*Original article*

# Production of ZFN-mediated *GGTA1* knock-out pigs by microinjection of gene constructs into pronuclei of zygotes

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## Abstract

Animals as a source of organs and tissues for xenotransplantation could become a backup solution for the growing shortage of human donors. The presence of human xenoreactive antibodies directed against Galα1,3Gal antigens on the cell surface of a pig donor triggers the activation of the complement leading to a hyperacute reaction. The development of genetic engineering techniques has enabled the modification of genomes by knocking in and/or knocking out genes. In this paper, we report the generation of modified pigs with ZFN mediated disruption of the *GGTA1* gene encoding the enzyme responsible for synthesis of Galα1,3Gal antigens. ZFN plasmids designed to target the exon 9 region of the pig *GGTA1* gene encoding the catalytic domain were injected into the pronuclei of fertilized egg cells. Among 107 piglets of the F0 generation analyzed, one female with 9-nt deletion in exon 9 of the *GGTA1* gene was found. 13 of 33 piglets of the F1 generation represented the +/- *GGTA1* genotype and 2 of 13 F2 piglets represented the -/- *GGTA1* genotype. No changes in the animals' behavior, phenotype or karyotype were observed. Analysis confirmed heredity of the trait in all animals. A complex functional analysis of the modified animals, including flow cytometry, human serum cytotoxicity test and immunohistochemical detection, was performed to estimate the phenotype effect of genetic modification and this indicated an efficient *GGTA1* knock-out in modified pigs.

**Key words:** Galα1,3Gal epitopes, pigs, xenotransplantation, genome edition, ZFNs, functional characteristics

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## Introduction

Techniques based on sequence-specific nucleases have revealed new possibilities for genome editing. Numerous genetically engineered animals have been generated using one of three rapidly developing nuclease dependent systems – ZFNs (zinc-finger nucleases), TALENs (transcription activator-like effector nucleases) and CRISPRs (clustered regulatory-interspaced short palindromic repeats). This type of engineering is based on nuclease-created DBSs (double-strand breaks) activating DNA repair mechanisms of the cell – HDR (homology-directed repair) and/or NHEJ (non-homologous end joining), which is imperfect and can create small indels (insertions or deletions) at the target site (Kwon et al. 2013, Sato et al. 2016). In this way knockout animals can be produced with higher efficiency as compared to other protocols relying on a homologous recombination (Hauschild et al. 2011, Whyte et al. 2011, Yang et al. 2011).

The Gal antigen (Galα1,3Gal) present in glycolipids and glycoproteins found on the surface of porcine cells is the main reason for a xenograft rejection in the pig-to-human system. The Galα1,3Gal antigen is formed as a result of galactose molecule attachment to N-acetyllactosamine (N-lac) with the α1,3-glycoside bond. The reaction is catalyzed by the α1,3-galactosyltransferase enzyme (*GGTA1*, α1,3GT). Neither the enzyme nor the sugar unit is found in humans or Old World monkeys. As *GGTA1* is expressed by many microorganisms, humans and Old World monkeys possess natural antibodies against this epitope, constituting approximately 1 % of all antibodies circulating in human blood. Binding of the Galα1,3Gal antigen by xenoreactive antibodies triggers a series of reactions, which lead to transplant rejection as a result of hyperacute rejection (HAR). The removal of the Galα1,3Gal antigen from the surface of porcine cells is believed to be a crucial condition in the production of organs suitable for interspecies transplantations.

The aim of this study was to produce a ZFN-mediated disruption of the porcine *GGTA1* gene encoding α1,3-galactosyltransferase with the support of a microinjection as a fairly rapid gene delivery method (Sato et al. 2016) and also to analyze the functional characteristics of genetically modified pigs with reference to the Galα1,3Gal antigen. We report efficient production of pigs with disrupted *GGTA1* gene by the ZFNs in plasmid format microinjection into pronuclei of fertilized egg cells. The results of complex functional analyses of the F0 pig founder and the F1 and F2 offspring indicated efficient *GGTA1* knock-out in modified pigs.

## Materials and Methods

### Animal welfare

The animals were maintained and handled according to the Polish guidelines for animal welfare. The experiments were approved by the ethics committee (Permission 1181/2015, 21st May 2015, II Local Ethic Commission in Krakow). All animals were under veterinary care. Observation of animal health and behavior was performed on a daily basis.

### ZFN design and construction

Custom ZFN plasmids were designed to target the exon 9 region of the *GGTA1* gene encoding the catalytic domain of the enzyme. The pZFN1 and pZFN2 gene constructs were designed and provided by Sigma-Aldrich (CompoZr). The pZFN1 and pZFN2 plasmids contained ZFN targeting reverse and forward strands, respectively. ZFNs bind to the TGGCAAGACAT CAGCATGatgcgCATGAAGACCATCGG binding site (ZFN cut site in lowercase). These ZFNs had a six- and five-finger protein recognizing 18 and 15 bases.

