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The Effect of Recombinant Human Alpha-1,2-Fucosyltransferase and Alpha-Galactosidase A on the Reduction of Alpha-Gal Expression in the Liver of Transgenic Pigs

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Original article

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Genetically modified pigs lacking Galα1→3Gal and other immunogenic carbohydrates are considered as the most promising, alternative source of various tissues and organs for human transplantation. Here, we tested the hypothesis that combining the expression of human α1,2-fucosyltransferase (h*FUT2*) and α-galactosidase A (h*GLA*) genes would allow for the removal of this specific carbohydrate in porcine transgenic livers. We investigated the expression profile of human α1,2-fucosyltransferase and α-galactosidase A proteins and the amount of Galα1→3Gal antigen in the liver of single transgenic h*FUT2* (n=5), h*GLA* (n=5), and double transgenic h*FUT2*×h*GLA* (n=5) pigs. Both human proteins, α1,2-fucosyltransferase and α-galactosidase A, were abundantly expressed in the liver tissue in respective transgenic lines as was revealed by confocal microscopy and Western blotting. The level of Galα1→3Gal epitope evaluated by lectin histochemistry and lectin blotting was significantly lower ($p<0.05$) in all genetically modified livers than that in the control non-transgenic porcine livers. Importantly, the double transgenic line expressed a significantly lower ($p<0.05$), but still detectable level of this antigen, compared to both single transgenic pigs, as shown by lectin blotting. Histological evaluation of the liver samples stained with haematoxylin and eosin showed no morphological evidence of hepatic abnormalities in all transgenic pigs. Our study indicates that the simultaneous expression of two protective transgenes h*FUT2*×h*GLA* indeed improves the removal of the Galα1→3Gal epitope in porcine liver. However, this modification alone is not sufficient enough for complete elimination of this antigen from porcine liver tissue.

Key words: Genetically modified pigs, liver, Galα1→3Gal epitope, human α1,2-fucosyltransferase, human α-galactosidase A.

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Pigs are considered to be the most suitable, alternative donors of tissues and organs for human patients with acute or chronic organ failure. They share many similarities in organ anatomy and physiology and share an approximately 96% genetic identity with humans (GALILI *et al.* 1988; COOPER *et al.* 2002; WHYTE & PRATHER 2011; HRYHOROWICZ *et al.* 2017). Pigs have good breeding potential and rapid

growth at a relatively low cost. In addition, porcine endogenous retroviruses could be effectively inactivated by CRISPR-Cas9 to prevent their transmission to human recipients (NIU *et al*. 2017).

Unfortunately, the phylogenetic distance between pigs and humans entails a complex immune response leading to hyperacute rejection as well as acute humoral and cellular rejection of transplanted pig organs (COOPER *et al*. 2015, 2016; LU *et al*. 2020). Hyperacute rejection is an immediate reaction of the human immune system to the major carbohydrate xenoantigen Galα1→3Gal, which is abundantly present on the surface of porcine cells, in particular endothelial cells. The formation of the Galα1→3Gal epitope is driven by the enzyme α1,3-galactosyltransferase, encoded by the *GGTA1* gene (SANDRIN & MCKENZIE 1994). *GGTA1* is functional in most mammals, including pigs, but not in humans and Old World apes (GALILI *et al*. 1988; LU *et al*. 2020). In turn, primates produce natural xenoreactive antibodies, of which about 90% recognise the Galα1→3Gal epitope (SANDRIN *et al*. 1993; MCMORROW *et al*. 1997).

A promising approach to overcome hyperacute rejection is generating homozygous α1,3-galactosyltransferase gene-knockout (GTKO) pigs using zinc finger nuclease (ZFN) technology, transcription activator-like effector (TALE) nucleases and modifications of the CRISPR/Cas system (LAI *et al*. 2002; PIERSON 2009; LADOWSKI *et al.* 2019). Another strategy to remove the Galα1→3Gal epitope from porcine cells involves the combined transgenic expression of recombinant human enzymes α1,2-fucosyltransferase (rhα1,2-FT) and α-galactosidase A (rhα-Gal A) (OSMAN *et al*. 1997). α1,2-fucosyltransferase is encoded by h*FUT2* and in humans occurs in the *cis* compartment of the Golgi apparatus. This human enzyme acts earlier than porcine endogenous α1,3-GT, which is present in the *trans* compartment. This strategy is based on the competition of these two enzymes acting on the same substrate during oligosaccharide processing in transgenic cells. As an oligosaccharide moves through the Golgi apparatus from the *cis* to the *trans* compartment, it is first fucosylated by rhα1,2-FT. Hence, this oligosaccharide cannot accept the terminal galactose residue in the subsequent reaction catalysed by α1,3-GT (HARTEL-SCHENK *et al*. 1991). In turn, the recombinant human α-galactosidase A, encoded by the h*GLA* gene, is responsible for the cleavage of terminal D-galactose residues (LUO *et al*. 1999). The expression of rhα1,2-FT or rhα-Gal A alone does not, however, allow for the complete elimination of the Galα1→3Gal epitope from pig cells. Thus, it was suggested that the co-expression of both rhα1,2-FT and rhα-Gal A would be more efficient method for this epitope reduction (LUO *et al*. 1999; ZEYLAND *et al*. 2014). Indeed, ZEYLAND *et al*. (2014) reported for the first time the successful production of double transgenic pigs that expressed human α1,2-fucosyltransferase and α-galactosidase and showed a considerable reduction of the α-Gal antigen level on the surface of skin fibroblasts. However, the effect of transgenic modification may vary among different cell types, due to their specific glycosylation pattern, and so far the impact of h*FUT2* and h*GLA* transgenes on carbohydrate antigen Galα1→3Gal expression has not been investigated in any solid organ. Liver is the largest internal organ in the body and exhibits remarkable regenerative capacity. The glycosylation of liver cells and secreted proteins play an essential role in regulating various metabolic and immune functions, performed by parenchymal hepatocytes, sinusoidal endothelial cells, hepatic stellate cells, resident macrophages (including Kupffer cells), and various lymphocytes.

In this context, in the present study we hypothesised that the combined actions of human α1,2-fucosyltransferase and α-galactosidase A enzymes in double transgenic h*FUT2*×h*GLA* pigs are more efficient in eliminating the Galα1→3Gal epitope in porcine liver than a single expression of either h*FUT2* or h*GLA* transgenes.

Materials and Methods

Animals

All animal procedures were conducted in accordance with the European Directive 2010/63/EU and approved by the Second Local Ethics Committee in Kraków, Poland (Permission 1181/2015 from 21 of May 2015). The liver tissue samples were obtained from the Department of Biotechnology of Reproduction and Cryopreservation, National Research Institute of Animal Production at Balice near Krakow. Fresh tissue specimens were taken immediately after slaughtering and the opening of the abdominal cavity from 15 transgenic pigs (n=5 for each transgenic variant) and from 8 non-genetically modified Polish Large White pigs. All liver samples were frozen in liquid nitrogen for further analyses. The transgenic pigs were designed to show the expression of recombinant human α1,2-fucosyltransferase (h*FUT2*), α-galactosidase (h*GLA*) and h*FUT2*×h*GLA* (LIPIŃSKI *et al*. 2010; ZEYLAND *et al*. 2014). The transgenic pigs and non-transgenic control pigs were healthy and normal in terms of reproductive capability. They were 12- to 18-months old and weighed 150-200 kg.

