Animal Science Papers and Reports vol. 39 (2021) no. 4, 379-392
Institute of Genetics and Animal Biotechnology
of the Polish Academy of Sciences, Jastrzębiec, Poland

Characteristics of human leukocyte antigen-E expression in transgenic porcine liver*

Jerzy Wiater[1], Janusz Karasiński[2], Ryszard Słomski[3], Zdzisław Smorąg[4], Kamil Wartalski[1], Barbara Gajda[4], Małgorzata Cybulska[5], Jacek Jura[4], Marek Romek[2]**

[1] Department of Histology, Jagiellonian University Medical College, Kopernika 7, 31-034 Krakow, Poland

[2] Department of Cell Biology and Imaging, Institute of Zoology and Biomedical Research, Jagiellonian University in Krakow, Gronostajowa 9, 30-387 Krakow, Poland

[3] Institute of Human Genetics Polish Academy of Sciences, Strzeszynska 32, 60-479 Poznan, Poland

[4] Department of Reproductive Biotechnology and Cryoconservation, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice/Krakow, Poland

[5] Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Jastrzębiec, Postępu 36A, Poland

(Accepted September 9, 2021)

Overexpression of human leukocyte antigen-E (HLA-E) in genetically modified pigs is a potential strategy to reduce immune responses generated by human natural killer (NK) cells after solid organ transplantation. Therefore, evaluating the effects of *in vivo* HLA-E expression is essential to clarify emerging issues of transgene efficiency in pig organs. This study aims to investigate the expression of HLA-E protein in the liver of transgenic pigs with reference to the hepatic lobular architecture and to determine whether the addition of the two protective transgenes (h*FUT2*×h*GLA*)

*This work was supported by The National Centre for Research and Development Poland (Grant No. INNOMED/I/17/NCBR/2014) in the framework of the "INNOMED" programme titled "Development of an innovative technology using transgenic porcine tissues for biomedical purposes". Acronym: "MEDPIG"
**Corresponding authors: marek.romek@uj.edu.pl

in pig cells affects the HLA-E expression pattern in triple transgenic liver. Here, we used confocal immunofluorescence and Western blotting to study the distribution and quantification of HLA-E protein in the liver of transgenic pigs carrying only the *HLA-E* transgene and from triple transgenic pigs (h*FUT2*×h*GLA*×*HLA-E*) designed to express *HLA-E* and human α1,2-fucosyltransferase (h*FUT2*) and α-galactosidase A (h*GLA*). In all transgenic animals the HLA-E protein was evenly distributed in hepatic lobules with no evidence of zonation pattern or gradient formation, and almost absent in interlobular connective tissue septa. Quantification of immunofluorescence and Western blot revealed a significantly higher expression of HLA-E protein in the liver of transgenic pigs compared to non-genetically modified pigs. However, no significant differences were observed in the level of HLA-E protein between the single and triple transgenic animals. This study demonstrates that expression of the *HLA-E* transgene in porcine liver is stable, not affected by h*FUT2*×h*GLA* genetic background and likely independent of mechanisms maintaining hepatic zonation of the liver lobules.

KEY WORDS: transgenic pigs / human leukocyte antigen E / human α-1,2-fucosyltransferase / human α-galactosidase / alpha-gal / liver zonation

Recent advances in xenotransplantation indicate that several genetic modifications must be introduced into pigs to make their organs more compatible with human immune systems and physiology [reviewed in Cooper *et al*. 2019, Ladowski *et al*. 2019, Sykes and Sachs 2019, Lu *et al*. 2020]. The principal modifications include a triple-knockout (TKO) of genes coding for the three known carbohydrate xenoantigens, namely galactose-α1,3-galactose (alpha-Gal), *N*-glycolylneuraminic acid (Neu5Gc), and Sda produced by the enzyme β-1,4-*N*-acetylgalactosaminyltransferase (B4GALNT2). As these TKO pigs constitute the basic genetic background, several transgenic proteins have been studied for optimal beneficial effects in overcoming the immune barriers.

The human leukocyte antigen-E (HLA-E) is classified as a non-classical major histocompatibility complex (MHC) class Ib molecule together with two additional members of this family HLA-F and HLA-G. Non-classical HLA molecules, in contrast to classical Ia antigens HLA-A, -B and -C, are characterized by limited polymorphism as well as by a particular expression pattern and functional profile [Kochan *et al*. 2013, Kraemer *et al*. 2014]. Unlike other class Ib proteins the HLA-E is present in all nucleated cells at low levels, although it is most abundantly expressed on endothelial cells and various types of immune cells [Lee *et al*. 1998, Coupel *et al*. 2007]. It is well known that HLA-E is a major ligand for the natural killer (NK) cells inhibitory receptor CD94/NKG2A [Lee *et al*. 1998, Matsunami *et al*. 2002, Lilienfeld *et al*. 2007] and has been demonstrated to suppress macrophage mediated cytotoxicity [Maeda *et al*. 2013, Esquivel *et al*. 2015]. The unique biological properties of HLA-E in suppressing immune responses have been successfully exploited for the development of various genetically engineered pig models. These animals were used for the study of mechanisms involved in human NK cell - porcine endothelial cell interactions [Weiss *et al*. 2009, Maeda *et al*. 2013, Puga Yung *et al*. 2017, 2018] as well as for the designing molecular strategies to prevent NK cell-dependent rejections of selected organs, such as kidneys [Weiss *et al*. 2009], heart (Abicht *et al*. 2018], lungs (Laird *et al*. 2017, Burdorf *et al*. 2018] and porcine forelimbs [Bongoni *et al*. 2014, Puga

Yung *et al*. 2018]. Collectively, the results from these studies clearly indicate that genetic modification of porcine cells to express HLA-E allows binding of HLA-E to the CD94/NKG2A inhibitory complex on circulating NK cells and results in potent inhibition of NK cell cytotoxicity responsible for endothelial damage. However, there is insufficient evidence to predict what the impact of transgenic HLA-E expression will be in a highly vascular porcine liver, which is also the largest internal organ in the body showing exceptional regenerative capacity. Moreover, liver sinusoidal endothelial cells are implicated in regulation of key metabolic functions [DeLeve 2013].

