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ScienceDirect

Journal of Genetics and Genomics 42 (2015) 437−444

JGG

ORIGINAL RESEARCH

Generation of B Cell-Deficient Pigs by Highly Efficient CRISPR/Cas9-Mediated Gene Targeting

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Received 9 February 2015; revised 1 May 2015; accepted 19 May 2015
Available online 27 May 2015

ABSTRACT

Generating B cell-deficient mutant is the first step to produce human antibody repertoires in large animal models. In this study, we applied the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system to target the J_H region of the pig *IgM* heavy chain gene which is crucial for B cell development and differentiation. Transfection of IgM-targeting Cas9 plasmid in primary porcine fetal fibroblasts (PFFs) enabled inducing gene knock out (KO) in up to 53.3% of colonies analyzed, a quarter of which harbored biallelic modification, which was much higher than that of the traditional homologous recombination (HR). With the aid of somatic cell nuclear transfer (SCNT) technology, three piglets with the biallelic *IgM* heavy chain gene mutation were produced. The piglets showed no antibody-producing B cells which indicated that the biallelic mutation of the *IgM* heavy chain gene effectively knocked out the function of the *IgM* and resulted in a B cell-deficient phenotype. Our study suggests that the CRISPR/Cas9 system combined with SCNT technology is an efficient genome-editing approach in pigs.

KEYWORDS: CRISPR/Cas9 system; *IgM* heavy chain; Pig genome editing; B cell-deficiency; Somatic cell nuclear transfer

INTRODUCTION

Polyclonal antibodies that can be used in clinical medicine are in desperately short supply because they are mainly obtained from human donors. Collecting antibodies from humans is limited by the volume of the obtained donated blood, the risk of transmission of infectious agents, and ethical concerns related to primary immunization with certain antigens to human donors (Ramsoondar et al., 2011). Thus, animal models for the development of humanized polyclonal antibodies (hpAbs) are urgently needed.

Although many studies have shown that transgenic mice can express human antibody repertoires by introducing large segments of the human genomic sequence into the mouse germline (Green et al., 1994; Lonberg et al., 1994), mice are not suitable for producing practical amounts of therapeutic hpAbs due to their small blood volume. Kuroiwa et al. (2002) have generated transgenic calves that can express correctly rearranged human heavy- and light-chain antibody transcripts. However, as the endogenous bovine immunoglobulin genes had not been inactivated, mixed bovine and human polyclonal antibodies were produced (Kuroiwa et al., 2002; Kuroiwa et al., 2009). Thus, large animal models with totally immunoglobulin genes inactivated are preferred.

Pigs have been used as large animal models for human diseases, because of their anatomic, nutritional, physiological and immunological similarities to humans (Carter et al., 2002;

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http://dx.doi.org/10.1016/j.jgg.2015.05.002

Rogers et al., 2008; Aigner et al., 2010). Pigs are easy to breed, have a short gestation period, and a large litter size. Moreover, compared to other large animals like nonhuman primates, pigs have advantages in biomedical research for ethical and economic reasons.

Previous researchers have reported that inactivation of the endogenous immunoglobulin gene(s) can enhance the production of hpAbs by exogenous human genes in animal models (Lonberg, 2005; Kuroiwa et al., 2009). J-region gene segment of the Ig heavy chain locus knockout/B cell-deficient pigs and Ig kappa chain-knockout pigs have been reported (Mendicino et al., 2011; Ramsoondar et al., 2011). However, these studies were based on traditional gene targeting technology, whose efficiency is notoriously poor due to the extremely low frequency of homologous recombination (HR) in somatic cells.

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system has emerged as a powerful and efficient tool for gene editing (Jinek et al., 2012; Wiedenheft et al., 2012; Wei et al., 2013; Bassett and Liu, 2014), and been successfully applied in many mammals, including mice, rats, pigs, and even monkeys (Li et al., 2013a; Shen et al., 2013; Tan et al., 2013; Wang et al., 2013; Hai et al., 2014; Ma et al., 2014; Niu et al., 2014). In this study, we combined this system with SCNT, and successfully generated three B-cell deficient piglets, which could be valuable models for further producing fully human antigen-specific polyclonal antibodies.

