

High efficiency of BRCA1 knockout using rAAV-mediated gene targeting: developing a pig model for breast cancer

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Abstract Germline inactivating mutations of the breast cancer associated gene 1 (BRCA1) predispose to breast cancer and account for most cases of familiar breast and/or ovarian cancer. The pig is an excellent model for medical research as well as testing of new methods and drugs for disease prevention and treatment. We have generated cloned BRCA1 knockout (KO) Yucatan miniature piglets by targeting exon 11 using recombinant adeno-associated virus (rAAV)-mediated gene targeting and somatic cell nuclear transfer by Handmade Cloning

(HMC). We found a very high targeting rate of rAAV-mediated BRCA1 KO. Approximately 35% of the selected cells were BRCA1 targeted. One BRCA1 KO cell clone (5D1), identified by PCR and Southern blot, was used as nuclear donor for HMC. Reconstructed embryos were transferred to three recipient sows which gave birth to 8 piglets in total. Genotyping identified seven piglets as BRCA1 heterozygotes (BRCA1^{+/ Δ 11}), and one as wild type. The BRCA1 expression was decreased at the mRNA level in BRCA1^{+/ Δ 11} fibroblasts. However, all BRCA1^{+/ Δ 11} piglets died within 18 days after birth. The causes of perinatal mortality remain unclear. Possible explanations may include a combination of the BRCA1 haploinsufficiency, problems of epigenetic reprogramming, presence of the marker gene, single cell clone effects, and/or the special genetic background of the minipigs.

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Introduction

Germline inactivation mutations of the breast cancer associated gene 1 (BRCA1) predispose to breast and/or ovarian cancer and account for approximately 5–10% of all breast cancer cases, including approximately 50% of familial breast cancer cases, and over

80% of familial breast and ovarian cancer cases (Rosen et al. 2003). More than 1,200 distinct documented variants have been found in the human BRCA1 gene, with a majority of variants leading to loss of BRCA1 expression or protein truncation and predisposition to cancers (Szabo et al. 2004). Although BRCA1 variants are rarely described in sporadic breast cancers, expression of BRCA1 is altered in some sporadic breast cancers or cell lines, mostly in association with abnormal DNA methylation status (Rice et al. 2000). The BRCA1 gene encodes a nuclear protein of 1,863 amino acids (aa) in human, 1,812 aa in mice and 1,863 aa in pigs (as in man) (Lane et al. 1995; Miki et al. 1994). It participates in a variety of biological processes including checkpoint control of cell cycle, transcriptional regulation, protein ubiquitination, homologous recombination and DNA damage repair (Deng 2006; Venkitaraman 2002; Yoshida and Miki 2004).

Attempts to create mouse models carrying targeted disruptions of BRCA1 revealed the crucial role of this gene in embryonic development and cell proliferation regulation (Hakem et al. 1996). Homozygous mice (BRCA1^{Δ11/Δ11}) died during the embryonic stages (E9.8–13.5) due to developmental delay and cellular proliferation defects (Gowen et al. 1996; Hakem et al. 1996). On the other hand, an additional P53 mutation (P53^{+/-}) could rescue the BRCA1^{Δ11/Δ11} mice embryos (Crook et al. 1997; Ludwig et al. 1997). Several presumptive mouse models were generated and tumors were developed in them with varying degrees of similarity to their human counterparts, as reviewed by Bouwman and Jonkers (2008). Heterozygous BRCA1 KO mice are developmentally and reproductively normal. However, heterozygosity for BRCA1 mutations in mice is not sufficient to predispose to breast cancer even at an advanced age while human BRCA1 mutation carriers have a 50–80% risk of developing breast cancer by the age of 70 (Alberg et al. 1999). Somatic loss of heterozygosity (LOH) of the remaining wild type allele is frequently found in human breast cancer cells but not in mice. The discrepancy between humans and mice could be due to differences related to the genetic constitution, environment and/or lifespan.

The pig is an excellent model for medical research including testing of new methods and drugs for disease prevention and treatment. A porcine genome survey revealed that the pig is genetically more

similar to man than conventional laboratory animals (Wernersson et al. 2005) in agreement with the similarities in organ development, physiology and metabolism. Factors including the size of organs, human-like physiology and longevity make the pig a good model for studying the pathological development of chronic or age-related illnesses, such as degenerative and malignant diseases.

Methods to develop genetically designed pig models of human diseases, especially targeted gene disruption by homologous recombination have been rapidly advanced during the last decade. The recombinant adeno-associated virus (rAAV)-mediated gene targeting was first reported by Russell et al. in 1998 (Russell and Hirata 1998) and improved by Kohli and co-workers (Kohli et al. 2004). A pig model of Cystic Fibrosis has been successfully generated using this method (Rogers et al. 2008). Handmade Cloning (HMC) is a cost-efficient technology for porcine somatic cell nuclear transfer (SCNT) without the use of micromanipulation (Du et al. 2007; Vajta 2007). A pig model expressing the dominant mutation APP^{sw} for Alzheimer's disease has been created using HMC at our institution (Kragh et al. 2009). In this study, we used rAAV-mediated gene targeting and HMC to generate BRCA1 KO piglets with the objective of creating a model for BRCA1-associated mammary carcinogenesis.

Materials and methods

Chemical information

Primers used in this study were synthesized by DNA Technology A/S, DK. PCR reactions were carried out using Platinum Pfx DNA Polymerase (Invitrogen, Cat#: 11708-039). Polyclonal rabbit Anti-human BRCA1 antibody (BRCA1 (D-20)) was purchased from Santa Cruz Biotechnology (Cat#: sc-641). Polyclonal goat Anti-rabbit immunoglobulins/HRP (Cat#: P0448) was purchased from DaKo. Rabbit polyclonal antibody for beta Actin—Loading Control (ab8227) was ordered from abcam, Cambridge, CB4 0FW, UK. DMEM was purchased from LONZA (Cat#: BE12-604F). G418 (Cat#: 10131027) was purchased from Invitrogen. Recombinant human basic fibroblast growth factor (bFGF) was purchased from Sigma (Cat#: F0291). Prime-It Random Primer

Labeling Kit was purchased from Stratagene (Cat#: 300385). Plasmid Mini-prep and Maxi-prep kits were purchased from QIAGEN (Cat#: 27104 and Cat#: 12163, respectively). Restriction enzymes used in this study were purchased from New England Biolabs. *XL2-Blue* Ultracompetent cells were purchased from Stratagene (Cat#: 200150).

Cell culture

Primary porcine fibroblasts were established from ear biopsies of fetal or newborn Yucatan minipigs. Fibroblasts were cultured in DMEM media supplemented with 15% FCS, penicillin/streptomycin, and glutamine. In one experiment the medium was additionally supplemented with bFGF (5 ng/ml).