Pig liver is the optimal model for translational studies due to anatomical and physiological similarities with human liver, and a high degree of genetic similarity between the two species [Eberlova *et al*. 2020]. However, compared to porcine kidney and heart, transplantation of genetically engineered liver in nonhuman primates have proven difficult, mostly due to profound thrombocytopenia and coagulopathy limiting graft survival to almost 30 days [Patel *et al.* 2017, Shah *et al.* 2017, Lamm *et al.* 2022].

The liver tissue architecture is highly complex and conserved among mammals, and displays a remarkable phenomenon known as metabolic zonation [Jungermann and Keitzmann 1996, Gebhardt and Matz Soja 2014, Ben-Moshe and Itzkovitz 2019]. At the microscopic level, the liver consists of hexagonal units called lobules. Hepatic cells are arranged within the lobules in concentric plates along the sinusoidal capillary network extending from the central vein to portal triad. Although morphologically similar, hepatocytes are highly diverse in their metabolic activities resulting in spatial separation of various metabolic pathways along the liver sinusoids. While hepatic cells located in the outer periportal zone express higher levels of enzymes involved in gluconeogenesis and β-oxidation, the centrilobular hepatocytes distributed around the central vein are specialized in glycolysis, lipogenesis, and detoxification. Metabolic zonation of endothelial cells and hepatic stellate cells is another prominent feature of liver biology [Lamers *et al*. 1989, Halpern *et al*. 2017, 2018, Dobie *et al*. 2019].

Here, we ask whether these intricate zonation pattern of hepatic functions has any impact on the HLA-E protein expression in transgenic pig liver. This is important issue when considering that efficient and stable expression of human transgenes is essential to promote long-term protection of vital organs with a low cell turnover, such as the heart, kidney, lung, pancreas, and liver [Hryhorowicz *et al*. 2017, Cooper *et al*. 2019].

Recently, transgenic pigs have been generated and extensively characterised by Hryhorowicz *et al*. [2018] for the purpose of studying the inhibitory effects of HLA-E on the human NK cell-mediated immune responses as a complementary strategy to protect porcine xenograft. This model was created by direct pronuclear microinjection of the DNA construct pHLAE-GFPBsd containing the HLA-E gene into fertilized oocytes. The strong constitutive promoter of the elongation factor 1α (EF-1α) was used to ensure systemic and high-level transgene expression. Moreover, a new pig model (h*FUT2*×h*GLA*×*HLA-E*) that express the *HLA-E* and two other human transgenes α1,2-fucosyltransferase (h*FUT2*) and α-galactosidase A (h*GLA*) associated with glycosylation pathways was also developed [unpublished, Słomski and Smorąg].

The aim of our study was to evaluate the expression of HLA-E protein in the liver of transgenic pigs by immunofluorescence and Western blotting approaches with reference to lobular structure. We also aimed to investigate whether the addition of the two protective transgenes h*FUT2*×h*GLA* in pig cells affects the HLA-E expression pattern in triple transgenic liver.

Material 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 Krakow, Poland. Permission 1181/2015 from 21 May 2015. Pigs were maintained under conventional conditions in the pigsty of the Department of Biotechnology of Reproduction and Cryopreservation, National Research Institute of Animal Production at Balice near Krakow. The veterinary care was provided by the institution. All transgenic pigs were generated for the purposes of xenotransplantation related studies. A detailed molecular, cytogenetic, and functional characteristic of single transgenic pigs with HLA-E expression has been previously reported in Hryhorowicz *et al*. [2018]. Fresh liver samples were collected from the single transgenic pigs (n=3) designed to express the human leukocyte antigen-E (*HLA-E*) and from recently generated triple transgenic pigs (n=3) expressing human α1,2-fucosyltransferase (h*FUT2*), α-galactosidase (h*GLA*) and human leukocyte antigen E (h*FUT2*×h*GLA*×*HLA-E*). Liver samples obtained from non-genetically modified pigs (Polish Large White) (n=2) served as a control group (CTR nTG). Liver tissue samples were obtained from 12- to 18-month-old pigs weighing 150-200 kg after slaughter of the animal and immediately frozen in liquid nitrogen for further experimental methods.

Immunofluorescence staining

Porcine liver samples were cryosectioned at 6 µm and collected onto poly-L-lysine coated microscopic slides. Sections were fixed with 4% paraformaldehyde 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 overnight at +4°C with the following primary antibodies: mouse monoclonal antibody against human leukocyte antigen E (ab11820, Abcam) diluted 1:200 in PBST, rabbit polyclonal antibody recognising human α1,2-fucosyltransferase (ab198712, Abcam) diluted 1:150, and rabbit polyclonal antibody against human α-galactosidase (PA5-27349, ThermoFisher Scientific, Waltham, MA, USA) diluted 1:200. The next day sections were washed several times in PBST and treated with appropriate secondary antibody Goat anti-Mouse or Goat anti-Rabbit labelled with Cy3 (Jackson Immuno Research) diluted 1:600 in PBST and incubated 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) and images were acquired using a 543 nm HeNe laser line at a resolution of 1024 × 1024 pixels. The fluorescence signal was detected from 583 to 593 nm. The relative fluorescence intensity was 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 as previously described [Romek *et al*. 2017]. For each genetically modified and control pig three sections were sampled by 70 ROI.