Histological analysis

The frozen liver samples were sectioned at 6 μm in Leica CM 1850 UV cryostat (Leica, Biosystems, Nussloch, Germany) and collected onto poly-L-lysine coated microscopic slides. Cryosections were fixed with 4% paraformaldehyde in PBS for 10 min, washed in PBS three times for 5 min and then stained with haematoxylin (Shandon, ThermoFisher Scien-

tific, Waltham, MA, USA) for 3 min. Subsequently, slides were washed 10 min in tap water and were then stained with eosin (ThermoFisher Scientific, Waltham, MA, USA) for 2 min. After dehydration in a graded series of ethanol (70%-100%), followed by two changes of xylene, sections were mounted with Consul-Mount™ (Shandon™, ThermoFisher Scientific, Waltham, MA, USA) and coverslipped. Finally, sections were analysed using a Nikon Optihot-2 bright field microscope (Nikon, Tokyo, Japan) equipped with a Nikon Digital Camera DXM 1200F (Nikon, Tokyo, Japan) and 10×, 20×, and 40× objective lenses.

Immunofluorescence staining

The porcine liver samples were cryosectioned at 6 μm and collected onto poly-L-lysine coated microscopic slides. Sections were fixed with a 4% paraformaldehyde solution in PBS for 10 min, washed in PBS and blocked in 5% Normal Goat Serum/PBST (Phosphate buffer saline with 0.1% v/v Triton X-100, Bioshop Inc., Burlington, Canada) for 45 min. Then sections were incubated with the primary antibodies against: human α1,2-fucosyltransferase (Rabbit polyclonal antibodies, ab198712, Abcam) diluted 1:150 in PBST; human α-galactosidase (Rabbit polyclonal antibodies, PA5-27349; ThermoFisher Scientific, Waltham, MA, USA) diluted 1:200, overnight at +4°C in a humidified chamber. In the next step, sections were washed several times in PBST and treated with appropriate secondary antibodies labelled with Cy3 (Goat anti Mouse or Goat anti Rabbit, Jackson Immuno Research) diluted 1:600 in PBST, for 1 h at room temperature. After final washes, sections were mounted in Fluoroshield with DAPI mounting medium (F6057, Sigma-Aldrich, St. Louis, MO, USA) and coverslipped. Fluorescently labelled sections were examined by Olympus FV1200 Confocal Microscope (Olympus, Tokyo, Japan). Relative intensities of fluorescence were quantified in each, randomly chosen region of interest (ROI) using ImageJ version 1.46r software (National Institutes of Health, Bethesda, MD, USA) in a greyscale of 256 levels (GAJDA *et al*. 2011; ROMEK *et al*. 2017). For each genetically modified and control pig, three sections were sampled by 70 ROI.

Double immunofluorescence staining was performed using primary antibodies against human α1,2-fucosyltransferase (Rabbit polyclonal antibodies, ab198712, Abcam) diluted 1:150 in PBST, and antibodies that recognize the Golgi apparatus (58K Golgi protein) (Mouse monoclonal antibodies, ab27043, Abcam) diluted 1:100 in PBST. The secondary antibodies were Goat anti Rabbit conjugated with Alexa Fluor 488 (ThermoFisher Scientific, Waltham, MA, USA) diluted 1:300 in PBST, and Goat anti Mouse Alexa Fluor 555 labeled (ThermoFisher Scientific, Waltham, MA, USA) diluted 1:600 in PBST.

Lectin histochemistry

The localization and semi quantitative comparison of Galα1→3Gal epitope expression in the liver of h*FUT2*, h*GLA,* and h*FUT2*×h*GLA* was assessed by using a specific lectin from *Griffonia simplicifolia* ($GS\text{-}IB_4$) conjugated with Alexa Fluor 647 fluorescent dye (I32450, Molecular Probes, Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA). Lectin histochemistry was carried out on 6 μm cryostat sections, which were fixed with 4% paraformaldehyde in PBS, washed in PBS and then blocked in 1% BSA (Bovine Serum Albumin, Bioshop Inc., Burlington, Canada) in PBST for 1 h. Samples were washed three times in PBS and treated with lectin $GS\text{-}IB_4$ diluted 1:200 in DPBS, at +4°C overnight in a dark humidified chamber. After final washes, sections were mounted in Fluoroshield with DAPI and coverslipped. Fluorescently labelled sections were examined as described in the paragraph Immunofluorescence staining.

Total protein isolation and Western/Lectin blot analyses

Total protein was extracted from frozen tissue samples by using a radioimmunoprecipitation assay lysis buffer (RIPA buffer, Thermo Fisher Scientific, Waltham, MA, USA) containing 1% of proteinase inhibitor cocktail (RIPA+PI; Bioshop Inc., Burlington, Canada). Liver samples were cut into small, approximately 2 mm^3 pieces and homogenized in 300 μl RIPA+PI. Tissue lysates were centrifuged at 14 000 × g for 15 min at +4°C and supernatants collected. Protein concentration was determined with microassay DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. Protein samples were stored at minus 80°C for further analyses.

Protein samples were diluted in 2× Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) with β-mercaptoethanol (BME; Sigma-Aldrich, St. Louis, MO, USA) and denatured at 100°C for 5 min. Then, proteins were separated in SDS-PAGE using 5% stacking and 10% resolving gels. Molecular weights were estimated with reference to standard proteins (Precision Plus Dual Color Protein Standard, Bio-Rad Laboratories, Hercules, CA, USA). For immunoblotting, lectin blotting proteins were electrotransferred onto PVDF membrane (Immobilon, Merck, Dermstadt, Germany) at a constant amperage of 250 mA for 120 min.

For immunoblotting, membranes were blocked for 1 h in 5% non-fat milk in TBST (Tris buffer saline with 0.1% v/v Tween20, Bioshop Inc., Burlington, Canada) and after several washes in TBST, incubated overnight at +4°C with the following primary antibodies (the same as for immunofluorescent labelling) against: human α1,2-fucosyltransferase diluted 1:1000

in TBST and human α-galactosidase diluted 1:1000 in TBST. Then, membranes were washed several times in TBST and incubated with appropriate secondary antibodies horse radish peroxidase (HRP) conjugated (Goat anti-Rabbit or Goat anti-Mouse ThermoFisher Scientific, Waltham, MA, USA) at a dilution of 1:6000 in TBST for 1 h at room temperature. The β-actin was used as a reference protein (ab8224 Abcam, Mouse monoclonal antibodies anti-β-actin) diluted 1:2000 in TBST.

For lectin blotting, membranes were blocked for 30 min in 1% BSA in TBST. After several washes in DPBS followed by TBS, membranes were incubated overnight at +4°C with lectin GS-IB_4 labelled with HRP (L5391, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in DPBS. Finally membranes were washed in TBS.

For both immunoblotting and lectin blotting, protein bands were detected by chemiluminescence using Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and visualized with the ChemiDocTM XRS+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were quantified using Image Lab™ 2.0 Software (Bio-Rad Laboratories, Hercules, CA, USA) by measurement of their relative optical densities. Following protein detection in Western blotting, membranes were stripped and reprobed with anti-β-actin antibody, which was used as a reference protein (ab8224 Abcam, Mouse monoclonal antibodies anti-β-actin) diluted 1:2000 in TBST.

