The knockout of the HMG- Box Domain of the porcine SRY-gene by CRISPR/Cas RNP microinjection causes sex reversal in gene-edited pigs

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Abstract

The sex-determining region on Y chromosome (SRY) is presumed as a main genetic switch of male sex development. Mutations within the SRY gene are associated with a male-to-female sex reversal syndrome in humans and other mammalian species such as mice and rabbits. However, the underlying mechanism causing this syndrome is not definitely known. To understand the genetic pathway of SRY, the investigation and analysis of morphological changes are essential. For the first time, we successfully generated a knockout of the SRY gene in pigs using microinjection of two Clustered regularly interspaced short palindromic repeats (CRISPR) – associated protein - 9 nuclease (Cas9) ribonucleoprotein (RNP) complexes targeting the centrally located "high mobility group"- box domain (HMG) of the SRY gene. Mutation within the porcine HMG- box domain resulted in the development of complete external and internal female genitalia in genetically male piglets. Uteri and oviducts displayed equal morphology compared to female wildtype controls, only the ovaries showed smaller size in sex reversal piglets. In contrast to the HMG- box domain deletion, a deletion within the 5' flanking region of the HMG domain was not associated with sex reversal. In summary, this study demonstrates for the first time the main role of the HMG- box domain of the SRY gene triggering the male sex determination in pigs.

Keywords

Porcine SRY gene; sex reversal; CRISPR/Cas9 RNPs; HMG domain

1 Introduction

In mammals, the male and female sex are determined by the presence or absence of the Y chromosome [1]. The sex-determining region on Y chromosome (SRY) located on the short arm of the Y chromosome is mainly involved in the gender determination during embryogenesis [2, 3]. It is expressed in the male genital ridge at the time of sex determination causing the formation of primary precursor cells of tubuli seminiferi, leading to the development of testicle from undifferentiated gonads [4]. Mutations within the SRY gene are associated with male-to-female sex reversal syndrome in humans (Swyer syndrome)[5]. Not all cases of sex reversal can be explained by alterations of the SRY gene, however 15 % of humans with male-to-female sex reversal syndrome show mutations or dysfunction of the SRY gene. Moreover, most of the mutations are detectable in the HMG- box domain of the SRY gene [6, 7]. The centrally located "high mobility group"- box domain (HMG) of the SRY protein

is responsible for DNA binding [8, 9] and thought to act as the main functional domain of the SRY gene [10]. Furthermore, it displays high similarity between different species [11, 12]. In previous studies in mice [13] and rabbits [14], the SRY gene was knocked out using different targets of the SRY sequence. However, the direct knockout of the HMG- box domain to elucidate the function of the HMG- box domain was not achieved. The pig is a perfect human disease animal model due to its high degree of physiological, genetic and anatomical similarity [15]. Therefore, to provide a basis for diagnosis of the sex reversal syndrome in humans, we target either the 5' flanking region of the HMG-box domain of the porcine SRY gene or directly the HMG- box domain to investigate its central function.

The porcine SRY gene consists of a single exon, similar to the human SRY gene, with an open reading frame of 624 bp representing 206 amino acids encoding the testis-determining transcription factor (TDF). It is expressed specifically in the male genital ridge at the time of sex determination. No extra gonadal expression of the porcine SRY gene is detectable [16]. First expression of the porcine SRY gene can be measured on day 21 postcoitum (p.c.) with its highest expression rate during day 21 and 23 p.c. Testis formation can be histologically observed by day 24 to 27 p.c. shortly after beginning of the SRY expression [17, 18]. According to this, the SRY gene is presumed to serve as the master gene causing formation of testicle from undifferentiated gonads. Furthermore, it is still unknown whether the SRY gene is the only sex-determining gene on the Y-chromosome or if other genes such as SOX9 [19-21] and SOX3 [8] are involved as well. In an effort to investigate the molecular mechanism of the sex determination in pigs, we envisaged to knockout different target sites on the porcine SRY gene by cell transfection followed by somatic cell nuclear transfer or intracytoplasmic microinjection of two CRISPR/Cas9 RNPs(Fig. 1).

2 Materials and Methods

Animal Welfare. Animals were maintained and handled according to the German guidelines for animal welfare and the German law regarding genetically modified organisms. The animal experiments were approved by an external animal welfare committee (Niedersaechsische Landesamt fuer Verbraucherschutz und Lebensmittelsicherheit, LAVES, AZ: 33.9-42502-04-17/2541).

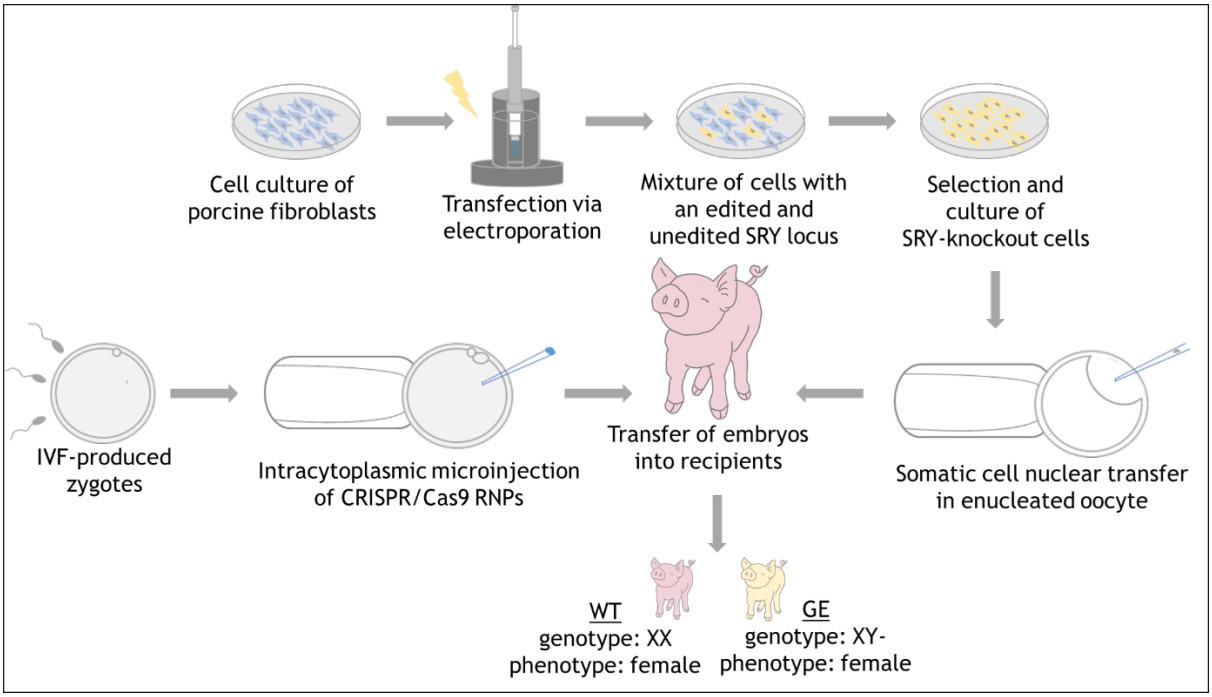
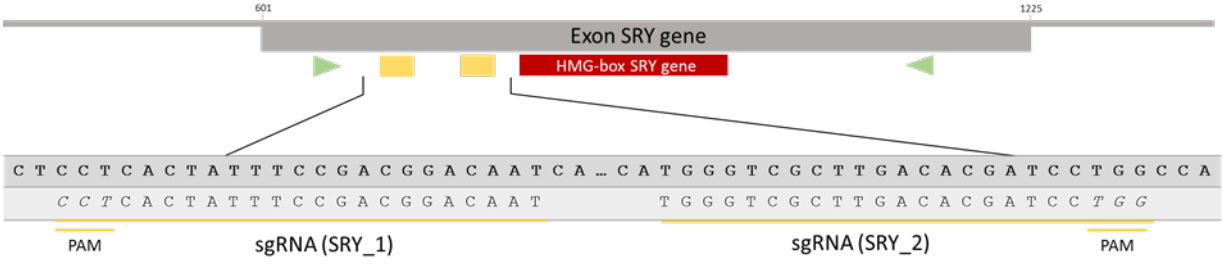


Fig. 1 Schematic illustration of the experimental design to generate SRY-KO piglets (XY-) by either intracytoplasmic microinjection of two CRISPR/Cas9 RNP complexes into IVF-produced zygotes or SCNT of SRY-knockout cells. Embryos were surgically transferred into hormonally synchronized recipients and the offspring were analyzed pheno- and genotypically.