Construction of rAAV/BRCA1 KO vector

The rAAV/BRCA1 KO vector was constructed as previously described (Kohli et al. 2004; Rogers et al. 2008). Two homology arms (~1 kb each), from exon 11 of the BRCA1 gene, were amplified by PCR using Yucatan minipig genomic DNA as template. These two homology arms were subsequently linked to a neomycin/zeocin gene cassette by a three-way fusion PCR. The neomycin/zeocin genes were comprised in a 4 kb *PvuI* fragment isolated from a pNeDaKO-Neo plasmid (a generous gift from Bert Vogelstein & Kenneth W. Kinzler, The Johns Hopkins University Medical Institutions, Baltimore, MD 21231, USA). The three-way fusion PCR was performed as described by Kohli et al. (2004) using the following PCR protocol: 1 cycle of 94°C for 1 min; 27 cycles of 94°C for 30 s, 57°C for 30 s, and 68°C for 4 min; 1 cycle of 68°C for 7 min. Fusion products were digested with *NotI* and ligated to a *NotI* cleaved pAAV-MCS plasmid backbone (Stratagene, Cat. #240071-5) containing an ampicillin (Amp) resistance conferring gene (*B-lactamase gene, bla*) and the viral inverted terminal repeat sequences (ITRs). The final rAAV/BRCA1 KO plasmid construct was verified by sequencing and *NotI* digestions (Fig. S1, S2). PCR primers for making the rAAV/BRCA1 KO constructs are listed in Table S1.

rAAV/BRCA1 KO virus packaging

Packaging of the rAAV/BRCA1 KO virus (serotype 1) was performed by the Vector Core Facility at the

Gene Therapy Center, University of North Carolina at Chapel Hill (UNC), USA. Briefly, the pAAV/BRCA1 KO plasmid was co-transfected into 293 cells with pXX680 and pHelper plasmids. Twenty-four hours post-transfection, medium from the transfected cells was removed and replaced with fresh medium. Virus was harvested 48–72 h post-transfection and purified using gradient purification. For a detailed protocol please refer to the protocol published by Grieger et al. (2006).

Transduction and selection

Primary fibroblasts (1×10^6) were seeded onto a gelatin coated 10 cm cell culture dish 1 day before transduction. The rAAV/BRCA1 KO virus particles (5×10^{11} particles) were added to the cells for 24 h, followed by trypsinization and seeding into gelatin-coated 96-well plates at a dilution of one 10 cm dish into fifty 96-well plates, which should give about 400 cells per well. Three days after transduction (2 days after splitting into 96-well plates), cells were selected with G418 (1 mg/ml) for 2 weeks with the medium changed every 3–4 days. After selection, G418-resistant cell clones were trypsinized, and 1/3 were transferred to 96-well PCR plates for PCR screening; 1/3 were cultured in gelatin-coated 96-well cell culture plates for Southern blot analysis; 1/3 were cultured in gelatin-coated 96-well plates for freezing and these cells were subsequently used as nuclear donor cells for SCNT.

PCR screening

G418-resistant cells in 96-well PCR plates were harvested, centrifuged, and re-suspended in 25 μ l lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-Cl, pH 8.5, 0.5% Nonidet P40, 0.5% Tween, 400 μ g/ml Proteinase K). The cells were lysed (65°C for 30 min, 95°C for 10 min) (McCreath et al. 2000) and 1 μ l lysate was used for PCR screening. Primers for screening of the BRCA1 targeting events are listed in Table S1. PCR conditions were as follows: (1) neo screening: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 20 s, 67°C for 30 s, and 68°C for 40 s; followed by 68°C for 7 min. (2) BRCA1 5' targeting screening: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 20 s, 61°C for 30 s, and 68°C for 3 min; followed by 68°C for 7 min. (3) BRCA1 3'

targeting screening: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 67°C for 30 s, and 68°C for 3 min; followed by 68°C for 7 min.

RT-PCR

Total RNA were extracted from fibroblasts (passage 4) using RNAeasy plus Mini Kit (QIAGEN, Cat #: 74134) according to the manufacturers' protocol. Synthesis of cDNA was performed with the iScript cDNA Synthesis Kit (BIO-RAD, Cat #: 170-8890) from 1 µg of total RNA. 1 µl (two times diluted) cDNA product was used as template for semi-quantitative or quantitative real-time PCR analysis. Quantitative RT-PCR was carried out using F-410 2× DyNAmo HS Master Mix (FINNZYMES) on LightCycler 480 (Roche). Primers are listed in Table S1.

Southern blot analysis

Genomic DNA (15 µg) was digested with *BlnI* restriction enzyme (6 U/µg DNA) over night, followed by gel electrophoresis and vacuum blotting. Primers for generating the BRCA1 probe labeling template and the Neo probe labeling template are listed in Table S1. Random probe labeling was performed using the Prime-It II Random Primer Labeling Kit according to the manufacturer's instructions. Pre-hybridization and hybridization were carried out at 42°C, and all washing procedures were performed at 53°C.

Western blot analysis

Western blots of the BRCA1 protein in porcine primary fibroblasts were performed with a BRCA1 (D-20) polyclonal antibody (Santa Cruz Biotechnology, Inc.) raised against a peptide mapping at the N-terminus of the BRCA1 protein. Fibroblasts were sequentially cultured in 10 cm cell culture dishes until ~80% confluence prior to being, washed twice with PBS, and lysed with 100 µl lysis buffer. Twenty micrograms of total protein lysates were electrophoresed in 10% Precise™ Proteom Gels and transferred onto nitrocellulose membranes by semidry-blotting using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell cassette. Membranes were incubated with a

1:1,000 dilution of anti-BRCA1 D-20 antibodies and a 1:10,000 dilution of anti-beta Actin antibodies, followed by HRP-conjugated goat anti-rabbit immunoglobulins. Chemiluminescence detection was achieved with SuperSignal West Dura extended Duration Substrate (Thermo, Cat#: 34076).

Oocyte collection and in vitro maturation

Except where otherwise indicated all chemicals for the embryological work were obtained from Sigma–Aldrich Co. (St Louis, MO, USA).

Cumulus–oocyte complexes (COC) were aspirated from 2 to 6 mm follicles from slaughterhouse-derived sow ovaries and matured in groups of 50 in 400 µl in vitro maturation (IVM) medium consisting of bicarbonate-buffered TCM-199 (GIBCO BRL, USA) supplemented with 10% (v/v) cattle serum (CS), 10% (v/v) pig follicular fluid, 10 IU/ml equine chorionic gonadotrophin, and 5 IU/ml human chorionic gonadotrophin (Suigonan Vet; Skovlunde, Denmark) in the Submarine Incubation System (SIS) for 41–44 h.

Handmade cloning and embryo culture

COCs were shortly treated with 1 mg/ml hyaluronidase and pipetted vigorously to remove the cumulus cells attached to the zonae pellucidae. Zonae pellucidae of oocytes were partially digested with 3.3 mg/ml pronase solution dissolved in T33 (T for HEPES buffered TCM199, GIBCO BRL, USA; while the number refers to the concentration of CS, here 33%) for 20 s, followed by quick washing in T2 and T20 drops. Oocytes with distended and softened zonae pellucidae were lined up in T2 drops supplemented with 2.5 µg/ml cytochalasin B. With a finely drawn and fire-polished glass pipette, oocytes were rotated to locate the polar body. Oriented bisection was performed manually with ultra-sharp splitting blades (AB Technology, Pullman, WA, USA) under a stereomicroscope. Less than half of the cytoplasm close to the polar body was removed from the remaining putative cytoplasm.