RESULTS

Cas9-single guide (sg) RNA effectively mediated gene disruptions in porcine fetal fibroblasts (PFFs)

J_H5 region of IgM heavy chain was selected as the Cas9-sgRNA targeting site, since it is the only single functional J_H region gene segment on VDJ rearrangements in pigs (Butler et al., 1996; Eguchi-Ogawa et al., 2010). The precise target of sgRNA for the J_H5 region is shown in Fig. 1A. The targeting ability of the Cas9-sgRNA vector was first tested on PFFs. PFFs co-transfected with the Cas9-sgRNA plasmid and a neomycin expression plasmid were selected with G418 for three days before harvested for genomic DNA extraction. PCR amplification of regions spanning the target site treated with or without "Surveyor" was subjected to PAGE electrophoresis analysis. The cleavage bands shown in Fig. 1B indicated that the constructed Cas9-sgRNA plasmid targeted the J_H5 region efficiently.

To obtain *IgM* locus knockout cells, we co-transfected the above two plasmids into early passage of male primary PFFs derived from a 35-day-old fetus. The transfected cells were selected with G418 for approximately 10 days before single cell colonies were collected. Genotyping analysis was performed on each colony by sequencing to determine whether the colony was an *IgM* gene monoallelic mutation or homozygous/heterozygous biallelic mutation. Of 45 single cell colonies that had been analyzed, 18 monoallelic and 6 biallelic modified ones were identified, respectively (Table 1). In these 6 biallelic mutation colonies, 8−109 bp indels were detected in the gene target region (Fig. 1C).

Production of *IgM*-modified pigs by somatic cell nuclear transfer (SCNT)

PFFs with biallelic mutation in the J_H5 region of the *IgM* gene were pooled as donor cells for SCNT. A total of 500 morula- and blastula-stage reconstructed embryos were transferred to five recipient gilts. B-ultrasonic examination was performed on each recipient about 30 days after surgery, and two of the five gilts were found to be pregnant, indicating a pregnancy rate of 40% (Table 2). Of the two pregnant recipients, one was terminated at 35 days to collect fetuses for PFF isolation and genotype analysis, and five fetuses were collected. The mutation patterns of four fetuses were found among the donor cell lines while there was one unexpected wild-type (WT) pig probably due to impure colonies that contained a few WT cells (Table S1).

The other recipient was maintained until term and delivered three live male piglets and two mummified corpses. Punched ear tissues of the three piglets were collected two days after birth for genotype analysis, which showed that the piglets #1 and #3 carried a 109 bp deletion and the piglet #2 carried an 8 bp deletion, corresponding to 7# and 1# donor cells, respectively (Fig. 1C and Table 3). Three days after birth, the piglet #1, which had the lowest birth weight (Table S2), died for unknown cause. The other two piglets were sacrificed at four weeks of age, when they remained healthy on full breast-feeding.

Phenotypic characteristic analysis of cloned piglets

To detect the IgM protein expression level of these gene-mutation piglets, Western blot was performed using full protein extraction of spleen. Compared to the WT piglet, the expression level of IgM light chain was dramatically decreased and that of IgM heavy chain was even undetectable in cloned piglets (Fig. 2A).

The disruption of the *IgM* J_H region prevents functional VDJ rearrangement at the *IgM* heavy chain locus, which is required for B cell survival during early development (Jakobovits et al., 1993; Yel et al., 1996; Jung et al., 2006). We therefore detected the membrane-bound IgM-positive B cells in the gated lymphocyte population of peripheral blood mononuclear cells (PBMCs). WT piglets possessed more than 40% IgM-positive B cells among their PBMCs. In contrast, IgM-positive B cells were barely present in *IgM*-knockout neonatal piglets (Fig. 3). Concomitantly, sandwich ELISA exhibited extremely low levels of IgM and IgA in the plasma of the cloned litters when compared to WT piglets (Fig. 2B). These results indicated that disruption of both J_H alleles prevented expression of *IgM*, because the loss of 109 or 8 bp in the J_H region created a frameshift mutation that resulted in the disruption of the IgM expression. As the elimination of IgM

