Xenotransplantation; characterization of transgenic porcine endothelial cells and in vitro assessment of the immunological effect of the multigene modification in a 3D-microfluidic system

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Abstract

The pig is the most suitable animal as organ donor to overcome the deficit in the need of human donor organs. With genetic engineering projects pig donor organs are genetically modified to bypass the great interspecies immunological barriers that lead to xenograft rejection. The aim of this diploma thesis is to characterize a specific genetic modification on porcine endothelial cells (pEC) with two porcine knock-out genes (GGTA1, CMAH) and five human transgenes (CD46, CD55, CD59, A20, HO1) and to evaluate their immunological effect in a 3D-microfluidic system. The characterization was made with immunofluorescence (IF) staining on chamber slides and compared to wildtype (wt) EC. Wt and transgenic (tg) pEC were cultured in 3D-microfluidic channels and perfused with medium for 72 hours followed by a 2 hours perfusion with or without normal human serum (NHS). The results show that all the genetic modifications are successfully deleted or implemented. Additionally, significant reduction of Complement 3b/c (C3c) and IgG-deposition in 2xKO 5xtg PVEC and PAEC is shown.

Introduction

Xenotransplantation is a future alternative to compensate the insufficient availability of human donor organs. The vascular endothelium is the first layer to be in contact with the recipient's blood. However, human already produce preformed anti-pig-antibodies against three pig carbohydrates (α-Gal, Neu5Gc, β4Gal) on the surface of pEC which lead to complement/coagulation activation and graft dysfunction [1]. In addition, molecular incompatibilities between human and porcine complement and coagulation regulatory genes also lead to xenograft rejection. Porcine donors can be genetically modified to delete xenoantigens and to express human transgenes to minimize the risk of a xenograft rejection based on human immune response [2].

Aims and leading questions

Aim 1

The characterization of the porcine EC to evaluate the expression of five human genes (CD46, CD55, CD59, A20, HO1) and the deletion of porcine GGTA1 and CMAH comparing two different cell types (arterial and venous).

- Do genetically modified pEC express these five human genes and are GGTA1 and CMAH genes successfully deleted on the EC surface on a protein level?
- Are there any differences in the tg expression level between artery and vena pEC?

Aim 2

The evaluation of different immune responses to the EC with or without human serum perfusion in a 3D-microfluidic system including the influence of genetic modifications of the porcine EC to the immunological effects.

- Do genetic modifications influence the human innate immune system responses such as complement activation to the transgenic pEC when perfused with human serum?
- How strong is the influence of the modifications of the EC to the human immune responses, in comparison to wt pEC?

Material and methods

EC isolated from death pig's vessels (1xwt and 1xtg) were expanded and arterial and venous 2xKO 5xtg and wt pEC were cultured in chamber slides for characterizing the genetic modifications on the protein level (Fig. 1). Arterial pEC (PAEC) and venous pEC (PVEC) were cultured in an 3D-microfluidic system and perfused with medium for 72h under a pulsatile flow . After human serum perfusion C3c and IgG-deposition were compared between wt and tg pEC using IF staining.

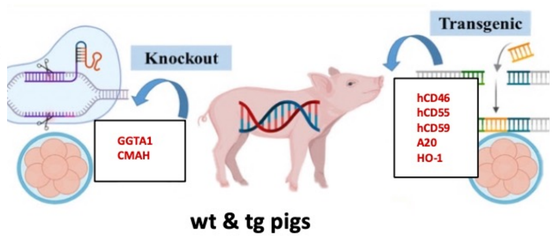

Figure 1: Genetic engineering of pigs for xenotransplantation (Lei, 2022, p.4) - adapted

Characterization of PAEC and PVEC

2xKO and 5xtg pEC were analyzed with IF staining. Chamber slides were stained with IF antibodies to make the two KO porcine genes (CMAH and GGTA1) and five human transgenes (CD46, CD55, CD59, A20 and HO1) apparent on a protein level to be compared to wt pEC. The signal of DAPI in blue is shown to see the distribution of the cells (Fig. 2 a-n). The representative image of wt and 2xKO 5xtg PAEC shows successfully knocked-out porcine genes and generated five human transgenes. In PVEC the same results are visible.