Fibroblasts (5D1) were trypsinized and re-suspended in 20 µl of T2. Fusion was performed in two steps, where the second one included the initiation of activation. For the first step, 50% of the available

cytoplasts were transferred into 1 mg/ml of phytohaemagglutinin (PHA; ICN Pharmaceuticals, Girraween, Australia) dissolved in T0 for 3 s, and then each one was quickly dropped over a single fibroblast cell. After attachment, cytoplast-fibroblast pairs were equilibrated in fusion medium (0.3 M mannitol and 0.01% polyvinyl alcohol; PVA) for 10 s and transferred to a fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450; BTX, San Diego, CA, USA). Using AC of 0.06 kV/cm and 700 kHz, pairs were aligned to the wire of the fusion chamber with the somatic cells farthest from the wire, then fused with a DC pulse of 2.0 kV/cm for 9 μ s. After the DC pulse, pairs were removed carefully from the wire, transferred to T10 drops and incubated further to observe whether fusion had occurred. Approximately 1 h after the first fusion, each pair was fused with another cytoplast in activation medium (0.3 M mannitol, 0.1 mM MgSO₄, 0.5 mM CaCl₂ and 0.1% PVA). By using an AC of 0.06 kV/cm and 700 kHz, one fused pair and one cytoplast were aligned to one wire of the fusion chamber, with fused pairs contacting the wire. A single DC pulse of 0.86 kV/cm was applied for 80 μ s. When fusion had been observed in T10 drops, reconstructed embryos were transferred into PZM-3 supplemented with 5 μ g/ml cytochalasin B and 10 μ g/ml cycloheximide. After a 4 h incubation at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂ with maximum humidity, embryos were washed three times and cultured in PZM-3 (Yoshioka et al. 2002) using the well of the well system (Vajta et al. 2000).

Embryo transfer

D5 and D6 fresh blastocysts were surgically transferred into both uterine horns of Danish Landrace sows on day 4 after weaning. Pregnancy was diagnosed by ultrasonography on day 21 and confirmed every second week. The farrowing was induced with an injection of prostaglandine if the sow did not show signs of labor as late as day 121. All animals were housed and cared for in strict accordance to the proposals for animal research reviewed by the Danish Institute of Agricultural Sciences and the Danish Centre for Bioethics and Risk Assessment. Experimental permission was given by the Danish Animal Ethics Committee.

Results

Porcine (*Sus scrofa*) BRCA1 gene

The human BRCA1 gene is located on chromosome 17 (17q21.33), contains 22 coding exons and encodes a protein of 1,863 aa. As illustrated in the human genome browser (<http://www.genome.ucsc.edu/>), most of the disease-related single-nucleotide mutations were found in the biggest exon (exon 11) of the BRCA1 gene. The sequences within exon 11 are unique (analyzed by RepeatMasker) in the genome (Fig. 1a). Only two transcripts of the porcine BRCA1 gene are registered in the NCBI database (AB271921.1 and AB355637.1) encoding a transcript for 1863 aa (BAF62296) and 1592 aa (BAG09491), respectively. By blasting the *Sus Scrofa* genome (Sscrofa_9b) with the transcript AB271921.1, we find that the pig BRCA1 gene is located in chromosome 12 and contains 22 coding exons, with exon 11 homologous to the human exon 11 (Fig. 1b). The encoded porcine BRCA1 protein shows an overall amino-acid identity to the human homolog of 74% (Fig. 1c). Since the mouse BRCA1 protein only shows 58% identity, the porcine BRCA1 protein is evolutionary closer to the human homolog.

A very high BRCA1 targeting efficiency is achieved by rAAV-mediated homologous recombination

An rAAV-based BRCA1 KO viral vector was generated as illustrated in Fig. 2a. The two homology arms, approximately 1 kb each, were amplified by PCR from exon 11 of the porcine BRCA1 gene. Fifty-five base pairs (CACCTAGTCATACTGAGT TACAAATTGATAGTTGTTCTAGCAGTGAAGA GATGAA) should in this way be replaced by a transgene cassette containing the Neomycin (*Neo*) selection gene. BRCA1 targeted events were determined by targeting PCRs with primer pairs F1 + R1 and F2 + R2 that amplify the 5' and 3' targeting products, respectively. Some PCR results with the primer pair F1 + R1 are presented in Fig. 2b, showing that the targeted events can be clearly detected in cell lysates. We measured the rAAV-mediated BRCA1 targeting events in three experiments (Fig. 2c). A rAAV-mediated BRCA1 targeting rate of at least 30% (% of BRCA1 KO clones out of

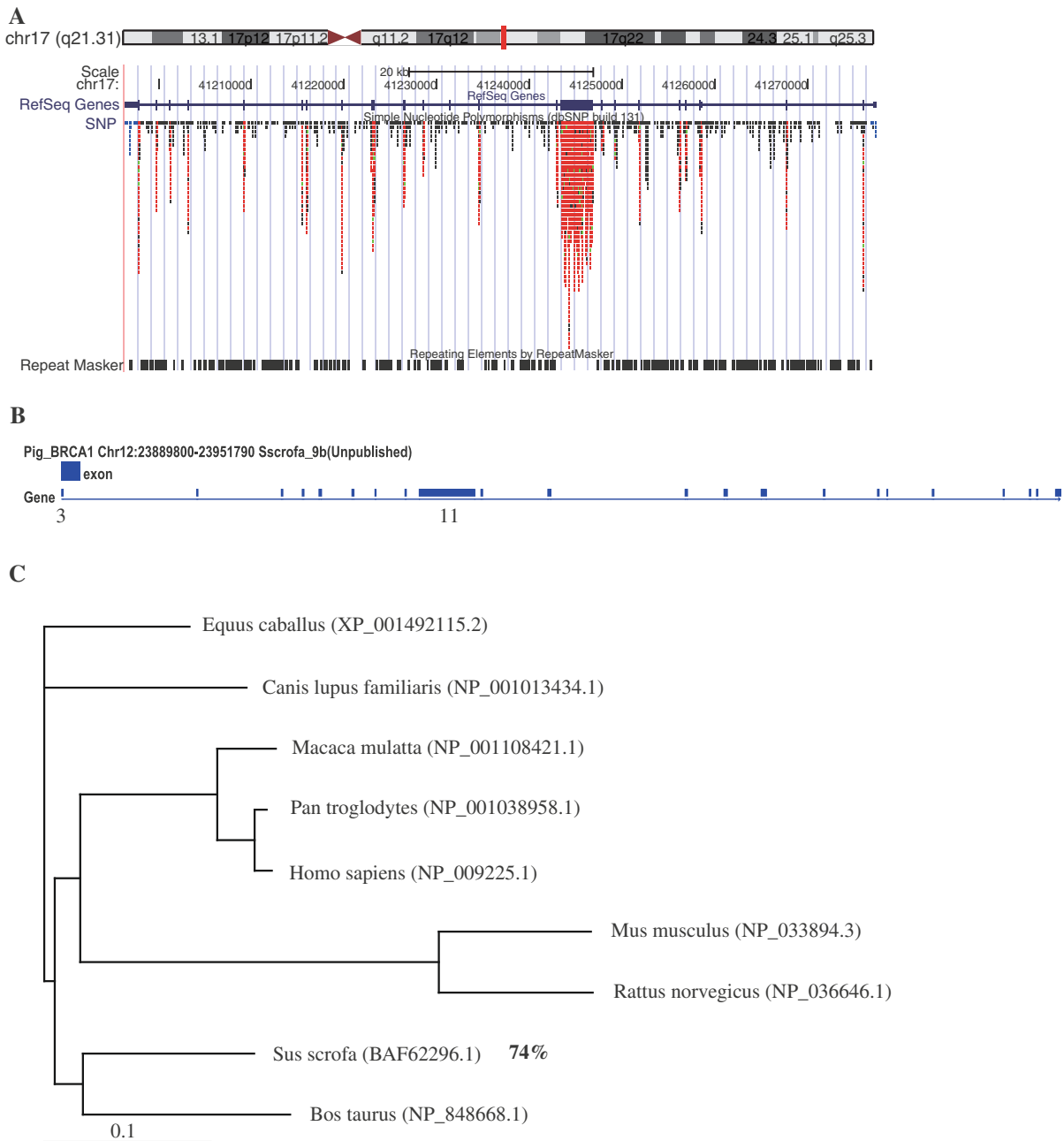


Fig. 1 Bioinformatics analysis of the porcine BRCA1 gene. **a** The human BRCA1 gene with information on chromosome location and presence of genetic variants and repeat sequences (from the Human Genome browser). **b** Classification of coding exons of the porcine BRCA1 gene (GenBank accession number: AB271921.1). The porcine BRCA1 gene is located

in chromosome 12 and comprised of 22 coding exons. **c** Phylogenetic analysis of the BRCA1 protein among major mammals. The porcine BRCA1 protein (GenBank accession number: BAF62296.1) has 74% amino acids sequences similarity to the human BRCA1 protein (NP_009225.1)

