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Generation of antibody- and B cell-deficient pigs by targeted disruption of the J-region gene segment of the heavy chain locus

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Abstract A poly(A)-trap gene targeting strategy was used to disrupt the single functional heavy chain (HC) joining region (J_H) of swine in primary fibroblasts. Genetically modified piglets were then generated via somatic cell nuclear transfer (SCNT) and bred to yield litters comprising J_H wild-type littermate (+/+), J_H heterozygous knockout (±) and J_H homozygous knockout (−/−) piglets in the expected Mendelian ratio of 1:2:1. There are only two other targeted loci previously published in swine, and this is the first successful poly(A)-trap strategy ever published in a livestock species. In either blood or secondary lymphoid tissues, flow cytometry, RT-PCR and ELISA detected no circulating IgM⁺ B cells, and no transcription or secretion of immunoglobulin (Ig) isotypes, respectively in J_H −/− pigs. Histochemical and immunohistochemical (IHC) studies failed to detect lymph node (LN) follicles or CD79α⁺ B cells, respectively in J_H −/− pigs. T cell receptor $(TCR)_\beta$ transcription and T cells were detected in J_H −/− pigs. When reared conventionally, J_H −/− pigs succumbed to bacterial infections after weaning. These antibody (Ab)- and B cell-deficient pigs have significant value as models for both veterinary and human research to discriminate cellular and humoral protective immunity to infectious agents. Thus, these pigs may aid in vaccine development for infectious agents such as the pandemic porcine reproductive and respiratory syndrome virus (PRRSV) and H1N1 swine flu. These pigs are also a first significant step towards generating a pig that expresses fully human, antigen-specific polyclonal Ab to target numerous incurable infectious diseases with high unmet clinical need.

Keywords Genetically engineered animal model · Immunoglobulin heavy chain locus · B cell and antibody deficiency · Somatic cell nuclear transfer ·

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Humoral versus cellular immunity · Human
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Introduction

B cell-deficient mice have long been used in research,
and multiple lines have been generated (Kitamura
et al. 1991; Jakobovits et al. 1993). In side-by-side
studies with wild-type mice and T- and B cell-
deficient mouse models, T cell (cellular) responses
can be separately studied from B cell (humoral)
responses for many diseases (bacterial, viral, parasitic,
inflammatory and autoimmune). In addition to study-
ing the basic immune response to a pathogenic
challenge, these animals have also been used to test
vaccines for effectiveness (McNeal et al. 2002; Wyatt
et al. 2004). Unfortunately, results from rodent
immunological models often do not translate to larger
mammals, such as livestock and humans (Melo et al.
2007; Rogers et al. 2008b; Butler et al. 2009).

The isolator piglet is an in vivo model for the fetal
immune system since piglets maintained under such
conditions remain, immunologically, like fetal ani-
mals (Sinkora and Butler 2009). Thus far, this model
has proven valuable in: (1) understanding the immu-
nopathology caused by the pandemic PRRSV in which
pathology is related to polyclonal B cell activation
(Butler et al. 2008), (2) swine influenza research
(Vincent et al. 2006), (3) studying the pathology of
colibacillosis (Erume et al. 2008), and (4) understand-
ing the intestinal absorption of maternal Igs (Butler
et al. 2009). By rearing B cell-deficient piglets
gnotobiotically, the relative roles of B cells and Ab
production, versus T cells, in the control of postnatal
viral and bacterial disease, might be better understood
for veterinary and agricultural applications.

Anatomic, nutritional, physiological and immuno-
logical similarities to humans have made the pig an
important biomedical research animal (Rogers et al.
2008b; Butler et al. 2009). For example, pigs are
omnivorous like humans, have a similar gut flora and
are infected by closely-related viral and bacterial
pathogens. For most major enteropathogens, such as
Helicobactor pylori, *Shigella flexneri* and *Salmonella
enterica*, mouse animal models do not reproduce the
tropism, immunopathology, or other hallmarks of the
corresponding human infections (Goodwin et al.
1986; Lecuit 2007). The dietary and mucosal

similarity of humans and swine has resulted in their
specific use as models for digestive, respiratory and/
or infectious diseases, and allergies (Krakowka et al.
1991; Saif et al. 1996; Helm et al. 2002; Charley et al.
2006; Souza et al. 2007; Dawson et al. 2009). A
prominent and recently described example of a
porcine model that more fully recapitulated a human
condition is the knock-out piglet model for cystic
fibrosis (Rogers et al. 2008b).

Swine are infected by closely-related viral and
bacterial pathogens often to the extent that they can
serve as zoonotic reservoirs for human pathogens,
and thus play a critical role in public health threats
worldwide. The H1N1 Influenza A virus (swine flu) is
a current, relevant and prominent zoonotic example.
Many zoonotic agents have potential bioterrorism
applications as well (Mattix et al. 2006). Thus, the
availability of Ab- and B cell-deficient piglets should
serve as valuable as models for certain human
diseases and would aid in vital vaccine development
by helping to ascribe protective immunity to T or B
cells, or both.

Toward an even more broad utility, generation of a
Hc −/− pig provides the initial critical step towards
the complete replacement of porcine immunoglobulin
(Ig) loci with their human equivalents. High volume,
pathogen-free, antigen-specific, fully-human Intrave-
nous Immunoglobulin (IVIG), produced in geneti-
cally-modified pigs, could be used not only for IVIG
therapy, but also as treatments for infectious diseases.
Comparisons of our strategy with technology under
development by other groups has been previously
described (Waltz 2006).

We tested the hypothesis that by targeting the J_H
region gene segment in the porcine HC locus, VDJ
rearrangement would be impaired, such that no IgM^+
B cells would develop; thus, preventing Ig expression
of any isotype and subsequent Ab secretion. There-
fore, we predicted that this pig would be Ab- and B
cell-deficient.

Materials and methods

Primary fibroblast isolation and vector
construction

Sequence data required for construction of a gene
targeting vector was generated using a bacterial

artificial chromosome (BAC) carrying a major portion of the porcine Ig HC locus (Eguchi-Ogawa et al. 2010). All subsequent steps involved amplification of relevant porcine HC sequences from a previously generated porcine primary fetal fibroblast (PPFF) cell line using primers sequences based on BAC sequence analysis. The PPFF cell line was isolated from a fetus at day 33 of gestation. After removing the head and viscera, the fetus was washed with Hanks' Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA), placed in 20 ml of HBSS and diced with small surgical scissors. The tissue pellet was resuspended in 50 ml tubes with 40 ml of DMEM + 100 U/ml collagenase (Invitrogen). Tubes were incubated for 40 min in a shaking water bath at 37°C. The digested tissue was allowed to settle for 3–4 min and the cell-rich supernatant was transferred to a new 50 ml tube and spun down. The cells were then resuspended in 40 ml of DMEM, 10% Fetal Calf Serum, 1× non-essential amino acids, 1 mM sodium pyruvate (Invitrogen) and 2 ng/ml basic fibroblast growth factor (Roche Molecular Biochemicals, Indianapolis, IN), then seeded into 10 cm dishes. All cells were cryopreserved upon reaching confluence. An amplimer, extending from the intron between the HC Diversity (D_H) and J_H regions, and ending 3′ of the Coding region of Mu constant ($C\mu$)1 of the porcine HC locus, was generated so that both homologous arms in the knockout vector could be generated from a contiguous fragment (refer to Fig. 1a for schematic of HC locus). The pKOTKneo vector (Lexicon Genetics, The Woodlands, TX), from which the poly(A) signal 3′ of the neomycin Resistance gene (neoR) had been excised, was used to generate the J_H targeting construct (pREV708). The single functional porcine J_H is found within 6 kb upstream of the coding sequence for $C\mu$ region (Butler et al. 1996). The 5.8 kb 3′ homologous targeting arm sequence was ligated into the pKOTKneo vector, downstream of the phosphoglycerate kinase (PGK) promoter-neoR cassette. The 2.0 kb 5′ homologous arm sequence was ligated into this same vector upstream of the PGK-neoR cassette. A 0.4 kb CMV enhancer sequence was inserted between the 5′ homologous arm and the PGK-neoR cassette, making a CMV-PGK-neoR cassette (lacking a poly(A) signal sequence), and completing the pREV708 J_H targeting construct.