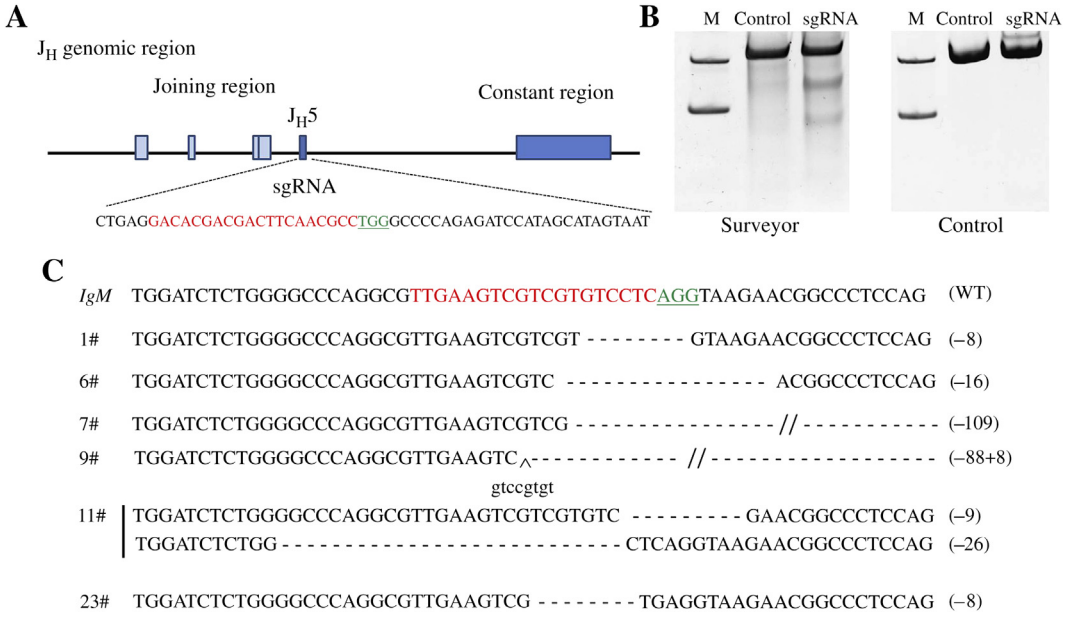

Fig. 1. Cas9-sgRNA-mediated gene targeting in PFFs.
A: Schematic diagram of sgRNA targeting at the J_H genomic region of *IgM*. PAM sequences are underlined and highlighted in green. sgRNA targeting sites are highlighted in red. **B:** Surveyor assay for Cas9-mediated cleavage at target site in PFFs. PCR products not subjected to Surveyor cleavage are on the right. **C:** Sequences of modified *IgM* J_H5 region locus detected in screened PFFs. Deletions (−) and insertions (+); lower case letters denote inserted base pairs.

protein may influence the production of the rearranged heavy chain immunoglobulin isotypes, the secretion of immunoglobulin isotypes was blocked. Significantly smaller amounts of IgG, which would come from breast milk, could be detected in the plasma of *IgM*-knockout piglets compared to WT piglets (Fig. 2B).

We further analyzed the organizational structure of the major secondary lymphoid organs, i.e., mesenteric lymph nodes (LNs), of our *IgM*-knockout piglets. Normally, the secondary lymphoid organs contain follicles that consist of a dense population of B cells. Hematoxylin and eosin (H&E) staining showed a complete lack of follicular structure and germinal center organization in the mesenteric LNs of all three cloned piglets (Fig. 4A). Immunohistochemistry was also performed on mesenteric LNs to detect T and B cell markers. $CD79a^+$ B cells were tightly packed in and around the germinal centers in WT pig LNs, and $CD3^+$ T cells dominated the paracortical region. In contrast, the *IgM*-knockout piglets showed only $CD3^+$ T cell staining, but totally lacked $CD79a^+$ B cell staining (Fig. 4B).

Table 1
Summary of mutant colonies of CRISPR/Cas9-mediated *IgM* gene targeting

Experiment	Monoallelic-KO	Biallelic-KO	Indel-positive
1	4/11	2/11	6/11
2	9/23	2/23	11/23
3	5/11	2/11	7/11
Total	18/45 (40%)	6/45 (13.3%)	24/45 (53.3%)

Mutant colonies/total colonies were tested in three independent tests.