Western blot analysis

Total protein was extracted from frozen liver tissue samples by using 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 pieces of approximately 2 mm^3 and homogenized in 300 µL RIPA+PI. Tissue lysates were centrifuged at 14 000 × g for 15 min at +4°C and supernatant 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 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 by using 5% stacking and 10% resolving gels. Molecular weights were estimated with reference to standard proteins (Precision Plus Dual Color Protein Standards, Bio-Rad Laboratories, Hercules, CA, USA). For immunoblotting proteins were transferred onto PVDF membrane (Immobilon, Merck) at 250 mA for 120 min. 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 primary antibodies (the same as those used for immunofluorescence labelling) against HLA-E diluted 1:1000 in TBST, human α1,2-fucosyltransferase at dilution 1:1000 or human α-galactosidase diluted 1:1000. The β-actin was used as a reference protein (Mouse monoclonal anti-β-actin antibody diluted 1:2000, ab8224, Abcam). Then membranes were washed in TBST and incubated with appropriate secondary antibodies horseradish peroxidase (HRP) conjugated Goat anti-Mouse or Goat anti-Rabbit (ThermoFisher Scientific, Waltham, MA, USA) at dilution 1:6000 in TBST for 1 h at room temperature. Finally, membranes were washed in TBS. 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 the Image Lab™ 2.0 Software (Bio-Rad Laboratories, Hercules, CA, USA) by measurement of their relative optical densities.

Statistical analysis

Quantitative data were expressed as the mean ± standard error of the mean and examined using the Shapiro–Wilks *W* test for normality. Comparisons between the appropriate means were performed by one-way analysis of variance (ANOVA), followed by Tukey HSD *post hoc* test. Statistical significance was marked accordingly: **p<0.01, ***p<0.001.

Results and discussion

In this study we applied immunofluorescence staining and confocal microscopy to assess the spatial pattern of human leukocyte antigen E expression in the liver of single *HLA-E* transgenic and triple h*FUT2*×h*GLA*×*HLA-E* transgenic pigs. In the liver tissue of control non-transgenic pigs (CTR nTG) we observed weak, barely detectable

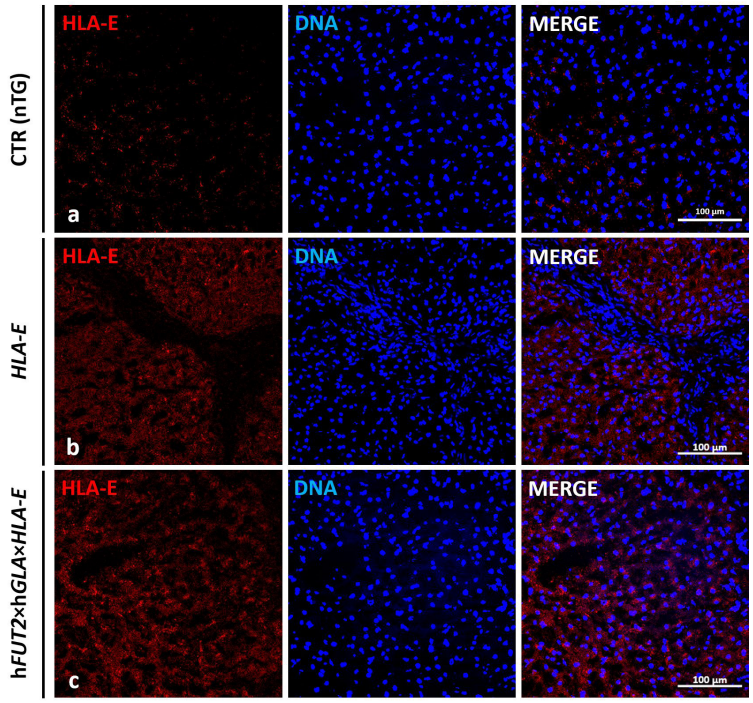

Fig. 1. Immunofluorescence labelling of HLA-E protein in liver tissue sections obtained from (a) control non-transgenic pigs CTR (nTG) and from (b) single HLA-E transgenic and (c) triple h*FUT2*×h*GLA*×*HLA-E* transgenic pigs. Immunofluorescent staining with Cy3 labelled secondary antibodies (red) and DAPI counterstaining (blue). Scale bars represent 100 µm. The immunofluorescence signal of HLA-E was detected in the lobules as a punctuate pattern distributed homogeneously along the porto-central axis and was very weak within interlobular septa. Analysis showed barely detectable positive background signal from HLA-E in the control group CTR (nTG) (a).

positive signal from the HLA-E immunostaining (Fig. 1a). In contrast, the HLA-E was brightly expressed in the liver tissue of both single and triple transgenic pigs and was evenly distributed between the central vein and portal triads in hepatic lobules and almost absent in interlobular connective tissue septa (Fig. 1b and c). Importantly, the HLA-E protein was present homogeneously throughout the liver lobules with no evidence of zonation pattern or gradient formation, indicating that this transgene expression is not influenced by factors regulating hepatic zonation. Quantification of fluorescence intensity showed significantly higher ($p<0.01$) expression of HLA-E protein in both single transgenic and triple transgenic porcine liver in comparison to control non-transgenic pigs, while there was no difference in HLA-E expression between the single transgenic and triple transgenic pigs (Fig. 2). Western blot analysis revealed the presence of HLA-E protein with an estimated molecular weight of about 43 kDa in the liver of all transgenic pigs (Fig. 3A). In a control group we observed a positive signal for HLA-E, well above the background. Quantification of Western blots showed that the relative expression of HLA-E protein was significantly higher ($p<0.01$) in transgenic variants than that in the control non-transgenic group (Fig. 3B). No significant difference in HLA-E expression was detected between the single transgenic and triple transgenic pigs.