Primary fibroblast transfection and screening

The vector was linearized and electroporated into the isogenic PPFFs. This procedure has been previously described (Dai et al. 2002). Linearized vector DNA pREV708 (0.5–10 μg) was introduced into 0.5–2 million PPFF cells by electroporation using an ECM2001 Electrocell Manipulator (BTX Inc., San Diego, CA). Forty-eight hours post-transfection, the transfected cells were seeded and selected with 250 μg/ml of G418 (Invitrogen, Carlsbad, CA). Cells from confluent wells were harvested from plates and split into two for PCR screening and cryopreservation for SCNT. Prior to screening, PPFF cells were lysed with of 40 mM Tris pH8.9, 0.9% Triton X100, 0.9% Nonidet P40, 400 μg/ml Proteinase K (Invitrogen or Sigma, St. Louis, MO) at 60C for 30 min. Primers used to detect 5′ targeting were: HCKOXba5′2: tctagaagacgctggagagaggccag; 5′armS: taaagcgcatgctccagactgcctt. PCR conditions were 65°C 15 min and 95°C 10 min then a cocktail was added to all samples containing water, primers, Takara 10× buffer, dNTPs, and Taq. PCR conditions continued as 94°C 2 min, [94°C 30 s, 66°C 30 s, 72°C 5 min (35 cycles)], 68°C 7 min. Primers used to detect 3′ targeting were: Neo442S: catcgccttctatcgccttctt; 650+ca: aagtacttgccgcctctcagga. PCR conditions were similar except denaturation was for 10 s, annealing temperature was 65°C, and extension was at 68°C for 10 min all for 30 cycles. Primers were made by Sigma (St. Louis, MO). PCR reagents were obtained from Takara, Japan and restriction enzymes from NEB, Ipswich, MA. Southern blot on PPFFs has been described previously (Dai et al. 2002), which is used to confirm PCR results. After electrophoresis, the DNA was transferred onto a nylon membrane using standard procedures, and probed with a digoxigenin labeled J_H probe for the XbaI digest, or probed with a digoxigenin labeled HC $C\mu$ probe for the NcoI digest. Bands were detected using a chemiluminescent substrate system (Roche Molecular Biochemicals, Indianapolis, IN). Primer and XbaI Southern blot probe locations in HC locus are shown in Fig. 1a.

Somatic cell nuclear transfer and piglet screening

Enucleation of in vitro matured oocytes (BoMed, Madison, WI and/or TransOVA, IA) was performed as described elsewhere (Dai et al. 2002; Ramsoondar
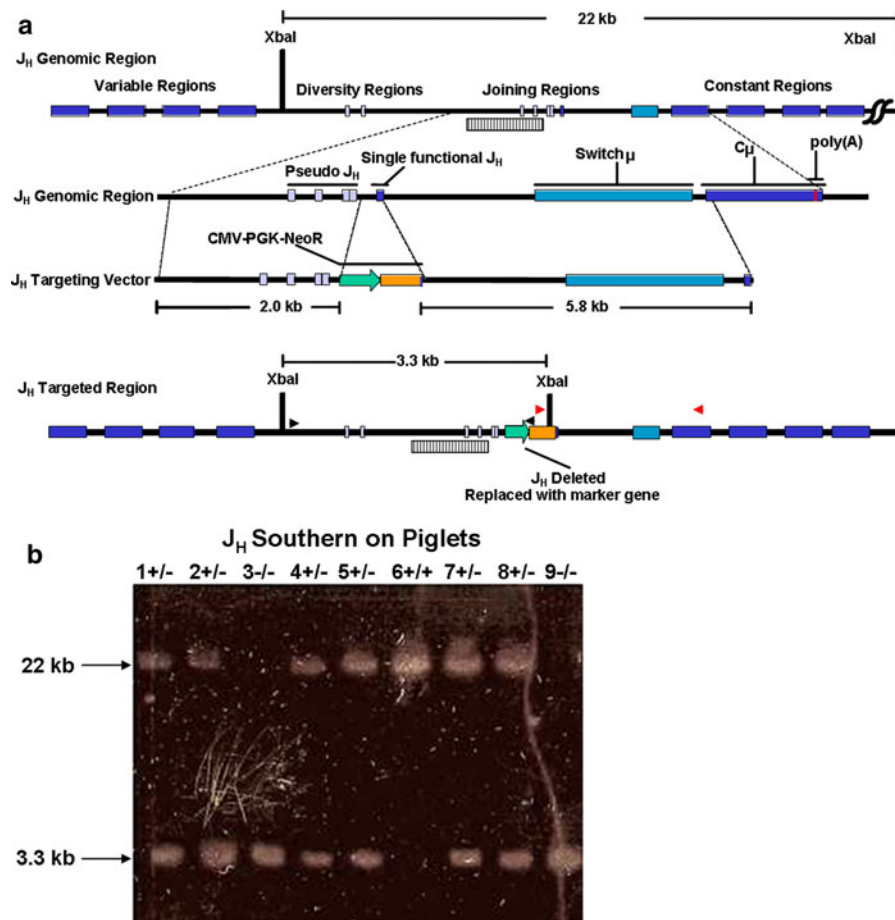
Fig. 1 Targeting strategy for the porcine J$_H$ region. **a** The *top* is a schematic of the porcine HC locus containing the J$_H$ region, showing one functional J$_H$ (the *dark box* most 3′ of the region) and four J$_H$-like pseudogenes. The *middle* is an enlarged version of the *top* focused on the genomic sequence to be targeted with the targeting vector underneath it. The 3′ targeting arm retained the complete J$_H$ to IgM constant region (Cμ) intron, and a few bp of the 3′ J$_H$ coding sequence, to insure normal splicing between the neoR transcript and the poly(A) signals found downstream of the Cμ coding region.

Locations of primers used for PCR screening of targeted clones are shown as well (*black arrowheads* for 5′ and *red arrowheads* for 3′ end targeting confirmation, respectively). **b** A representative Southern (S) blot is shown for the nine piglets from the first litter of a J$_H$ ± × J$_H$ ± mating. The Southern probe used for the *Xba*I digestion is shown as ⅢⅢⅢⅢⅢ in *A*. A 22 kb band represents a non-targeted allele (see **a**). A 3.3 kb band represents a targeted allele (see **a**). The genotype is given along with the piglet number along the top of the blot

et al. 2009; Phelps et al. 2009). A single targeted PPFF cell was placed under the zona pellucida in contact with each enucleated oocyte. Fusion and activation followed. Reconstructed embryos were cultured in NCSU-23 medium for 1–4 h at 39°C, and then transferred to the oviduct of an estrus-synchronized recipient gilt. Crossbred gilts (large white/Duroc/Landrace) (280–400 lbs) were synchronized as recipient animals by oral administration of 18–20 mg Regu-Mate (Altrenogest, Hoechst, Warren, NJ) mixed into their feed. Regu-Mate was fed for 14 consecutive days. Human Chorionic Gonadotropin (hCG, 1,000 units; Intervet America, Millsboro, DE) was administered intramuscularly 105 h after the last Regu-Mate treatment. Embryo transfers were done 22–26 h after the hCG injection. Pregnant Mare Serum Gonadotropin (PMSG, 1,000 IU) and hCG (500 IU) we used on day 10 and 13 post transfer for maintenance of pregnancy. Pregnancy was confirmed via ultrasonography 28 days post-transfer. Pregnancies were

monitored thereafter on a weekly basis. All piglets were born via natural parturition.