Statistical analysis

For each genetically modified and non-modified group of pigs and for all analyses, three repeats were performed. Quantitative data were expressed as the mean ± standard error of the mean and examined using the Shapiro-Wilk (SW) test for normality. Comparisons between the appropriate means were performed by one-way analysis of variance (ANOVA), followed by the Tukey HSD *post hoc* test. Statistical significance was marked accordingly: *p<0.05, **p<0.01, ***p<0.001.

Results

Histological analysis

Histological analysis of transgenic and non-transgenic porcine livers was performed to examine any possible adverse effects of transgenesis on the morphology of porcine liver. Routine staining with haematoxylin and eosin showed no visible differences between livers from transgenic and non-transgenic pigs in the histological organisation of tissue structures such as a portal space, bile ducts, hepatic veins, hepatic arteries, and parenchymal cells. Figure 1 shows representative

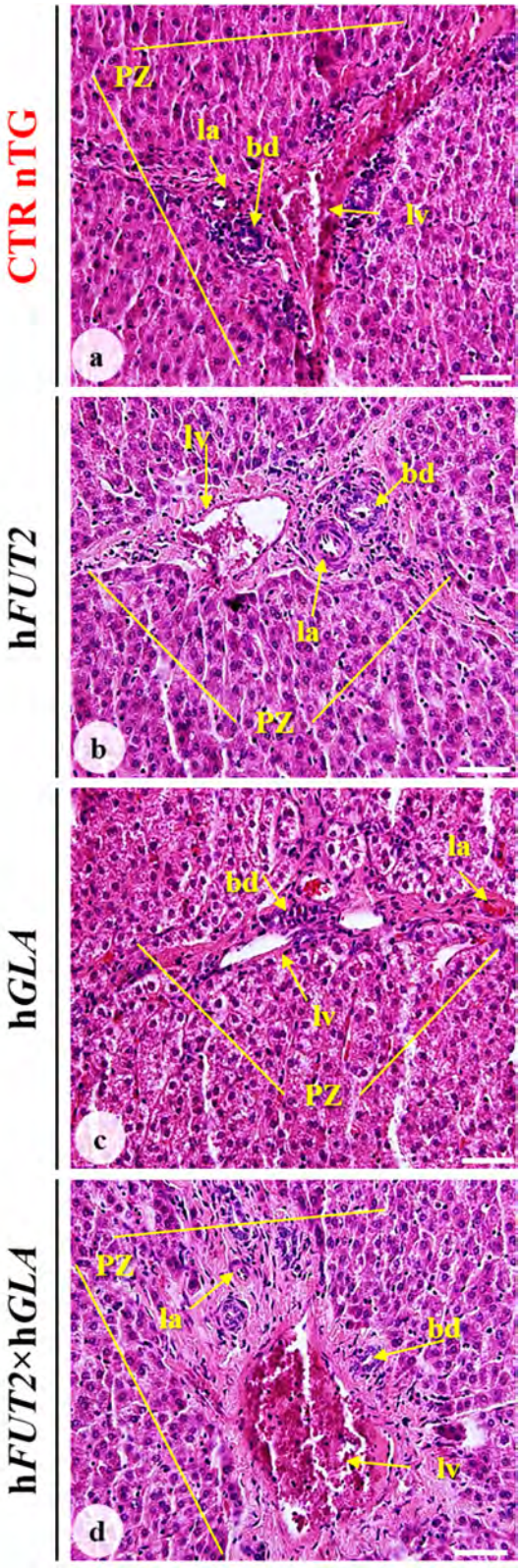

Fig. 1. Histological analysis of transgenic and non-transgenic (control) porcine livers. Representative microphotographs of control non-transgenic CTR nTG (a), single transgenic h*FUT2*, h*GLA* (b, c), and double transgenic h*FUT2*×h*GLA* (d) porcine liver cryostat tissue sections. Haematoxylin-eosin staining. The most typical elements of the liver structure were marked as follows: lv – liver vein; PZ – portal venous zone; la – hepatic artery; bd – bile duct. The analysis showed no histological differences between the control and the transgenic samples. Scale bars = 50 μm.

images of liver tissue sections stained with H&E from single transgenic h*FUT2* and h*GLA*, double transgenic h*FUT2*×h*GLA*, and non-transgenic pigs. The analysis showed no histological differences between the control and the transgenic samples.

Immunofluorescence staining

The localization of recombinant human α1,2-fucosyltransferase (rhα1,2-FT) and α-galactosidase A (rhα-Gal A) was examined by the immunofluorescence staining of liver cryostat sections derived from single transgenic h*FUT2* and h*GLA*, double transgenic h*FUT2*×h*GLA,* and non-transgenic pigs. The positive immunofluorescence signal from rhα1,2-FT was evenly distributed in the hepatic lobules of the porcine liver derived from both transgenic lines h*FUT2* and h*FUT2*×h*GLA* (Fig. 2a and 2c). In turn, rhα-Gal A immunostaining was less homogenous and detected in small clusters of hepatocytes as well as in the interlobular space in the porcine liver of transgenic variants h*GLA* and h*FUT2*×h*GLA* (Fig. 2b and 2d). In the control non-transgenic livers the rhα1,2-FT was barely detectable by immunofluorescence (Fig. 2e), while we did not observe any positive signal from recombinant human α-galactosidase (Fig. 2f). Double immunofluorescence labeling experiments demonstrated colocalization of the recombinant human α1,2-fucosyltransferase with the Golgi resident 58K protein in the perinuclear region of hepatocytes originated from both transgenic pig models, h*FUT2* and h*FUT2*×h*GLA*. Figure 4 shows representative microphotographs of immunofluorescence colocalization of recombinant human-α1,2-fucosyltransferase (rhα1,2-FT) and the Golgi apparatus (58K Golgi protein) in porcine liver cryostat sections derived from single (h*FUT2*) and double transgenic pigs (h*FUT2*×h*GLA*).

The semi-quantitative analysis of fluorescence intensity showed statistically significant, inter-individual differences in the expression of recombinant human α1,2-fucosyltransferase between the control (CTR nTG) and both transgenic variants, h*FUT2* ($p<0.01$) and h*FUT2*×h*GLA* ($p<0.001$) (Fig. 3a). Moreover, significant differences were also detected between h*FUT2*×h*GLA* and h*FUT2* pigs ($p<0.01$). Considering recombinant human α-galactosidase expression, we did observe statistically significant differences between the control (CTR nTG) and both transgenic variants, h*GLA* ($p<0.01$) and h*FUT2*×h*GLA* ($p<0.001$), but there was no difference between the transgenic animals (Fig. 3b).

Western blot

Liver tissue samples were derived from single (h*FUT2*, h*GLA*) and double (h*FUT2*×h*GLA*) transgenic pigs as well as from non-transgenic, control pigs (CTR nTG). Western blot analysis of total protein extracts from porcine liver samples showed the presence of recombinant human α1,2-fucosyltransferase and α-galactosiadase A proteins in all corresponding transgenic samples (Fig. 5a-c). In the control group (Fig. 5d), we observed a weak positive signal for rhα1,2-FT and a barely detectable signal for human rhα-Gal A. The signal intensities of the analysed proteins were normalized to beta-actin, which was used as a loading control. Quantitative analysis of the Western blot showed that the relative expression of both tested proteins was significantly higher (at least $p<0.01$) in all transgenic variants than that in the control group (CTR nTG) (Fig. 5e and 5f). The specificity of primary antibodies was determined by the presence of a single band in the liver samples, at the expected molecular weight, on a Western blot.