Transfection of gRNAs. The CRISPR/Cas9 system was employed to induce defined deletions within the SRY gene (Ensembl transcript: ENSSSCG00000037443). Guide RNAs (gRNAs) targeting either the 5' flanking region of the HMG- box domain of the SRY gene (SRY_1 and SRY_2) or encompassing the HMG- box domain (SRY_1 and SRY_3) were designed using the web-based design tool *CRISPOR* (http://crispor.tefor.net/) (Fig.2). Target sequences were further analyzed via BLAST to reduce the off-target probability. The gRNA oligos with a BbsI overhang were cloned into the linearized CRISPR/Cas9 vector pX330 (addgene #42230). Afterwards, two CRISPR/Cas9 plasmids were co-transfected (with a final concentration of 5µg/µl) into male porcine fibroblasts by electroporation (Neon™ Transfection System, ThermoFisher Scientific) to test the efficacy of the plasmids to induce double-stranded breaks at the targeted locus. The electroporation conditions were as following: 1350 V, 20 mm, 2 pulses. After lysis of transfected cells, the cell lysate was analyzed using SRY specific primer (SRY-F: 5'-TGAAAGCGGACGATTACAGC and SRY-R: 5'-GGCTTTCTGTTCCTGAGCAC-3'). The purified PCR product (10ng/µl) (Invisorb® Fragment CleanUp – Startec) was sanger sequenced to detect mutations at the target site (Supplements Fig. 1/4/5).

A



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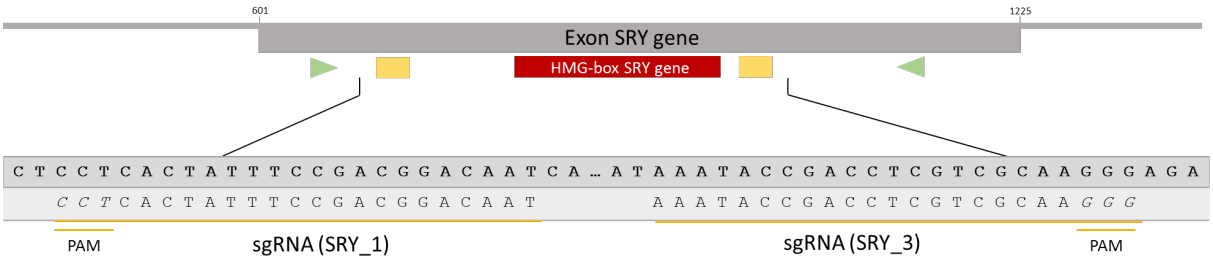

Fig. 2 a Schematic illustration showing the guide RNAs (yellow underlined) targeting an approx. 72 bp segment in the 5' flanking region of the HMG- box domain (red box) of the SRY gene. Primer amplifying the SRY exon are indicated with green arrows. **b** Location of two sgRNA target sites (yellow underlined) flanking the HMG- box domain (red box) of the SRY gene.

In-Vitro-Fertilization and In-Vitro-Maturation. Fresh boar semen was thawn for 30 sec. in a water bath (37 °C). Motility of sperm was analyzed using microscopy. After washing with Androhep® and centrifugation for 6 minutes at 600 g, approx. 75 to 100 sperm per oocyte (depending on semen capacity) were used for fertilization (no sexed sperm was utilized for fertilization). After four hours of incubation, the fertilized oocytes were cultured in porcine-zygote-medium (PZM).

Somatic cell nuclear transfer. SCNT was performed as previously described [22]. Fetal fibroblasts transfected with gRNA SRY_1 and SRY_2 targeting the flanking region of the HMG-box domain of the SRY gene were used as donor cells (Supplement Fig 1). Eighty-two and eighty-six one- to two-cell embryos were surgically transferred into two hormonally synchronized (20mg/day/gilt Altrenogest for 12 days, followed by an injection of 1500 IU PMSG on day 13 and ovulation was induced by intramuscular injection of 500 IU hCG 78 h after PMSG administration) German Landrace gilts (7-9 months of age) (Supplements Table 1).

Preparation of RNP complexes for microinjection. The Alt-R CRISPR/Cas9 system (IDT) consists of two CRISPR RNA components (crRNA and tracrRNA). The crRNA was individually designed to target the HMG- box domain of the SRY gene (SRY_3: 5' – AAATACCGACCTCGTCGCAA – 3'). To generate an active gRNA, both components (crRNA and tracRNA) were annealed (95 °C for 5 min and then ramp down to 25 °C at 5 °C/min) in a ratio of 1:1 to reach a final concentration of 1 µg/µl. Afterwards, the gRNA complex was mixed with Alt-R S.p. Cas9 nuclease 3NLS and incubated for 10 minutes at room temperature to form an active RNP complex with a final concentration of 20 ng/µl. The second RNP complex was prepared using the individually designed synthetic single-guide RNA (SRY_1: 5' – ATTGTCCGTCGGAAATAGTG – 3') from Synthego. The sgRNA was mixed with purified 2NLS-Cas9 nuclease using a ratio of approximately 1:1,5 (0,84 µl sgRNA [25pmols] and 1,25µl Cas9 protein [25pmols]) and incubated for 10 minutes at room temperature. After a centrifugation step at 10.000 rpm for 10 minutes (4°C), the supernatant was transferred into a new tube. Both RNP complexes were mixed in a ratio of 1 (SRY_1) to 1,7 (SRY_3) and directly used for microinjection.

Microinjection. The RNPs targeting the SRY gene were intracytoplasmatically co-injected into IVF-produced zygotes obtained from slaughterhouse ovaries. Therefore, approx. 10 pL of the RNP solution was injected with a pressure of 1 bar into the IVF-produced zygotes (Eppendorf transjector 5246). The injected oocytes were cultured in PZM-3-Medium (porcine-zygote-

medium) at 37°C, 5% CO_2 and 5% O_2. At day 6 when embryos reached the blastocyst stage, 31 or 32 embryos were surgically transferred into three recipients (Table 1).

Establishing cell cultures from SRY-KO piglets. Porcine fibroblasts were isolated from ear tissue of the piglets and cultured in Dulbecco´s modified Eagle's medium (DMEM) with 2% penicillin/streptomycin, 1% non-essential amino acids and sodium pyruvate and 30% fetal calf serum (FCS). When the cells reached confluency, they were lysed and genomic DNA was used for further analysis (PCR and karyotyping).

PCR-based genotyping. Genomic DNA of the piglets was extracted from tail tips. Cells were isolated from ear tissue. The DNA concentration was determined by a NanoDrop photometer (ThermoScientific, Waltham, USA). For genotyping of the piglets, polymerase chain reaction (PCR) was employed using specific primer (SRY-F: 5'-TGAAAGCGGACGATTACAGC-3' and SRY-R: 5'-GGCTTTCTGTTCCTGAGCAC-3') flanking a 498 bp segment of the SRY gene (Fig. 2). PCR amplification was performed in a total volume of 50 µl : 20 ng DNA, 0,6 µM reverse and forward primer, 1,5 mM $MgCl_2$, 0,2 mM dNTPs and 1,25 U *Taq* Polymerase (Program conditions for 32 cycles with denaturation at 94°C for 30 sec, annealing at 59 or 60 °C for 45, extension at 72°C for 30 sec and a final extension at 72°C for 5 minutes). Standard conditions for electrophorese were 80 V, 400 mA and 60 min using 1 % agarose gel. The PCR-product was purified (Invisorb®Fragment CleanUp-Kit, Startec) and sanger sequenced (Fig. 4/5 and Supplement Fig 2/3). To further analyze the genotype of the piglets Y-chromosome-specific genes such as KDM6A, DDX3Y, CUL4BY, UTY, UBA1Y and TXLINGY were amplified with specific primer sets (Supplement Table 2).

Karyotyping of the cells. Karyotyping was accomplished on porcine fibroblasts isolated from ear tissue of the piglets. After treatment of cells for 30 minutes with colcemide (Invitrogen), cells were trypsinised and metaphases were prepared according to standard procedures. Fluorescence R-banding using chromomycin A3 and methyl green was performed as previously described in detail [23]. At least 15 metaphases were analysed per offspring. Karyotypes were described according to Gustavsson, 1988 and the International System for Human Cytogenetic Nomenclature (ISCN).

Off-target analysis. The top ten off-target effects were selected from the gRNA design tool *CRISPOR* (http://crispor.tefor.net/). PCR primers used for amplifying the PCR product are listed in Supplements Table 3 for SRY_1 and Table 4 for SRY_3. The PCR product was purified (Invisorb®Fragment CleanUp-Kit, Startec, Germany) and analyzed via Sanger sequencing.