Off-target analysis

A certain level of off-target cleavage by the CRISPR/Cas9 system has been reported in several studies (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). We thus sought to test the possible off-target effects in these genome-modified pigs. The potential off-target sites were identified (Table S3) by screening the pig genome based on sequence homology to the 23 bp sequence (sgRNA + PAM), allowing for ungapped alignments with up to four mismatches in the sgRNA target sequence (Niu et al., 2014). To analyze the potential off-target effects in the three biallelic mutant piglets, four potential off-target sites were randomly selected for PCR using punched ear genomic DNA as templates. The primers for amplifying the off-target sites are listed in Table S4. Sanger sequencing of the PCR products indicated that none of the sequencing reads had mutations, suggesting no off-target effects at these sites in our cloned pigs.

Table 2
Efficiency of somatic cell nuclear transfer in generating *IgM*-mutant pigs

Recipient	Donor cells	Number of transferred embryos	Pregnancy	Number of fetuses or newborn piglets
1	7#, 9#	96	No	0
2	7#, 9#	10	No	0
3	1#, 7#, 9#	97	Yes	5[a]
4	1#, 6#, 7#, 9#	105	Yes	3[b]
5	1#, 6#, 7#, 9#	102	No	0
Total		500	2/5 (40%)	8

[a] 35-day-old fetuses; [b] three live piglets and two mummified corpses.

Table 3
Target site analysis of *IgM*-knockout piglets

Piglet ID	Target site mutation	Indel
	AGGCGTTGAAGTCGTCGTGTCCTCAGGTAAGAAC	(WT)
Piglet #2	AGGCGTTGAAGTCGTCGT− − − − − − − −GTAAGAAC	(−8/−8)
Piglet #1, #3	AGGCGTTGAAGTCGTCG− − − − −//− − − − − − − − − −	(−109/−109)

DISCUSSION

In this study, we generated B cell-deficient pigs by targeting the J_H5 region of the *IgM* heavy chain with the aid of the CRISPR/Cas9 system and SCNT. The gene targeting efficiency of the Cas9-sgRNA in PFFs reached 53.3% (Table 1), which is much higher than that of the poly (A)-trap strategy (0.63% in PFFs) (Mendicino et al., 2011). In addition, we obtained homozygous *IgM* heavy chain-knockout pigs by just one round of transfection, clone screening and SCNT, while in the study of Mendicino et al. (2011) two rounds of cross-breeding with heterozygous knockout pigs were needed to obtain homozygous knockout pigs. The high efficiency and relative simplicity of the Cas9-sgRNA technology enabled the rapid production of gene-knockout pigs.

Unlike most of the other studies which employ the co-injection of *Cas9* mRNA and sgRNA in one-cell stage embryos to establish gene-modified animals (Li et al., 2013b; Wang et al., 2013; Hai et al., 2014; Niu et al., 2014), we chose PFFs as target cells as reported (Zhou et al., 2015). It has also been confirmed that the CRISPR/Cas9 system can be used to generate pigs with specific genetic modifications either by modifying somatic cells followed by SCNT or by directly modifying the *in vitro* produced zygotes (Whitworth et al., 2014; Zhou et al., 2015). Screened cells with specific mutations were used as donor nuclei to generate gene-targeted pigs carrying stable and identical mutations, which avoided the non-determinacy of the genotypes of cloned piglets (Zhou et al., 2015) and the problem of embryo toxicity (Whitworth et al., 2014) when using the mRNA injection strategy. Besides, in our study, we delivered the CRISPR/Cas9 system plasmids into the cell nucleus by using the nucleofection method, which may enhance the expression of CRISPR/Cas9 components and lead to higher targeting efficiency.

IgM is the first isotype to be expressed on B cell after successful rearrangements of a heavy and a light chain and is essential for B cell development (Kitamura et al., 1991; Yel et al., 1996; Tomizuka et al., 2000). Animals with disrupted *IgM* genes