Lectin histochemistry

The expression profile of the Galα1→3Gal epitope was identified in porcine liver tissue sections by labelling with the specific lectin GS-IB_4 Alexa Fluor 647 conjugated (Fig. 6). The lectin GS-IB_4 strongly labelled Galα1→3Gal in all the liver tissue sections of the control group (Fig. 6a). In contrast, liver samples from the single transgenic pigs h*FUT2*, h*GLA* (Fig. 6b and 6c) and from the double transgenic pigs h*FUT2*×h*GLA* (Fig. 6d) displayed a much lower Galα1→3Gal fluorescence intensity compared to the control group.

Semiquantitative analysis of fluorescence intensity of the AlexaFluor 647 dye revealed that the Galα1→3Gal epitope expression in the liver of all three transgenic lines was significantly lower as compared to the control non-transgenic pigs (Fig. 7) at $p<0.01$ for both h*FUT2* and h*GLA*, and $p<0.001$ for h*FUT2*×h*GLA*. However, no significant differences were detected among the individual groups of genetically modified animals ($p>0.05$).

Lectin blot analysis

Lectin blot analysis was used to determine the expression profile of Galα1→3Gal at the total protein level using lectin GS-IB_4 labelled with HRP. The results showed the presence of the Galα1→3Gal epitope in all analysed porcine liver samples (Fig. 8a). Beta-actin served as a loading control. The quantitative analysis of the Galα1→3Gal epitope showed statistical differences between the control group (CTR nTG) and all genetically modified liver samples (Fig. 8b). The lowest significant expression of the Galα1→3Gal epitope was observed in samples from double transgenic porcine livers (h*FUT2*×h*GLA*) and this was statistically different from both, h*FUT2* ($p<0.05$) and h*GLA* ($p<0.01$) (Fig. 8b). GALILI *et al*. (1988) dem-

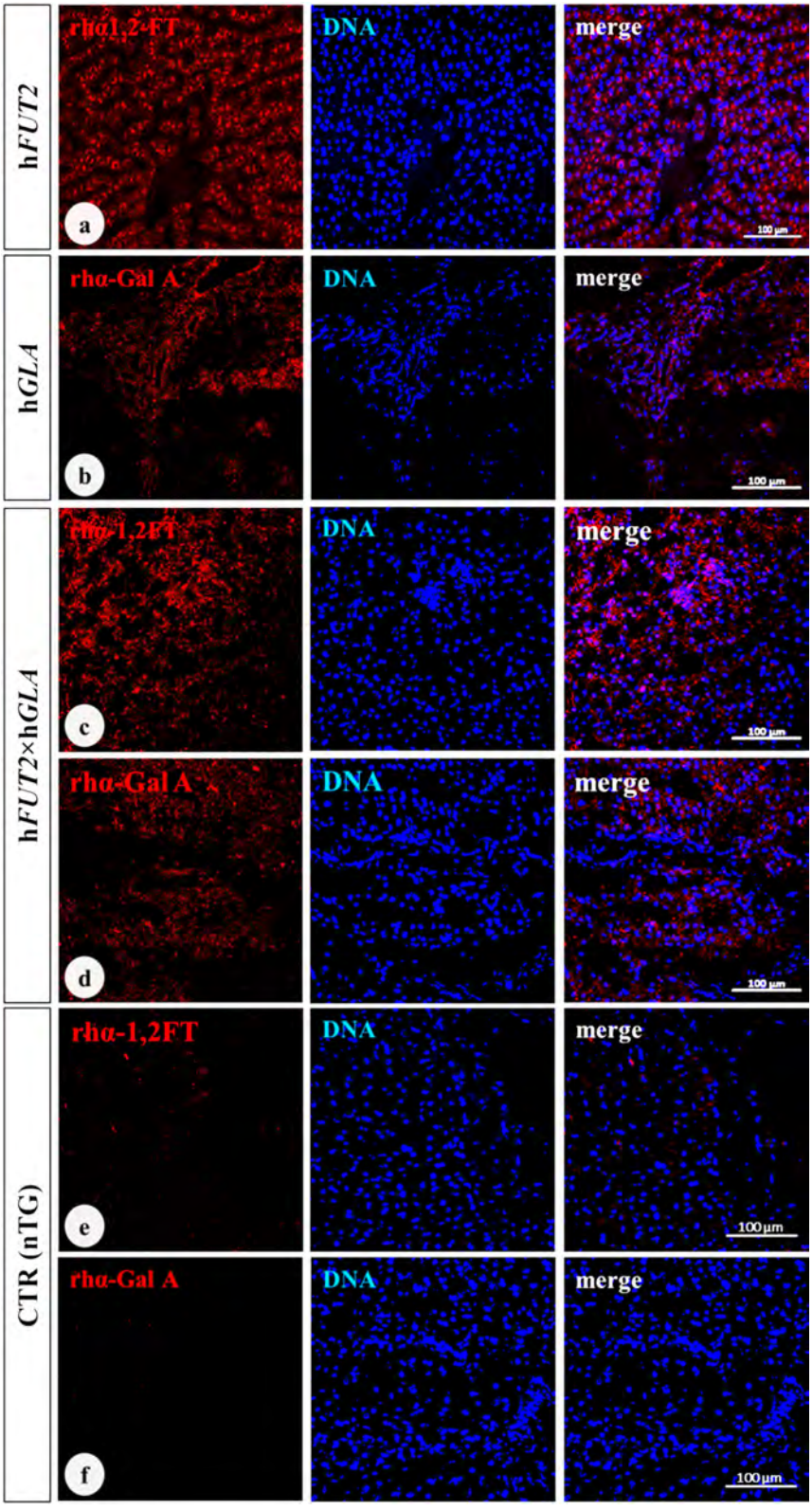

Fig. 2. Immunofluorescence analysis of porcine liver cryostat tissue sections. Representative microphotographs of immunofluorescence localization of recombinant human: α1,2-fucosyltransferase (rhα1,2-FT) and α-galactosidase A (rhα-Gal A) in porcine liver cryostat sections derived from single (a, b) and double transgenic pigs (c and d). Liver cryostat sections from non-transgenic pigs were used as a control group (CTR nTG) (e and f). Immunofluorescent staining with Cy3 labelled secondary antibodies (red) and DAPI counterstaining (blue). Scale bars = 100 µm. Immunoreaction was performed on liver serial sections from at least three pigs of each experimental group. The immunofluorescence signal from α1,2-rhFT was distributed homogeneously in all lobules of porcine liver derived from each analysed transgenic variant (a and c). The recombinant human α-galactosidase A was located less homogeneously in single clusters of hepatocytes as well as in the interlobular space in the porcine liver of all variants (b and d). Analysis showed a barely detectable positive signal from rhα1,2-FT (e) in the control group (CTR nTG). No positive signal from recombinant human α-galactosidase A in the control (CTR nTG) is seen (f).

onstrated an abundance of this epitope on mammalian cells including pig cells (1×10^6-30×10^6 epitopes/cell). Since in our study lectin blotting was performed on total protein samples, we postulate that the large number of bands positive for Galα1→3Gal result from a great quantity of this epitope in pig liver cells.

