine which are derived from three different germ layers. WT: wild type pig samples as control.
Figure 5. FACS assay of peripheral lymphocytes from the F1 piglets stained with FITC-conjugated SLA1-antibody. Fresh peripheral lymphocytes were collected from the piglets with bi-allelic mutation, mono-allelic mutation and no mutation (wild type pig), and stained with FITC-conjugated SLA-1 antibody and FITC-conjugated IgG isotype control antibody in parallel. The red histogram is for lymphocytes stained with SLA-1 antibody, and the blue one for IgG isotype control antibody.

Figure 6. Survival of skin grafts. Skin grafts were prepared from the bi-allelically mutant F1 pig (#P2) and the F1 pig (#P214) without mutation of the same litter, respectively, and grafted into wounds made on the back of mice of FVB/N inbred strain. (a) The procedure of skin grafting. (b) The skin grafts of the F1 B2m-null pig (#P2) on different days post grafting; PG: post grafting. (c) The skin grafts of the F1 pig containing wild type target sequence (#P214) on different days post grafting; PG: post grafting. (d) Skin graft survival rates on different days post grafting; *indicates statistical significance.
To wash the stained cells and eliminate the unbound antibodies, the blood cells were re-suspended with 1 mL of FACS buffer and spin at 1400 rpm for 5 min. This washing process was repeated twice, and then the stained blood cells were subjected to flow cytometry analysis.

**Skin grafting.**  Skin grafting was performed as previously described. Briefly, Split-thickness skin grafts were made from the full thickness skin pieces surgically collected from pigs after anesthesia. The hair on the back of anesthetized recipient mice of FVB/N strain was shaved off, and a 1 × 1 cm² wound was made on the back by shaving off the dorsal skin carefully while leaving the subcutaneous vasculature intact. Porcine skin graft with the size of 1 × 1 cm² was planted into the wound and sutured with the host skin. To fasten the touch of skin graft with the wound, the porcine skin grafts were covered with a dressing of several layers of gauze pieces and secured with a transparent medical film dressing to provide a sustainable pressure. The survival of skin grafts was examined every other day, and the survival time was defined as the first day on which more than 90% area of skin grafts was necrotic as described by Wang et al.33.

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**Acknowledgements**

The authors also thank the entire Huang Lab and Wei Lab for their support and advice. This work is supported by National Natural Science Foundation of China (31171377, 31171280 & 31201081).
**Author Contributions**
Y.W., X.H. and H.W. designed the experiments and performed the analysis of data. Y.W. wrote the manuscript. Y.D. designed the TALENs and performed genetic assay. X.Z. and L.W. performed embryo microinjection and transfer. J.L. performed FACS assay. F.W. performed immunohistochemical assay. Z.H. performed skin grafting.

**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:** Wang, Y. et al. Efficient generation of B2m-null pigs via injection of zygote with TALENs. *Sci. Rep.* 6, 38854; doi: 10.1038/srep38854 (2016).

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