G418-resistant clones) was consistently observed in these three experiments with an average targeting rate of ~35%. Due to the limited proliferative capacity of

primary fibroblasts, almost all the BRCA1 KO clones identified from experiment 1 and 2 stopped growing when expanded for Southern blot analysis. In

experiment 3, we added basic fibroblast growth factor (bFGF2, 5 ng/ml) to the cell culture medium since this growth factor has been reported to improve the proliferative capacity of primary cells without affecting cell functions. Indeed, addition of bFGF2 to the cell culture medium can improve the proliferative

capacity of primary porcine fibroblasts without affecting the targeting efficiency (Fig. 2c).

Eight BRCA1 KO clones and two random integration clones (identified by PCR screening) were expanded for Southern blot analysis. Targeting screening PCRs (5', 3' and Neo gene) were performed

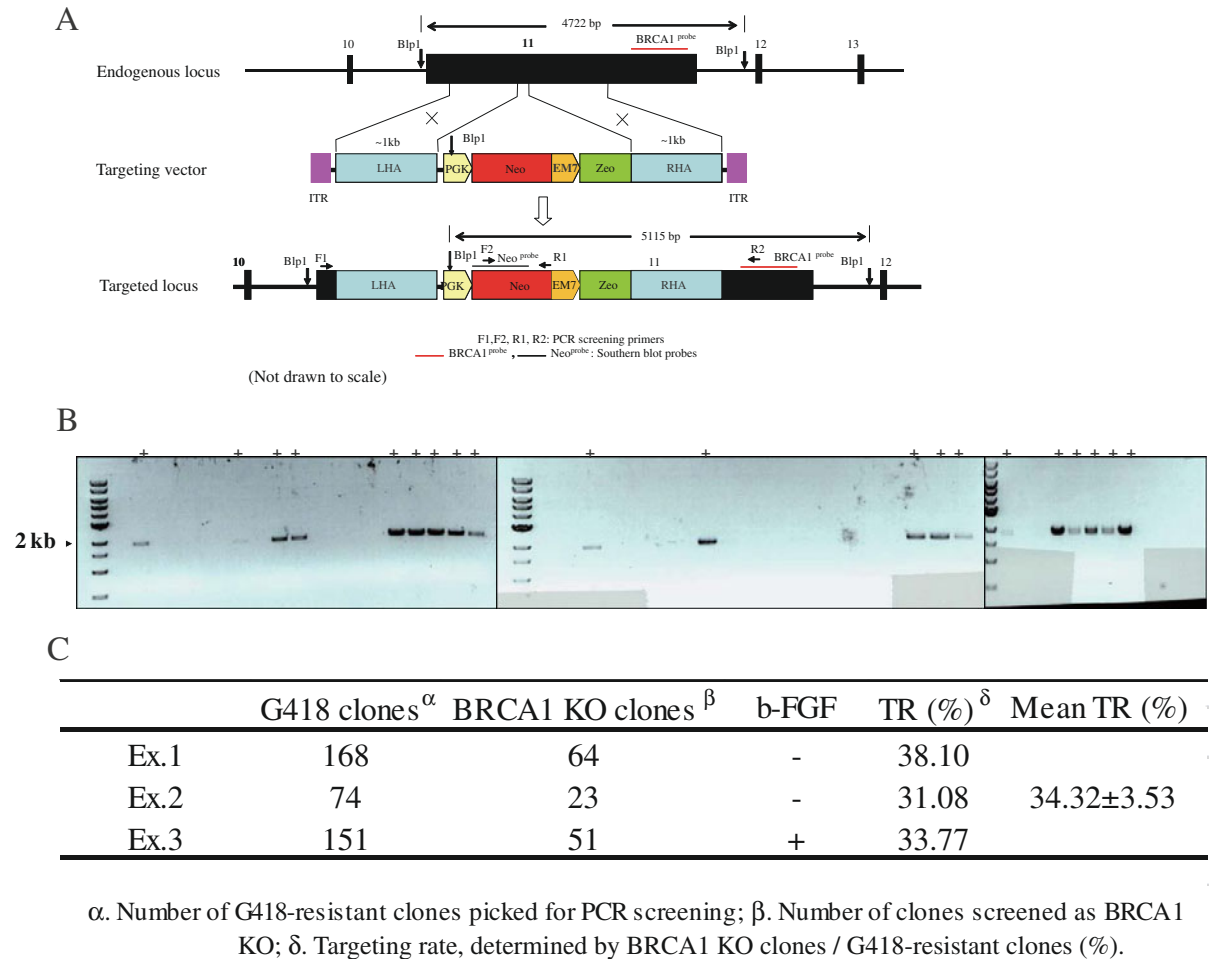


Fig. 2 Generation of BRCA1 KO fibroblasts for SCNT. **a** Schematic representation of the endogenous BRCA1 locus, targeting vector and the targeted locus. Two homology arms (~1 kb each) are homologous with sequences in exon 11 of the endogenous BRCA1 gene. Fifty-five base pairs in exon 11 should be replaced by the “PGK-Neo-EM7-Zeo” selection cassette from the targeting vector, resulting in a targeted fragment of 5,115 bp when *B*l*p*1 digested genomic DNA is hybridized with Neo- or BRCA1-specific probes. Hybridization of *B*l*p*1 digested genomic DNA with a BRCA1-specific probe yields a WT fragment of 2,722 bp. F1, R1, F2, and R2 are primers used for targeting PCR screening. **b** PCR (F1 and R1) screening for BRCA1 targeted events in cell lysates. An amplicon of ~2 kb indicates that a specific clone is correctly

targeted, marked as “+”. **c** Summary of the rAAV-mediated BRCA1 targeting rate in three independent experiments. **d** Screening PCR on genomic DNA from the expanded cells. *Lanes 1–8* represent cell clones that were positive in PCR-screening of cell lysates before being expanded. *Lanes 9–10* represent clones with random integration of the targeting vector. *Lane 11* is a positive BRCA1 KO control. *Lane 12* is genomic DNA from WT cells. **e** Southern blot analysis of the genomic DNA samples that were used in (**d**). *Lanes 13–15* represent 1, 3, and 10 copies of the neo control. Southern blot analysis showed that all BRCA1 KO clones (*Lanes 1, 4–7*) were BRCA1 KO only, without any random integration. Cells represented in *lane 4* were used as nuclear donor cells for SCNT to generate the BRCA1 KO piglets