### Production of porcine zygotes

Sixty pubertal Polish Landrace gilts (approx. 8 months old, 90-120 kg bodyweight) served as *in vivo* zygote donors. Donor gilts were superovulated:1500 IU of PMSG (Folligon, Intervet) and 1000 IU hCG administered 72 hours after PMSG injection (Choluron, Intervet). Following detection of oestrus signs (24 h after hCG administration) the donor gilts were artificially inseminated twice at 12 h intervals. 18-20 hours after the second insemination, zygotes were recovered surgically by flushing the oviducts with phosphate-buffered saline solution containing 1% of bovine albumin.

### Microinjection and embryo transfer

Zygotes were transfected with pZFN1 and pZFN2 plasmid DNAs [4 ng/μl] by microinjection into one of the visualized pronuclei. The transfected zygotes were then morphologically evaluated. Damaged zygotes were removed. Positively evaluated transfected zygotes suspended in PBS (phosphate-buffered saline) supplemented with 20% FCS (fetal calf serum) were expelled into the recipient oviduct by insertion of a fine plastic cannula connected to a Hamilton syringe (Jura et al. 2007). Transfer procedures were performed under full surgical anesthesia. Recipient gilts were checked for pregnancy after 52 days.

### Screening Analysis and Sequencing

Genomic DNA was extracted from the ear biopsy specimens of pigs. PCR was performed using ZFN-F (5'-TGCGTTCCTTTAAAGTGTTTGA-3') and ZFN-R (5'-CTGTAGCTGAGCCACCGACT-3') primers under the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 30 s, and a final extension at 72°C for 6 min. The 200-bp PCR product was sequenced with automated genetic analyzers (Applied Biosystems Prism) using ZFN-R primer. The 200-bp PCR products (using ZFN-R primer) from different individuals that showed in direct sequencing mixed base calls were cloned into pSC-A-amp/kan cloning vector (Agilent Technologies) according to the manufacturer's protocol. The white colonies were picked for recombinant plasmids analysis. Plasmid DNA minipreps were prepared and sequenced using an automated genetic analyzer (Applied Biosystems Prism) using M13F primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'). For sequence analysis 10-20 clones per individual were used. Only two types of alleles were detected (wild type and with deletion).

### Porcine Skin Fibroblast Isolation and Cultivation

Primary fibroblast cell lines were started from ear biopsy specimens of pigs and culture as described in Zeyland et al. (2013).

### Methaphase Preparation and Karyotype Evaluation

Karyotype evaluation of pigs was based on the GTG band pattern and available patterns of porcine karyotypes (Gustavsson 1988). The samples were observed under fluorescent microscope and photographic documentation was processed using MetaSystems 2004 IKAROS software, Version 5.0.

### Human Serum Cytotoxicity Test

To analyze the viability of primary porcine fibroblasts in the presence of human serum as a source of the complement components, a human serum cytotoxicity assay was applied according to the procedure described earlier (Zeyland et al. 2013). The analysis was performed on the following cell lines: +/-*GGTA1*, -/-*GGTA1* and double transgenic (human α1,2-fucosyltransferase and α-galactosidase) generated earlier (Zeyland et al. 2014).

### Flow Cytometry

Porcine skin fibroblast from cultures, showing full cellular confluence, were washed with Hanks solution and trypsinized (0.25 % trypsin, 0.02 % EDTA). Fibroblasts were collected by centrifugation. The cells were washed 2-times with PBS 0.2% Tween 20 and treated with 5% BSA for 30 min. After washing with PBS 0.2% Tween 20 the cells were incubated with antibodies for 45 min at 4°C in the dark (1 $\mu$g/ml) to detect the Galα1,3Gal epitopes presented on the surface of porcine fibroblasts. Primary antibodies (M86, α-Gal Epitope Monoclonal Antibody, My Biosource) specifically bind to Galα1,3Gal epitopes which are presented on the surface of pig cells. Secondary antibodies (Goat anti-Mouse IgM Cross Adsorbed Secondary Antibody, DyLight 488 conjugate" (Thermo Fisher Scientific)) were labeled with FITC (fluorescein isothiocyanate). The primary-secondary antibodies complexes enabled detection and quantification of Galα1,3Gal epitope levels. After a washing step (PBS 0.2% Tween 20) the samples were analyzed using a BD FACS Aria™ III (Becton Dickinson) flow cytometer. The cells were characterized by forward scatter (FSC) and side scatter (SSC), and one fluorescent parameter: green fluorescence (FITC detector - 530/30 band pass filter) from FITC labeled antibodies. Flow cytometric analyses were performed using logarithmic gains and specific detector settings (10 000 events were recorded per analysis). Data were acquired in a four-decade logarithmic scale as area signals (FSC-A, SSC-A, FITC-A) and analyzed with FACS DIVA software (Becton Dickinson). Analysis of fluorescence signals from FITC labeled antibodies preceded a doublets discrimination procedure with the use of height versus width scatter signal measurement, in order to discriminate single events (cells) from conglomerates. The single cells are thus designated population P3. Each sample was analyzed in triplicate. The determination of Galα1,3Gal epitope levels on the surface of analyzed cells was based on the median values of green fluorescence signals from the FITC detector.