3 Results

Investigation of the 5' flanking region of the HMG domain. In order to generate SRY-KO pigs, a deletion in the 5' flanking region of the HMG- box domain of the SRY gene was introduced. One recipient went to term and delivered two healthy genetically male offspring (704/1 and 2) with a deletion of approx. 70bp (Supplement Table 1, Fig. 2). Sequencing of the target site detected a genetic modification of -72bp and -73bp in both piglets (Supplement Fig. 3). The piglets showed male external and internal genitalia. No sex reversal did occur. Therefore, we conclude, that mutations within the 5' flanking region of the HMG- box domain does not affect

196 the formation of the SRY protein. Moreover, these results give evidence of an assumed
197 duplication of the porcine SRY gene.
198
199 **Production of SRY-KO pigs.** In the next step, we induced a deletion of approx. 300bp
200 encompassing the HMG- box domain of the SRY gene (Fig. 2b). Two recipients went to term
201 and delivered in total twelve healthy piglets with a female phenotype (Tab. 1/Fig. 3). No sexed
202 sperm was used for fertilization. Three of the piglets showed a deletion of approx. 300bp
203 within the HMG- box domain motif of the SRY gene (Fig 4). Sequencing of the target region
204 revealed in-frame mutations of -292bp in piglet 715/2 and -267bp in piglet 715/7. In piglet
205 714/1 two different mutations with a deletion of 298 bp and an indel formation including a
206 deletion of 298 and an insertion of 1 bp were detected (Fig. 5). Furthermore, six different Y-
207 chromosome-specific genes (KDM6A, TXLINGY, DDX3Y, CUL4BY, UBA1Y, UTY) were analyzed.
208 Detection of these genes approved a male genotype and sex reversal in these three piglets
209 (Supplements Fig. 6). To further confirm the male genotype of the piglets, isolated cells from
210 ear tissue were karyotyped. Standard karyotype of the pig includes 38 chromosomes[24]. The
211 results of the karyotyping confirmed the male genotype of the piglets by detection of the Y
212 chromosome (Fig. 6, Supplements Fig 7/8). No chromosomal abnormalities were detected in
213 the sex-reversed pigs, except for pig 714/1, in which an inversion of chromosome 7 was
214 detected. If this inversion occurred through the use of CRISPR/Cas or naturally occurred
215 remains unclear. Further analysis is required to answer this question.
216 In total, 34 putative off-target sites within the porcine genome were determined
217 (http://crispor.tefor.net/). We designed primers for the top ten off-target events for each
218 gRNA (Supplements Tab. 2/3). The analyzes of the off-target sites is still in process.
219 Our SRY-KO study underline the role of the SRY gene in the male sex development during
220 porcine embryogenesis.
221

Recipients	Transferred Embryos	Pregnancy	Piglets	Phenotype	Sex reversal
8018	32	-	-		-
714	32	+	1	female	1
715	31	+	11	female	2

222
223 **Table 1** Results of the embryo transfer of microinjected zygotes into recipients. Three of twelve piglets
224 showed sex reversal with a female phenotype and a male genotype.
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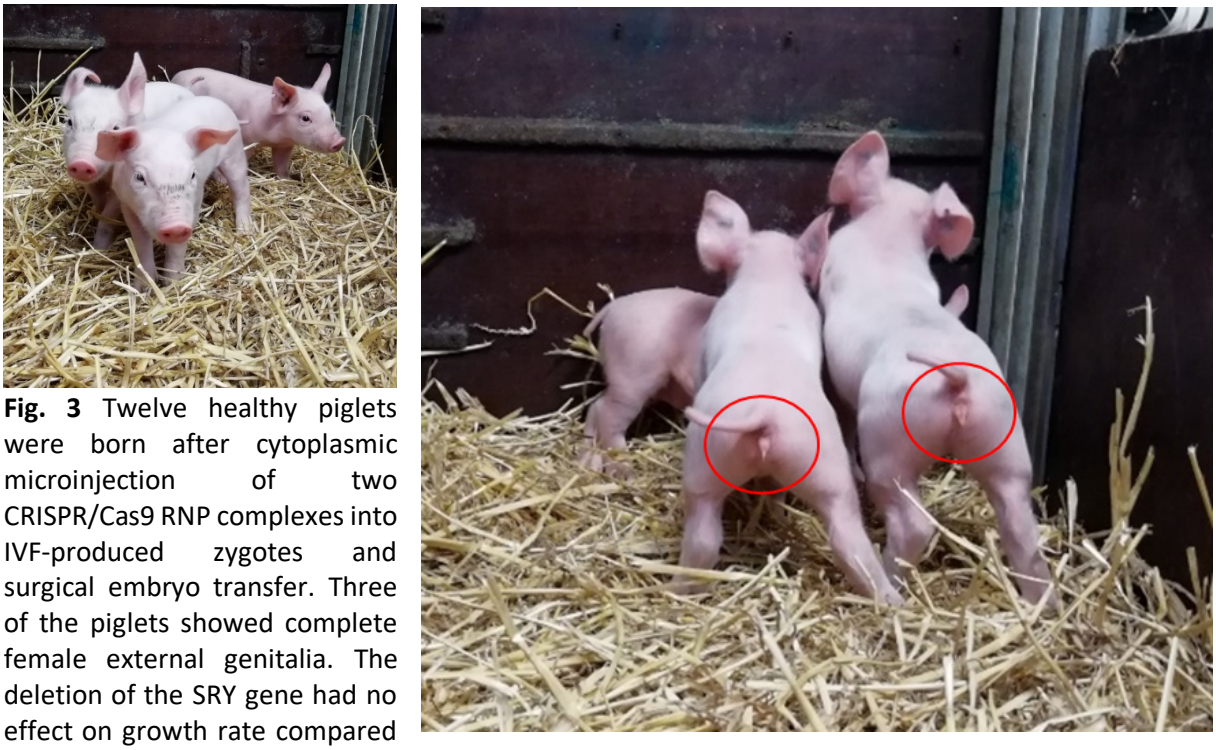

Fig. 3 Twelve healthy piglets were born after cytoplasmic microinjection of two CRISPR/Cas9 RNP complexes into IVF-produced zygotes and surgical embryo transfer. Three of the piglets showed complete female external genitalia. The deletion of the SRY gene had no effect on growth rate compared to wildtype. All piglet developed normal.

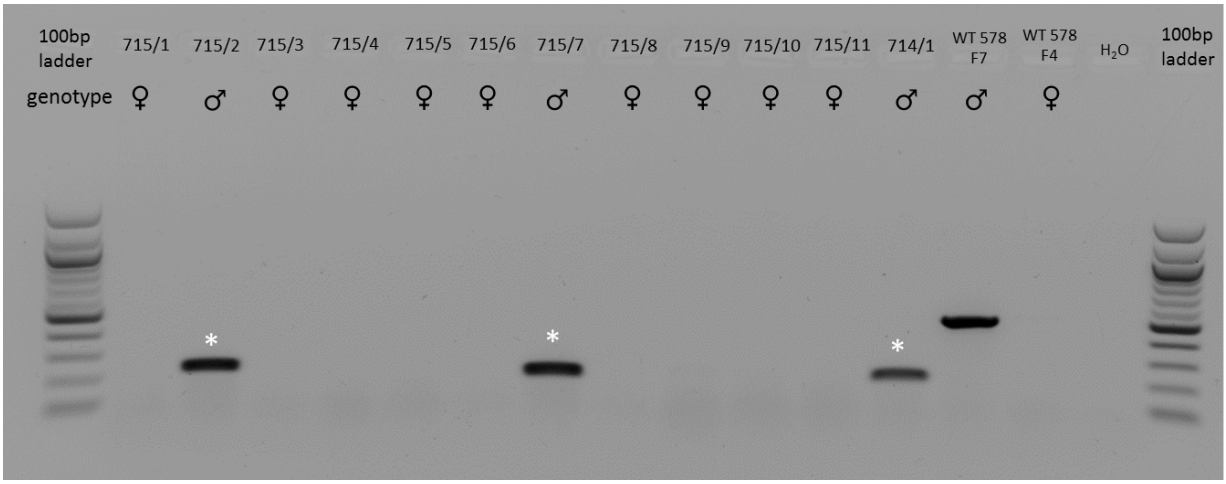

Fig. 4 PCR detection of the mutated SRY gene in piglets generated via microinjection of CRISPR/Cas9 RNP complexes. Three piglets (715/2, 715/7 and 714/1, indicated with white asterisk) showed deletions of approx. 300 bp within the SRY gene compared to a male wildtype control (WT 578 F7). The male WT control showed an expected band of approx. 500 bp. Female WT control (WT 578 F4) is negative as expected for SRY amplification.