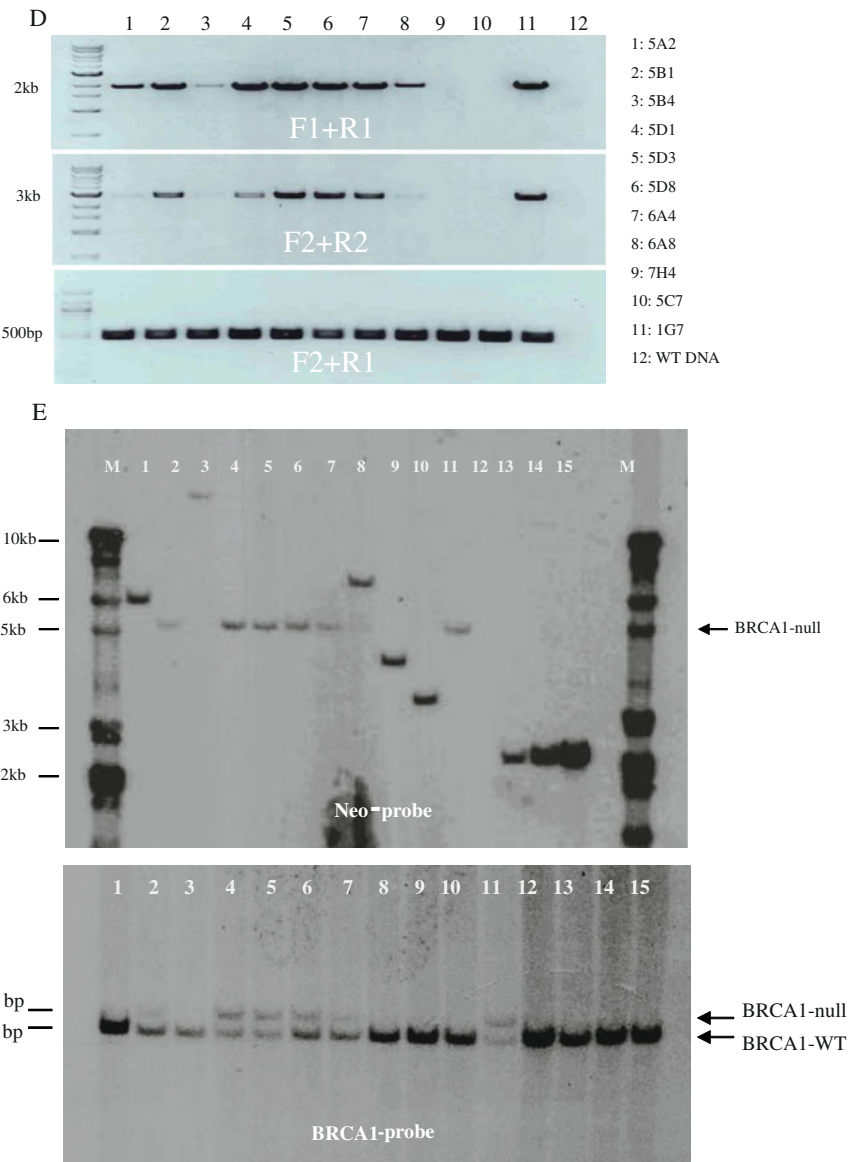


Fig. 2 continued

on the genomic DNA from expanded cells. However, the 3' and 5' targeting PCR production for some clones were rather weak compared to other clones (Fig. 2d). Southern blot results further showed that such clones (e.g. lane 1, 3 and 8 in Fig. 2d) are heterogeneous, consisting of BRCA1 KO cells as well as cells with random integration of the construct with the selectable marker. Clones represented in lane 2, 4, 5, 6 and 7 of Fig. 2d were judged as BRCA1 KO. The clone represented in lane 4 (cell clone ID: 5D1) gave a very clear 1:1 ratio of the BRCA1-null

and BRCA1-WT bands when hybridized with the BRCA1-probe suggesting that 5D1 should be a pure BRCA1^{+/ Δ 11} cell clone.

Transgenic piglets generated by HMCs

The 5D1 fibroblasts were used as nuclear donor cells for somatic cell nuclear transfer by HMC. Seventy-nine, 110 and 158 reconstructed embryos were transferred to three recipient sows which subsequently gave birth to 1, 2 and 5 piglets, respectively

Table 1 Summary of embryonic transfers and their outcome

Donor cells	Breed	Recipient sow ID	Embryos transferred	Live birth	Still birth	Mummies	Piglet ID	Genotype	Survival period
5D1	Yucatan	2083	79	1	0	0	104	BRCA1+/-	18 days
5D1	Yucatan	2199	110	2	0	0	38	BRCA1+/-	3 days
					0	0	39	WT	Still alive
5D1	Yucatan	3139	158	2	1	2	199	BRCA1+/-	3 days
							200	BRCA1+/-	4 days
							S1	BRCA1+/-	0
							M1	BRCA1+/-	0
							M2	BRCA1+/-	0

(Table 1). One stillborn piglet and two mummies were also retrieved. Pictures of the live cloned piglets were taken at the age of 1 week (Fig. 3).



cloned piglet (No. 39), 1 week



cloned piglet (No. 104), 1 week

Fig. 3 Cloned pigs. Photos of live cloned piglets, taken at 1 week of age

Genotyping identified seven piglets as BRCA1 KO

PCR and Southern blot based genotyping were carried out using genomic DNA from fibroblasts established from ear biopsies obtained from the cloned piglets. As illustrated in Fig. 4a, 5' targeting, 3' targeting and transgene PCR are expected to give products of 1,953, 2,901 and 4,068 bp, respectively. Results are shown for the genotyping of piglets No. 38, No. 39 and No. 104. The PCR-based genotyping shows that piglets No. 38 and No. 104 were BRCA1 KO, while piglet No. 39 was wild type (WT) (Fig. 4b). The genotypes of these piglets were further confirmed by Southern blotting using the Neo- and BRCA1-specific probes (Fig. 4c). In addition, all the five piglets from the third transfer were BRCA1 KO (Table 1/genotyping data not showed). The birth of piglet No. 39 suggests that there must have been some WT cells that survived the G418 selection together with the BRCA1 KO cells. A similar observation has previously been published in relation to the development of a cystic fibrosis pig model (Rogers et al. 2008). Unexpectedly, all the BRCA1 KO piglets survived no more than 18 days while the WT pig still is alive after half a year (Table 1).

BRCA1 mRNA amounts are decreased in BRCA1 KO cells

Although all the BRCA1 KO piglets died within 18 days, primary fibroblasts were established from each piglet allowing us to initiate analyses of their properties. First, we checked whether the deaths were due to cytogenetic abnormalities. However, all the