### Immunohistochemical analysis

Tissue sections from different organs of pigs (six controls and six modified animals) were harvested immediately following termination of the animals, cut into smaller fragments and transferred to buffered 10% formalin. After several days of fixation in formalin, all tissue samples were embedded in paraffin using a standard procedure. For detection of Galα1,3Gal antigen, a primary monoclonal anti-Galα1,3Gal antibody (MyBiosource) with streptavidin-biotinylated complex method using the Novolink Polymer Detection System was applied. The reaction was visualized with 3-3' diaminobenzidine (DAB). The studies were conducted on serial, 4 $\mu$m paraffin sections, on SuperFrost/Plus microscopic slides. Fixed specimens were exam-
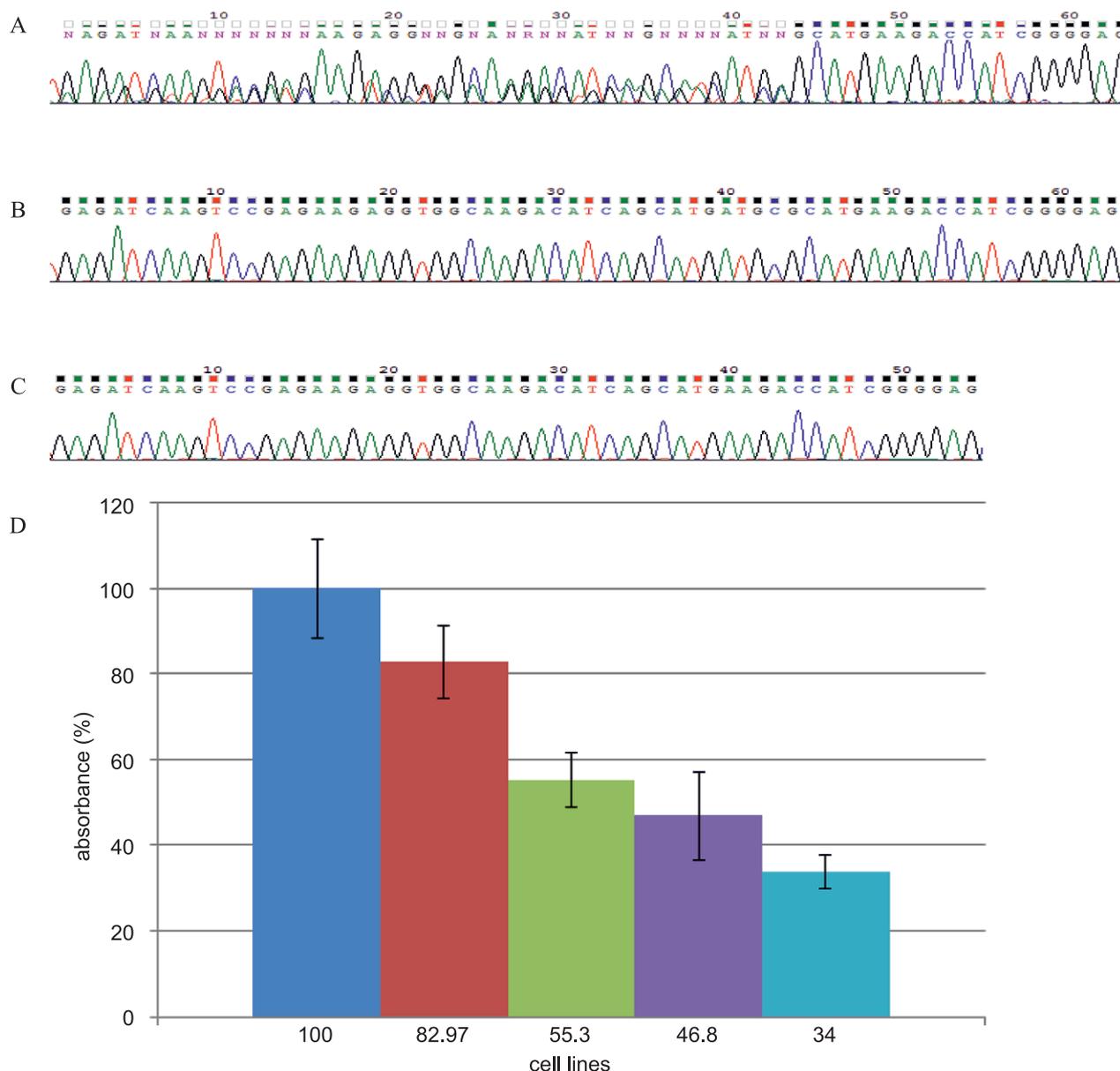
**Fig. 1.** Sequencing of the *GGTA1* gene fragment of exon 9 from founder sow and survival rate analysis examined in human serum cytotoxicity test. First, the PCR product was sequenced and two overlapping sequences of two alleles were detected (A). The PCR product was then cloned and sequenced: sequences B and C represent plasmids containing amplicon derived from the wild type allele of the *GGTA1* gene (B) and plasmids containing amplicon derived from the allele with 9-nt deletion (5'-GCATGATGC-3') (C). Sequencing was performed on 10-20 clones per individual. (D) The survival rate was measured after 2h incubation by an estimation of the average number of live cells compared to the total number of cells counted in 3 repetitions. The absorbance value (Y axis) is directly proportional to the number of live cells. Cells from the wild type animal were measured in the basic medium without human serum (blue) as a control, whereas cells from -/-*GGTA1* (red), +/-*GGTA1* (green), double transgenic (purple) and wild type (light blue) were measured in test medium containing 50 % human serum. Standard deviation is plotted for every cell line.

ined using a Leica DM 1000 Led microscope with a Leica Mc170 digital camera.