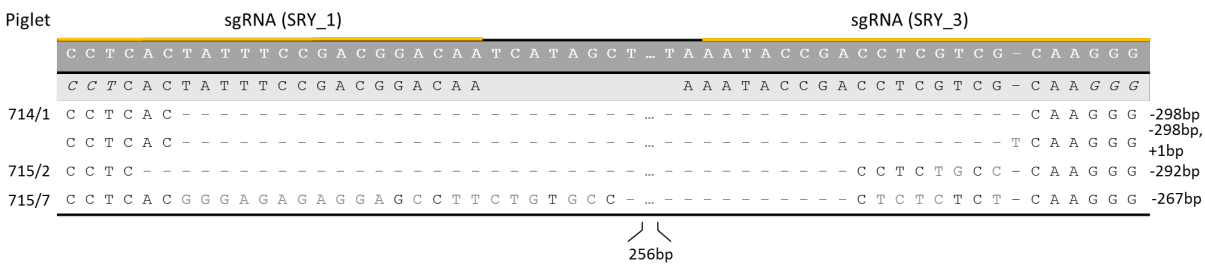

Fig. 5 Sanger Sequencing of the purified PCR product of SRY-KO piglets (715/2, 715/7 and 714/1) showing genetic modification of the SRY locus. Piglet 715/2 with a deletion of -292bp and in piglet 715/7 a deletion of -267bp. Piglet 714/1 show two different mutations with a deletion of -298bp and an indel formation of -298bp and +1bp .

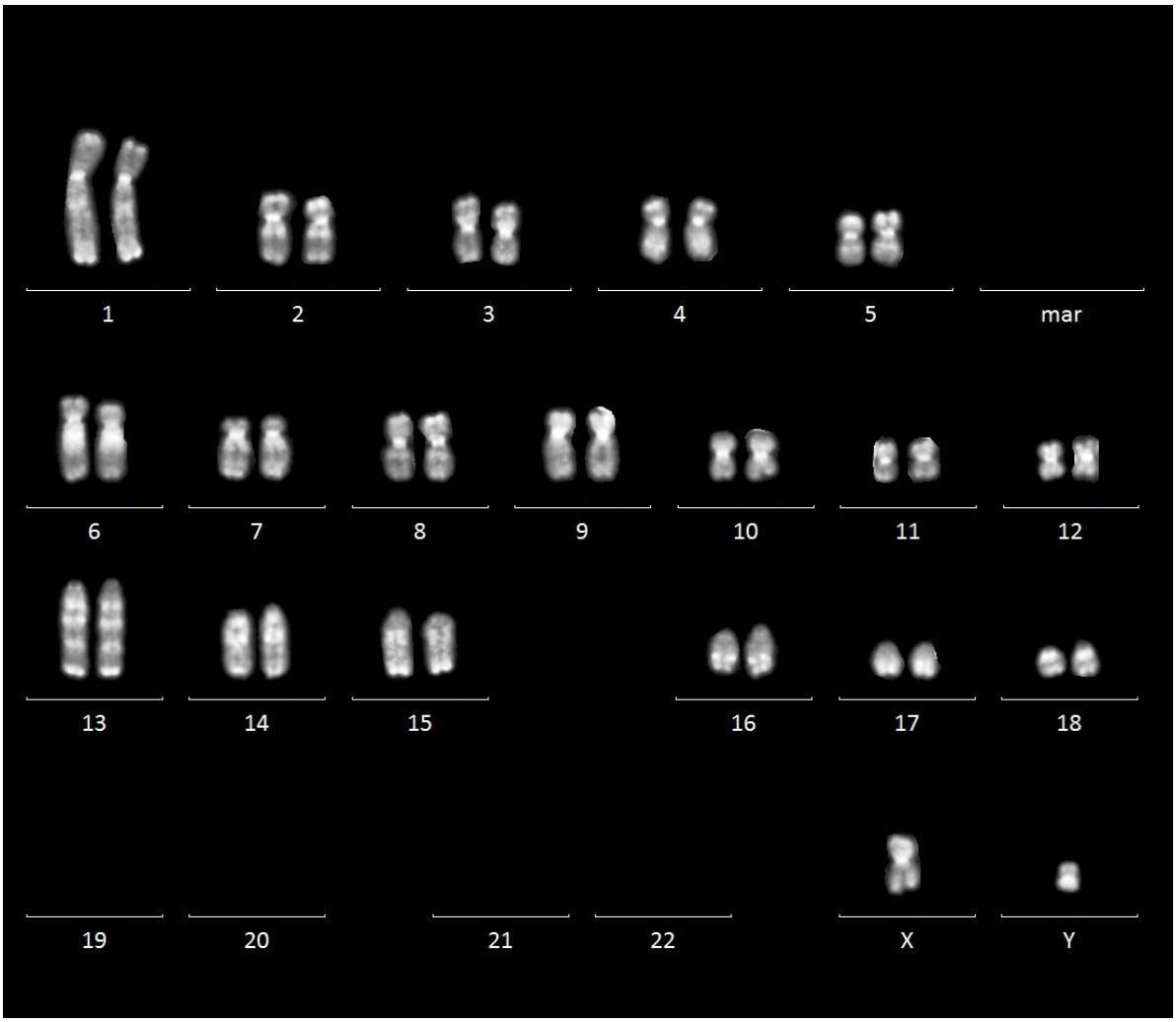

Fig. 6 Karyotyping of cells from SRY-KO piglet 715/2 confirming the male genotype of this piglet. The karyotypes of piglet 715/7 and 714/1 are shown in the Supplement Fig. 6.

External and internal genitalia of SRY-KO pigs. We explored the external and internal genitalia of the SRY-KO piglets. Wildtype (WT) females from conventional artificial insemination and age-matched WT females generated at the same time as the KO pigs using microinjection served as controls. The external genitalia of the SRY-KO piglets at the age of a few days and one month were equal to the external genitalia of WT females from microinjection and conventional artificial insemination. To further investigate the internal genitalia, the ovaries, oviducts and uteri of the SRY-KO piglets and female controls were prepared at the age of 34 days. As shown in Fig. 7 the SRY-KO piglet had complete female internal genitalia including ovaries, oviducts and uterus. Interestingly, the ovaries were approx. 2-fold smaller compared to the ovaries of the female WT control. There were no differences in size and morphology in oviducts and uteri between SRY-KO and WT females. These results approved that the SRY-KO causes a sex reversal in genetically male pigs with development of complete female external and internal genitalia.