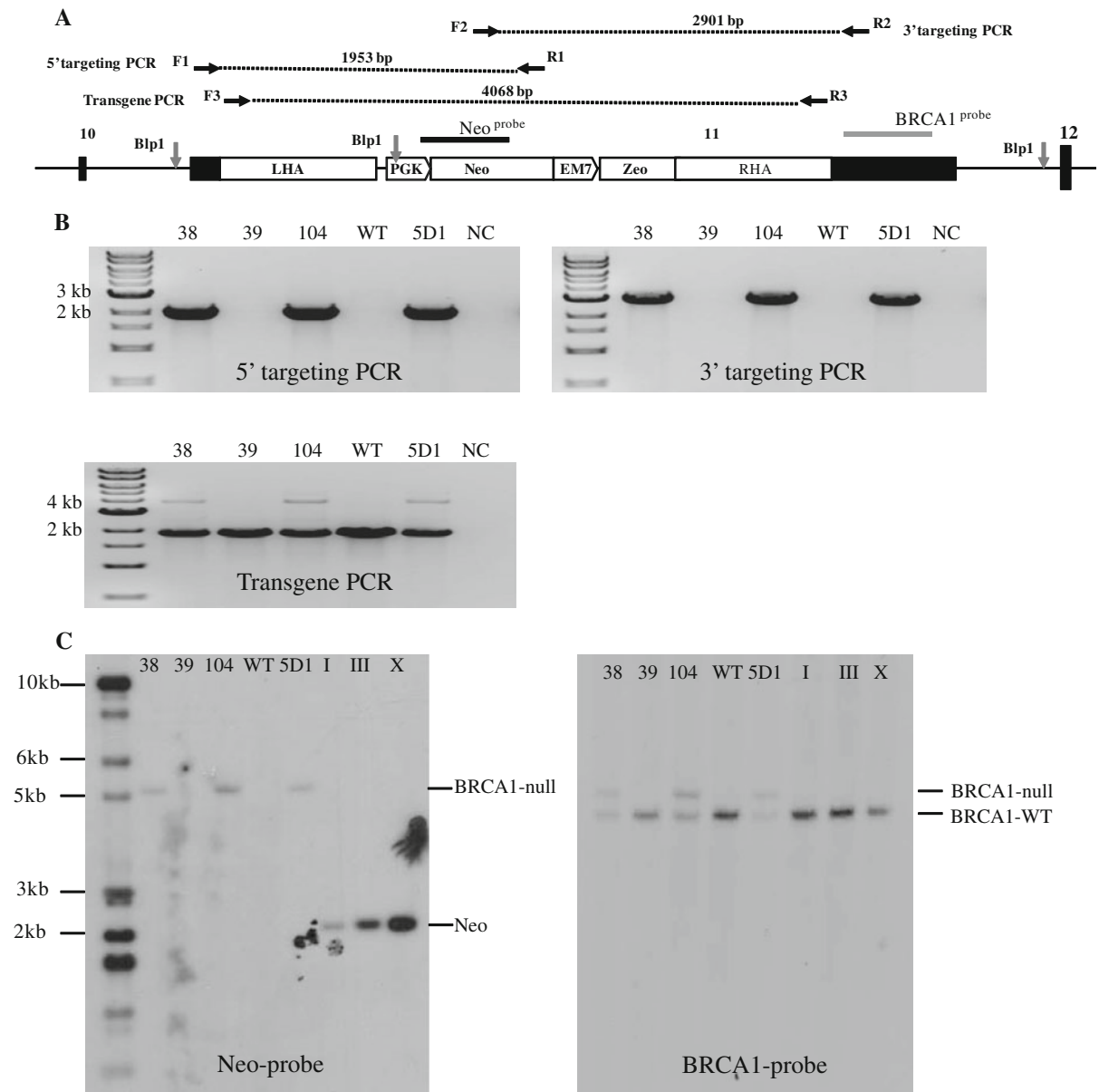


Fig. 4 Genotyping of the cloned piglets. **a** Schematic representation of the genotyping PCR (5', 3' and transgene) and the localization of the Neo and BRCA1 probes used for Southern blot analysis on *Bsp1* digested genomic DNA. **b** PCR-based genotyping. Cloned piglets No. 38 and No. 104 are BRCA1 KO and piglet No. 39 is WT. 5D1 represents genomic DNA

from the donor cells. WT, wild type genomic DNA; NC, negative control, H₂O. **c** Southern blotting-based genotyping confirms that piglets No. 38 and No. 104 are BRCA1 KO without random integrations. I, III, and X represent 1, 3, and 10 copies of the neo control

piglets have the correct number of chromosomes ($2n = 38, XX$) and seemingly normal karyotype (Fig. S3). We then measured the BRCA1 expression of the targeted locus (P1 + P3, P2 + P3) as well as the endogenous BRCA1 gene (P4 + P5) as shown in Fig. 5a. A fusion transcript expression from the

transgene was detected by both of P1 + P3 and P2 + P3 (Fig. 5b). Expression of endogenous BRCA1 mRNA, detected by P4 + P5, was altered in the BRCA1 KO cells. Q-PCR showed that the amounts of endogenous BRCA1 mRNA were about 1.5-fold and fourfold lower in No. 38 and No. 104

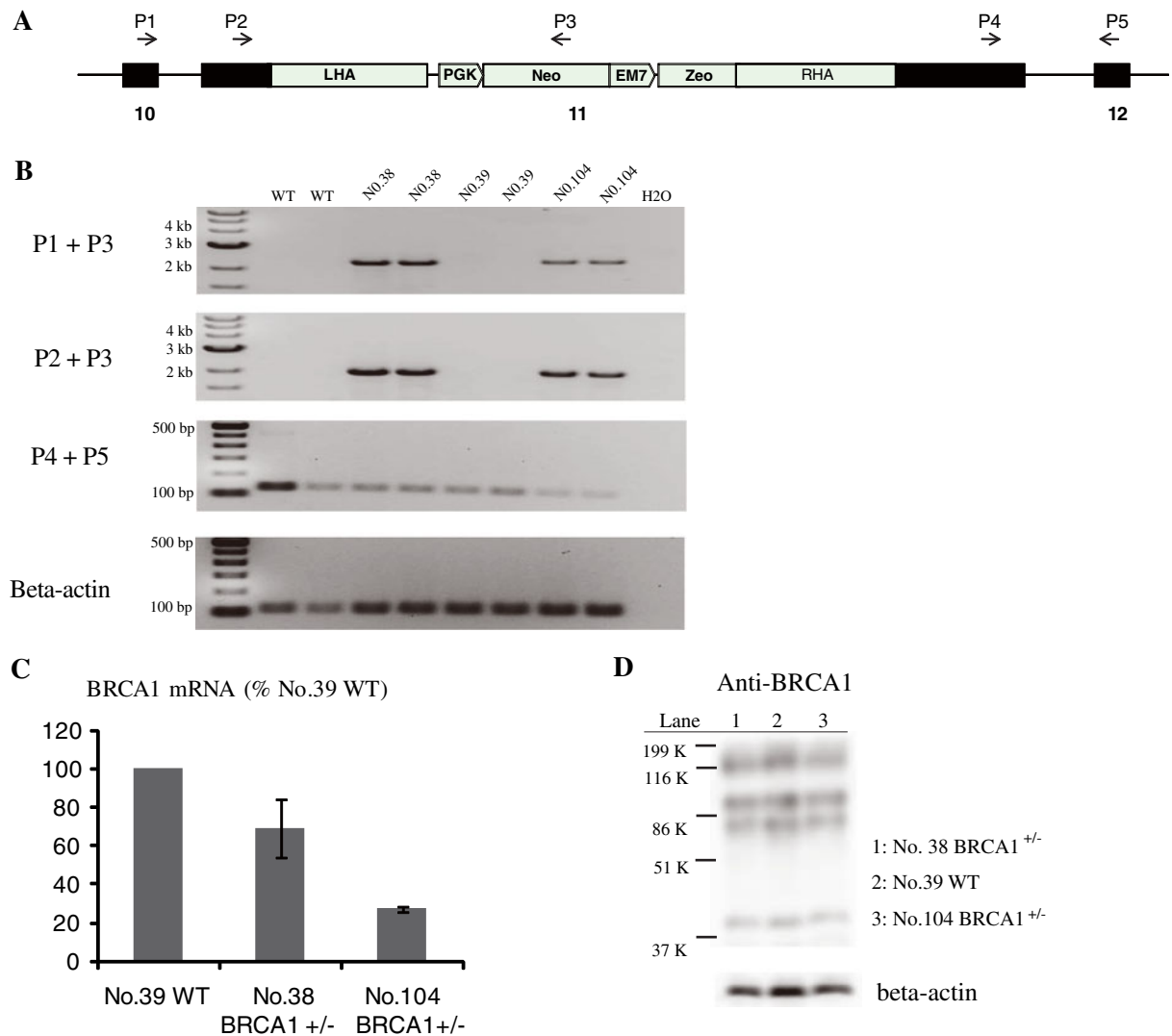


Fig. 5 BRCA1 expression. **a** Schematic representation of the primers (P1, P2, P3, P4, and P5) used for semi-quantitative PCR or quantitative PCR (Q-PCR). **b** Semi-quantitative PCR detection of BRCA1 mRNA from the targeted and WT alleles. P1 + P3 and P2 + P3 detected transcripts from the targeted allele. P4 + P5 measure the amount of transcripts from the endogenous BRCA1 gene. Beta-actin was used as internal