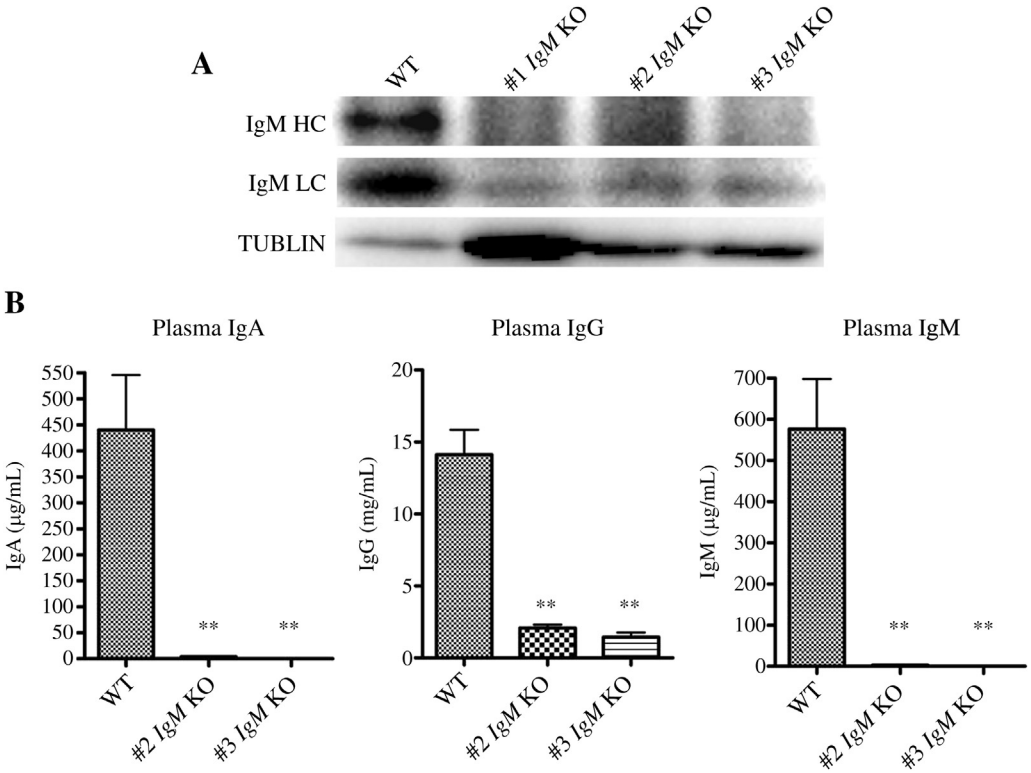

Fig. 2. IgM protein and plasma Ig expression analysis of the *IgM*-mutant piglets.
A: Western blot analysis demonstrated IgM heavy and light chain expression in spleens of the *IgM*-mutant piglets compared to a WT piglet. **B:** Plasma immunoglobulin levels in #2, #3 $IgM^{-/-}$ piglets and a WT piglet were assessed by ELISA (**$P < 0.01$).