### Quantitative and statistical analysis of immunohistochemical detection of Galα1,3Gal

Microscopic specimens were scanned using a Mirax Midi Slide Scanner (Carl Zeiss) and then converted by Pannoramic Viewer software from digital format (mrxs) to picture format (tif). Quantitative analysis was performed using Definiens Developer XD software. For selection of the reaction and calculation of the surface area multi panel segregation was applied. Two classes of pixels were created: pixels ascribed to tissue with positive immunohistochemical reaction (Reaction) and pixels ascribed to tissue without reaction (Tissue).

Pixels were ascribed to defined classes according to level of brightness and the image object fusion function was then applied. For calculation of the percentage of reaction to whole tissue specimen the following equation was used: Percentage of reaction [%]=surface area of Reaction/[(surface area of Reaction + surface area of Tissue without Reaction)x100%]. The results for every organ in control and modified pigs were estimated using number (N), mean, median, minimum and maximum values and standard deviation. The Shapiro-Wilk test, U Mann Whitney test and t-Student test were used for analysis, performed using STATISTICA v. 10.0. Differences were accepted as statistically significant at $p < 0.05$.

# Results

## Generation of *GGTA1*-KO pigs

Introduction of the pZFN1 and pZFN2 plasmids into zygotes was performed using a standard microinjection method into one of the pronuclei of the zygote. 830 fertilized egg cells from 60 donors were subjected to microinjection. In total, 780 (94%) zygotes were qualified and transferred into the oviducts of 25 synchronized recipient gilts. The F0 generation obtained from 24 recipients consisted of 107 piglets that were subjected to DNA screening analysis in order to detect ZFN-induced changes. The analysis detected one female piglet (F0 generation) with 9-nt deletion (5'-GCATGATGC-3') in the exon 9 region of the *GGTA1* gene (1/107; 0.93 %). The sequence screening results are presented in Fig. 1. When the gilt founder reached puberty, artificial insemination (AI) was performed using sperm of wild type boars. 33 piglets were obtained, including 13 +/- *GGTA1* heterozygotes (13/33; 39 %). F1 heterozygotes were crossbred and 13 piglets were generated including 9 modified (9/13; 69%). Two males were confirmed as -/- *GGTA1* homozygotes. The line founder (F0) and its offspring (F1, F2) showed no changes in phenotype and behavior.

## Karyotype evaluation

The band pattern of chromosomes from the modified pigs was compared with the standard karyotype of the domestic pig (Gustavsson 1988). Cytogenetic analysis showed no chromosomal aberrations. All analyzed animals showed no changes in the karyotype.

## Human Serum Cytotoxicity Test

To verify the functionality of the *GGTA1* gene knock-out the survival rate of the cell lines derived from the modified pigs (+/-*GGTA1*, -/-*GGTA1*) was compared with wild type cells in the presence of human serum (complement system). Activation of the complement system results in a number of reactions leading to formation of a membrane attacking complex responsible for forming channels in the cellular membrane. The destruction of the recognized cells is a consequence of membrane malformations through changes of lipid orientation and release of liposomes. The survival rate analysis was performed by accurate determination of the percentage of the functional and viable cells using MTT colorimetric assay. The absorbance value (axis Y) was directly proportional to the number of live cells. The results of the absorbance measurements of cells incubated with human serum showed that the highest cell viability occurred in the -/-*GGTA1* line (survival rate 82.97%), followed by the +/-*GGTA1* line (55.3%) and double transgenic cell line with combined expression of human α1,2-fucosyltransferase and α-galactosidase (46.8%). In contrast, the wild type cells had the lowest viability (survival rate 34%) (Fig. 1D.)

## Flow cytometry

Flow cytometric analysis was used to evaluate the Galα1,3Gal epitope levels on the surface of pig cells. Fibroblasts were established from the wild type, +/- *GGTA1* and -/-*GGTA1* pigs. M86 primary antibodies were used with secondary FITC conjugates to reveal the differences in fluorescence intensity between cells from +/-*GGTA1*, -/-*GGTA1* and wild type pigs (Fig. 2 A,B,C). The mean values of green fluorescence intensity medians (measured as a fluorescence signal from the FITC detector of gated population P3 and expressed as relative fluorescence units (RFU)) from FITC-stained secondary IgM antibody conjugated to anti-Galα1,3Gal monoclonal antibodies, were 1244, 957 and 151 RFU for the wild type, +/-*GGTA1* and -/-*GGTA1* pig, respectively. The standard deviation (SD) values were 39.02, 11.14 and 9.87 RFU for the wild type, +/-*GGTA1* and -/-*GGTA1* pig, respectively. The results demonstrated significant reduction in Galα1,3Gal epitope levels in -/-*GGTA1* pigs as the median values of green fluorescence intensity for this sample were close to the instrument noise. This may indicate a lack of Galα1,3Gal epitope molecules on the surface of tested cells in -/-*GGTA1* pigs.