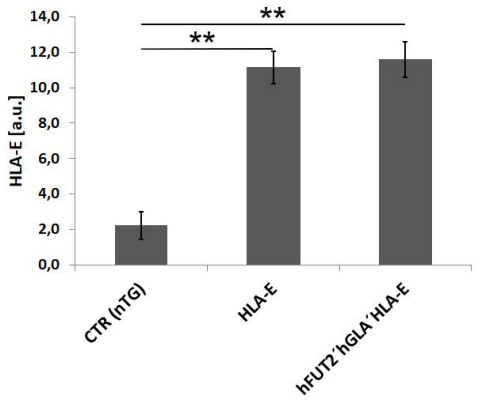

Fig. 2. Semi-quantitative analysis of immunofluorescence staining of human leukocyte antigen E (HLA-E) in the liver cryostat sections from the control non-transgenic pigs CTR (nTG) and from single HLA-E transgenic and triple h*FUT2*×h*GLA*×*HLA-E* transgenic pigs. Bar graphs show mean ±SEM. p-values are denoted as **$p<0.01$.

The liver tissue is highly regenerative and is also known for its structural stability with remarkable low cellular turnover. Under normal, homeostatic conditions less than 1-2% of rodents hepatocytes are cycling, with the remainder persisting for weeks to months in a quiescent G0 state [Stranger 2015, MacDonald 1961]. Hence, in view of our results, we hypothesise that the uniform, non-zonal distribution of HLA-E would be beneficial for immune protection of lobular structures when considering its potential translational value in suppressing the primate cellular response to porcine antigens.

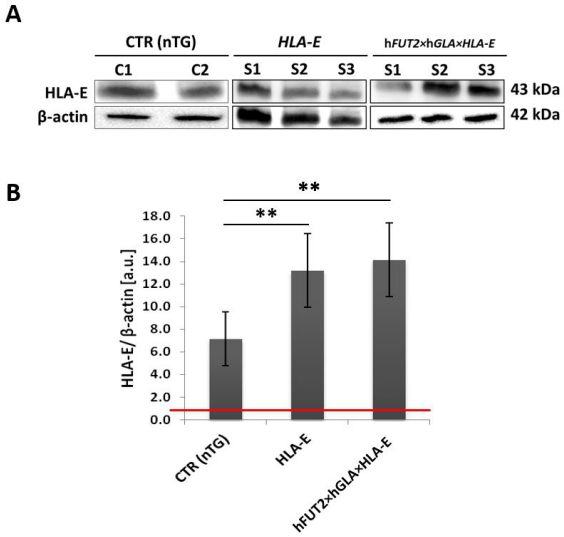

Fig. 3. Western blot analysis of the relative expression of human leukocyte antigen E (HLA-E) proteins in single HLA-E transgenic, triple h*FUT2*×h*GLA*×*HLA-E* transgenic and non-transgenic porcine liver. (A) Representative blots of the expression of HLA-E proteins in the liver samples derived from single and triple transgenic as well as non-transgenic, control pigs (CTR nTG). The β-actin was a loading control for all analysed proteins. (B) The results of relative expression in arbitrary units of HLA-E. Bar graph show mean ±SEM. The red line is taken as the cut-off value 1.0. p-values are denoted as: **p<0.01.

Recent reports have provided evidence that in normal human liver HLA-E protein was expressed in hepatocytes and Kupffer cells and it was significantly upregulated in patients with chronic hepatitis C [Araújo *et al*. 2018, 2021]. In a comparative study Coupel *et al*. [2007] investigated the HLA-E protein expression in normal human organs such as kidney, skin, liver, salivary gland, urinary bladder, thyroid, stomach, and endometrium. These authors have shown that HLA-E immunostaining was mainly restricted to endothelial cells and was consistently observed on endothelial cells from all types of vessels, including arteries, veins, capillaries, and lymphatics. They also reported that HLA-E was basally expressed by B and T lymphocytes, natural killer (NK) cells and by macrophages. In human liver, NK cells are the major lymphocyte population and make up to 50% of hepatic lymphocytes [Fahrner *et al*. 2016, Yamagiwa *et al*. 2009]. In another study, Weiss *et al*. [2009] generated transgenic pigs expressing HLA-E with an HLA-B7 signal sequence and human β2-microglobulin (huβ2m) and revealed by immunohistochemistry the strong HLA-E protein expression in endothelia of all investigated organs, that is, heart, kidney, pancreas, lung, liver, and brain of transgenic pigs. Together, all these results indicate that transgenic HLA-E expression in porcine liver and other vascular organs is highly consistent with the endogenous HLA-E gene expression pattern found in human organs. It follows, therefore, that this transgene expression in endothelial cells is inherently stable *in vivo*.