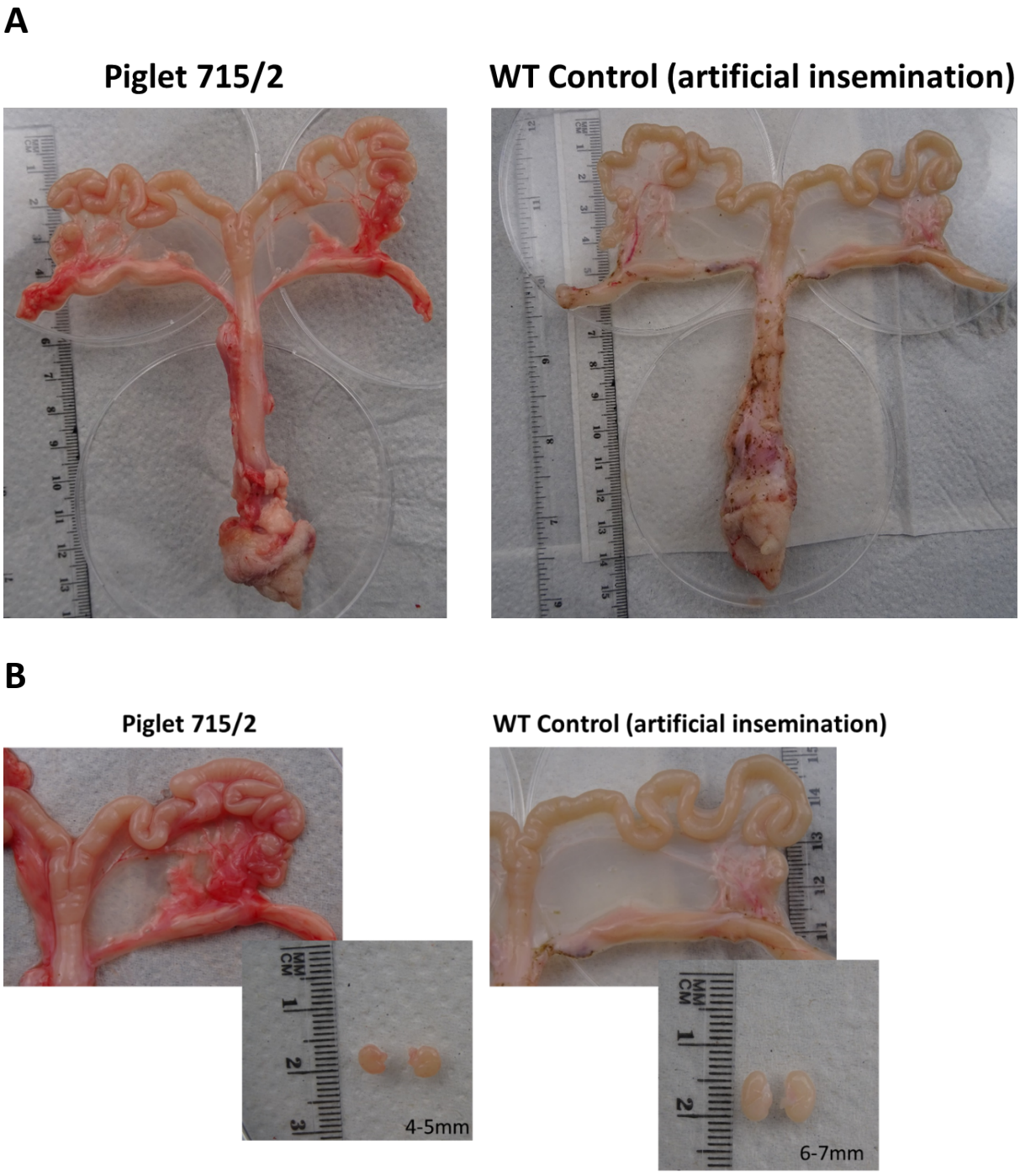

Fig. 7 The uteri and ovaries of the SRY-KO, XY piglet (715/2) and the WT,XX piglet (control from artificial insemination) at day 34. **a** No differences were shown in size of oviduct and uteri. **b** Solely the ovaries in SRY-KO, XY piglets are approx. 2-fold smaller than the ovaries of the WT, XX piglet (B).

4 Discussion

A promising candidate gene presumably involved in the male sex development is the sex-determining region on Y chromosome (SRY) [17]. Nevertheless, the molecular function and

the question if the SRY gene is the main switch for male sex development in all mammals is still unknown. Previous studies in mice [13] and rabbits [14] were performed to investigate the potential role of the sex-determining region. The murine SRY gene was knocked out by introducing a two base pair insertion in the 5' part of the ORF (open reading frame) of the SRY gene causing a frameshift mutation of the sequence. In one genetically male offspring the mutation led to a female phenotype [25]. While, in rabbits a disruption of the Sp1-binding site in the 5' flanking region of the SRY gene resulted in a sex reversal [14]. Interestingly, in our experimental setting in pigs a mutation upstream of the HMG- box domain of the SRY gene via SCNT did not result in the generation of genetically male offspring with a female phenotype. It is thought that the porcine SRY gene is present in two palindromic head-to-head copies[26], as in rabbits [27]. But it is still unknown, if both copies of the porcine SRY gene are active and required for male sex development and if there is the need of reaching a certain threshold to induce the development of a male gender as it was previously described in mice [28-30]. In both studies, the HMG- box domain as the main functional domain was not examined. Here, we report for the first time the successful knockout of the HMG- box domain of the porcine SRY gene using intracytoplasmic microinjection of two CRISPR/Cas9 RNP complexes resulting in genetically male piglets with a female phenotype. CRISPR/Cas9 RNP components exist only temporary in the cells limiting the guide RNA and Cas9 expression to a short time frame. Therefore, the use of CRISPR/Cas9 RNP complexes enable efficient genome editing while significantly reducing the risk of possible off-targets and undesired random integration of DNA segments into the host genome compared to DNA plasmids. Furthermore, mosaicism can be reduced to a minimum by using RNPs in microinjection technique [31-33]. The CRISPR/Cas9 system has become the genome editing technology of choice for most application due to its ease of use, cost-effectivity and highly specific way to introduce mutations at the targeted loci [34, 35]. Nevertheless, there is the possibility of off-target cleavages at undesired genomic sites. Therefore, it is necessary to increase the specificity of the CRISPR/Cas system regarding gRNA design [36].

In the present study, healthy SRY-KO piglets showing normal development and growth rate were born. Moreover, the knockout of the SRY HMG- box domain resulted in piglets with a female phenotype including complete female external and internal genitalia. Only the ovaries were smaller compared to the female WT controls. A previous study in rabbits reported that SRY-KO rabbits had a dramatically reduced number of follicles. Although, a normal copulatory behavior existed, no pregnancy was established by mating of an SRY-KO rabbit and a wildtype male rabbit. However, transfer of embryos at the blastocyst stage from wildtype female rabbits into pseudo pregnant SRY-KO rabbits results in a successfully pregnancy with the birth of twelve kitten. Therefore, they assume that the abnormal development and reduced number of follicles are responsible for the subfertility in the sex-reversal rabbits [14]. We suggest that SRY-KO pigs could also develop a reduced fertility because of the smaller size of ovaries. Nevertheless, in regard of the fertility rate and copulatory behavior more studies are needed after the piglets reach sexual maturity. In conclusion, these results described here, provide first evidence that the centrally located HMG- box domain on SRY gene is the functional domain of the porcine SRY protein responsible for male sex determination. The pig serve as a suitable animal model to investigate the male-to-female sex reversal syndrome in humans due to its physiological and anatomical closer relation to human as mice. In addition to its importance for sex reversal studies, the pre-determination of sex using CRISPR/Cas9 system in pigs could be a great benefit due to its impact on economic profit. The pig production would extremely benefit from a shift to generate more female offspring as the male-specific boar taint remains a big obstacle. Our results could pave the way to generating

a boar, which produces only female offspring by integrating a CRISPR/Cas9 vector targeting the HMG- box domain on SRY gene into the porcine genome.

5 Conflict of interest statement

The authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence this work.

6 Acknowledgements

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9 Supplements

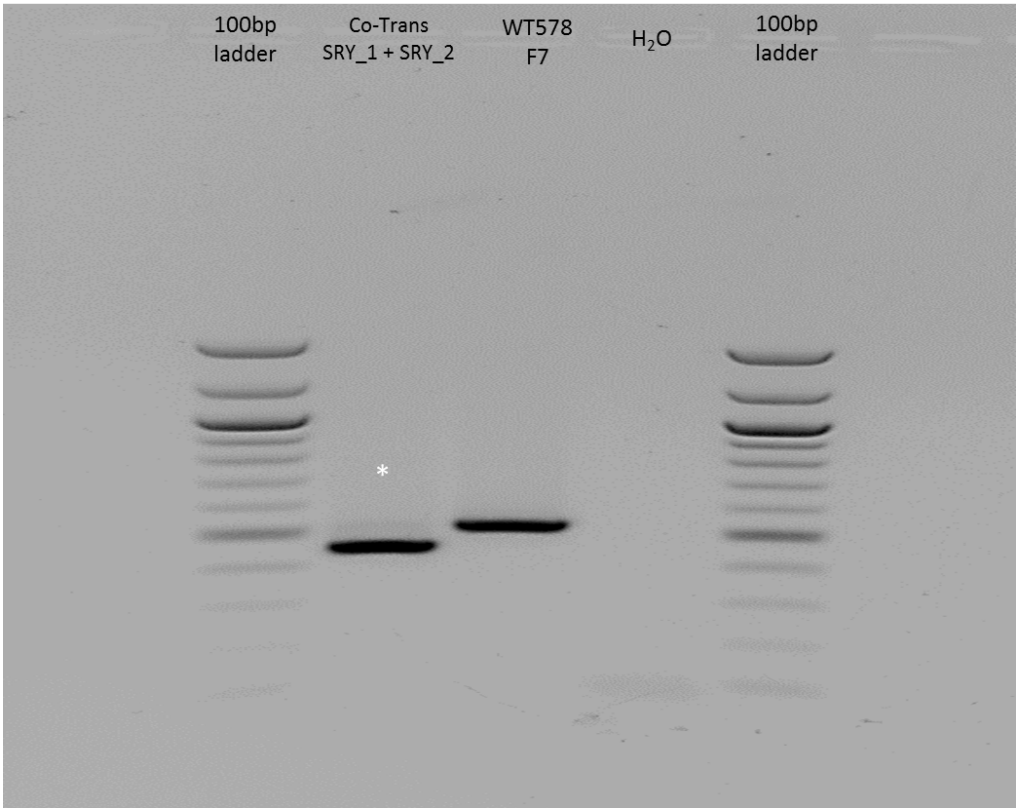


Fig. 1 PCR after co-transfection of two plasmids (SRY_1 and SRY_2) in male fetal fibroblasts and selection of the edited cells via single cell dilution (lower band). The edited cells revealed a mutation of approx. 70bp (indicated with a white asterisk) compared to WT control. The male WT control (WT 578 F7) showed an expected band of approx. 500bp. The edited cells were further employed for SCNT.