KO cells, respectively, when compared to No. 39 WT cells (Fig. 5c). Finally, we searched for truncated BRCA1 proteins, since several in-frame stop codons were introduced into the targeted locus. However, Western blot analysis did not reveal any truncated BRCA1 protein. Four different BRCA1 isoforms were found using the anti-BRCA1 antibody D20. No significant differences were observed with respect to BRCA1 protein levels (Fig. 5d).

control. PCRs were carried out in duplicate. WT refers to wild type fibroblasts established from non-cloned newborn piglets. **c** Q-PCR using primers P4 and P5. BRCA1 mRNA amounts were significantly lower in the BRCA1 KO cells than in the WT cells of piglet NO. 39. **d** Western blot analysis of BRCA1 proteins. Four BRCA1 isoforms were detected using the BRCA1 antibody D-20. Beta-actin was chosen as loading control

Discussion

We have generated the first BRCA1 knockout piglets by targeting exon 11 (BRCA1^{+/-Δ11}) using an rAAV-mediated gene targeting strategy. This is the first large animal with genetically designed BRCA1 deficiency. The ultimate goal of this work is to develop an appropriate model for investigating the

pathogenesis of certain types of breast cancer, as well as for testing of drugs and other anti-cancer therapies.

After the identification of the major breast cancer susceptibility gene, BRCA1, a range of BRCA1 KO mice with a variety of mutations have been generated. These mice have greatly contributed to the discovery of the basic biological functions of BCRA1 in embryonic development, maintenance of genomic integrity, cell cycle checkpoint regulation, and cell proliferation control. Unfortunately, heterozygous BRCA1 KO mice did not contribute to the elucidation of the pathogenesis of BRCA1-associated breast cancer since none of the heterozygous mice showed a tumor predisposing phenotype. A possible explanation might be the limited lifespan of the mice and the protected environment that these mice experience. Transgenic mice are normally kept in a germ-free environment without genotoxic challenges such as radiation and major oxidative stress. Consequently, they may not accumulate enough genetic alterations to develop breast cancer before they die for other reasons. Compared to mice, pigs have a longer lifespan and are genetically closer to man. They can be exposed to environments and life styles that are more similar to those of humans. Thus, a BRCA1 KO pig model may contribute to a better understanding of the specific pathogenesis of human BRCA1-associated breast cancer, and help us to expose the genetic drivers of breast carcinogenesis.

A major methodological improvement in the present study is the very high gene targeting rate ($\sim 35\%$) that could be achieved by using the rAAV-mediated homologous recombination strategy with optimal design of the vector. The high targeting rate obtained may thus in part be due to the complete lack of repetitive sequences in the BRCA1 KO targeting construct that consists of exon sequence only (Fig. 1a). A targeting efficiency in the range of 0.4–13% has previously been reported in human cell lines (Kohli et al. 2004). In the original study using rAAV-based homologous recombination in porcine primary fibroblasts a targeting frequency of 0.27–10.93% was observed (Rogers et al. 2008). In another set of experiments, we have further improved the gene targeting efficiencies of rAAV-mediated BRCA1 KO (up to 76%) by cell cycle synchronization of the targeted fibroblasts by serum starvation followed by a serum shock (submitted for publication). This approach might eventually allow us to

obtain correctly designed cells without selection procedures based on drug resistance.

The most unexpected observation in the present study was the perinatal mortality of the BRCA1^{+/ Δ 11} piglets. Although BRCA1 mRNA levels in fibroblasts established from the BRCA1^{+/ Δ 11} piglets were lower than that in WT fibroblasts (Fig. 5), no major genetic aberrations were detected in the heterozygous animals (Fig. S3). BRCA1^{+/-} mice are developmentally and reproductively normal and only mice homozygous for BRCA1 mutations have a compromised embryonic development (Gowen et al. 1996; Hakem et al. 1996). Our BRCA1^{+/ Δ 11} piglets were able to develop to term but had a high perinatal mortality.

The reason for the early deaths of the BRCA1^{+/ Δ 11} piglets is unclear, but SCNT cloning in general has been found to be associated with various problems including lower pregnancy rates (Campbell et al. 2005), gene expression alterations (Tian et al. 2009), abortions, stillbirths, and a high incidence of perinatal death (Young et al. 1998). Failure to thrive is frequently observed in cloned pigs (WT or transgenic) generated by HMC (Schmidt et al. 2010). The mean rate of piglets/transferred embryos has been reported to be $7.3 \pm 0.6\%$ using landrace or Göttingen/Yucatan minipigs. However, the perinatal mortality rate is higher for Göttingen and Yucatan minipigs than for Large White pigs (Schmidt et al. 2010). Insufficient epigenetic reprogramming has been suggested to be associated with these perinatal deaths. The perinatal mortality of our BRCA1^{+/ Δ 11} pigs may thus partly be explained by the genetic background of the specific breed of miniature pigs as we in other cloning experiments, involving other transgenes, experience similar perinatal losses of cloned Yucatan piglets. Embryonic mortality of 129/B6 mice with a hypomorphic BRCA1 mutation (Brca1- Δ 11) can be completely rescued when backcrossed onto 129/Sv or outcrossed using MF1 (Ludwig et al. 2001). Likewise, the embryonic survival of mice with lethal BRCA2 mutations was much shorter with 129/SvEv than with BALB/cJ genetic background (Bennett et al. 2000). Furthermore, BRCA1 may not be as crucial for embryonic development in humans, since a constitutionally homozygous patient with a nonsense mutation in both BRCA1 alleles has been described (Boyd et al. 1995). Accordingly, loss of BRCA1 function may have different consequences in different species and in individuals with different

genetic backgrounds. In order to investigate whether the mortality of BRCA1^{+/ Δ 11} pigs is associated with the Yucatan genetic background, we are currently generating Göttingen minipigs that are BRCA1^{+/ Δ 11} as we have observed that the pregnancy rate and the piglets/transferred embryos rate are both higher when using Göttingen fibroblasts as donor cells compared to using Yucatan fibroblasts. Many other factors, such as presence of the marker gene, SCNT itself, and use of a single cell clone, may contribute to the perinatal mortality observed. These issues will have to be investigated further if targeting using Göttingen fibroblasts results in similar perinatal losses.

Although BRCA1^{+/-} mice did not show increased predisposition to breast cancer (Hakem et al. 1996), shortened life span and more frequent occurrence of ovarian tumors have been observed when such mice were subjected to ionizing irradiation (Jeng et al. 2007). Thus, BRCA1 haploinsufficiency might be a predisposing factor to certain diseases when combined with exogenous stress factors. The fibroblasts used as nuclear donors in HMC are subjected to a range of stress factors such as G418 selection, long-term cell culture, and a severe reprogramming process during HMC.