The functional significance of stable HLA-E expression in the vascular endothelial cell compartment has already been explored in genetically modified pig organs perfused *ex vivo* with human blood [Bongoni *et al*. 2014, Laird *et al*. 2017, Abicht *et al*. 2018, Puga Yung *et al*. 2018]. In general, the transgenic HLA-E expression on the background of α1,3-Galactosyltransferase knockout (GalTKO) and human membrane cofactor protein (hCD46) was associated with decreased NK-mediated cytotoxicity supporting the xenoprotective value of HLA-E. The endothelial cells derived from HLA-E transgenic pigs, which were used in the present study, were previously tested in NK cell cytotoxicity assays, and showed greater viability *in vitro* in comparison to the wild-type controls [Hryhorowicz *et al*. 2018].

In this study we have also investigated the hepatic HLA-E protein expression in recently developed [unpublished, Słomski and Smorąg] triple transgenic pigs (h*FUT2*×h*GLA*×*HLA-E*) expressing *HLA-E* and human α1,2-fucosyltransferase (h*FUT2*) and α-galactosidase (h*GLA*). These multi-transgenic pigs were chosen due to the following reasons: (i) cooperative activity of h*FUT2* and h*GLA* in the Golgi apparatus can ultimately modify the cell surface glycosylation by removing alpha-gal carbohydrate, although, as we have shown recently, the simultaneous expression of these two transgenes improves the removal of the Galα1→3Gal epitope in porcine liver, it is not sufficient enough for complete elimination of this major xenoantigen from liver tissue [Wiater *et al.* 2020]; (ii) the minimally polymorphic HLA-E is a highly active molecule expressed on the surface of endothelial cells

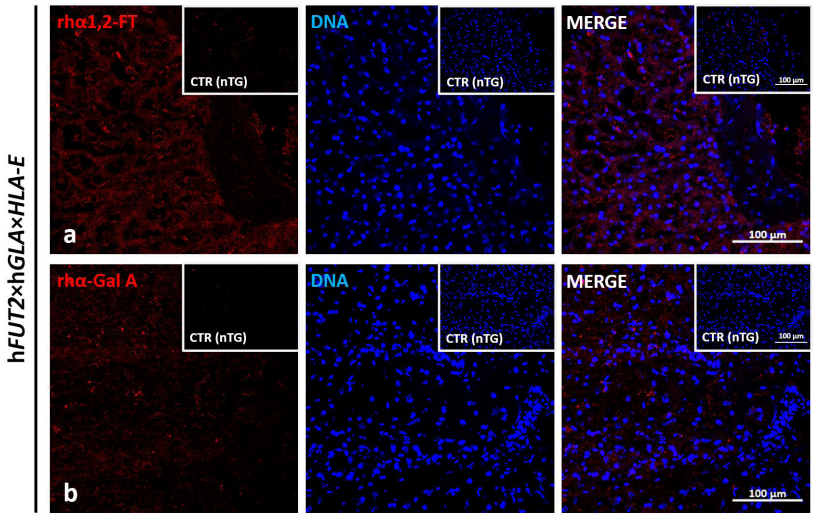

Fig. 4. Immunofluorescence labelling of human α1,2-fucosyltransferase (a) and α-galactosidase A (b) in cryostat tissue sections obtained from triple hFUT2×hGLA×HLA-E transgenic porcine livers as well as from non-transgenic, control pigs CTR (nTG) (inserts). Immunofluorescent staining with Cy3 labelled secondary antibodies (red) and DAPI counterstaining (blue). Scale bars represent 100 µm. Analysis showed barely detectable positive background signal from α1,2-fucosyltransferase in the control group (CTR nTG).

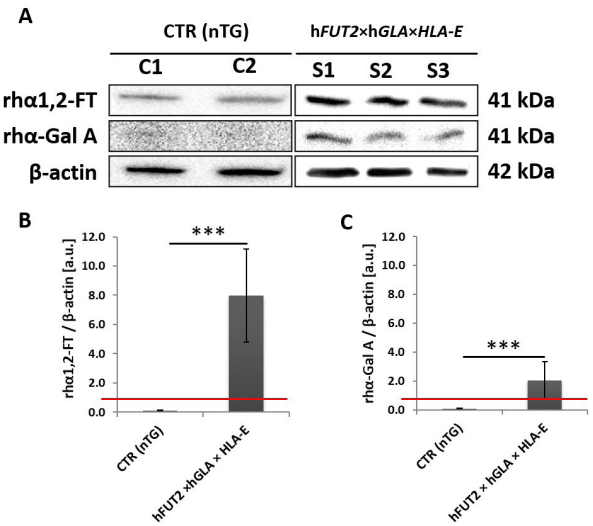

Fig. 5. Western blot analysis of the relative expression of recombinant human α1,2-fucosyltransferase and α-galactosidase A proteins in triple hFUT2×hGLA×HLA-E transgenic and non-transgenic porcine liver. (A) Representative blots of the expression of rhα1,2-FT and rhα-Gal A proteins in the liver samples derived from triple transgenic as well as non-transgenic, control pigs (CTR nTG). The β-actin was a loading control for all analysed proteins. (B and C) Bar graph show mean ±SEM. The red line is taken as the cut-off value 1.0. p-values are denoted as: ***p<0.001.

and other cell types. To confirm the functional expression of h*FUT2* and h*GLA* transgenes in the liver of triple transgenic pigs we examined the expression of human α1,2-fucosyltransferase and α-galactosidase A proteins by immunofluorescence (Fig. 4) and Western blotting (Fig. 5). The immunofluorescence signal from α1,2-fucosyltransferase was almost homogeneously distributed in hepatic lobules (Fig. 4a) while that of α-galactosidase A was less homogenous and detected in small clusters of hepatocytes and in the interlobular space (Fig. 4b). In the control non-transgenic liver, the α1,2-fucosyltransferase and α-galactosidase A were barely detectable by immunofluorescence (Fig. 4 inserts). Our Western blot analysis also revealed the presence of both human α1,2-fucosyltransferase and α-galactosidase A proteins with molecular weight of approximately 41 kDa in triple transgenic pig liver (Fig. 5A). The relative expression of these proteins was significantly higher ($p<0.001$) in transgenic variants than that in the control non-transgenic group (Fig. 5B and C). Beta-actin used as a loading control was detected at 42 kDa. Hence in this study, our immunofluorescence and Western blot data showed that *hFUT2×hGLA* genetic background had no effects on the expression of HLA-E protein in hepatic tissue.