Phenotypical characterization methodology for $J_H +/+$, $J_H \pm$ and $J_H -/-$ neonatal and weaned piglets

$J_H \pm$ pigs were mated and the offspring of one litter (piglets 1–9) were not given the opportunity to suckle. Blood was collected from all nine animals in the litter, which were subsequently euthanized for detailed phenotypical analyses. WBC from these piglets were analyzed by flow cytometry for the presence of mature IgM^+ B cells, and sera were analyzed by sandwich ELISA for the presence of IgM, IgG and IgA. Tissues were collected for RT-PCR, histology and IHC as described. Subsequent litters were allowed to suckle and reared conventionally. These litters were weaned at approximately 4 weeks of age (a total of 5 $J_H -/-$ pigs were obtained from these litters) and blood samples were collected after 4 additional weeks for flow cytometry and ELISA (there were only 3 surviving $J_H -/-$ pigs at this timepoint). Animals in poor health were euthanized, and histology and IHC were performed on collected tissues. Animals were handled in accordance with reviewed and approved IACUC protocols.

Flow cytometry

WBC were recovered from heparinized blood using standard procedures. One million WBC were stained per tube for 45 min at 4°C using mouse IgG1 anti-porcine IgM (M160; K. Nielsen, ADRI, Canada) and a mouse IgG1 isotype control (MOPC-31C, BD Biosciences). Anti-IgM was detected using goat anti-IgG1 conjugated to RPE for 30 min at 4°C (Jackson ImmunoResearch, West Grove, PA). Samples were analyzed on the FACSAria flow cytometer using BD FACSdiva software (BD, Franklin Lakes, NJ). The lymphocyte gate was determined by scatter plot in relation to monocyte and granulocyte gates. At least 10,000 lymphocytes were counted per run.

Measurement of plasma Igs

Sandwich ELISAs were performed on heparinized plasma samples to quantify levels of porcine IgM, IgG and IgA using well-established procedures (Butler et al. 2000). These procedures have lower limits of detection of 0.4, 0.4 and 0.6 ng/ml, respectively for the three porcine isotypes. Each sample was assayed in triplicate and the Standard Error of the Mean (SEM) is given for each.

Measurement of IgM, IgG, IgA, and TCR_β transcription

Recovery of rearranged Ig transcripts by RT-PCR from splenic or WBC RNA has been described previously (Butler et al. 2001). Two forward and one reverse PCR primers were designed for each Ig isotype, and were used in two rounds of hemi-nested PCR. The primer sequences used are given below. Primers used for amplification of rearranged TCR_β transcripts included a mix of three forward primers and one reverse primer that together amplify all TCR families (Table 1). Due to the vast number of TCR_β family members, and the multiple primer sequences used here for TCR_β RT-PCR, many bands with different sizes were expected, preventing the formation of a distinctly sharp banding pattern.

Table 1 Primer sequences used for presence or absence of Ig isotype and TcR transcription

Target	Forward primer		Reverse primer	
IgM	V_HFR15'	gaggagaagctggtggagt	CH4	ctgcacgaacacgtccgc
IgG	V_HFR13'	tctcctgtgtcggctctgg	CH2	ccaccaccacgcacgtga
IgA			CH2	gagcccaggagcaggtct
$TCR\beta$	$V_\beta L1$	kgcaycgggstkctctg	Constant1	gcggagtcattgcggct
	$V_\beta L2$	ctcascggramcctttgc	Constant2	gtggtctcacctgctgcag
	$V_\beta L3$	ssytccaksctcytytgctrtg		

Necropsies

Necropsies were performed at the Virginia-Maryland Regional College of Veterinary Medicine (we acknowledge Dr. Kevin Pelzer for his expert advice). Necropsy reports included gross, microbiological, histopathological, and immunohistological findings, and cause of morbid illness.

Histology and IHC

Mesenteric (M)LNs were removed and were either fixed in 10% formalin or frozen in blocks of OCT (Electron Microscopy Sciences, Hatfield, PA). Formalin fixed tissues were embedded in paraffin blocks and cut at 5 μm for staining with Hematoxylin and Eosin (H + E) and for use in enzyme-based IHC studies using the automated Ventana™ system. This approach was used for the detection of two cross-species T and B cell markers, i.e. the Igα marker using mouse monoclonal Ab anti-human CD79α (mouse IgG1; clone HM57; DakoCytomation, Carpinteria, CA), and rabbit anti-human CD3 (rabbit IgG; DakoCytomation). Both markers cross-react with the homologous proteins in the pig. Representative histological and IHC images were taken using a Digital Sight DS-L1 camera on a Nikon Eclipse E400 microscope, and analyzed using Nikon ACT-1 software (Nikon, Melville, NY).

Fluorescence IHC was done using frozen sections cut at 5 μm and incubated for 1 h at room temperature in a humidified chamber with mouse mAbs to porcine kappa light chain (27.2.1), porcine lambda light chain (27.7.1) and porcine IgM (M160) (Sinkora et al. 2001). All were mouse IgG1 monoclonal Abs so mouse IgG1 (MOPC-31C) was used as an isotype control. Frozen sections were dried and fixed in cold acetone (Sigma, St. Louis, MO), followed by avidin–biotin blocking (Invitrogen, Carlsbad, CA). Secondary Ab blocking steps were included (Jackson ImmunoResearch) prior to the addition of biotinylated donkey anti-mouse IgG and subsequently fluorescein-conjugated streptavidin (Jackson ImmunoResearch). Slides were washed in PBS between steps, were cover-slipped using 22 × 30 mm coverslips (VWR, West Chester, PA), and were preserved using Slowfade with DAPI (Invitrogen). Representative photos were taken using an Olympus DP71 camera on a Provis microscope, and analyzed using DP controller software (Olympus, Center Valley, PA).

Results

Genomic targeting of the J_H region of the porcine HC locus by a poly(A) trap and production of pigs with a J_H targeted allele

Figure 1a is a map of the porcine HC locus between the 3′ V_H genes and C_μ. A large body of sequence data on VDJ rearrangements in swine indicated that swine appear to possess only a single functional J_H region gene segment (Butler et al. 1996; Eguchi-Ogawa et al. 2010). Sequence analyses confirmed the presence of one functional J_H region fragment (J_H5), and also revealed the presence of four J_H-like fragments 5′ of J_H5 (J_H1–J_H4) that, while possessing some sequence homology, due to truncation or inadequacy of their recombination signal sequences, would be regarded as J_H pseudogene segments. A schematic of the genomic region and the poly(A)-trap targeting strategy described in Materials and Methods is also shown in Fig. 1a. A successfully targeted event necessitated that the poly(A) from $C\mu$ be utilized as a targeting trap to ensure transcription and subsequent translation of the neoR gene product for G418 resistance. Of 476 G418-resistant colonies obtained from a total of 9 transfections, 3 were correctly targeted as determined by PCR and Southern blot (data not shown), inactivating one J_H allele. This gave a targeting efficiency of 0.63% in this particular PPFF cell line utilizing the poly(A)-trap strategy described in this report in the absence of any enhancement (i.e. negative selection with gancyclovir). The location of the primers and the XbaI probe are indicated in Fig. 1a.

