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EARLY GRAFT FAILURE OF GalTKO PIG ORGANS IN BABOONS IS REDUCED BY EXPRESSION OF A HUMAN COMPLEMENT PATHWAY-REGULATORY PROTEIN

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Abstract

Background—We describe the incidence of early graft failure (EGF, defined as loss of function from any cause within 3 days after transplant) in a large cohort of GalTKO pig organs transplanted into baboons in three centers, and the effect of additional expression of a human complement pathway-regulatory protein, CD46 or CD55 (GalTKO.hCPRP).

Methods—Baboon recipients of life-supporting GalTKO kidney (n = 7) or heterotopic heart (n = 14) grafts received either no immunosuppression (n = 4), or one of several partial or full immunosuppressive regimens (n = 17). Fourteen additional baboons received a GalTKO.hCPRP kidney (n = 5) or heart (n = 9) and similar treatment regimens. Immunologic, pathologic, and coagulation parameters were measured at frequent intervals.

Results—EGF of GalTKO organs occurred in 9/21 baboons (43%). hCPRP expression reduced the GalTKO EGF incidence to 7% (1/14; p<0.01 vs GalTKO alone). At 30 min, complement deposits were more intense in organs in which EGF developed (p<0.005). The intensity of peri-transplant platelet activation (as β -thromboglobulin release) correlated with EGF, as did the cumulative coagulation score (p<0.01).

Conclusions—We conclude that (i) the transgenic expression of a hCPRP on the vascular endothelium of a GalTKO pig reduces the incidence of EGF, and reduces complement deposition, (ii) complement deposition and platelet activation correlate with early GalTKO organ failure, and

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DISCLOSURES

David Ayares is an employee of Revivacor. The other authors declare they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: DKCC, RNP, AMA, RNB. Data collection: AMA, SSK, MBE, AKS, TS, HI, TZ, LB, ES, CA, XC, KAH, PCC, MMM, RNB. Critical scientific contributions: AMA, SSK, LB, MMM, RNB, DKCC, RNP. Data analysis and interpretation: AMA, SSK, MBE, HI, LB, MMM, RNB, DKCC, RNP. Statistics: AMA. Drafting article: AMA, DKCC, RNP, HI. Critical revision: AMA, MMM, DKCC, RNP. Approval of article: AMA, SSK, LB, MMM, DKCC, RNP.

(iii) the expression of a hCPRP reduces EGF but does not prevent systemic coagulation activation. Additional strategies will be required to control coagulation activation.

Keywords

α 1,3-galactosyltransferase gene-knockout; baboon; complement-regulatory protein; pig; xenograft rejection; xenotransplantation

INTRODUCTION

Pig organ xenografts transplanted into humans or nonhuman primates can be injured through a number of immunologic mechanisms that include antibody-mediated activation of the complement and/or coagulation cascades, activation of innate immune cells and platelets, and initiation of various pathogenic adaptive immune responses [1]. The initial humoral response is largely directed towards galactose- α 1,3-galactose (Gal) antigens expressed on pig vascular endothelial cells [2], and is significantly attenuated by α 1,3-galactosyltransferase gene-knockout (GalTKO). However non-Gal carbohydrate, protein and lipid antigens on porcine endothelial cells can bind anti-nonGal antibodies in blood from humans and nonhuman primates, and in theory could pose a significant residual humoral immunological barrier.

Although hyperacute rejection (i.e., within 24 h) has been infrequently reported in association with heart [3,4] and kidney [5, 6] xenografts from pigs homozygous for GalTKO in published series, we observed that a substantial proportion of GalTKO organs exhibit either hyperacute rejection or relatively rapid graft demise within 3 days after transplant, a phenomenon we term “early graft failure” (EGF). Based on *in vitro* studies indicating that the expression of a human complement pathway-regulatory protein (hCPRP) on GalTKO pig aortic endothelial cells affords significant protection [7], GalTKO pigs that express hCPRP genes, such as human membrane cofactor protein (hCD46) or decay accelerating factor (hCD55), have been produced [8–11].

Here we report a retrospective multi-center study comparing outcomes of heart or kidney grafts from GalTKO pigs and GalTKO.hCPRP pigs to evaluate whether a survival benefit is conferred in association with the expression of a hCPRP, and assess mechanistic correlates.