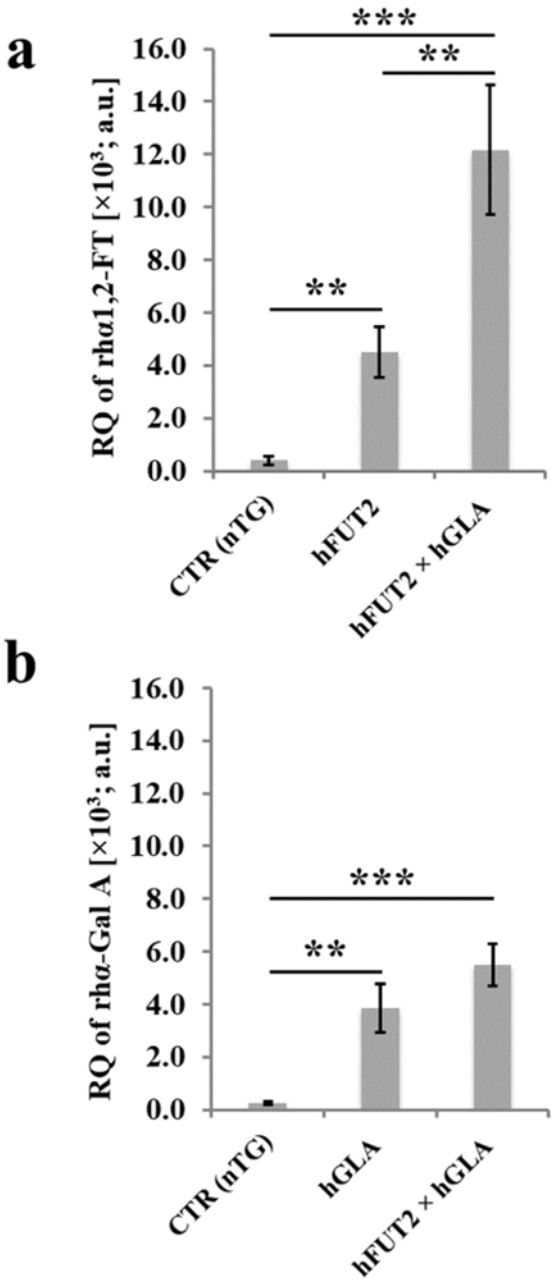

Fig. 3. Relative expression of recombinant human: α1,2-fucosyltransferase (a) and α-galactosidase A (b). For each genetically modified and control animal, three sections were sampled by 70 ROI. Data are presented as mean ± SEM and displayed as arbitrary units in exponential notation ($\times10^3$). One-way ANOVA and *post hoc* Tukey HSD test, values are denoted as **p<0.01, ***p<0.001.

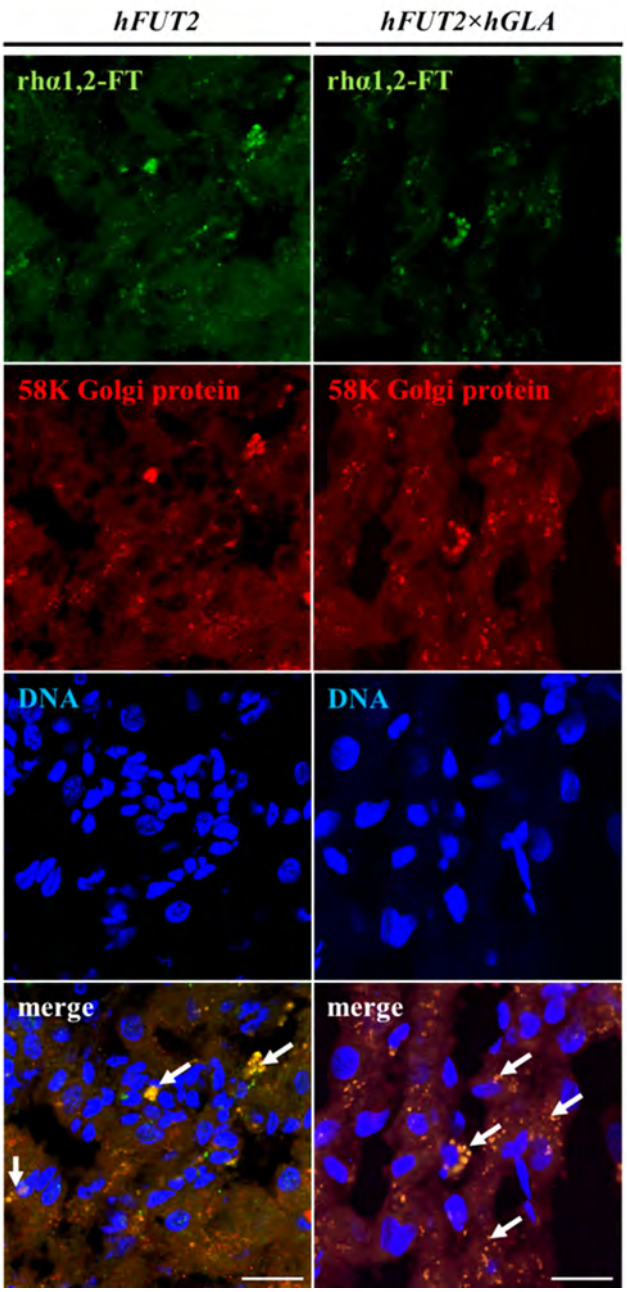

Fig. 4. Immunofluorescent colocalisation of recombinant human α1,2-fucosyltransferase and the Golgi apparatus in porcine liver cryostat tissue sections. Representative microphotographs of the immunofluorescence colocalization of recombinant human: α1,2-fucosyltransferase (rhα1,2-FT) and the Golgi apparatus (58K Golgi protein) in porcine liver cryostat sections derived from single (h*FUT2*) and double transgenic pigs (h*FUT2*×h*GLA*). Immunofluorescent staining with Alexa Fluor 488 labelled secondary antibodies (green) for rhα1,2-FT, Cy3 labelled secondary antibodies (red) for the 58K Golgi protein, and DAPI counterstaining for nuclei (blue). Immunoreaction was performed on liver serial sections from at least three pigs from each experimental group. White arrows on the merged pictures show the colocalization of both analyzed proteins. Scale bars = 20 μm.