The liver functions performed in homeostatic conditions are intimately related with zonal activity of hepatic genes, as most of them exhibit spatially graded expression patterns [Jungermann and Keitzmann 1996, Gebhardt and Matz-Soja 2014, Ben-Moshe

and Itzkovitz 2019]. These are primary regulated by the canonical Wnt pathway and unidirectional blood flow from the portal area to central vein, which creates several gradients of oxygen, hormones, nutrients, and metabolites along the porto-central axis of liver lobule. In addition, Cheng *et al*. [2018] generated glucagon deficient (*Gcg*$^{-/-}$) mice and demonstrated that glucagon also contributes to liver metabolic zonation through a counterplay with the Wnt/β-catenin signalling pathway. Interestingly, the expression of glucagon receptor (*Gcgr*) and insulin receptor (*Insr*) did not reveal zonation pattern but was evenly distributed throughout the liver lobules in both *Gcg*$^{-/-}$ and WT mice [Cheng *et al*. 2018]. Recently, Halpern *et al*. [2017] measured the entire transcriptome of mouse liver cells by employing single-cell RNA-sequencing and single molecule fluorescence *in situ* hybridization and found that around 50% of liver genes are significantly zonated. Their analysis also revealed that about two thirds of these zonated genes were not predicted targets of either Wnt, hypoxia, Ras signalling or pituitary hormones. Another recent study reported the presence of non-metabolic zonation of tight junction protein claudin-2 and cell adhesion protein E-cadherin in the liver of transgenic mouse model *Cldn2-EGFP* and demonstrated that endothelial Wnt signalling is required for this phenomenon [Ma *et al.* 2020]. However, to our knowledge no data is available regarding the physiological regulation of the *HLA-E* gene expression in human liver in the context of liver zonation. In the present study, we used immunofluorescence staining to show the localization of HLA-E protein in the liver of single transgenic and triple transgenic pigs. Since we did not detect any signs of zonal distribution of the HLA-E protein in liver lobules we would suggest that transgenic *HLA-E* expression is likely independent of signalling pathways involved in metabolic and non-metabolic liver zonation.

In conclusion, our results show the uniform distribution of HLA-E protein in hepatic lobules of transgenic porcine liver and suggest that HLA-E expression is apparently stable in different genetic backgrounds and regulated independently of mechanisms responsible for liver zonation. Further research of HLA-E and other human transgenic proteins expressed in porcine liver should consider in more detail the aspect of liver zonation as this feature is fundamental for liver biology.

REFERENCES

1. ABICHT J.-M., SFRISO R., REICHART B., LÄNGIN M., GAHLE K., PUGA YUNG G.L., SEEBACH J.D. RIEBEN R., AYARES D., WOLF E., KLYMIUK N., BAEHR A., KIND A., MAYR T., BAUER A., 2018 – Multiple genetically modified GTKO/hCD46/HLA-E/hβ2-mg porcine hearts are protected from complement activation and natural killer cell infiltration during ex vivo perfusion with human blood. ***Xenotransplantation*** 25, e12390.
2. ARAÚJO R.C., BERTOL B.C., CÉSAR DIAS F., DEBORTOLI G., ALMEIDA P.H., FERNANDES SOUZA F., VILLANOVA M.G., ZAMBELLI RAMALHO L.N., CANDOLO MARTINELLI A.L., DA CRUZ CASTELLI É., TEIXEIRA MENDES JUNIOR C., DONADI E.A., 2021 – HLA-E gene polymorphisms in chronic hepatitis C: Impact on HLA-E liver expression and disease severity. ***Human Immunology*** 82, 177-185.