Importantly, the survival of the WT cloned pig (No. 39) which has been subjected to exactly the same procedures as the dead heterozygous littermates strongly suggests that the loss of function of one BRCA1 allele has, to some extent, affected the developments of the genetically modified pigs generated by SCNT.

Still, the reasons for the prenatal deaths of BRCA1^{+/ Δ 11} piglets are unknown. To investigate this problem, fibroblasts established from the dead BRCA1^{+/ Δ 11} piglets have been used as nuclear donors for SCNT (re-cloning) as this approach may exclude epigenetic effects, and succeed due to improved reprogramming. We have made two transfers, in which 100 and 97 reconstructed embryos were transferred to two recipient sows. Unfortunately, though both recipient sows were tested pregnant, they aborted within few weeks. Eventually, we aim at creating a conditional pig model of mammary-specific BRCA1 KO (Liu et al. 2007).

In conclusion, we have produced cloned piglets with haploinsufficiency of the BRCA1 gene (BRCA1^{+/ Δ 11}) via highly efficient rAAV-mediated gene targeting. None of the piglets survived more

than 18 days, but pathological analyses and new experiments are under way to understand and/or eliminate the reason for the perinatal lethality.

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References

- Alberg AJ, Lam AP, Helzlsouer KJ (1999) Epidemiology, prevention, and early detection of breast cancer. *Curr Opin Oncol* 11:435–441
- Bennett LM, McAllister KA, Blackshear PE, Malphurs J, Goulding G, Collins NK, Ward T, Bunch DO, Eddy EM, Davis BJ, Wiseman RW (2000) BRCA2-null embryonic survival is prolonged on the BALB/c genetic background. *Mol Carcinog* 28:174–183
- Bouwman P, Jonkers J (2008) Mouse models for BRCA1 associated tumorigenesis: from fundamental insights to preclinical utility. *Cell Cycle* 7:2647–2653
- Boyd M, Harris F, McFarlane R, Davidson HR, Black DM (1995) A human BRCA1 gene knockout. *Nature* 375: 541–542
- Campbell KH, Alberio R, Choi I, Fisher P, Kelly RD, Lee JH, Maalouf W (2005) Cloning: eight years after Dolly. *Reprod Domest Anim* 40:256–268
- Crook T, Crossland S, Crompton MR, Osin P, Gusterson BA (1997) p53 mutations in BRCA1-associated familial breast cancer. *Lancet* 350:638–639
- Deng CX (2006) BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Res* 34:1416–1426
- Du Y, Kragh PM, Zhang Y, Li J, Schmidt M, Bogh IB, Zhang X, Purup S, Jorgensen AL, Pedersen AM, Villemoes K, Yang H, Bolund L, Vajta G (2007) Piglets born from handmade cloning, an innovative cloning method without micromanipulation. *Theriogenology* 68:1104–1110
- Gowen LC, Johnson BL, Latour AM, Sulik KK, Koller BH (1996) Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. *Nat Genet* 12:191–194

- Grieger JC, Choi VW, Samulski RJ (2006) Production and characterization of adeno-associated viral vectors. *Nat Protoc* 1:1412–1428
- Hakem R, de la Pompa JL, Sirard C, Mo R, Woo M, Hakem A, Wakeham A, Potter J, Reitmair A, Billia F, Firpo E, Hui CC, Roberts J, Rossant J, Mak TW (1996) The tumor suppressor gene *Brcal* is required for embryonic cellular proliferation in the mouse. *Cell* 85:1009–1023
- Jeng YM, Cai-Ng S, Li A, Furuta S, Chew H, Chen PL, Lee EY, Lee WH (2007) *Brcal* heterozygous mice have shortened life span and are prone to ovarian tumorigenesis with haploinsufficiency upon ionizing irradiation. *Oncogene* 26:6160–6166
- Kohli M, Rago C, Lengauer C, Kinzler KW, Vogelstein B (2004) Facile methods for generating human somatic cell gene knockouts using recombinant adeno-associated viruses. *Nucleic Acids Res* 32:e3
- Kragh PM, Nielsen AL, Li J, Du Y, Lin L, Schmidt M, Bogh IB, Holm IE, Jakobsen JE, Johansen MG, Purup S, Bolund L, Vajta G, Jorgensen AL (2009) Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. *Transgenic Res* 18:545–558
- Lane TF, Deng C, Elson A, Lyu MS, Kozak CA, Leder P (1995) Expression of *Brcal* is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice. *Genes Dev* 9:2712–2722
- Liu X, Holstege H, van der Gulden H, Treur-Mulder M, Zevenhoven J, Velds A, Kerkhoven RM, van Vliet MH, Wessels LF, Peterse JL, Berns A, Jonkers J (2007) Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proc Natl Acad Sci USA* 104:12111–12116
- Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A (1997) Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of *Brcal*, *Brc2*, *Brcal/Brc2*, *Brcal/p53*, and *Brc2/p53* nullizygous embryos. *Genes Dev* 11:1226–1241
- Ludwig T, Fisher P, Ganesan S, Efstratiadis A (2001) Tumorigenesis in mice carrying a truncating *Brcal* mutation. *Genes Dev* 15:1188–1193
- McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* 405:1066–1069
- Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W et al (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266:66–71
- Rice JC, Ozcelik H, Maxeiner P, Andrulis I, Futscher BW (2000) Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. *Carcinogenesis* 21:1761–1765
- Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS (2008) Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J Clin Invest* 118:1571–1577
- Rosen EM, Fan S, Pestell RG, Goldberg ID (2003) BRCA1 gene in breast cancer. *J Cell Physiol* 196:19–41
- Russell DW, Hirata RK (1998) Human gene targeting by viral vectors. *Nat Genet* 18:325–330
- Schmidt M, Kragh PM, Li J, Du Y, Lin L, Liu Y, Bogh IB, Winther KD, Vajta G and Callesen H (2010) Pregnancies and piglets from large white sow recipients after two transfer methods of cloned and transgenic embryos of different pig breeds. *Theriogenology* 74:1233–1240
- Szabo CI, Worley T, Monteiro AN (2004) Understanding germ-line mutations in BRCA1. *Cancer Biol Ther* 3:515–520
- Tian XC, Park J, Bruno R, French R, Jiang L, Prather RS (2009) Altered gene expression in cloned piglets. *Reprod Fertil Dev* 21:60–66
- Vajta G (2007) Handmade cloning: the future way of nuclear transfer? *Trends Biotechnol* 25:250–253
- Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, Callesen H (2000) New method for culture of zona-included or zona-free embryos: the well of the well (WOW) system. *Mol Reprod Dev* 55:256–264
- Venkitaraman AR (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108:171–182
- Wernersson R, Schierup MH, Jorgensen FG, Gorodkin J, Panitz F, Staerfeldt HH, Christensen OF, Mailund T, Hornshoj H, Klein A, Wang J, Liu B, Hu S, Dong W, Li W, Wong GK, Yu J, Bendixen C, Fredholm M, Brunak S, Yang H, Bolund L (2005) Pigs in sequence space: a 0.66X coverage pig genome survey based on shotgun sequencing. *BMC Genomics* 6:70
- Yoshida K, Miki Y (2004) Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci* 95:866–871
- Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S (2002) Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biol Reprod* 66:112–119
- Young LE, Sinclair KD, Wilmut I (1998) Large offspring syndrome in cattle and sheep. *Rev Reprod* 3:155–163