One of the 3 targeted PPFF cell lines (which was female) was utilized for SCNT. A total of 682 embryos were reconstructed and transferred to 3 recipient gilts. One pregnancy was utilized to collect day 45 fetuses, from which PPFFs were isolated for future manipulation and re-cloning while the remaining 2 recipients carried their pregnancies to term, producing 9 female cloned piglets. This gave an average efficiency of live births/total transferred embryos of 1.98%. Five of the 9 piglets were confirmed to be $J_H \pm$ by Southern blot analysis using

a digoxigenin (dig) labeled probe for the *Xba*I digest (see Fig. 1a for location), and a dig labeled J_H probe for the *Nco*I digest (latter not shown in this report, used as confirmation only). The 4 J_H +/+ piglets can be attributed to bystander effects, where neoR colonies selected for SCNT cloning contained a mixture of targeted and bystander unmodified cells, which would be subsequently observed in fetuses from litters originating from these colonies. J_H ± cloned founder females were outbred to wild-type male pigs of a similar large white breed in order to generate J_H ± F1 offspring of both sexes. F1 male and female J_H ± pigs were subsequently used for cross breeding resulting in the birth of multiple F2 litters. Their genotypes approximated the expected 1:2:1 Mendelian ratio, which was confirmed after subsequent litters (data not shown).

J_H −/− piglets are devoid of mature B cells and Ab of any isotype

J_H −/− piglets lack Ig transcription and protein production

Flow cytometry was used to detect the percentage of membrane-bound IgM^+ B cells out of the gated lymphocyte population of circulating white blood cells (WBC). Within a litter (genotyped in Fig. 1b), before colostrum exposure, no IgM^+ B cells were detected in WBC from either of the two J_H −/− piglets; in contrast, the J_H +/+ piglet showed 30.8% IgM^+ B cells in the lymphocyte gate, and the six J_H ± piglets had a range of 16.8% to 25.9% IgM^+ B cells in the lymphocyte gate (Fig. 2a). This constitutes a range of 55% to 84% the number of IgM^+ B cells in J_H ± piglets in comparison to a J_H +/+ piglet. As confirmation, flow cytometry was performed on pigs from a subsequent litter at 8 weeks of age that were reared normally (∼4 weeks post-weaning), where again none of the three J_H −/− pigs had any detectable IgM^+ B cells, in stark contrast to the 39.3% IgM^+ B cells in the lymphocyte gate in a J_H +/+ littermate control pig (Fig. 2b).

Within the litter genotyped in Fig. 1b, when assayed by RT-PCR, all J_H −/− piglets lacked transcripts for IgM, IgG and IgA in spleen (Fig. 3a) and MLNs (data not shown), unlike in J_H +/+ and J_H ± piglets where transcripts for all these Ig isotypes were detectable (Fig. 3a). Transcripts for IgM were

also lacking in WBC of J_H −/− piglets, but not in J_H ± piglets (Fig 3b). In contrast, in WBC, RT-PCR showed that normal rearrangements were observed in T cells for the TCR_β chain of all genotypes tested (Fig. 3c). Sandwich ELISAs showed that colostrum-deprived J_H −/− piglets also lacked serum IgM, IgG, and IgA (Fig. 3d), while J_H +/+ and J_H ± piglets had a relatively wide range of levels of serum Ig (Fig. 3d). Focusing on the predominant isotype, the IgG levels in the 6 J_H ± piglets were all less than the IgG level in the J_H +/+ piglet, over a wide range (from 30% to 83% of J_H +/+ levels). Therefore, disruption of both J_H alleles prevents expression of membrane-bound IgM, the transcription of productive rearranged HC Ig isotypes, and the secretion of Ig isotypes as described.

The MLNs of J_H −/− piglets lack follicles, Ig and are devoid of CD79α$^+$ B cells

MLNs were analyzed by Hematoxylin & Eosin (H & E) and IHC staining within the genotyped litter described in Fig. 1b. Fig. 4a shows the presence of numerous follicles, all with normal morphology in a J_H +/+ piglet, while a range of follicular morphologies were observed in the MLNs of the J_H ± piglets. These ranged from nearly normal follicles and germinal centers to some with smaller follicles and seemingly underdeveloped germinal centers (the latter is represented in Fig. 4a). In some cases, there were scattered leukocyte-containing fields that showed little resemblance to follicles. In general, the number of follicles, diameter of the average follicle and size of the average germinal center was smaller in the J_H ± piglets compared to the J_H +/+ piglets (data not shown). Most striking was the complete lack of follicular structure and germinal center organization in the MLNs of all J_H −/− piglets (Fig. 4a). Fluorescence IHC failed to detect IgM HC, or kappa and lambda light chain proteins in any J_H −/− piglets; in contrast, follicles in J_H +/+ and J_H ± piglets showed positive staining for Ig chain proteins (Fig. 4b).