## Quantitative and statistical analysis of immunohistochemical detection of Galα1,3Gal

Tissue sections from the heart, liver, kidney and aorta from wild type and *GGTA1* modified pigs (+/- *GGTA1*, -/- *GGTA1*) were used. The results of quantitative and statistical analysis of the wild type *vs*. +/- *GGTA1* pigs were plotted on the right side of immunohistochemistry detection images. The results
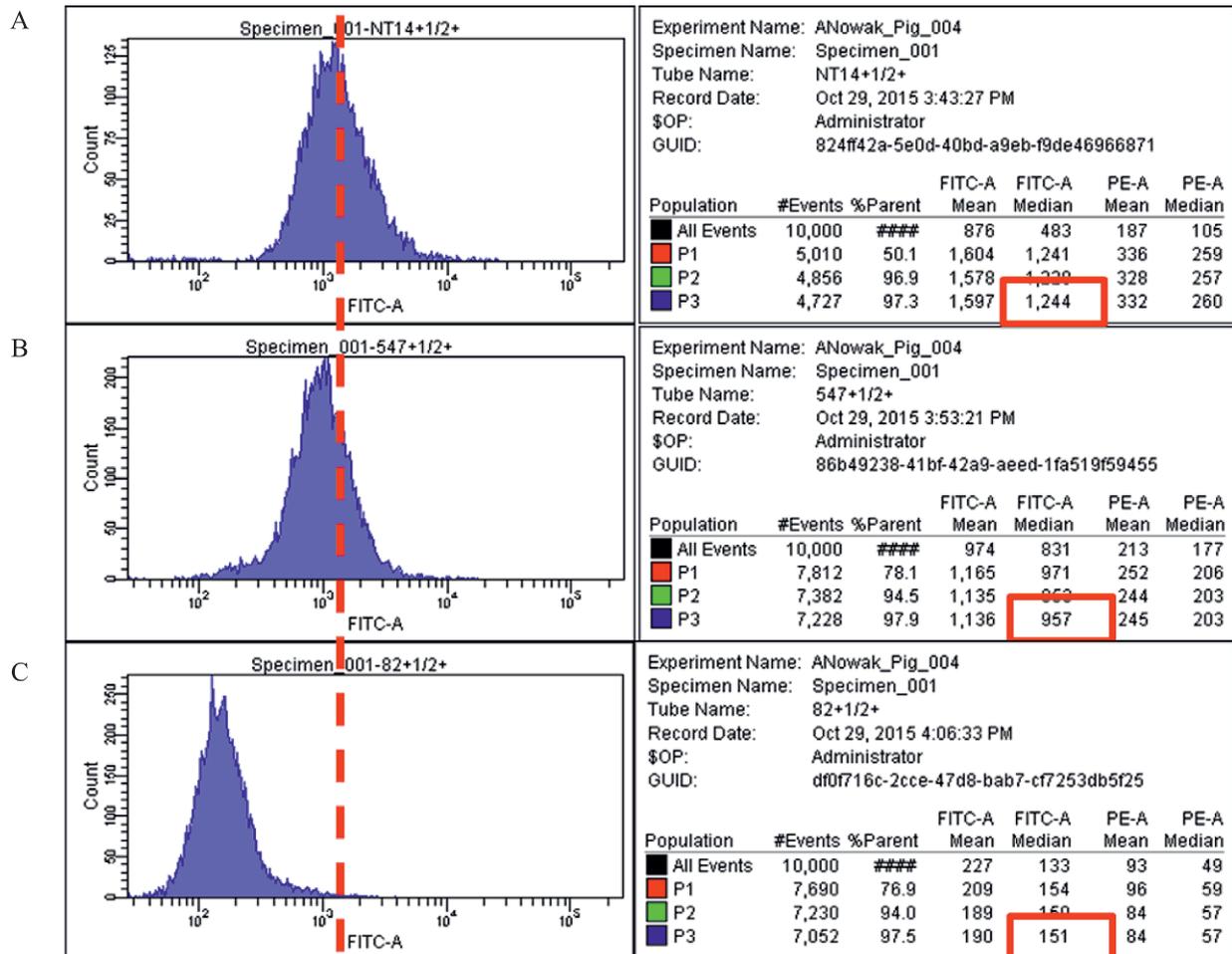
A



B



C



Table 1. Results of statistical analysis of Gala1,3Gal antigen expression in tissue sections from heart, liver, kidney and aorta in modified (+/- *GGTA1*) *vs.* wild type (WT) pigs. Six controls and six modified (+/- *GGTA1*) pigs were analysed. (-/- *GGTA1*) pigs were not included because of the too small number of animals.