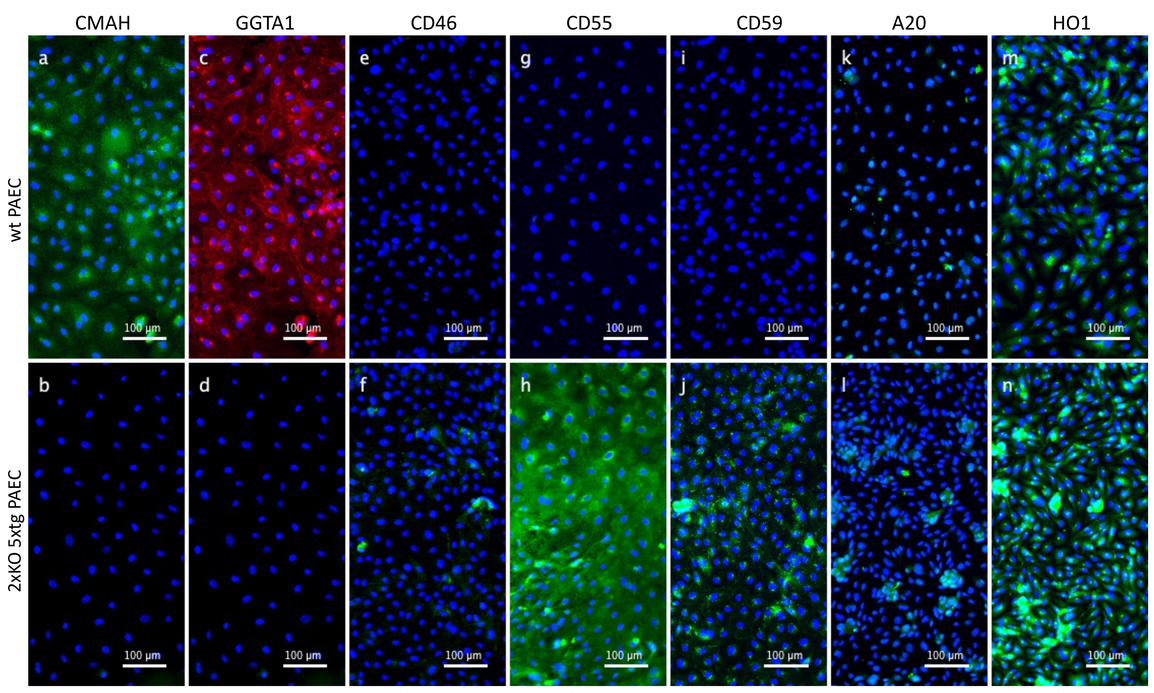

Figure 2: IF stained images of chamber slides for characterization of 2xKO and 5xtg porcine EC. wt PAEC shows no or low signal for human transgenes, but strong signal for KO pig genes CMAH and GGTA1. All five human transgenes are visible in 2xKO 5xtg PAEC. Additionally, A20 and HO1 are visible in both cell types (wt and 2xKO 5xtg) of PAEC with a stronger signal in 2xKO 5xtg PAEC. (Büttiker, 2023)

Microfluidic of PAEC and PVEC

The 72-hour microfluidic run was followed by a two-hour activation for channels with 10% diluted NHS and pure DMEM for control channels. An IF staining for all channels was made for DAPI, C3c (yellow) and IgG (red). All control channels and the serum perfused 2xKO 5xtg channels show almost no reaction. In all wt serum perfused channels C3c (Fig. 3) and IgG deposition (Fig. 5) is apparent. In C3c deposition the difference is only significant on the 2xKO 5xtg PVEC (Fig. 4). From the statistical analysis the difference in IgG deposition is significant for both PAEC and PVEC (Fig. 6).