IHC for T and B cell markers was performed in MLN (Fig. 5) and spleen (data not shown). Clear, well-defined follicular structures were observed in the MLNs of J_H +/+ piglets, with CD79α$^+$ B cells tightly packed in and around the germinal centers, and CD3$^+$ T cells dominating the paracortical region
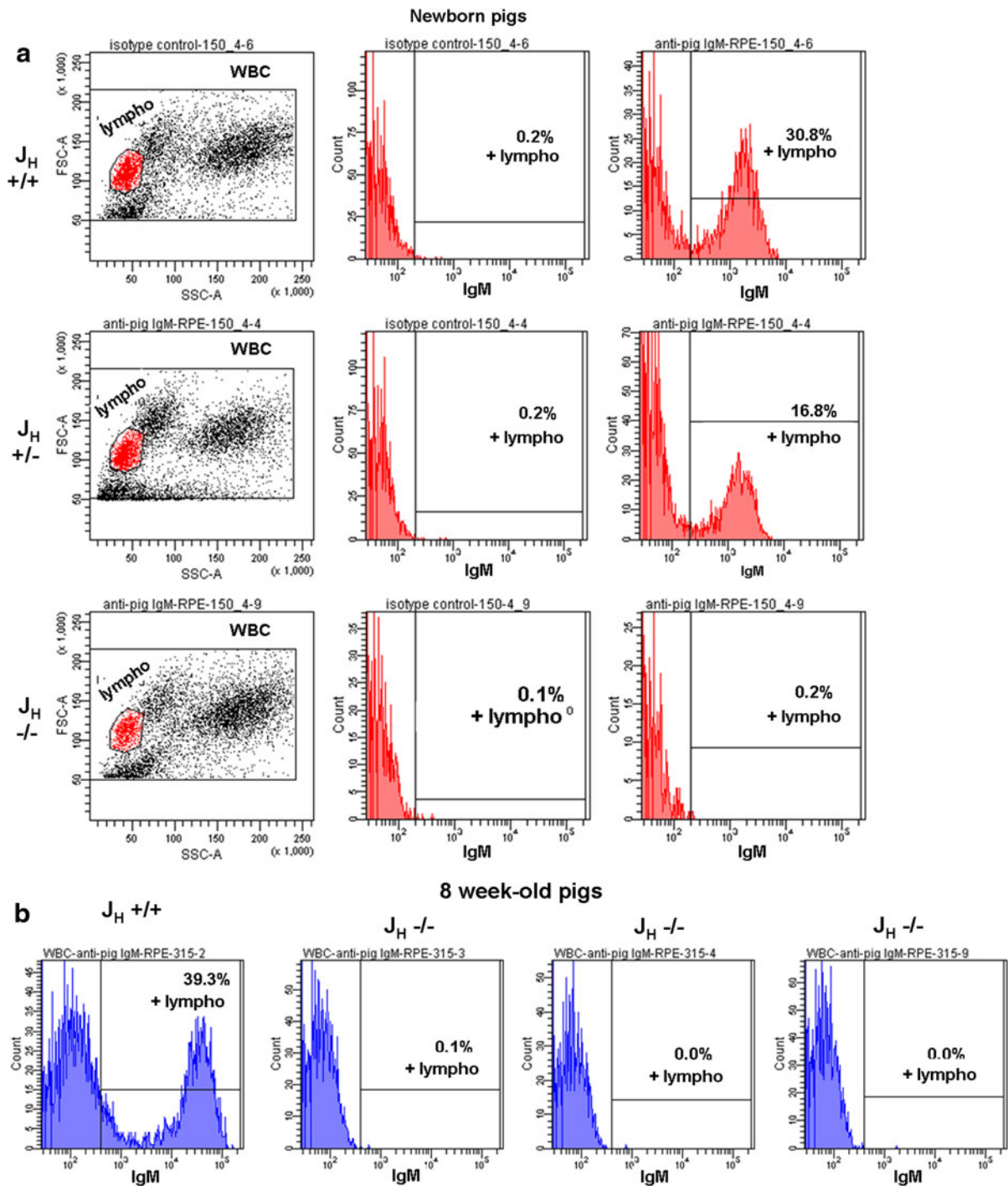
Fig. 2 Expression of IgM on J_H +/+, J_H ± and J_H −/− pigs on WBC. **a** Histogram of a J_H +/+ piglet (*top*, piglet # 6), a J_H ± piglet (*middle*, representative piglet # 4) and a J_H −/− piglet (*bottom*, representative piglet # 9) after birth. **b** Shown are data of a J_H +/+ pig (pig # 315-2) in comparison to 3 surviving J_H −/− pigs (pig #s 315-3, 315-4 and 315-9) within a litter reared normally at 8 weeks of age. No J_H −/− pigs have IgM+ lymphocytes at either age
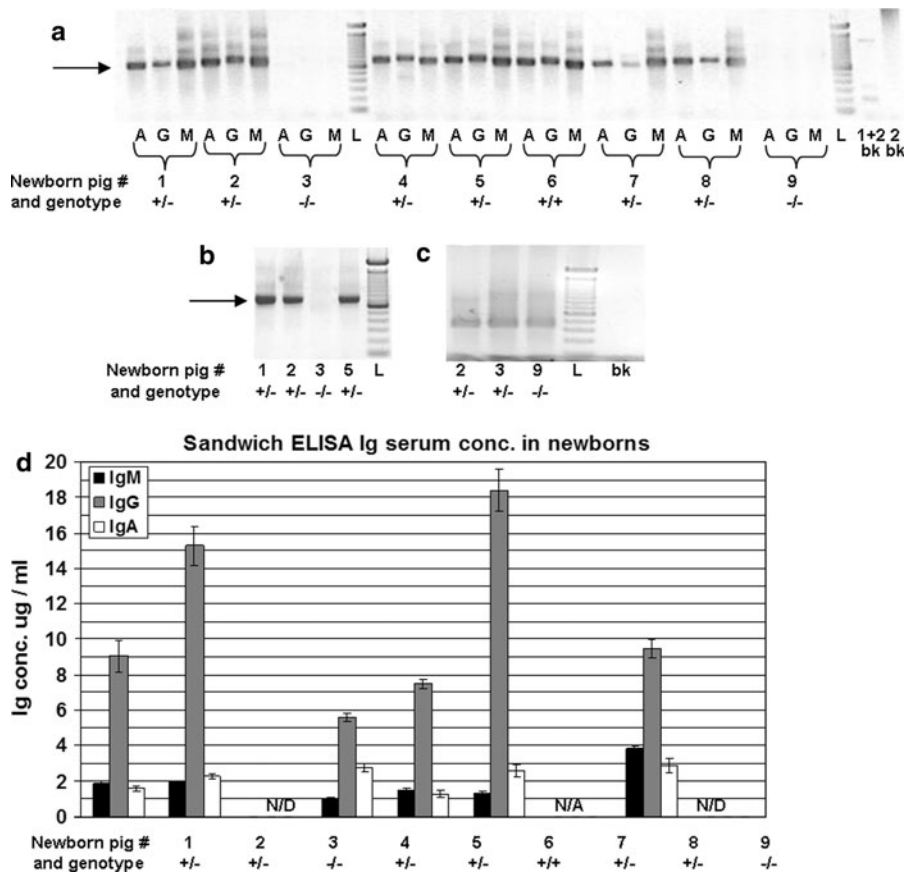
Fig. 3 Transcription and serum titers of major Ig isotypes in colostrum-deprived $J_H +/+$, $J_H \pm$ and $J_H -/-$ newborn piglets. **a** RT-PCR recovery of splenic transcripts encoding rearranged IgA (A) IgG (G) and IgM (M) in piglet piglets 1–9 genotyped in Fig. 1b. Negative controls for the first round PCR (bk 1+2) and the second round (bk 2) are indicated together with a polynucleotide ladder (L). *Arrow points* out expected band.

b RT-PCR recovery of transcripts for IgM from WBC of representative piglets. *Arrow points* out expected band. **c** RT-PCR recovery of rearranged TCR_β transcripts from WBC of representative piglets. **d** Ig levels in the sera of littermates of various genotypes. N/D = below the detection limit of 0.4–0.6 ng/ml; N/A = serum not available for testing. Values are ±Standard Error of the Mean

between follicles. Note that the pig contains MLNs with an inverted microanatomical structure (Jonjic et al. 1987). The MLNs from $J_H +/+$ and $J_H \pm$ piglets showed similar T and B cell zone features. In contrast, $J_H -/-$ MLNs and spleens were completely devoid of B cells. T cells appeared largely unaffected in the $J_H -/-$ piglets in comparison to the stains of the other genotypes. Thus, disrupting both J_H alleles prevents follicular and germinal center formation and displays a lack of Ig isotypes and $CD79\alpha^+$ B cells in secondary lymphoid organs. These data are consistent with the lack of transcription and secretion of Ig isotypes and IgM^+ B cells described earlier.