3. ARAÚJO R.C., DIAS F.C., BERTOL B.C., SILVA D.M., ALMEIDA P.H., TEIXEIRA A.C., SOUZA F.F., VILLANOVA M.G., RAMALHO L.N.Z., DONADI E.A., MARTINELLI A.L.C., 2018 – Liver HLA-E Expression Is Associated with Severity of Liver Disease in Chronic Hepatitis C. ***Journal of Immunology Research*** 2018, 2563563.
4. BEN-MOSHE S., ITZKOVITZ S., 2019 – Spatial heterogeneity in the mammalian liver. ***Nature Reviews. Gastroenterology & Hepatology*** 16, 395–410.
5. BONGONI A.K., KIERMEIR D., JENNI H., BÄHR A., AYARES D., KLYMIUK N., WOLF E., VOEGELIN E., CONSTANTINESCU M.A, SEEBACH J.D, RIEBEN R., 2014 – Complement dependent early immunological responses during ex vivo xenoperfusion of hCD46/HLA-E double transgenic pig forelimbs with human blood. ***Xenotransplantation*** 21, 230-243.
6. BURDORF L., AZIMZADEH A.M., PIERSON R.N 3RD., 2018 – Progress and challenges in lung xenotransplantation: an update. ***Current Opinion in Organ Transplantation*** 23, 621-627.
7. CHENG X., KIM S.Y., OKAMOTO H., XIN Y., YANCOPOULOS G.D., MURPHY A.J., GROMADA J., 2018 – Glucagon contributes to liver zonation. ***Proceedings of the National Academy of Sciences of the United States of America*** 115, E4111-E4119.
8. COOPER D.K.C., HARA H., IWASE H., YAMAMOTO T., LI Q., EZZELARAB M., FEDERZONI E., DANDRO A., AYARES D., 2019 – Justification of specific genetic modifications in pigs for clinical organ xenotransplantation. ***Xenotransplantation*** 26, e12516.
9. COUPEL S., MOREAU A., HAMIDOU M., HOREJSI V., SOULILLOU J.P., CHARREAU B., 2007 – Expression and release of soluble HLA-E is an immunoregulatory feature of endothelial cell activation. ***Blood*** 109, 2806-2814.
10. DELEVE L.D., 2013 – Liver sinusoidal endothelial cells and liver regeneration. ***The Journal of Clinical Investigation*** 123, 1861-1866.
11. DOBIE R., WILSON-KANAMORI J.R., HENDERSON B.E.P., SMITH J.R., MATCHETT K.P., PORTMAN J.R., WALLENBORG K, PICELLI S., ZAGORSKA A., PENDEM S.V., HUDSON T.E., WU M.M., BUDAS G.R., BRECKENRIDGE D.G., HARRISON E.M., MOLE D.J., WIGMORE S.J., RAMACHANDRAN P., PONTING C.P., TEICHMANN S.A., MARIONI J.C., HENDERSON N.C., 2019 – Single-Cell Transcriptomics Uncovers Zonation of Function in the Mesenchyme during Liver Fibrosis. ***Cell Reports*** 29, 1832-1847.
12. EBERLOVA L., MALECKOVA A., MIK P., TONAR Z., JIRIK M., MIRKA H., PALEK R., LEUPEN S., LISKA V., 2020 – Porcine Liver Anatomy Applied to Biomedicine. ***The Journal of Surgical Research*** 250, 70-79.
13. ESQUIVEL E.L., MAEDA A., EGUCHI H., ASADA M., SUGIYAMA M., MANABE C., SAKAI R., MATSUURA R., NAKAHATA K., OKUYAMA H., MIYAGAWA S., 2015 – Suppression of human macrophage-mediated cytotoxicity by transgenic swine endothelial cell expression of HLA-G. ***Transplant Immunology*** 32, 109-115.
14. FAHRNER R., DONDORF F., ARDELT M., SETTMACHER U., RAUCHFUSS F., 2016 – Role of NK, NKT cells and macrophages in liver transplantation. ***World Journal of Gastroenterology*** 22, 6135-6144.
15. GEBHARDT R., MATZ SOJA M., 2014 – Liver zonation: novel aspects of its regulation and its impact on homeostasis. ***World Journal of Gastroenterology*** 20, 8491-8504.
16. HALPERN K.B., SHENHAV R., MASSALHA H., TOTH B., EGOZI A., MASSASA E.E., MEDGALIA C., DAVID E., GILADI A., MOOR A.E., PORAT Z., AMIT I., ITZKOVITZ S., 2018 – Paired-cell sequencing enables spatial gene expression mapping of liver endothelial cells. ***Nature Biotechnology*** 36, 962-970.
17. HALPERN K.B., SHENHAV R., MATCOVITCH-NATAN O., TOTH B., LEMZE D., GOLAN M., MASSASA E.E., BAYDATCH S., LANDEN S., MOOR A.E., BRANDIS A., GILADI A., AVIHAIL A.S., DAVID E., AMIT I., ITZKOVITZ S., 2017 – Single-cell spatial reconstruction reveals global division of labour in the mammalian liver. ***Nature*** 542, 352-356.