Recipients	Transferred Embryos	Pregnancy	Piglets	Sex reversal
704	82	+	2	-
7266	86	-	-	-

Table 1 Transfer of embryos generated by SCNT into recipients. Two piglets were born. Both of them showed a male phenotype and no sex reversal.

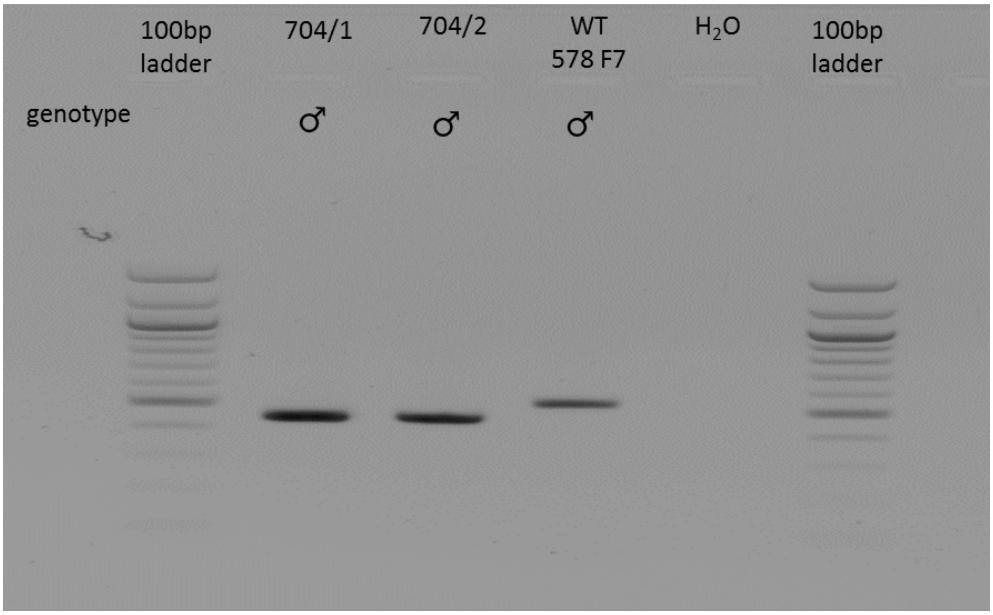


Fig. 2 PCR to detect genetic mutations in the SRY gene of the two piglet generated via SCNT. Both piglets showed a mutation of approx. 70bp compared to the male WT control (WT 578 F7). The WT control displayed an expected band of approx. 500bp.

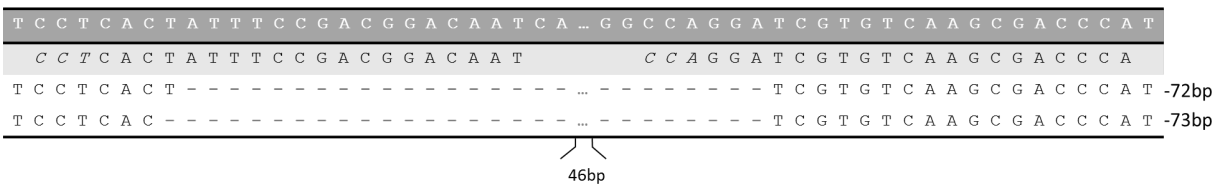


Fig. 3 Both piglets (704/1 and 704/2) generated via SCNT of two gRNAs (SRY_1 and SRY_2) targeting the 5' flanking region of the HMG- box domain of the SRY gene showed two different mutation including a deletion of -72bp and -73bp.

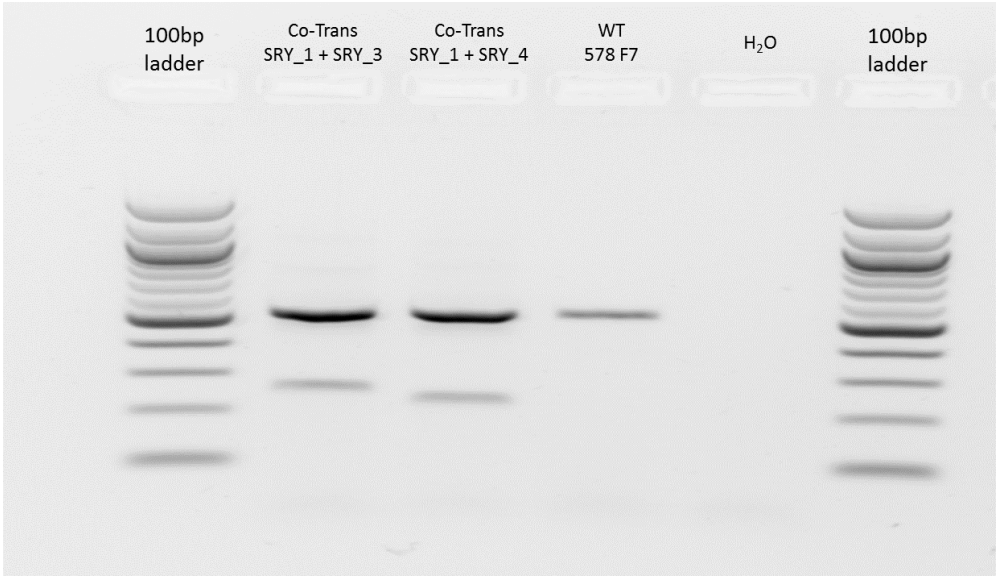


Fig. 4 PCR of cells after co-transfection of two gRNAs targeting the porcine SRY gene using electroporation (Neon ™ Transfection System, ThermoFisher Scientific). Male WT control showed an expected band of approx. 500bp. The additional lower band indicate a deletion of approx. 300 bp caused by the CRISPR/Cas9 system at the SRY locus. For further experiments the combination of SRY_1 and SRY_3 were used.

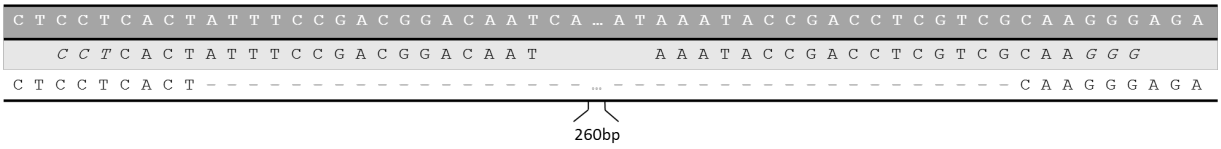


Fig. 5 Sequencing of the PCR product from co-transfection of SRY_1 and SRY_3 revealed a deletion of 298 bp compared to the wildtype in fetal fibroblasts.

Genloci	Primer	Sequence (5´- 3´)	Annealing Temp. (°C)	Length (bp)
KDM6A	KDM6A_1f	AAATCAAGGTATTACTTCACTATCCTG	60	442
	KDM6A_1r	ACCATGGAGATTTGACATCACCA		
CUL4BY	CUL4BY_1f	AGCCAGCCGGATAGAAAGTT	60	353
	CUL4BY_1r	ACAAAAGACACTCAGTTAAAACTTACC		
DDX3Y	DDX3Y_1f	ACTGGATTCTGTGTTCTTTGGA	60	291
	DDX3Y_2r	TTGGGGTGTTCTGTGCATGA		
TXLINGY	TXLINGY_1f	CCTCTAGCTTGTGGTTGGCA	60	249
	TXLINGY_1r	GTGGCCCTCTGAATCTTGCT		
UTY	UTY_1f	TCGTAAAGTGCTAAGTGGAGAAGA	60	345
	UTY_2r	TCAGGAACACACTGACGCTC		
UBA1Y	UBA1Y_1f	GCTGACACACTCACTGACCA	59	438
	UBA1Y_1r	AGCCATCAGAATCGTGTGGG		

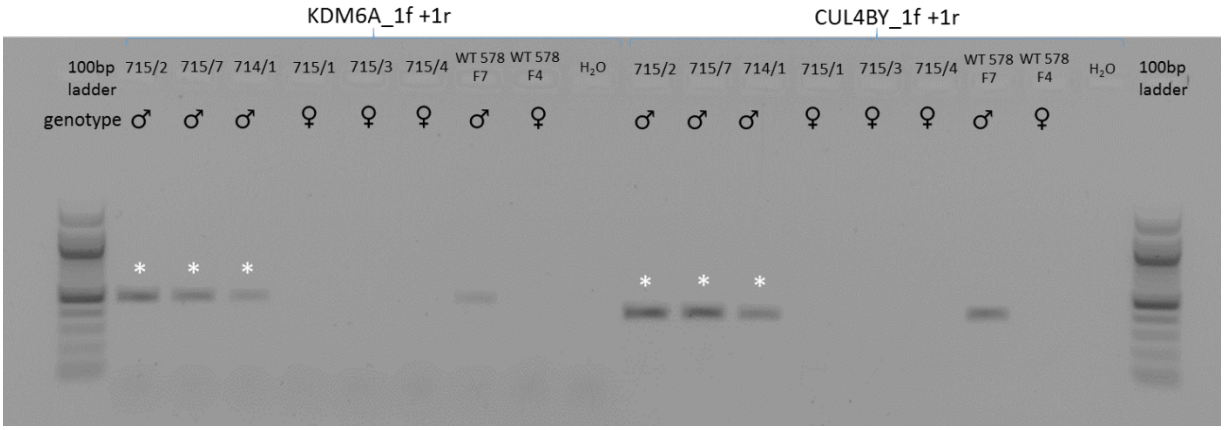
Table 2 Primer set for six different Y-chromosome-specific genes are listed. They can be used for Y chromosome detection.