$J_H -/-$ piglets succumb to infection after conventional weaning

A second litter was allowed to develop past the neonatal age. Five conventionally reared $J_H -/-$ piglets that were allowed to suckle all remained healthy until weaning at ~ 4 weeks postpartum. Thereafter they suffered from a wasting-like syndrome that is characteristic of bacterial infection over a period of two months (Table 2). Just as the $J_H -/-$ piglets, these $J_H -/-$ pigs were devoid of IgM^+ B cells in the bloodstream (3 of which are shown in Fig. 2b). Normally, serum IgG levels rise to ~ 30 mg/ml after
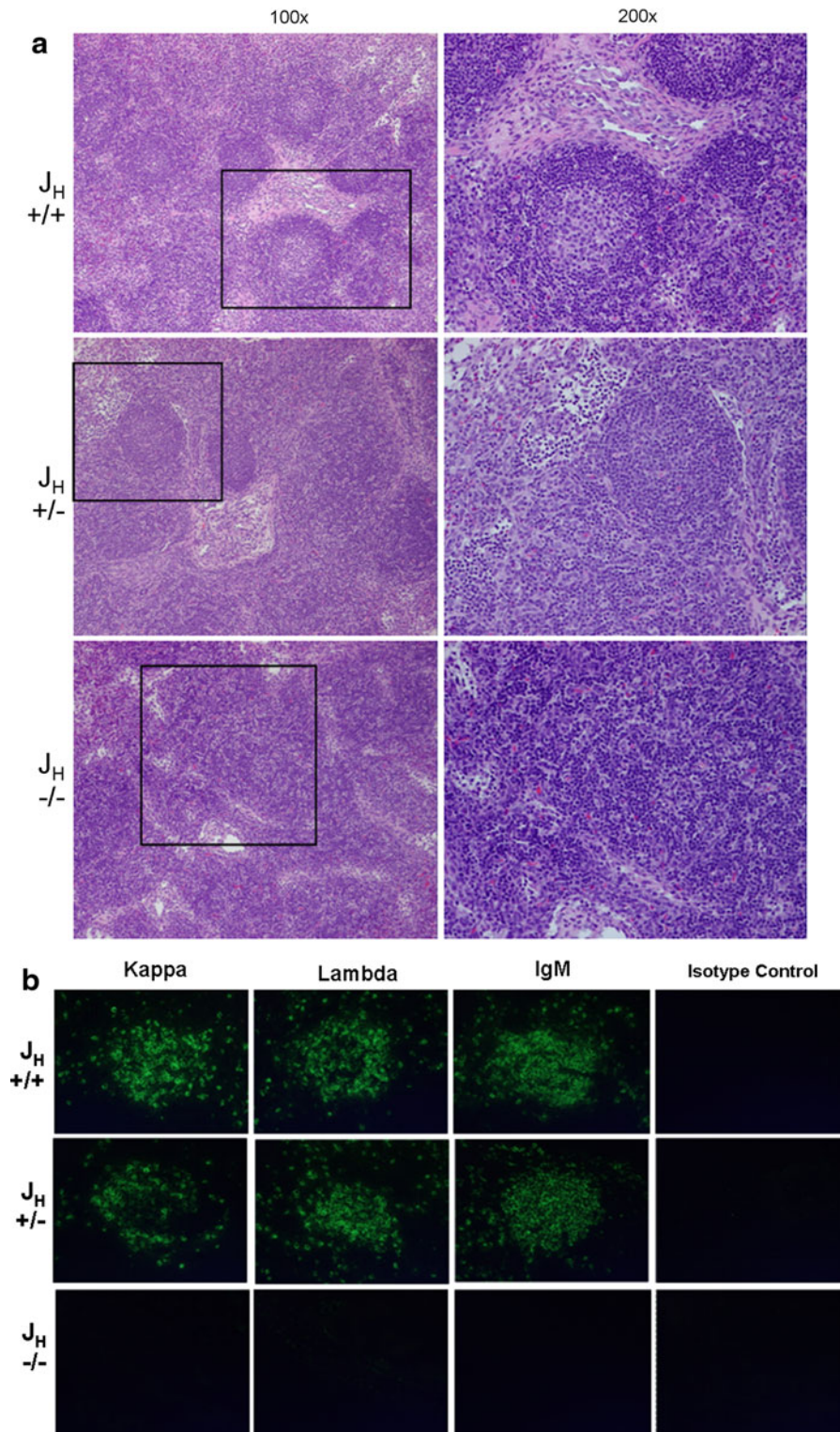
◀ **Fig. 4** Histochemistry and fluorescence IHC of the LNs of J$_H$ +/+, J$_H$ ± and J$_H$ −/− newborn piglets. **a** MLN were H and E stained and areas of follicular development were analyzed. In a J$_H$ +/+ piglet (*top*, piglet # 6), follicles were developed and have a distinct germinal center, which is visible at both *lower* magnification (100×, *left side*) and at *higher* magnification (200×, *right side*, centered on *black box* in 100×). In one example of a J$_H$ ± piglet (*middle*, piglet # 1), follicles have structure but are not fully developed, and lack intact germinal centers. In all J$_H$ −/− piglets (*bottom*, representative piglet # 9), there is no distinguishable follicular or germinal center development. **b** MLNs were removed and stained with anti-porcine kappa light chain, anti-porcine lambda light chain, anti-porcine IgM, or isotype control Abs. In the J$_H$ +/+ piglet (*top*, piglet # 6), staining for either light chain and for IgM are positive in MLN follicles. In a J$_H$ ± piglet (*middle*, representative piglet # 1), a similar staining pattern can be found. In all J$_H$ −/− piglets (*bottom*, representative piglet # 9), there is no staining for either light chain or IgM. Magnification 200×

suckling and then decline with a half-life of 10 days. However, since some de novo synthesis continues after birth, levels at 8 weeks postpartum do not normally drop below 6–8 mg/ml (Klobasa et al. 1981). Although, in the J$_H$ −/− piglets, the IgG level at 8 weeks for the same 3 pigs tested in Fig. 2b was as low as ∼7% of the levels of their J$_H$ +/+ littermates (i.e. 0.5–1.7 mg/ml versus 7–8 mg/ml, data not shown). These low levels are well below the cutoff for survival. Although IgG absorption by J$_H$ −/− piglets had not been determined, these low post-weaning levels suggest the lack of *de novo* synthesis in these conventionally reared piglets. As seen in the earlier tested J$_H$ −/− piglets, all 5 weaned J$_H$ −/− pigs of this litter were devoid of CD79α$^+$ B cells and follicular/germinal center development in MLNs and spleens, with no effect on staining of T cells (Table 2). The cause of illness was determined by necropsy performed by an expert veterinary pathologist. Thus, J$_H$ −/− piglets are severely susceptible to bacterial infection and cannot be conventionally maintained after weaning.

Discussion

Fibroblasts are currently the most commonly used cell type for genetic engineering in livestock, including pigs. However, gene targeting using a promoter-trap strategy, which requires expression of the target gene, may not always be feasible due to a restricted repertoire of genes expressed in fibroblasts. In this study, a poly(A)-trap strategy was successfully used

to obtain targeted recombination events in fibroblasts where promoter trapping [previously performed by our group (Dai et al. 2002)] would not be feasible (Rogers et al. 2008a). Knockout of the single functional porcine J$_H$ region gene segment, which is not actively transcribed in fibroblasts, was demonstrated in primary somatic cells. There are only two other targeted loci previously published in swine (Dai et al. 2002; Rogers et al. 2008a), and this is the first successful poly(A)-trap strategy ever published in a livestock species. Rodents and humans can utilize 4–6 J$_H$ gene segments, but swine only utilize one (Butler et al. 1996; Jung et al. 2006). J$_H$ −/− pigs were devoid of IgM$^+$ B cells, IgM transcripts and serum Ig isotypes in the blood. J$_H$ −/− pigs also lacked follicular architecture, Ig isotype transcripts and heavy and light chain proteins, and B cells in secondary lymphoid organs. These findings are consistent with studies on B cell-deficient mice and humans (Chen et al. 1993; Jakobovits et al. 1993; Busslinger 2004; Jung et al. 2006; Blom and Spits 2006; LeBien and Tedder 2008).

Regardless of the resulting Ig HC isotype, either kappa or lambda light chain must be associated with a HC in order to produce a functional Ab. In our study, the blood and secondary lymphoid organs of J$_H$ −/− piglets were devoid of detectable transcription and secretion of all major Ig isotypes and all Ig protein chains (heavy or light chains), respectively, proving that J$_H$ −/− pigs are completely deficient of Ab. Productive VDJ rearrangement at the HC locus is required for the generation of the pre-B-cell receptor, which signals differentiation from the pro-B-cell to the pre-B-cell stage during B cell development (Jung et al. 2006). Essentially, functional VDJ rearrangement at the HC locus is required for B cell survival during early development (Jakobovits et al. 1993). This was confirmed for pigs by this study, since no IgM$^+$, kappa or lambda light chain$^+$, or CD79α$^+$ B cells were found in the blood or peripheral lymphoid organs of the J$_H$ −/− pigs. Also, the lack of B cells and follicular architecture in the J$_H$ −/− piglets supports previous contentions that development of follicles is B cell-dependent (Randall et al. 2008). We showed that normal rearrangements were observed in T cells for the TCR$_β$ chain of all pigs tested, which, like the HC locus, undergoes VDJ recombination (Chowdhury and Sen 2004). Our data suggest that targeting both alleles of the J$_H$ region of the HC locus