| Organ | Group | No. | Average | Fold of change | P |
|---|---|---|---|---|---|
| Heart | WT | 6 | 20.41 | 3.2 | 0.0477* |
| | +/- *GGTA1* | 6 | 6.35 | | |
| Liver | WT | 6 | 26.83 | 3.4 | 0.0002** |
| | +/- *GGTA1* | 5 | 7.78 | | |
| Kidney | WT | 6 | 44.78 | 1.5 | 0.0006** |
| | +/- *GGTA1* | 6 | 30.01 | | |
| Aorta | WT | 6 | 13.96 | 2.3 | 0.021* |
| | +/- *GGTA1* | 6 | 6.14 | | |

*,** level of statistically significant $p < 0.05$.

of immunohistochemical detection of Galα1,3Gal antigen are presented in Fig. 2D. In Table 1 the results of the quantitative and statistical analysis of six wild type animals and six (+/- GGTA1) pigs are presented in more detail. Its a result of knocking out one allele of *GGTA1,* a decreased level of the Galα1,3Gal antigen expression was observed in all analyzed tissues. It was shown that the Galα1,3Gal antigen expression level was decreased in +/- *GGTA1* pigs (from 1.5x in kidneys to 3.4x in liver). All results were statistically significant.

## Discussion

Extremely rapid progress in genome editing techniques has caused a major increase in successful reports of the generation of transgenic animals, including domestic animals. In this study, we report the generation of pigs with a disrupted *GGTA1* gene by application of ZFNs in plasmid format microinjection into the pronuclei of fertilized egg cells. The first to use the ZFN-mediated *GGTA1* knock-out gene in pigs was
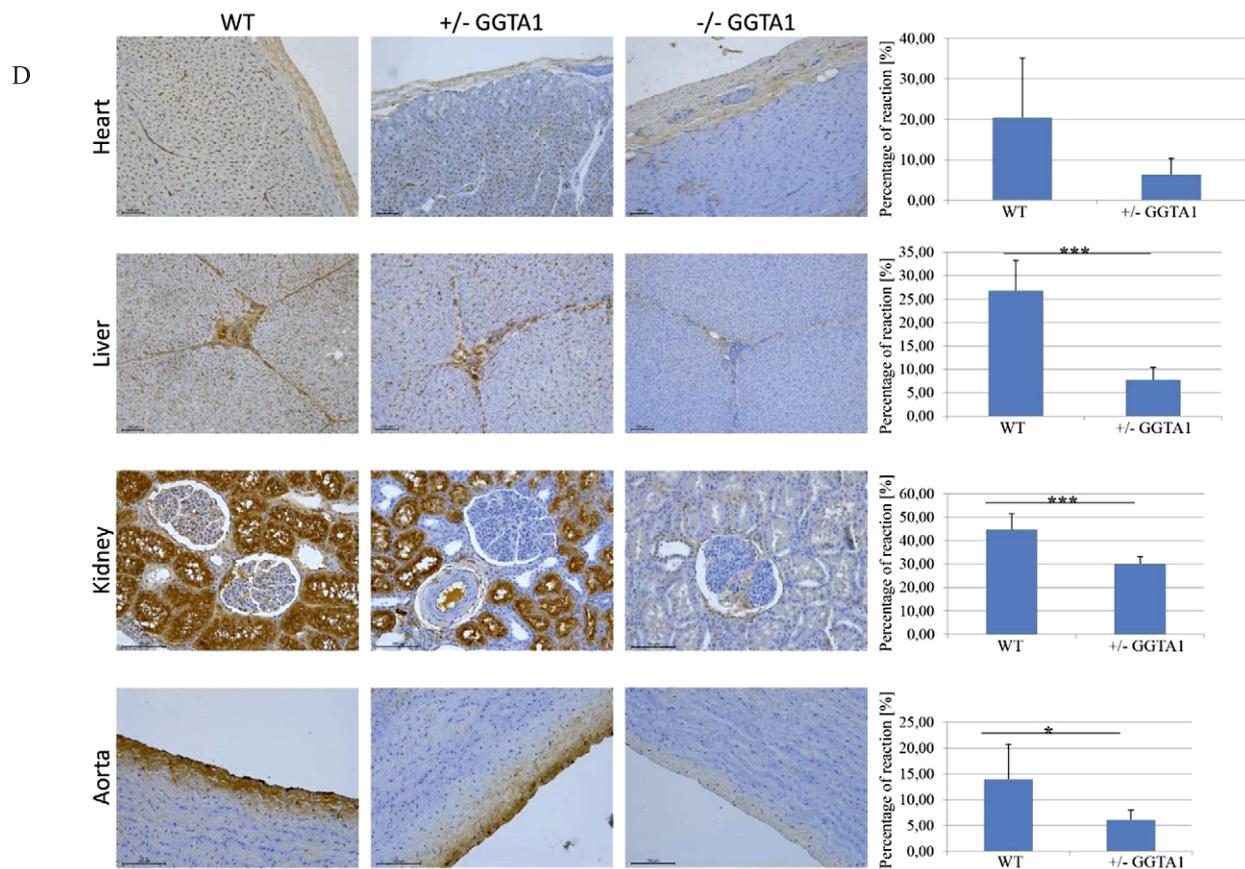
**Fig. 2.** Galα1,3Gal epitope expression analyzed in cell lines by flow cytometry (A, B, C) and tissues of modified pigs by immunohistochemistry (D). Fibroblasts cultured from ear bioptats of wild type, +/-*GGTA1* and -/-*GGTA1* pigs were analyzed. They were labeled with anti-Galα1,3Gal monoclonal antibodies conjugated with FITC-stained secondary IgM antibody. The X axis represents the mean values of green fluorescence intensity medians from an FITC detector (expressed as relative fluorescence units (RFU)) and the Y axis representsthe number of labelled cells. Analysis of fluorescence signals from FITC labeled antibodies preceded a doublets discrimination procedure with the use of height versus width scatter signals measurement, in order to discriminate single events (cells) from conglomerates. The single cells are thus designated population P3. Analysis showed a decreased level of Galα1,3Gal antigen in modified animals as compared to the wild type. A fluorescence intensity shift towards low RFU values indicated decrease in Galα1,3Gal epitope levels by 23% for the +/-*GGTA1* pig (B) and 88% for the -/-*GGTA1* pig (C) in comparison with the wild type (A). Each representative sample was analyzed in 3 repetitions. All modified animals were analyzed. D/ Galα1,3Gal antigen expression in the different organs (heart, liver, kidney and aorta) of genetically modified (+/- *GGTA1*, -/- *GGTA1*) vs. wild type (WT) pigs was analyzed by immunohistochemistry with anti-Galα1,3Gal monoclonal antibody. The images of WT and +/- *GGTA1* pigs represent six controls and six heterozygotes. Homozygote -/- *GGTA1* was represented by two individuals. Images were obtained using 100 x (heart and liver) or 200 x magnification (kidney and aorta). The scale bars are equal to 100 $\mu$m. The results of the quantitative analysis of the Galα1,3Gal antigen expression are presented on the right side. -/- *GGTA1* pigs were not estimated using statistical methods due to the lower number of individuals available for histological analysis.