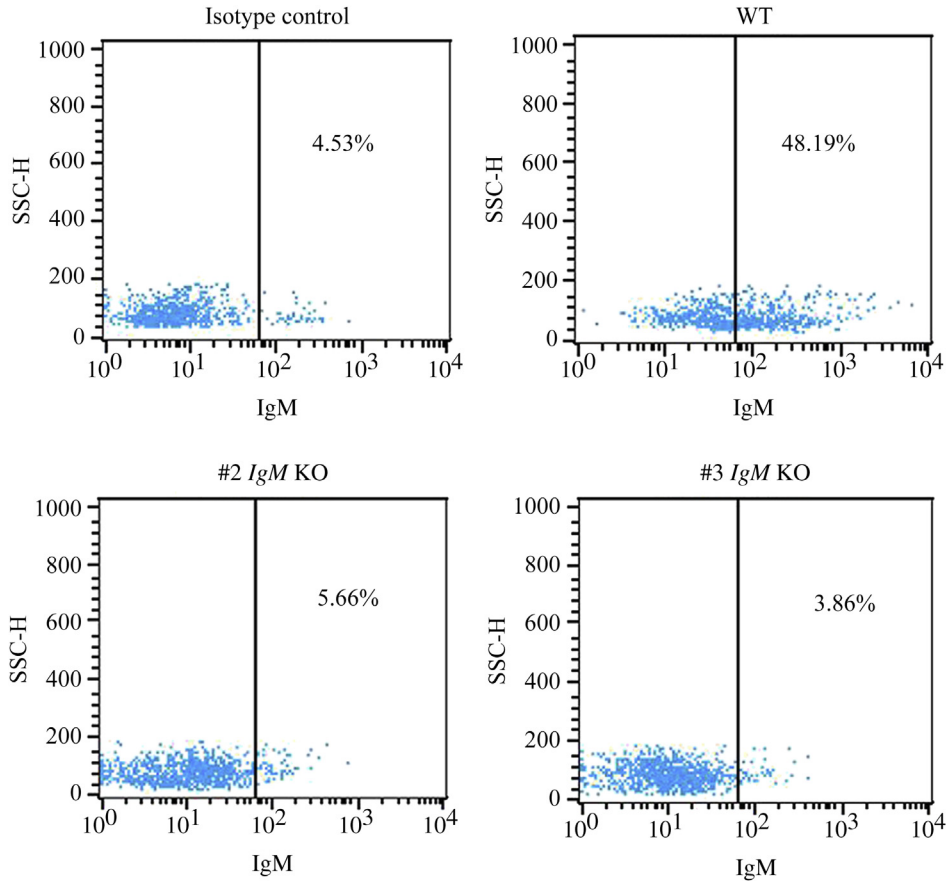

Fig. 3. Fluorescence-activated cell sorting analysis of peripheral blood mononuclear cells.
Mouse IgG1 antibody was used as isotype control.

have been proven to be B cell- and antibody-deficient (Mendicino et al., 2011; Tesson et al., 2011). Unlike other large farm animals, such as sheep, goats, and cattles, pigs have only one *Ig* heavy chain locus that needs to be inactivated for complete *Ig* replacement (Kuroiwa et al., 2009; Ramsoondar et al., 2011). Our data proved that the frameshift mutation in the J_H region of the *IgM* heavy chain created by the CRISPR/Cas9 system could disrupt IgM protein expression (Fig. 2A), causing depletion of both B cells and Igs in the blood (Fig. 2B and Fig. 3). Furthermore, histological examination of LNs showed a lack of $CD79a^+$ B cells and follicular architecture in the cloned piglets (Fig. 4). All these data further confirmed the previous contention about the necessity of productive VDJ rearrangement at the heavy chain locus for the development of B cells (Jakobovits et al., 1993; Jung et al., 2006; Randall et al., 2008). This study provides a solid foundation to further genetically modify pigs to achieve our ultimate goal of producing pathogen-specific and high-titer human polyclonal antibodies in pigs.

MATERIALS AND METHODS

Animals

All pig experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Nanjing Medical University, China. Every effort was made to minimize animal suffering.

Plasmid construction

The plasmid pX330 (Addgene plasmid 423230) encodes an optimized *hSpCas9* gene and the chimeric guide RNA under mammalian promoters. A pair of phosphorylated oligos for the *IgM* heavy chain J_H region (*IgM*-Cas9_oligo1: CACCGTTGAAGTCGTCGTGTCCTC; *IgM*-Cas9_oligo2: AAACGAGGACACGACGACTTCAAC) were designed based on the target site sequence, and synthesized by Genscript (China). The oligos were heated at 37°C for 30 min, 95°C for 5 min, and annealed by decreasing 5°C/min to 25°C on a PCR machine (ABI Veriti, USA). The pX330 was digested with *Bbs* I (Fermentas, Canada) and ligated with the annealed oligos to generate the *IgM* targeting Cas9 plasmid.

Cell lines and culture

PFFs used throughout this study were primary cultures from male fetuses of Chinese Landrace pigs at 35 days after insemination. The PFFs were isolated as previously described (Lai et al., 2006). Fibroblasts were cultured in medium consisting of high glucose DMEM (Gibco 11995-065, USA), 15%