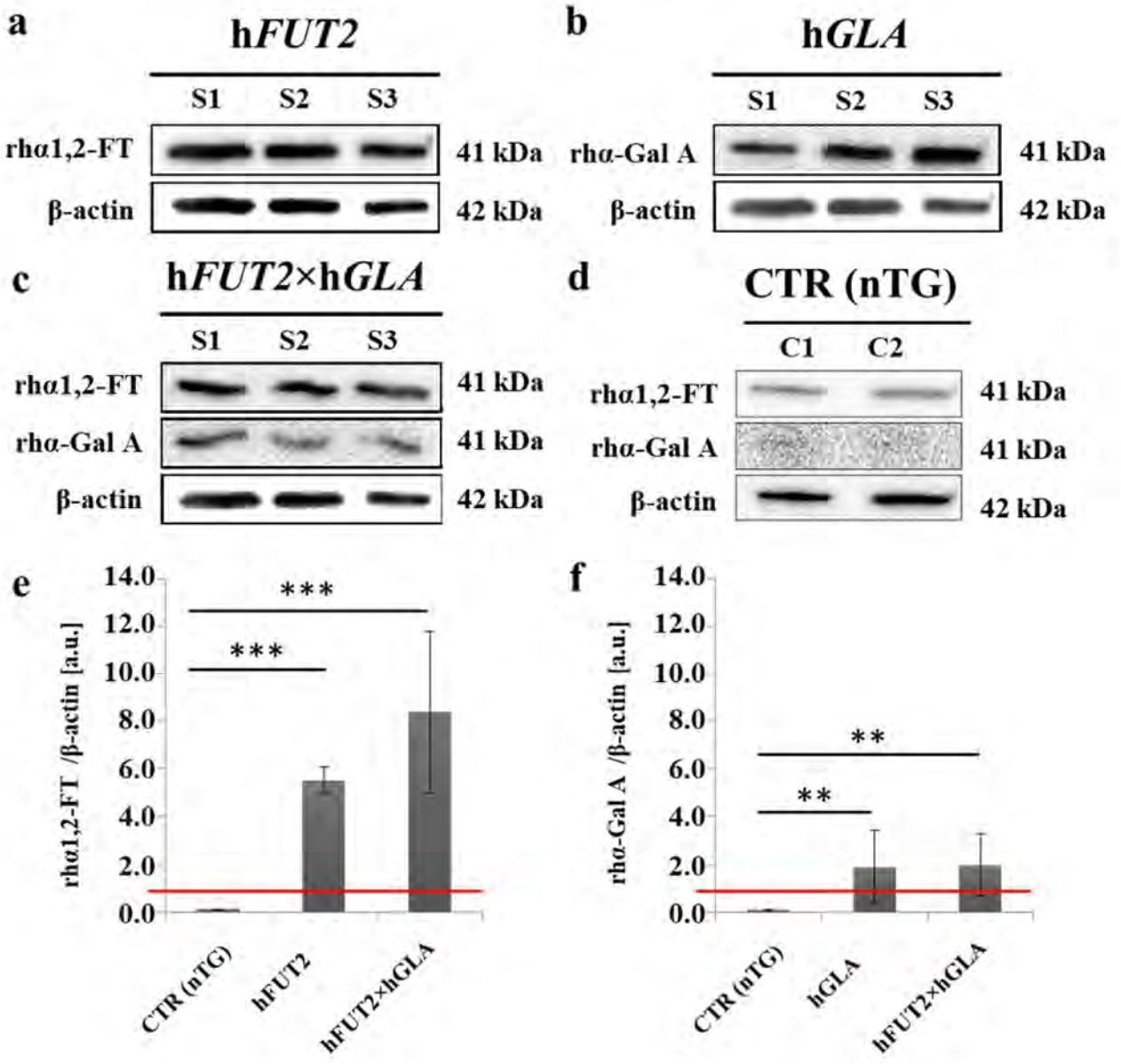

Fig. 5. Western blot analysis of the relative expression of recombinant human: α1,2-fucosyltransferase (rhα1,2-FT) and α-galactosidase (rhα-Gal A) proteins in transgenic and non-transgenic porcine livers. Representative blots of the expression of: rhα1,2-FT and rhα-Gal A proteins in the liver samples derived from single (a, b) and double transgenic (c) porcine livers. The liver samples of non-transgenic animals served as a control group (CTR nTG) – panel d. The β-actin was a loading control for all analyzed proteins. The results of the relative expression (in arbitrary units) of: rhα1,2-FT and rhα-Gal A were shown in panels e and f, respectively. Bar graphs show mean ±SEM of ROD from three separate analyses of three animals for each variant. The red line is taken as the cut-off value 1.0. Statistics: One-way ANOVA and *post hoc* Tukey HSD test, values are denoted as **p<0.01 and ***p<0.001.

Discussion

In this study we investigated the effects of the overexpression of human α1,2-fucosyltransferase and α-galactosidase A on the amount of Galα1→3Gal antigen and hepatic histology in porcine liver from three transgenic lines. As genetically modified pigs were produced to avoid hyperacute rejection (LIPIŃSKI *et al*. 2010; ZEYLAND *et al*. 2013, 2014), we discuss this aspect in relation to a liver model. Our immunofluorescence and lectin blot analyses revealed a significant reduction of Galα1→3Gal expression in the liver tissue in all three transgenic lines, h*FUT2*, h*GLA,* and h*FUT2*×h*GLA*, in comparison to non-transgenic pigs. More importantly, the double transgenic animals showed a significantly lower, but still