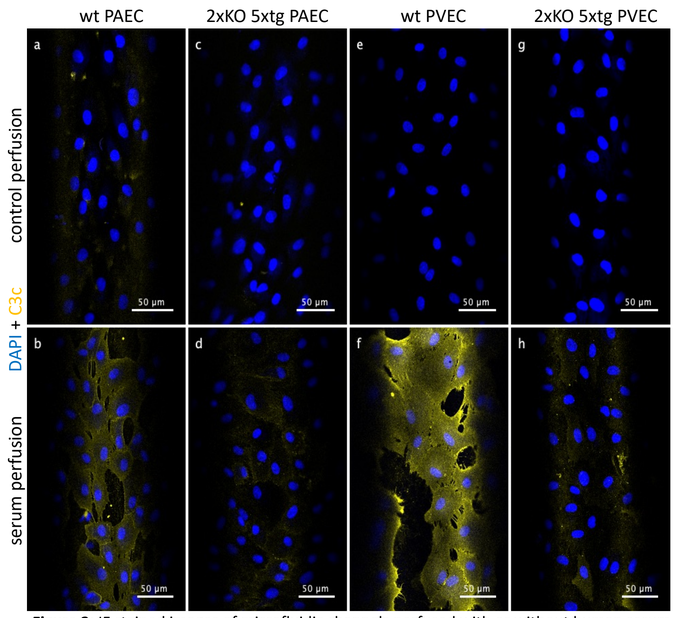

Figure 3: IF stained images of microfluidic channels perfused with or without human serum for two hours after 72-hour perfusion with DMEM under pulsatile flow of 590µl/min (shear stress=12dyn/cm^2. Comparison between wt and 2xKO 5xtg PAEC and PVEC for C3c. (Büttiker, 2023)

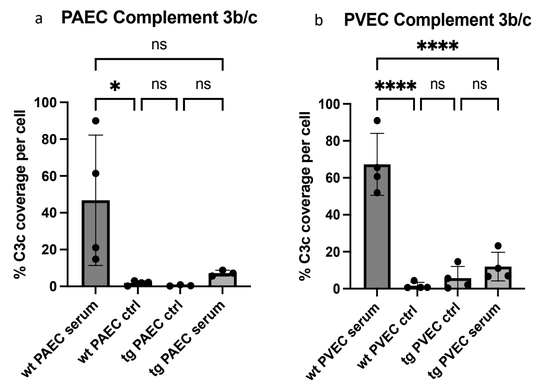

Figure 4: The percentage of C3c coverage (PAEC C3c p* = 0.0356, PVEC C3c p****< 0.0001) was statistically analyzed with ordinary one-way ANOVA test with multiple comparisons. (Büttiker, 2023)

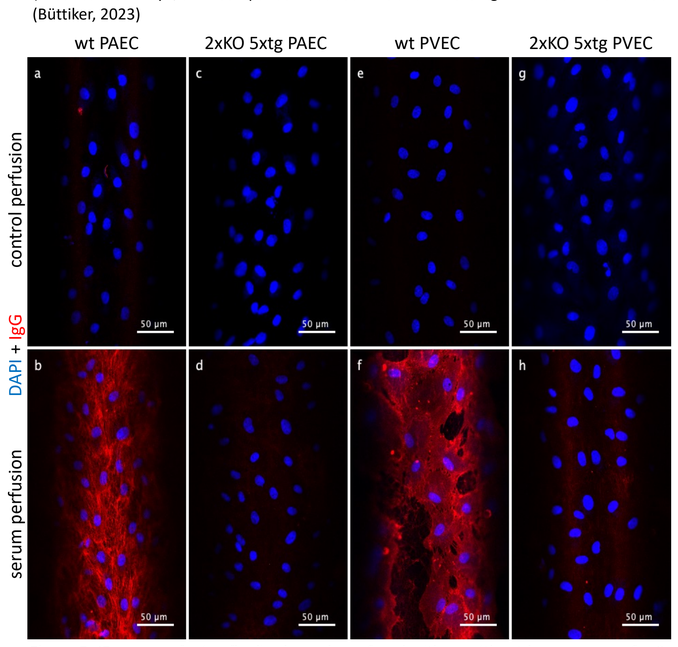

Figure 5: IF images of microfluidic channels perfused with or without human serum for 2 hours after 72 hours perfusion with DMEM under pulsatile flow (white arrow) of 590µl/min (shear stress=12dyn/cm^2). (Büttiker, 2023)