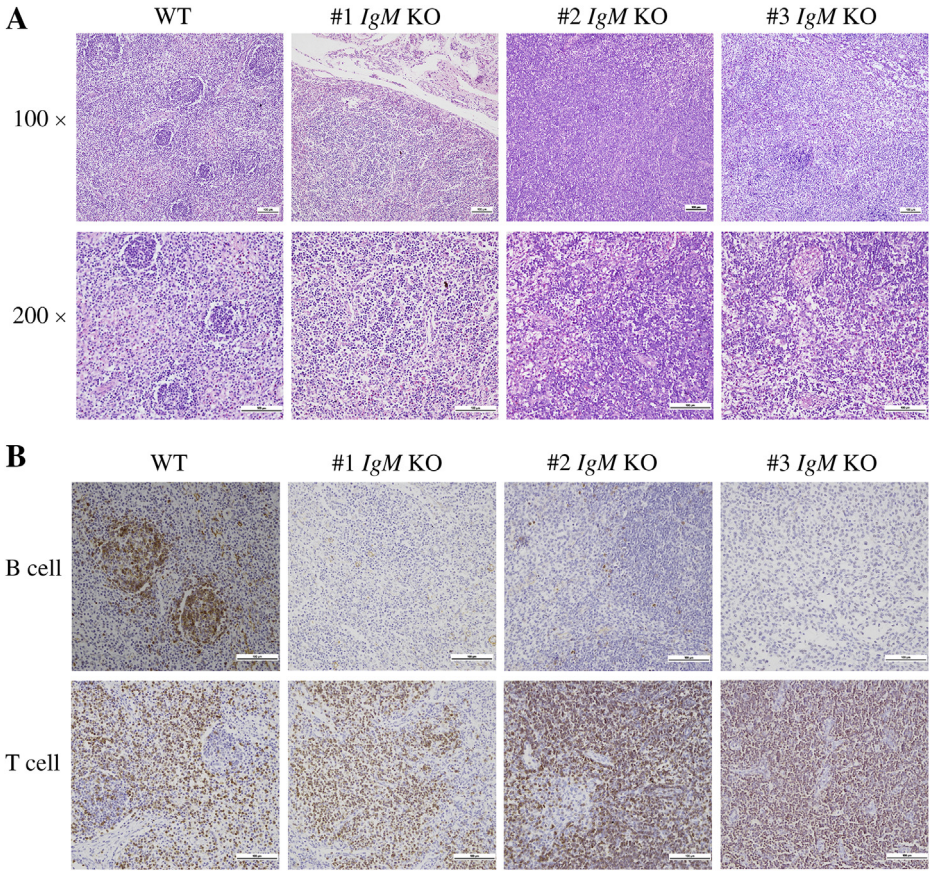

Fig. 4. Histochemistry and immunohistochemistry analysis of the LNs of *IgM* heavy chain-knockout newborn piglets.
A: Organizational structure of LNs shown by H&E staining. In all three *IgM* heavy chain-knockout piglets, no distinguishable follicles (as seen in the WT control) were seen, and no fully developed germinal center was observed at either lower (100×, upper row) or higher (200×, lower row) magnification. **B:** Staining with anti-CD79a or anti-CD3 antibody demonstrated the distribution of B or T cells in the LNs in newborn piglets. The three cloned piglets lacked CD79a-positive B cells, while the T cells marked with CD3 were largely unaffected (in comparison to the WT pig cells).

FBS (Gibco 10099) and 1 × Penn/Strep solution (Gibco 15140-122).

Transfection and subsequent clone screening

Approximately 1 × 10^6 PFFs were transfected with 5 μg *IgM*-targeting Cas9 plasmid and 1 μg neomycin-expression plasmid (pCMV-tdTomato) by using the basic fibroblast nucleofection kit (Amaxa Biosystems/Lonza, Germany) and nucleofection program U-023. Electroporated cells were replated in a 10 cm dish (Corning, USA) at a density of 1 × 10^5/10 mL after 48 h recovery. G418 (800 μg/mL) was added to the culture medium, and the selection medium was changed every 3−4 days. After 10−14 day selection, single colonies were isolated to 24-well plates. Cells from confluent wells were trypsinzed with 100 μL 0.25% trypsin and suspended in 350 μL culture medium. Cells (200 μL) were plated in a 12-well plate, and the remaining cells were collected and lysed as previously described for PCR screening (Mendicino et al., 2011). The primers used for amplifying the target region were: 5′-gccgcttcacttgggcgtc-3′and 5′-cgcttccaacataccccgtct-3′. PCR conditions were 95°C for 300 s, followed by 35 cycles of 98°C for 10 s, 68°C−0.6°C/cycle for 30 s, 72°C for 60 s, and finally 72°C for 7 min. The PCR products of each colony were sequenced and analyzed. PCR products with mutations were further analyzed by 1% agarose gel electrophoresis and purified from the gel using the NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany) and then subcloned into pMD18-T vector (Takara, Japan) according to the manufacturer's instructions. Fifteen to twenty individual clones were picked and sequenced.