Fig. 5 Distribution of T and B cells determined by IHC using anti-CD3 and anti-CD79α in newborn piglets. In a J_H +/+ piglet (*top*, piglet # 6), follicles were developed and have a distinct germinal center, which is visible at both *lower* magnification (*low*, 100×, *left side*) and at *higher* magnification (*high*, 200×, *right side*, centered on *black box* in *low*). In one example of a J_H ± piglet (*middle*, piglet # 1), follicles have structure but are not fully developed, and lack an intact germinal center. In all J_H −/− piglets (*bottom*, representative piglet # 9), there is no distinguishable follicular or germinal center development
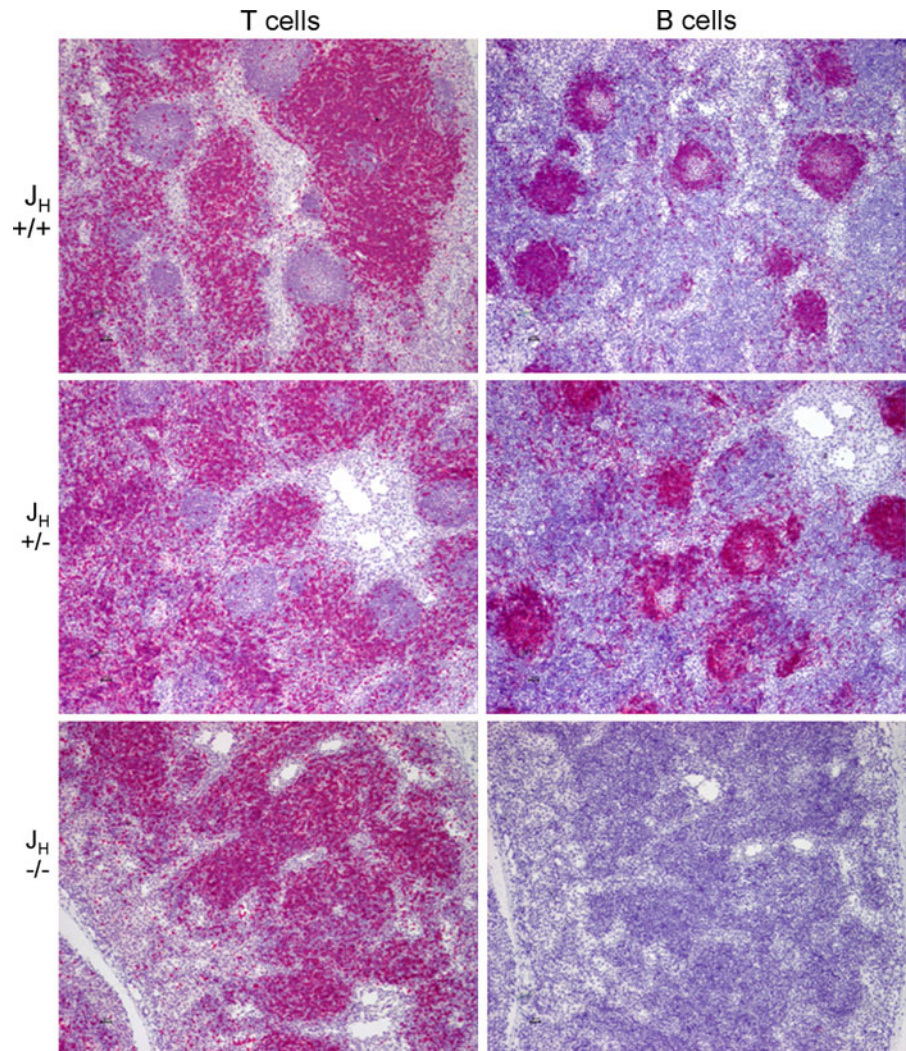
has no significant observed effect on VDJ recombination at T cell loci or subsequent generation of CD3$^+$ T cells.

With the absence of Ab and B cells in the J_H −/− pigs, they succumb to apparent bacterial infections when Ab supplied by the sow's colostrum was significantly diminished. It is known from studies on conventionally reared piglets that when serum IgG levels are < 5 mg/ml, piglets become highly susceptible, and die primarily from bacterial infections

(Klobasa et al. 1981). In this report, the 3 J_H −/− piglets that survived to 8 weeks of age (∼4 weeks post-weaning) showed a range of IgG levels of 0.5–1.7 mg/ml. Although bacteria were not seen in the lesions, the characteristics of the inflammation were always consistent with a bacterial infection. The absence of detectable levels of bacteria is not surprising, since the bacterial numbers were likely too low to be seen by light microscopy. Based on the fact that no immunocompetent pigs of the same age

Table 2 Summary of necropsy results from J_H −/− pigs reared normally that fell ill post-weaning

Pig #	Post-weaning (weeks)	T lym.	B lym.	Foll. Arch.	Cause of illness
315-10	1	+	−	Absent	Bacterial Enterocolitis
315-6	1.5	+	−	Absent	Interstitial Pneumonia (Septicemia)
315-3[a]	5	+	−	Absent	Bacterial Bronchopneumonia
315-4[b]	6	+	−	Absent	Bacterial Pneumonia
315-9[c]	7	+	−	Absent	Bacterial Enteritis and Septicemia

J_H −/− pigs were culled upon veterinarian-diagnosed signs of failing health. All J_H −/− pigs fell ill within 7 weeks post-weaning. Full necropsies were performed to determine cause of illness. Specimens were collected from MLN and splenic tissue to determine presence or absence of follicular architecture (Foll. Arch.) as described in Fig. 4a, and were stained for T and B cell markers as described in Fig. 5, recorded as either positive (+) or negative (−). MLN and spleen results were qualitatively equal for each pig

[a, b, c] Same J_H −/− pigs as shown in Fig. 2b that lacked IgM$^+$ B cells, and had as low as ∼7% of IgG levels of J_H +/+ littermates

range (including wild-type littermates) weaned normally had any evidence of infectious diseases, and the pigs were housed in clean facilities, the lesions were most likely caused by opportunistic bacteria. The slight temporal differences in post-weaning illness onset among J_H −/− pigs is likely due to each piglets' ability to suckle ample quantities of Ab-containing colostrum from the sow soon after birth. Based on this phenotype, for some disease models, facilities with gnotobiotic capabilities would be required.

Interestingly, the range of IgM$^+$ B cell surface expression, IgG production, and follicular development in the MLNs of J_H ± piglets was less than in a J_H +/+ piglet, over a fairly wide range. Thus, it may be useful to study these heterozygotes to confirm the apparent effect of the loss of one J_H allele on the ability to make productive VDJ rearrangements. According to allelic exclusion, if the rearrangement on the first chromosome fails, rearrangement on the second chromosome and subsequent expression can still occur (Jung et al. 2006). Since J_H ± B cells have only one functional allele, fewer productive rearrangements and, therefore, fewer B cells may be formed. However, this is in contrast to earlier reports from one group on B cell numbers in IgM +/+ versus IgM ± mice (Kitamura et al. 1991; Kitamura and Rajewsky 1992). This may be related to the species differences, targeting strategies, or any potentially different mechanisms of generating Ab diversity between mice and swine. In previous studies, it was observed that in fetal liver and yolk, 90% of rearrangements were productive, whereas this dropped to the expected 67% in late-term bone

marrow (Sinkora et al. 2003). Accordingly, this reported observation in piglets, and the histological and IHC observations reported here, suggest the need to learn more about VDJ rearrangement in swine.