Genloci	Primer	Sequence (5´- 3´)
intergenic:XM_021091945.1	101rev1_f	AGGGCTTTACATGGCTTCTCC
	101rev1_r	TGGAGGCGCAAGTCATAGG
intron:XM_021075830.1	101rev2_f	GTCCTCATGCTCTCGGCTAC
	101rev2_r	CCAACTGGACCCCTAGCTTG
intron:XM_005663347.3	101rev3_f	GTAGGTGTGGCCTTCGTCTT
	101rev3_r	CCATCAGGGGGCTATCACAC
intron:XM_021091957.1	101rev4_f	CTCTGACTTGCACCCTGCTT
	101rev4_r	ACTTCTCAATCCGCCCTATGC
exon:XM_021086306.1	101rev5_f	AACAACATGCGTCCAAACCG
	101rev5_r	GCATCAGCACTCACCTGGAT
exon:XM_021069624.1	101rev6_f	AGTGACTGGGTTTGGGGTTG
	101rev6_r	CGCCAGAGTCCCATACACTC
intergenic:XM_021090127.1	101rev7_f	TCCATAAGGAGTGCACGACG
	101rev7_r	TTTGCCCAGGTTCTGTCTGG
intergenic:XM_021082147.1	101rev8_f	GGGTGAATCCTGACCACGTC
	101rev8_r	AGTGTCCTTAGGGGGCAGAA
intergenic:XM_021101305.1	101rev9_f	TAGCACCCCCAGAAACTCCT
	101rev9_r	GGTGGATACTGTCAGCTGGG
intergenic:NM_001315615.1	101rev10_f	GCCTGCGCTTCTCATTTAGC
	101rev10_r	ATCCTGGACCTGGGCCTTAT

Table 3 Top ten off-target sites for gRNA SRY_1 on the porcine genome. Primer pair for sequencing of the off-target loci are listed.

Genloci	Primer	Sequence (5´- 3´)
intergenic:XM_021073683.1	408fw1_f	CTCCCCACAGCTGCTCTTTT
	408fw1_r	GAATTGGGCACTTGCTGGAC
intergenic:XM_003358165.4	408fw2_f	CTGTGCTACGTCCCCCAAAT
	408fw2_r	GCTTGTGGTCTTTGCCAGTG
intergenic:XM_021065302.1	408fw3_f	AGAGGAATGGGCAAACAGCA
	408fw3_r	CCGTGGTTAGCACCTGGTAG
intergenic:XM_021093786.1	408fw4_f	TTGGTCTGGGGAAGAAACCG
	408fw4_r	TCTGCCCTACCACTTCAGGA
intergenic:XM_003134254.5	408fw5_f	AGCCTTATCCAATGAGGCCG
	408fw5_r	CTAATGCCAGGGCAGTTTGC
intron:XM_021070776.1	408fw6_f	CCTTACCTGGTCCAGTTGGC
	408fw6_r	GCATTCATTTCCCCACGCAT
intron:XM_021069725.1	408fw7_f	ACAAAGTCCCTAGCTCACGC
	408fw7_r	GCTCCGCATCCTCAGATGTT
intergenic:XM_013983698.2	408fw8_f	GTCACCTGCCACTCTCTCAC
	408fw8_r	GGTCTGGAGTTCCTGTCGTG
intron:XM_021090110.1	408fw9_f	CTTCTCTGGTAACTGGCCCC
	408fw9_r	TCCCGCAGATCCATTCCAAC
intergenic:NM_001204768.1	408fw10_f	GACAGTGTTGGCGTATGCAC
	408fw10_r	GGAAAGTCAGTAGGGCCGAG

Table 4 Top ten off-target sites for gRNA SRY_3 on the porcine genome. Primer pair for sequencing the off-target loci are listed.



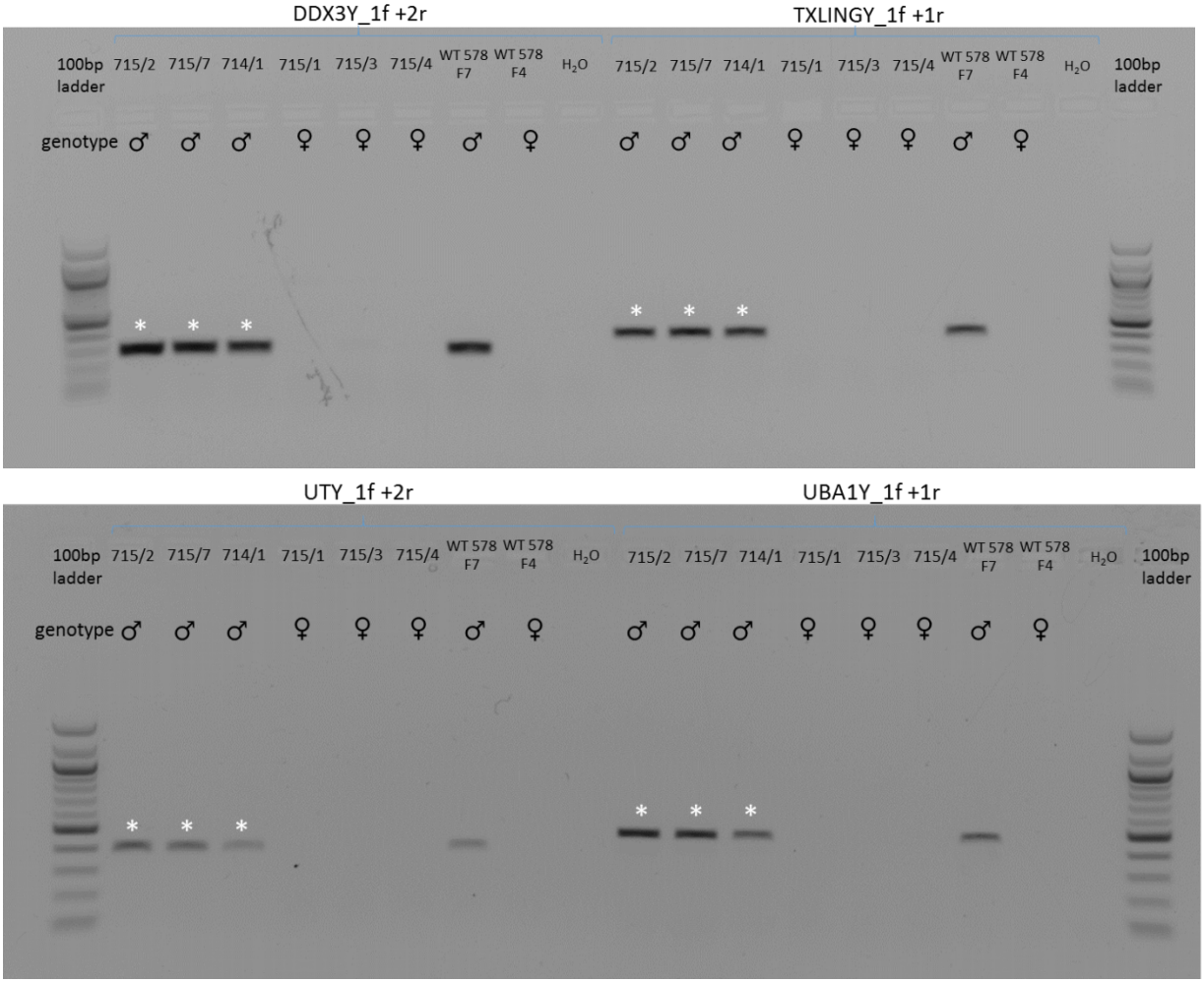


Fig. 6 PCR of six different Y-chromosome-specific genes (KDM6A, CUL4BY, DDX3Y, TXLINGY, UTY and UBA1Y) for detection of Y chromosome in SRY-KO piglets (715/2, 715/7 and 714/1, indicated by a white asterisk) compared to female wildtype controls (715/1, 715/3 and 715/4) from same litter generated by microinjection. Moreover, a male (WT 578/F7) and a female (WT 578/F4) DNA sample was used as control.