SCNT and embryo transfer

To produce mature pig embryos for SCNT, ovaries from six-month old gilts were purchased from a local slaughterhouse. The oocytes were collected and cultured for 42−44 h for maturation. The *in vitro* matured oocytes were used as recipient cytoplasts after removing cumulus cells and their nuclei. A single pig fibroblast with a biallelic *IgM* mutation was injected into the perivitelline space of the enucleated oocyte as the nucleus donor cell. After electrofusion of membranes between the donor cell and recipient cytoplast, the reconstructed embryos were electrically activated and then cultured in embryo-development medium at 38.5°C for 5 days until the blastocyst formed. About 110 blastocysts were transplanted

into the uterus of the surrogate pigs in estrus. Pregnancy status of the surrogates was monitored by ultrasound weekly starting one month after embryo transfer, and the cloned piglets were delivered by natural birth.

Surveyor nuclease assay

Fibroblasts transfected with or without Cas9-sgRNA plasmids (as described above) were cultured with G418 selection for 3 days. Genomic DNA was extracted and the genomic region surrounding the CRISPR/Cas9 target site for each gene was amplified by PCR. The Surveyor nuclease assay was performed using a Surveyor mutation detection kit (Transgenomic, USA) and analyzed on 10% PAGE gel as previously described (Cong et al., 2013; Hsu et al., 2013).

Flow cytometry

PBMCs were recovered from heparinized peripheral whole blood of wild-type and cloned newborn piglets using standard procedures. 1×10^6 PBMCs per tube were stained for 60 min at 4°C using mouse IgG1 anti-porcine IgM (MCA637, AbD Serotec, Germany) and a mouse IgG1 isotype control (MCA928, AbD Serotec). Anti-IgM was detected using goat anti-mouse IgG: FITC for 30 min at 4°C (STAR70, AbD Serotec). Samples were analyzed on a flow cytometer (BD, Franklin Lakes, USA).

Measurement of plasma immunoglobulins

Plasma samples were collected from heparinized pig blood to quantify IgM, IgG, and IgA levels using sandwich ELISA kit (Abnova, USA). Diluted samples were added to the blocked antibody wells, and the value of OD_{450nm} was read following kit instructions. A four-parameter logistics curve was made using MasterPlex 2010 software to calculate the immunoglobulin concentration of each sample. Each sample was assayed in triplicate, and the standard error of the mean (SEM) was calculated for each sample.

Western blot analysis

Spleens and LNs from euthanized piglets were dissected and frozen in liquid nitrogen. Whole cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Sweden). The antibody used was IgM [CM7] (GTX75506, GeneTex, USA).

Histology and immunohistochemistry

Mesenteric LNs were isolated from euthanized piglets and processed as described previously (Mendicino et al., 2011). Briefly, paraffin-embedded 5 μm sections were cut for staining with H&E and for use in enzyme-based immunohistochemistry studies. Tissue slides were incubated with anti-CD3 monoclonal antibody (1:50, Abcam, UK) or anti-CD79a monoclonal antibody (1:50, Abcam) overnight. Images were taken using a Digital Sight DS-Ri1 camera on a Nikon Eclipse 80i microscope.

Statistical methods

Data are presented as mean ± SEM and analyzed with GraphPad Prism. P values were determined by Student's t-tests and ANOVA for all quantifications. P values <0.05 were regarded as statistically significant.

ACKNOWLEDGMENTS

This work was supported by a grant from the Jiangsu Key Laboratory of Xenotransplantation (BM2012116). YD, RL and YW are Fellows at the Collaborative Innovation Center for Cardiovascular Disease Translational Medicine, Nanjing Medical University. We thank Dr. D.K.C. Cooper for reviewing the manuscript.

SUPPLEMENTARY DATA

Table S1. Details of the 35-day fetuses collected from the third recipient.
Table S2. Summary of *IgM*$^{-/-}$ piglets regarding their health status and origin of donor cells.
Table S3. List of putative off-target sites homologous to *IgM*-sgRNA.
Table S4. Primers for amplifying off-target sites and sequencing.
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2015.05.002.

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