18. HRYHOROWICZ M., ZEYLAND J., SŁOMSKI R., LIPIŃSKI D., 2017 – Genetically Modified Pigs as Organ Donors for Xenotransplantation. ***Molecular Biotechnology*** 59, 435-444.
19. HRYHOROWICZ M., ZEYLAND J., NOWAK-TERPIŁOWSKA A., JURA J., JUZWA W., SŁOMSKI R., BOCIANOWSKI J., SMORĄG Z., WOŹNIAK A., LIPIŃSKI D., 2018 – Characterization of three generations of transgenic pigs expressing the HLA-E gene. ***Annals of Animal Science*** 18, 919-935.
20. JUNGERMANN K., KEITZMANN T., 1996 – Zonation of Parenchymal and Nonparenchymal Metabolism in Liver. ***Annual Review of Nutrition*** 16, 179-203.
21. KOCHAN G., ESCORS D., BRECKPOT K., GUERRERO-SETAS D., 2013 – Role of non-classical MHC class I molecules in cancer immunosuppression. ***OncoImmunology*** 2, e26491.
22. KRAEMER T., BLASCZYK R., BADE-DOEDING C., 2014 – HLA-E: a novel player for histocompatibility. ***Journal of Immunology Research*** 2014, 352160.
23. LADOWSKI J., MARTENS G., ESTRADA J., TECTOR M., TECTOR J., 2019 – The desirable donor pig to eliminate all xenoreactive antigens. ***Xenotransplantation*** 26, e12504.
24. LAIRD C.T., BURDORF L., FRENCH B.M., KUBICKI N., CHENG X., BRAILEANU G., SUN W., O'NEILL N.A., CIMENO A., PARSELL D., SO E., BÄHR A., KLYMIUK N., PHELPS C.J., AYARES D., AZIMZADEH A.M., PIERSON R.N., 2017 – Transgenic expression of human leukocyte antigen-E attenuates GalKO.hCD46 porcine lung xenograft injury. ***Xenotransplantation*** 24, e12294.
25. LAMERS W.H., HILBERTS A, FURT E., SMITH J., JONGES G.N., VAN NOORDEN C.J.F, JANZEN J.W.G., CHARLES R., MOORMAN A.F.M., 1989 – Hepatic enzymic zonation: a reevaluation of the concept of the liver acinus. ***Hepatology*** 10, 72-76.
26. LAMM V., EKSER B., VAGEFI P.A., COOPER D.K.C., 2022 – Bridging to Allotransplantation - Is Pig Liver Xenotransplantation the Best Option? ***Transplantation*** 106, 26-36.
27. LEE N., LLANO M., CARRETERO M., ISHITANI A., NAVARRO F., LÓPEZ-BOTET M., GERAGHTY D.E., 1998 – HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. ***Proceedings of the National Academy of Sciences of the United States of America*** 95, 5199-5204.
28. LILIENFELD B.G., CREW M.D., FORTE P., BAUMANN B.C., SEEBACH J.D., 2007 – Transgenic expression of HLA-E single chain trimer protects porcine endothelial cells against human natural killer cell-mediated cytotoxicity. ***Xenotransplantation*** 14, 126-134.
29. LU T., YANG B., WANG R., QIN C., 2020 – Xenotransplantation: Current Status in Preclinical Research. ***Frontiers in Immunology*** 10, 3060.
30. MA R., MARTÍNEZ-RAMÍREZ A.S., BORDERS T.L., GAO F., SOSA-PINEDA B., 2020 – Metabolic and non-metabolic liver zonation is established non-synchronously and requires sinusoidal Wnts. ***Elife*** 9, e46206.
31. MACDONALD RA., 1961 – "Lifespan" of liver cells. Autoradio-graphic study using tritiated thymidine in normal, cirrhotic, and partially hepatectomized rats. ***Archives of Internal Medicine*** 107, 335-343.
32. MAEDA A., KAWAMURA T., UENO T., USUI N., EGUCHI H., MIYAGAWA S., 2013 – The suppression of inflammatory macrophage-mediated cytotoxicity and proinflammatory cytokine production by transgenic expression of HLA-E. ***Transplant Immunology*** 29, 76-81.
33. MATSUNAMI K., MIYAGAWA S., NAKAI R., YAMADA M., SHIRAKURA R., 2002 – Modulation of the leader peptide sequence of the HLA-E gene up-regulates its expression and downregulates natural killer cell-mediated swine endothelial cell lysis. ***Transplantation*** 73, 1582-1589.
34. PATEL S.M., LOURAS N., VAGEFI P.A., 2017 – Liver xenotransplantation. ***Current Opinion in Organ Transplantation*** 22, 535-540.

35. PUGA YUNG G., BONGONI A.K., PRADIER A., MADELON N., PAPASERAFEIM M., SFRISO R., AYARES D. L. WOLF E., KLYMIUK N., BÄHR A., CONSTANTINESCU M.A., VOEGELIN E., KIERMEIR D., JENNI H., RIEBEN R., SEEBACH J.D., 2018 – Release of pig leukocytes and reduced human NK cell recruitment during ex vivo perfusion of HLA-E/human CD46 double transgenic pig limbs with human blood. ***Xenotransplantation*** 25, e12357.
36. PUGA YUNG G., SCHNEIDER M.K.J., SEEBACH J.D., 2017 – The role of NK cells in pig-to-human xenotransplantation. ***Journal of Immunology Research*** 2017, 4627384.
37. ROMEK M., GAJDA B., KRZYSZTOFOWICZ E., KUCIA M., UZAROWSKA A., SMORAG Z., 2017 – Improved quality of porcine embryos cultured with hyaluronan due to the modification of the mitochondrial membrane potential and reactive oxygen species level. ***Theriogenology*** 102, 1-9.
38. SHAH J.A., PATEL M.S., ELIAS N., NAVARRO-ALVAREZ N., ROSALES I., WILKINSON R.A., LOURAS N.J., HERTL M., FISHMAN J.A., COLVIN R.B., COSIMI A.B., MARKMANN J.F., SACHS D.H., VAGEFI P.A., 2017 – Prolonged survival following pig-to-primate liver xenotransplantation utilizing exogenous coagulation factors and costimulation blockade. ***American Journal of Transplantation*** 17, 2178-2185.
39. STANGER B.Z., 2015 – Cellular Homeostasis and Repair in the Mammalian Liver. ***Annual Review of Physiology*** 77, 179-200.
40. SYKES M., SACHS D.H., 2019 – Transplanting organs from pigs to humans. ***Science Immunology*** 4, eaau6298.
41. WEISS E.H., LILIENFELD B.G., MÜLLER S., MÜLLER E., HERBACH N., KESSLER B., WANKE R., SCHWINZER R., SEEBACH J.D, WOLF E., BREM G., 2009 – HLA-E/human β2-microglobulin transgenic pigs: protection against xenogeneic human anti-pig natural killer cell cytotoxicity. ***Transplantation*** 87, 35-43.
42. WIATER J., KARASINSKI J., SLOMSKI R., SMORAG Z., WARTALSKI K., GAJDA B., JURA J., ROMEK M., 2020 – 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. ***Folia Biologica (Krakow)*** 68, 121-133.
43. YAMAGIWA S., KAMIMURA H., ICHIDA T., 2009 – Natural killer cell receptors and their ligands in liver diseases. ***Medical Molecular Morphology*** 42, 1-8.