Hauschild et al. (2011) using SCNT (somatic cell nuclear transfer). In their experiment, 1% of ZFN treated cells were Galα1,3Gal negative (indicated by FACS analyses of isolectin B4-FITC stained cells).

We decided to apply direct microinjection of ZFNs plasmids into the pronuclei of zygotes. This strategy has an advantage over SCNT because the procedure for the generation of a modified animal is easier and faster in comparison with SCNT. SCNT is thought to be limited by an increased risk of abnormal animal production, a higher frequency of perinatal deaths and low

offspring percentage and offspring with low birth weight (Wang et al. 2015). It has been reported that after somatic cloning of transgenic porcine cells, an observed high rate of embryonic loss was caused by the SCNT procedure. The low efficiency of pig cloning ranges between 3-5% (Petersen et al. 2008, 2009). The finite proliferative capacity of somatic donor cells is also a significant disadvantage. Although our efficiency was not higher (one F0 founder out of 107 piglets), we were able to generate modified animals by crossbreeding.

No PSCs (pluripotent stem cells) with germline competency have been generated directly from large animals (Sato et al. 2014). In future, the use of somatic cells for producing modified farm animals will probably be replaced by application of genetically engineered pluripotent stem cells (iPSCs – induced pluripotent stem cells). iPSCs are generally generated by viral-based (retroviral or lentiviral) or piggybac transposons-based or non-integrating episome-based transduction of pig fibroblasts (Ogorevc et al. 2016). These studies must be continued, especially to examine the pluripotent character of reprogrammed somatic cells and the potential risk of tumorigenesis (Zhang et al. 2012). Until then, direct microinjection of DNA/mRNA should be the preferred tool for generating modified pigs, since it shortens time, reduces costs and does not require transfection followed by selection of donor cells. It also eliminates the risk of erroneous reprogramming. The establishment of cell lines with targeted disruption is crucial but sometimes challenging. The use of antibiotics as a selective tool requires long-time *in vitro* culture, resulting in a worse condition of cells with high passages. This makes experiments extremely inefficient as the somatic cells have a limited lifespan. Other techniques, not affecting the biological functions of cells, should be implemented (Kang et al. 2016). Recently, the production of biallelic *GGTA1* knock-out pigs by cytoplasmic microinjection of CRISPR/Cas9 was reported by Petersen et al. (2016).