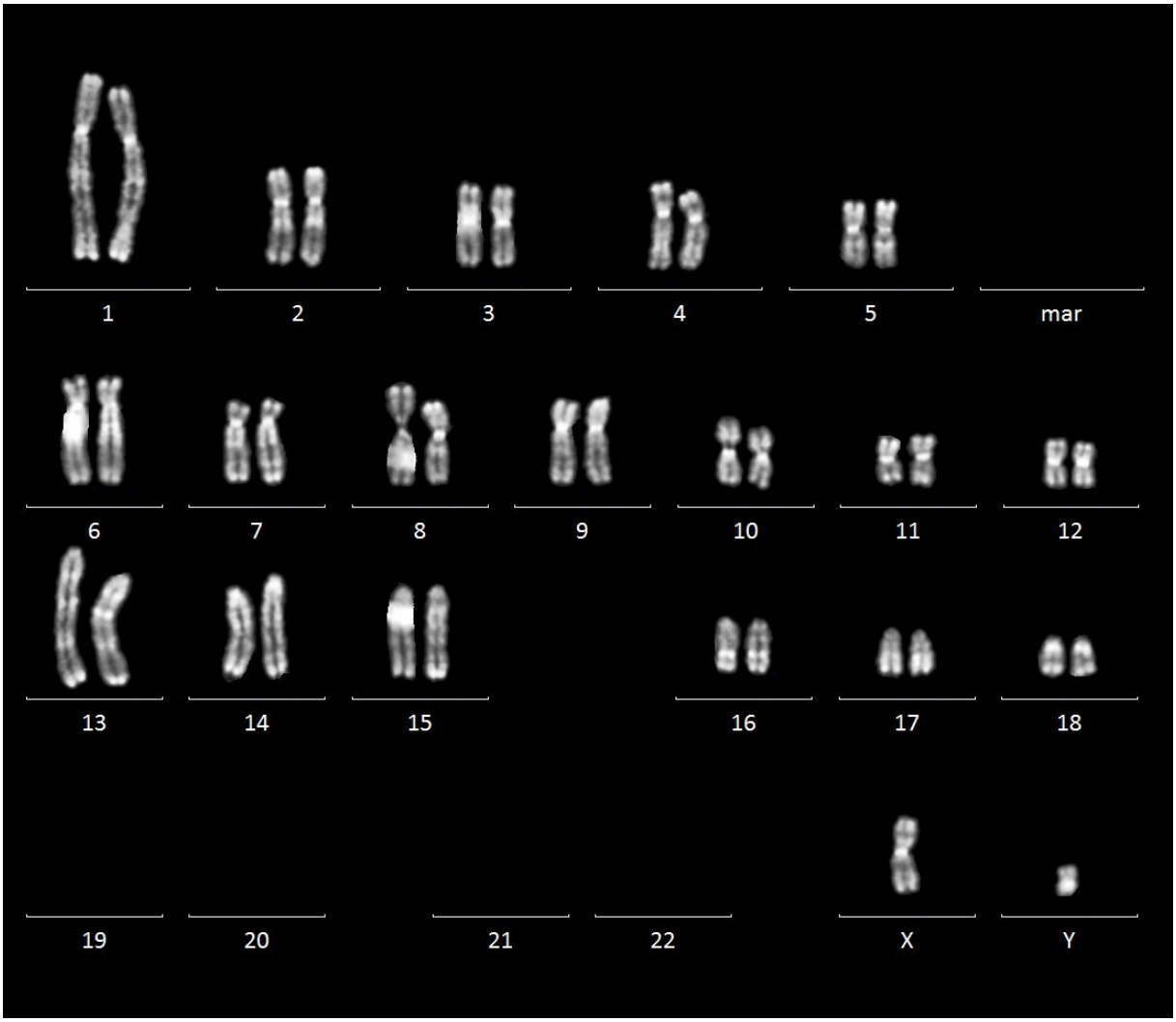


Fig. 7 Karyotyping of the SRY-KO piglet 715/7 confirming the male genotype by analysis of the sex chromosomes.

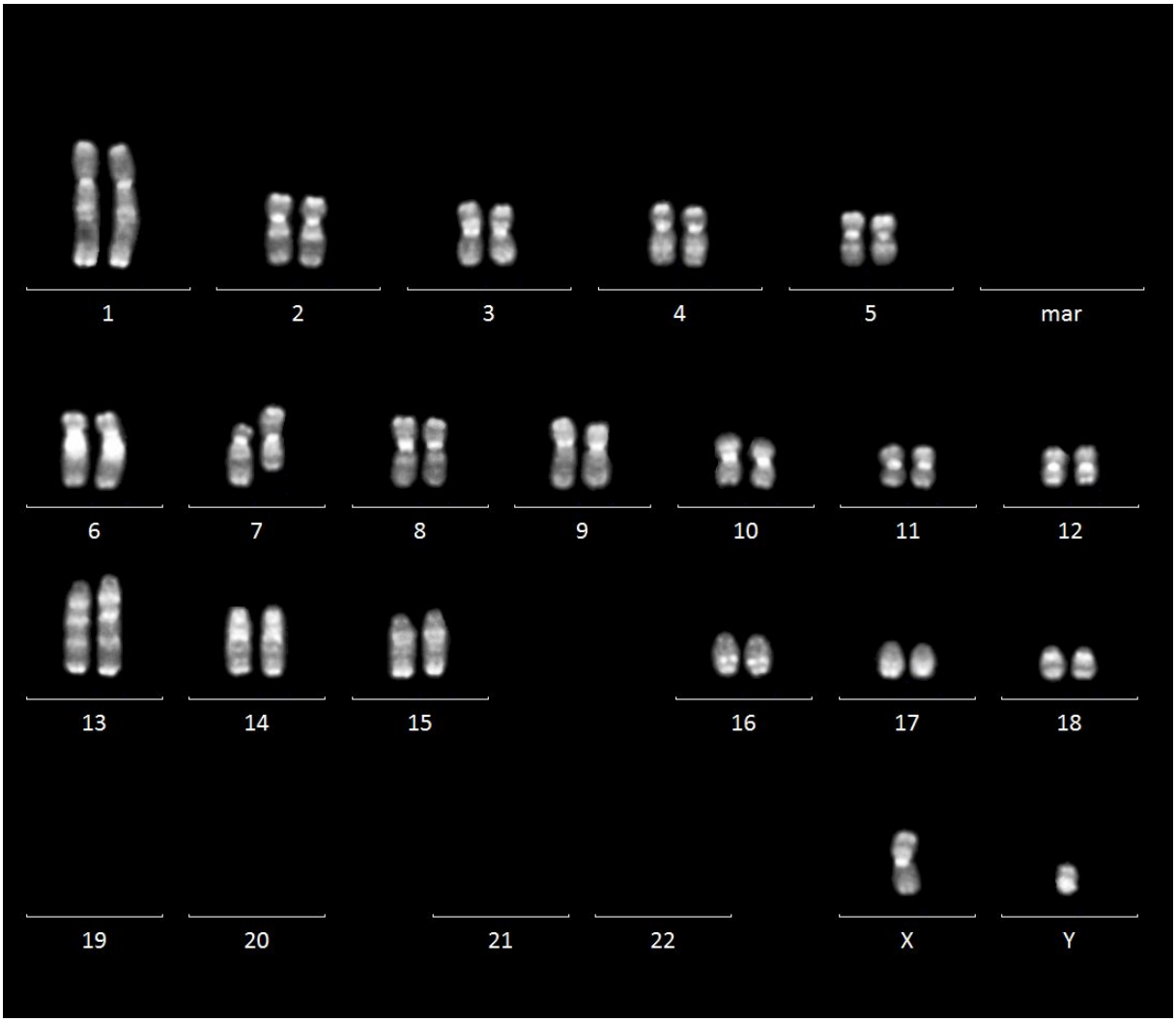


Fig. 8 Karyotyping of the SRY-KO piglet 714/1 confirming the male genotype by analysis of the sex chromosomes. In this piglet, a clonal aberration (inversion) could be found in chromosome 7.