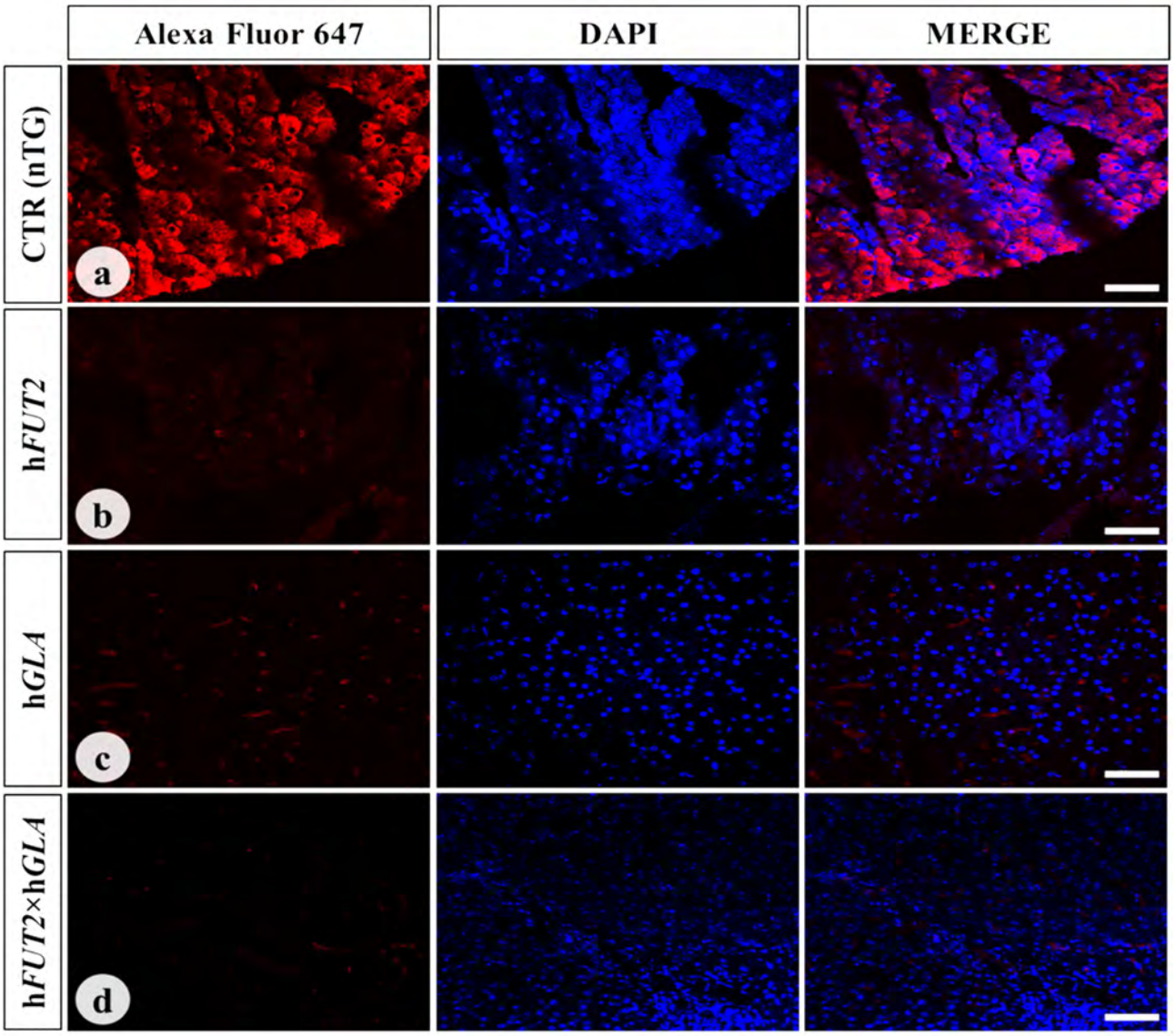

Fig. 6. Lectin histochemistry analysis of the Galα1→3Gal epitope expression in porcine liver cryostat tissue sections. Representative microphotographs of lectin GS-IB_4 labelled sections derived from: non-transgenic control (a), single transgenic (b and c), and double transgenic animals (d). The lectin histochemistry was performed with lectin GS-IB_4 Alexa Fluor 647 conjugated (red) and DAPI counterstained (blue). Scale bars = 50 μm.

detectable, level of the Galα1→3Gal epitope than two other genetically modified pig lines, as was evidenced by lectin blotting. Collectively, our results indicate that the constitutive co-expression of cooperating enzymes such as recombinant human α1,2-fucosyltransferase and α-galactosidase A is more efficient in removing the Galα1→3Gal antigen from porcine cells than the individual function of either h*FUT2* or h*GLA*. However, at present, we cannot exclude the possibility that the more effective removal of the Galα1→3Gal epitope in the double transgenic line is also due to a higher level of h*FUT2* expression when compared to the single transgenic h*FUT2* line (see Fig. 3a). Therefore, quantification of transgene copy number and mRNA level of h*FUT2* and h*GLA* in the liver should be carried out to verify this conclusion. Unfortunately, incomplete removal of the Galα1→3Gal in double transgenic h*FUT2*×h*GLA* pigs indicates that this genetic modification alone is not to be considered for liver xenotransplantation. Hence, our data support emerging evidence that multi-transgenic pig models with a larger number of deleted, humanized, or added genes are required for the effective elimination of major xenoantigen to prolong vascularised xenograft survival (Niemann & Petersen 2016). Undoubtedly, GTKO pigs represent the basis for further opti-

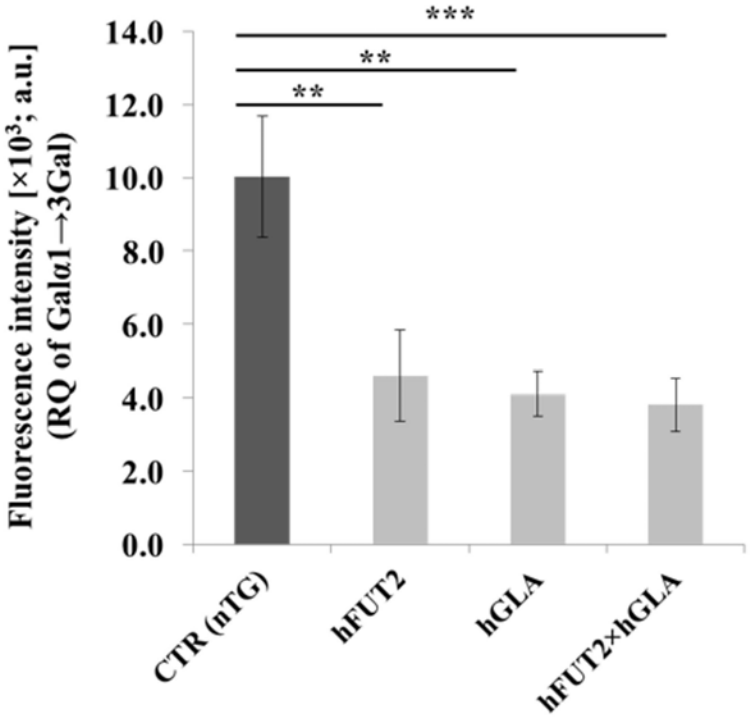

Fig. 7. Semiquantitative analysis of fluorescence intensity of AlexaFluor 647 dye for the detection of Galα1→3Gal epitope expression. For each genetically modified and control animal, three sections were sampled by 70 ROI. Data are presented as mean ± SEM and displayed as arbitrary units in exponential notation (×10^3). Statistics: One-way ANOVA and *post hoc* Tukey HSD test, values are denoted as **p<0.01 and ***p<0.001. A significantly lower relative expression of the Galα1→3Gal epitope in all tested samples compared to the control group (CTR nTG) is noted.