In our study disruption of both alleles was obtained by interbreeding +/- *GGTA1* pigs giving an expected efficiency of almost 25% (2 -/- *GGTA1* piglets and 7 +/- *GGTA1* in the F2 generation). The number, structure and architecture of the chromosomes were unaffected in the F0, F1 and F2 generation. We performed a set of functional tests to evaluate the quality of *GGTA1* (+/-) and *GGTA1* (-/-) animals in terms of their usefulness as potential donors for xenotransplantation. Flow cytometric based detection of Galα1,-3Gal epitopes demonstrated that the 9-nt deletion in the sequence encoding catalytic domain of the enzyme was sufficient to knock out the *GGTA1* gene and turn off its function. *GGTA1*-KO fibroblasts derived from genetically modified animals were protected against complement-mediated lysis in *in vitro* assay. The immunohistochemical detection of the Galα1,3Gal antigen with monoclonal antibody showed a statistically significant decrease in the Galα1,3Gal epitope level in the kidney, aorta, heart and liver (up to 3.4-fold) in +/- *GGTA1* pigs. All these tests were consistent: the removal of the *GGTA1* allele or both of alleles had a similar trend in lowering the Galα1,3Gal antigen level, but the most visible effect was obtained by immunohistochemical detection in liver. This relatively high decrease of the Galα1,3Gal epitope expression for heterozygotes in liver may be surprising and we are not sure how to explain this. It was perhaps influenced by a specific batch of monoclonal antibody or by the nature of liver, which is rich of metabolites. We presented the results of selected organs potentially important as grafts. We also observed a visible positive signal in the liver of the -/- *GGTA1* individual. According to Sharma et al. (2003) and also in our opinion, this may be caused by either incomplete inactivation of the *GGTA1* gene or by another glycosyltransferase with the same specificity.

Immunohistochemistry is a semi quantitative method; however, we applied complex evaluation based on a whole scan of microscope slides. Elaborated quantitative analysis was performed using dedicated software (Pannoramic Viewer and Definiens Developer XD), multi panel segregation with two classes of pixels and a number of statistical tests. Therefore presented images of IHC should be treated only as representative pictures of complex quantitative digital analysis.

In our studies, we observed no changes in the health and behavior of genetically modified pigs. The normal growth and health of the piglets indicated no negative influence of ZFN technology on ontogenic development. All animals were also examined for the presence and copy number of porcine endogenous retroviruses (PERVs) according to methods used previously (Mazurek et al. 2013), (data not published here). According to the International Xenotransplantation Association careful screening of the source herds for PERVs, selection of animals that exhibit low level expression of PERV A and PERV B and selection of animals that do not contain PERV C must be carried out in terms of xenotransplantation procedures (Denner et al. 2009). Genome editing tools (GETs) including ZFN-mediated as well as TALEN-mediated mechanisms contain a recognition and a cleavage domain for sequence-specific nuclease (SSN). Double-strand breaks mediated by ZFN technology reduce the risk of off-target occurrence (Ran et al. 2013). Comparative analysis of ZFN and TALEN showed differences caused by experimental variation, higher fidelity of PCR detection methods and DNA-dependent repair pathway (Kwon et al. 2013). It has been shown that ZFN-mediated alterations can occur during early embryonic development, resulting in 100% mutant cells in the organism (Huang et al. 2014). GETs are able to increase gene disruption *via* mutagenic DNA repair more than 10,000-fold (Petersen and Niemann 2015).

Donor shortage is the main factor that limits the number of transplantations treating severe organ failure. NHPs (non-human primates) are being consequently excluded as potential donors, since their medi-

cal utility causes strong ethical resistance. Numerous attributes indicate that pigs should fill the supply gap. The large phylogenetic distance is the main limitation to pig-to-human xenotransplantation. Only organs from genetically modified pigs will be able to overcome the severe immunological rejection responses occurring after xenotransplantation.

In this paper we showed efficient generation of pigs with a disrupted *GGTA1* gene by ZFNs in plasmid format directly microinjected into the pronuclei of fertilized egg cells. One of our goals was to establish a set of standard procedures to carry out functional testing of genetically modified animals to be used for xenotransplantation. We made an attempt to perform a number of tests to produce a complete characteristic of modified animals. To characterize the phenotype effect of *GGTA1* knock-out in modified pigs we performed a number of functional tests including karyotyping, flow cytometry, human serum cytotoxicity test, immunohistochemical detection and screening for PERV expression. We showed efficient *GGTA1* knock-out in the generated pigs and characterized some biological properties of these animals. The combining pronuclear microinjection and ZFN-mediated *GGTA1* gene disruption led to significantly decreased susceptibility to complement-mediated lysis. The fibroblasts derived from the -/- *GGTA1* pig had higher survival rates than those from wild-type pigs, which suggests that the organs and tissues from these pigs can overcome hyperacute rejection after xenotransplantation into humans. A similar result for complement-mediated lysis assay was reported by another research team during the analysis of fibroblasts derived from pigs with disrupted *GGTA1* gene by ZFNs/TALENs and somatic cell nuclear transfer (Hauschild et al. 2011, Bao et al. 2014, Kang et al. 2016).

The presented data on generating ZFN mediated *GGTA1* knock-out pigs is a transitional step for us to test one of the methods of gene editing in our facilities. We expect that these results will help us to establish procedures for the generation of polyallelic modified pigs for the study of pig-to-human-xenotransplantation.

The rapid progress of genome editing methods give hope that generation of genetically modified animals will be easier, faster and cheaper, and they may become in future a prospective source of more compatible xenografts. However, what is most important is that whatever method is applied, there should be a list of necessary procedures to test for functional accuracy and compatibility for using the organs or tissues of generated animals in biomedicine.

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