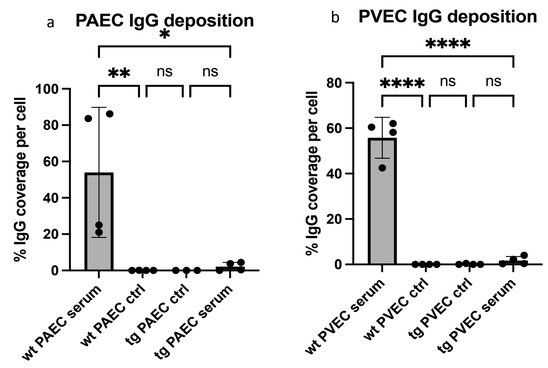

Figure 6: Wt and 2xKO 5xtg PAEC and PVEC were compared (PAEC IgG p* = 0.0110, p** = 0.0085, PVEC IgG p****< 0.0001). The percentage of coverage was statistically analyzed with one-way ANOVA test (Büttiker, 2023)

Conclusion and Discussion

Aim 1

All genetic modifications are successfully implemented in both cell types of interest and can be detected with IF staining on the protein level. Statistical differences between arterial and venous pEC were only shown for CD46 and CD59. A20 and HO1 show positive results in both types. Therefore, specific antibodies should improve the actual results.

Aim 2

The genetic modifications influence the human innate immune system responses by reducing C3c and IgG deposition. After serum perfusion a statistically significance for C3c and IgG deposition in PVEC is shown compared to wt pEC. For IgG deposition also a low significance for PAEC is shown, but not for C3c deposition. The stronger PVEC results can be due to the vessel type and predominant shear stress.

The results are a good basic for further projects. There are still some immune responses to take care of, maybe by using additional human transgenes or porcine KO genes.

References

[1] Fischer, K., Rieblinger, B., Hein, R., Sfriso, R., Zuber, J., Fischer, A., Klinger, B., Liang, W., Flisikowski, K., Kurome, M., Zakhartchenko, V., Kessler, B., Wolf, E., Rieben, R., Schwinzer, R., Kind, A., Schnieke, A. (2019). Viable pigs after simultaneous inactivation of porcine MHC class I and three xenoreactive antigen genes GGTA1, CMAH and B4GALNT2. *Xenotransplantation*, *27*. https://doi.org/10.1111/xen.12560

[2] Lei, T., Chen, L., Wang, K., Du, S., Gonelle-Gispert, C., Wang, Y., Buhler, L. (2022). Genetic engineering of pigs for xenotransplantation to overcome immune rejection and physiological incompatibilities: The first clinical steps. *Frontiers in Immunology, 13*, 1-14. https://doi.org/10.3389/fimmu.2022.1031185

Figures

Fig. 1: Lei, T., Chen, L., Wang, K., Du, S., Gonelle-Gispert, C., Wang, Y., Buhler, L. (2022). Genetic engineering of pigs for xenotransplantation to overcome immune rejection and physiological incompatibilities: The first clinical steps. *Frontiers in Immunology, 13*, 1-14. https://doi.org/10.3389/fimmu.2022.1031185

Fig. 2: Büttiker, S. (2023). *IF stained images of chamber slides for characterization of 2xKO and 5xtg porcine EC.* Berne: own figure, created with Fiji.

Fig. 3: Büttiker, S. (2023). *IF images of microfluidic channels for C3c perfused with or without serum after 72 hours perfusion.* Berne: own figure, created with Fiji.

Fig. 4: Büttiker, S. (2023). *The percentage of C3c coverage statistically analyzed with one-way ANOVA test.* Berne: own figure, created with Prism.

Fig. 5: Büttiker, S. (2023). *IF images of microfluidic channels for IgG perfused with or without serum after 72 hours perfusion.* Berne: own figure, created with Fiji.

Fig. 6: Büttiker, S. (2023). *The percentage of IgG coverage statistically analyzed with one-way ANOVA test.* Berne: own figure, created with Prism.