The availability of a B cell-deficient large animal model would be a valuable tool for studies aimed at determining whether protection from veterinary or human infectious agents is mediated by humoral or cellular immunity, or both. In infectious disease research and vaccine development, it is important to study the cellular and humoral immune response, both together and separately. Robl et al. first reported the targeted disruption of one functional HC locus in a large animal, namely cattle (Kuroiwa et al. 2004). No immunological characterization was performed to show a B cell and Ab deficiency at that time. More recently, the same group reported that cows express two functional HC loci (Kuroiwa et al. 2009) in contrast to the one functional locus expressed in mice (Kitamura et al. 1991), pigs (Butler et al. 2009) and humans (Yel et al. 1996). They showed that both bovine loci (four alleles in total) must be targeted for the cattle to become Ab- and B cell-deficient (Kuroiwa et al. 2009). The time and resources required to generate and house sufficient B cell-deficient cattle for research studies is significantly more than for the B cell-deficient pig model described herein. Specifically, for bovine B cell deficiency, 4 rounds of targeting and SCNT is required by their strategy, where cows are only produced by cloning (Kuroiwa et al. 2004; Kuroiwa et al. 2009). The method to generate B cell-deficient pigs has fewer burdens on molecular biology and animal cloning teams, with only 1 round of targeting

and 1 round of cloning required, following by standard breeding of heterozygous knockout animals. Also, since B cell-deficient pigs are: (1) generated by breeding or cloning, (2) litter bearing (cows usually produce one offspring versus over 10 on average for pigs naturally bred at our facility), (3) smaller than cows, and (4) less difficult to raise under gnotobiotic conditions (Butler and Sinkora 2007), producing and utilizing B cell-deficient pigs as models is much more time and cost efficient in the short- and the long-run.

J_H −/− pig models can have special applications in veterinary research on such agents as PRRSV, and other infections. PRRSV is a world-wide pandemic pig disease, which causes over a billion dollar per year impact on the commercial pig industry. Since PRRSV seems to cause virulence by subverting humoral immunity through polyclonal activation of the pre-immune B cell repertoire (Butler et al. 2008), the B-cell deficient pig model will likely be of significant value in disease etiology. Theoretically, if B cell-deficient piglets are reared in isolator units to protect them from bacterial infection, the degree to which polyclonal B cell activation contributes to PRRSV-induced immune dysregulation could be assessed. These same piglets could serve as a model to determine the relative role of cell-mediated and Ab-mediated viral immunity not only to PRRSV but other troublesome neonatal swine viruses such as influenza, parvovirus, and circovirus, which would have valuable agricultural applications (Butler et al. 2008). These include aiding in vaccination strategies for pigs (Welter and Welter 1990; Halbur et al. 1996).

There are also a number of human diseases that cannot be properly modeled in rodents (Forsberg 2005; Butler et al. 2008, 2009; Melo et al. 2007; Rogers et al. 2008a). For example, because of similarities with humans, pigs that lack the expression of the ion transport gene associated with cystic fibrosis (CFTR) have been generated to study this disease, since CFTR knockout mice fail to develop any clinically relevant form of cystic fibrosis (Rogers et al. 2008b). In the case of CF, there are always questions of whether bacterial infections like Pseudomonas that are often associated with CF are primary or secondary effects. This can be tested using germ-free CFTR knockout animals. It is yet unclear whether some CF pathology or CF protection is Ab mediated. Perhaps by crossbreeding CFTR −/− to J_H −/− pigs to obtain a double knock-out pig some

insight can be gained. Since pigs resemble humans in gastrointestinal physiology, dietary requirements and mucosal immunity, they have also been utilized as gnotobiotic models in studies involving rotavirus (Saif et al. 1996; Yuan et al. 2008), norovirus (Cheetham et al. 2006; Wang et al. 2006; Souza et al. 2007) and sapovirus (Wang et al. 2006), and both *Heliobacter pylori* (Krakowka et al. 1991) and *Escherichia coli* (Gunzer et al. 2003) infections. Therefore, the roles of humoral immunity in these and other infections, as well as in potential zoonotic infections [e.g. swine flu, Hepatitis E (Meng 2003)], can be addressed with the J_H −/− pigs (Butler et al. 2008, 2009). Findings from such studies should be highly useful to researchers designing vaccination strategies in humans to meet unmet clinical need.

A pig lacking a functional HC locus should be devoid of Ig expression and B cells, and, therefore, completely deficient in Ab-mediated humoral immune responses. We have shown the generation of a functional knockout of the HC locus herein. Further characterization experiments of this model are planned, and include evaluation of the stages involved in the B cell maturation block in the bone marrow of the J_H −/− pigs. Results would be expected to be similar to those reported for mice and humans, where it is known that an IgM knockout cannot ablate pro-B-cell generation (Kitamura et al. 1991; Jakobovits et al. 1993; Jung et al. 2006; LeBien and Tedder 2008). In addition, analysis of B and T cell numbers in blood, and in primary and secondary lymphoid organs will be performed, to confirm our findings and quantitatively show that targeting both alleles of the J_H region of the porcine HC locus has no effect on T cells. Further studies include whether IVIG from wild-type pigs could rescue the J_H −/− pig's susceptibility to common bacterial pathogens, and finally, quantitative testing of the J_H −/− pig's immune system via challenge with T-cell dependent and T cell-independent vaccines, followed by analyzing subsequent cellular responses in comparison to wild-type pigs under gnotobiotic conditions. This would complete the validation of the J_H −/− pig model for aiding in vaccination strategies.

If B cell-deficient pigs are later constituted with the human Ig locus such that they develop B cells and secrete human Abs, much more significant value will be added; however, certain issues will have to be addressed. These include whether there will be proper

communication between "humanized B cells" and porcine T cells so that T cell-dependent B cell responses and antigen-specific Ab production are realized. Also, since these animals likely would be reared in a conventional or Specific Pathogen- Free (SPF) environment to provide a practical source of IVIG, it remains unknown whether the human Abs produced in the pig will provide protection for the pig in such environments. Finally, significant purification steps during isolation of the humanized Abs will be required (as for any transgenic protein production system), to avoid any contamination with other porcine proteins or the potential horizontal transfer of zoonotic agents. Proof of concept for human Ig locus recombination in other species in production of 'humanized' Abs has already been provided, including mice and cattle (Lonberg et al. 1994; Nicholson et al. 1999; Tomizuka et al. 2000; Kuroiwa et al. 2009). Mice lack sufficient blood volume to make 'humanized mouse IVIG' practical. Pigs have several advantages over cattle, including shorter gestation length and time to puberty, and being large litter bearing provide a significantly larger blood volume to utilize per generation. In addition, a more efficient genetic engineering strategy can be utilized to generate the genetic platform. The latter is due to the fact that the pig only has one functional HC locus to target, and the targeted disruption of the porcine kappa (Ramsoondar et al. submitted) and lambda light chains is well underway. Therefore, 100% of all Ab produced in the pig would be fully human, generating more Ab overall and requiring less costly and less difficult purification processes. High volume, pathogen-free, fully-human IVIG from genetically-modified pigs could improve current IVIG immunotherapy by alleviating safety and supply issues, and targeting production to specific viral infections (HIV, hepatitis C, SARS, Ebola, swine flu, etc.), antibiotic-resistant bacterial infections (e.g. MRSA) and bio-warfare agents (e.g. Anthrax, Smallpox, etc.) (Waltz 2006; Butler et al. 2009).

In conclusion, the J_H −/− pig (1) can be immediately utilized as a pre-clinical model to study porcine and human diseases in the absence of B cells to aid vaccine development for agricultural and clinical applications, and (2) provides a starting point for generating pigs capable of producing fully human, antigen-specific, pathogen-free polyclonal Abs for immunotherapy to prevent or treat numerous diseases and pathogens with unmet clinical need.

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