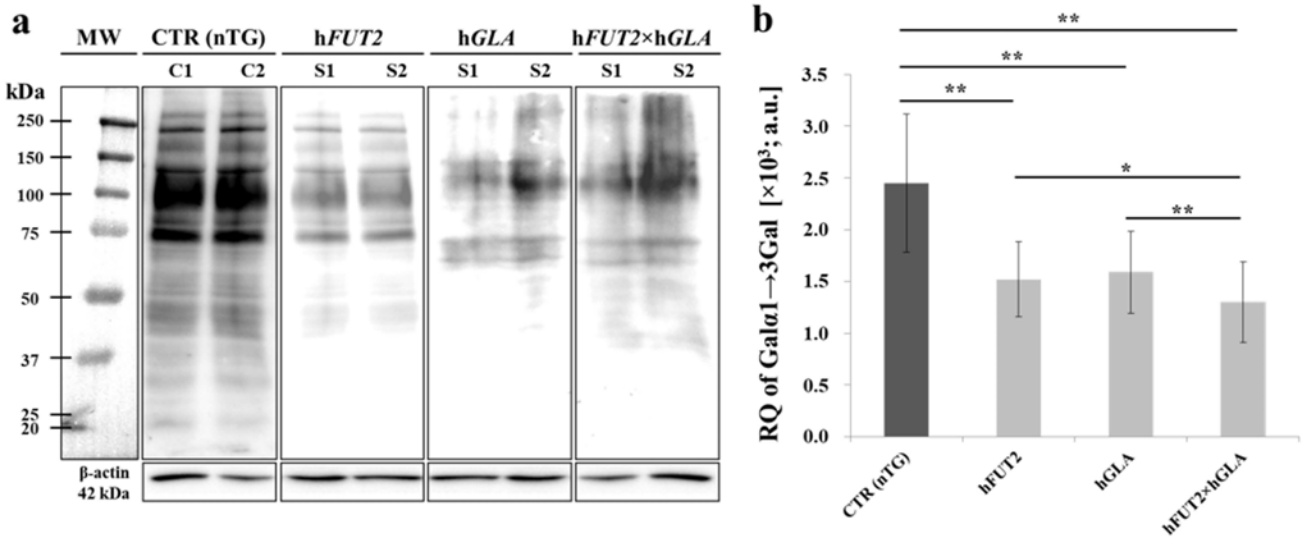

Fig. 8. Eastern blotting (lectin blotting) analysis of Galα1→3Gal epitope expression in transgenic and non-transgenic porcine liver samples at the protein level. Panel a: Representative lectin blots of the expression of the Galα1→3Gal epitope in liver samples derived from: non-transgenic (CTR nTG) control, single- (h*FUT2*, h*GLA*), and double transgenic (h*FUT2*×h*GLA*) pigs. MW indicates the molecular weight of protein standards. C1, C2, S1, S2 – control 1, control 2, sample 1, sample 2, respectively. Each band represents a glycosylated protein containing the Galα1→3Gal epitope. The β-actin served as a loading control for all analyzed samples. Panel b: The quantitative analysis of Galα1→3Gal epitope relative expression, data are presented as mean ± SEM from three separate ROD analyses of three animals for each variant, and displayed as arbitrary units in exponential notation (×10^3). One-way ANOVA and *post hoc* Tukey HSD test, values are denoted as: *p<0.05 and **p<0.01. The relative expression of the Galα1→3Gal epitope was significantly lower in the liver of all transgenic pigs compared to the control (CTR nTG) non-transgenic animals. Within the transgenic animals, the lowest significant expression of the Galα1→3Gal epitope showed liver samples derived from double transgenic (h*FUT2*×h*GLA*) pigs. In other samples (h*FUT2*, h*GLA*) the expression remained at a similar level with no significant differences.

mal genetic modifications. Recently, SHAH *et al*. (2017) established an orthotopic model of GTKO pig-to-primate liver xenotransplantation with continuous human coagulation factor infusion and costimulation blockade, in which baboon recipients survived up to 25 and 29 days while maintaining hepatic functions. And this is the longest survival time of liver xenografts to date. Moreover, two non-alpha Gal epitopes have been identified in pigs, namely *N*-glycolylneuraminic acid (Neu5Gc) and the SDa produced by the porcine β1,4-N-acetylgalactosaminyltransferase-2 (*B4GALNT2*), which may be a barrier to the clinical application of xenotransplantation (BYRNE *et al*. 2018; LADOWSKI *et al.* 2019; LU *et al*. 2020; TECTOR *et al*. 2020). Hence, additional genome editing is crucial to overcome the problems associated with immune rejection.

The single transgenic pigs were obtained by microinjection of linearized gene construct composed of human cytomegalovirus immediate early promoter and human α1,2-fucosyltransferase or human α-galactosidase coding sequences (LIPIŃSKI *et al*. 2010; ZEYLAND *et al*. 2013). In turn, double transgenic pigs h*FUT2*×h*GLA* were generated by interbreeding of appropriate single transgenic individuals (ZEYLAND *et al*. 2014). Using PCR, Southern blot and flow cytometry LIPIŃSKI *et al*. (2010) and ZEYLAND *et al*. (2013, 2014) confirmed successful incorporation of h*FUT2* and h*GLA* gene sequences in the porcine genome in skin fibroblasts derived from single transgenic and double transgenic pigs. Additionally, fluorescent in situ hybridization (FISH) analysis demonstrated that these gene sequences were located in specific regions of the genome, h*FUT2* on chromosome 14q28 and h*GLA* on chromosome 11p12. They also showed that both recombinant human α1,2-fucosyltransferase and α-galactosiadase A may individually reduce the expression of the Galα1→3Gal epitope. Our finding that the single expression of human α1,2-fucosyltransferase may decrease the amount of the Galα1→3Gal epitope in transgenic liver is consistent with earlier studies using different tissues (SANDRIN *et al*. 1995; SHARMA *et al*. 1996; COSTA *et al*. 2008; LIPIŃSKI *et al*. 2010). A previous study by JIA *et al*. (2004) proved that α-galactosidase alone lowered epitope Galα1→3Gal expression by 78%, while the co-expression of α-galactosidase and α1,2-fucosyltransferase reduced this epitope almost to zero on the surface of SV40-immortalised aortic porcine endothelial cells. Another study by ZEYLAND *et al*. (2014) estimated the Galα1→3Gal epitope reduction in porcine ear skin fibroblasts at 60% for α1,2-fucosyltransferase, 58.9 % for α-galactosidase, and 66.9 % for both α1,2-fucosyltransferase and α-galactosidase. Here we demonstrated by lectin histochemistry and lectin blotting that Galα1→3Gal epitope expression in porcine liver was decreased by 54% (histochemistry) and 38% (blotting) in the h*FUT2* pigs, 59% and 35% in the h*GLA* animals, and finally by 62% and 47% in the h*FUT2*×h*GLA* pigs. Therefore, it is clear that the most effective approach to eliminating this epitope is generating homozygous GTKO pigs lacking the gene for α1,3-galactosyltransferase.

We report here the abundant hepatic expression of recombinant human proteins α1,2-fucosyltransferase and α-galactosidase A in transgenic pigs, indicating an efficient and stable expression of human transgenes in porcine livers. Our study also revealed the predominant, perinuclear localization of human α1,2-fucosyltransferase in porcine hepatocytes, as evidenced by the immunofluorescence analysis of porcine livers derived from single h*FUT2* and double h*FUT2*×h*GLA* transgenic animals. Moreover, double immunostaining with antibodies against human α1,2-fucosyltransferase and Golgi 58K protein provide evidence for the colocalization of these two proteins and support the view of α1,2-fucosyltransferase distribution within the Golgi apparatus (MILLAND *et al*. 2001). Indeed, in human cells α1,2-fucosyltransferase is localized within the *cis* compartment of the Golgi complex, while in the porcine cells α1,3-galactosyltransferase is present in the *trans* compartment (HARTEL-SCHENK *et al*. 1991). Hence, distinctive distribution of these two enzymes in the Golgi network, which compete for the same substrate N-acetyllactosamine, allows for the lowering of the Galα1→3Gal epitope in the porcine hepatocytes by rhα1,2-FT.

Considering possible adverse effects of the overexpression of human transgenes on hepatic tissue integrity and cell morphology, we performed a routine histological analysis of the livers from the transgenic pigs. We found no histological evidence of liver tissue abnormalities in the analyzed pigs, which may suggest that these genetic modifications do not have deleterious effects on the functional morphology of porcine hepatic tissue.

In conclusion, we have shown that the overexpression of recombinant human α1,2-fucosyltransferase and α-galactosidase A in single and double transgenic pig models significantly reduces, but does not eliminate the Galα1→3Gal epitope from hepatic tissue. Hence, the efficacy of this approach for liver modification in xenotransplantation-related studies is limited as even hyperacute rejection remains a hurdle.

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Author Contributions

Research concept and design: J.W., J.K., R.S., Z.S., M.R.; Collection and/or assembly of data: J.W., K.W.; Data analysis and interpretation: J.W., J.K., M.R.; Writing the article: J.W., J.K., M.R.; Critical revision of the article: J.W., J.K., R.S., Z.S., J.J., B.G., M.R.; Final approval of article: J.W., J.K., R.S., Z.S., K.W., B.G., J.J., M.R.

Conflict of Interest

The authors declare no conflict of interest.

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