METHODS

Animals

GalTKO and GalTKO.CD46 pigs (Revivicor, Blacksburg, VA), and GalTKO.CD55 pigs (National Swine Resource and Research Center, Columbia, MO, Stock # NSRRC:009) served as sources of hearts (n=23) and/or kidneys (n=12) (Table 1). Baboons (*Papio anubis*; n=35) were from the Division of Animal Resources Oklahoma University Health Sciences Center, Oklahoma City, OK, the Tulane National Primate Research Center, Covington, LA, the Southwest National Primate Research Center, San Antonio, Texas, or the NIH animal holding facility, Poolesville, MD.

Baboons received either heterotopic pig heart (GalTKO = 14, GalTKO.hCPRP = 9) or life-supporting pig kidney (GalTKO = 7, GalTKO.hCPRP = 5) transplants. Cardiac graft function was monitored by palpation x3 weekly or palpation and telemetry (which continuously monitored heart rate, blood pressure, and body temperature). Kidney graft function was monitored by measurement of serum creatinine x3 weekly.

All animal care was in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). Protocols were approved by the Institutional Animal Care and Use Committees of all centers.

EGF was defined as loss of graft function within 3 days after transplantation from any cause except technical error (which was not observed in this series). Delayed xenograft rejection (DXR) was defined as loss of graft function from any cause after day 3. Loss of graft function was defined by the loss of palpable contraction for heart xenografts and by serum creatinine >5 mg/dL on 3 consecutive days for kidney xenografts. Death of a renal xenograft recipient in association with uremia was considered as graft failure. At graft failure, the recipient baboon was euthanized and necropsy was carried out. Euthanasia with a functioning graft was excluded from graft failure.

Immunosuppressive therapy (IS)

Four baboons received no IS. Thirty one baboons received IS, of which 18 received IS based on induction with anti-thymocyte globulin (ATG), and maintenance therapy with an anti-CD154 mAb, mycophenolate mofetil, and corticosteroids. Seven of the 31 received this 'core' IS regimen with additional anti-CD20mAb (n = 5) or CTLA4-Ig (n = 2). A "partial core regimen" (with omission or reduced dosing of one or more of these agents) was administered to 6 of the 31 baboons. Cobra venom factor was administered to 17/21 baboons that received GalTKO organs and to all 14 recipients of GalTKO.hCPRP grafts.

CH50 complement activity

Inhibition of complement by cobra venom factor was assessed in 16 baboons for which serum samples were available at the time of transplantation by using EZ Complement CH50 test kits (Diamedix, Miami Lakes, FL).

Monitoring of serum anti-nonGal IgM and IgG

Anti-nonGal IgM and IgG levels were monitored pre-transplant, and then at twice-weekly intervals post-transplantation by flow cytometry using donor pig aortic endothelial cells, as reported previously [12, 13]. Pooled human sera were used to indicate 'control' levels.

Histopathological examination of grafts

Biopsies were taken in the majority of grafts pre-transplantation, at 30min after transplantation, and at necropsy. Histopathological examination of the graft was carried out (staining with hematoxylin and eosin, immunofluorescence for anti-human IgM and IgG

antibody, C4d, C3 and C5b-9 complement fragments, fibrin, and CD41 deposition, as previously described [14].

Complement deposition was assessed semi-quantitatively by using a scoring system in which C4d, C3, and C5b-9 deposition were scored separately, with 0 = absence of complement, 1 = mild (1–10%) deposition, 2 = moderate (11–50%) deposition, and 3 = strong (>50%) deposition. The combined score (C4d+C3+C5b-9) was then calculated as a cumulative index reflecting complement pathway activation in the graft.

Monitoring of coagulation activation

In some recipients plasma was drawn pre-transplantation and at regular intervals throughout the post-transplant period in CTAD tubes (Becton Dickinson). Measurement of thrombin-antithrombin (TAT) was performed by ELISA (Enzygnost TAT micro, Dade Behring/Siemens AG, Marburg/Munich, Germany). Platelet activation was monitored by measurement of β -thromboglobulin (Asserachrom β -TG, Diagnostica Stago, Parsippany, NJ). To summarize activation of the coagulation cascade in each animal semi-quantitatively, we calculated a cumulative coagulation score based on the intensity of deposition of fibrin and CD41 on graft biopsies obtained 30min post-transplant (each scored on a scale of 3), and on plasma β -thromboglobulin and TAT measurements categorized based on independent scales as 0 = none, 1 = mild, 2 = moderate, and 3 = strong in blood samples obtained between 30 min and one day following transplantation. A cumulative coagulation score was calculated for each recipient as the sum of the four coagulation parameters, with 0 indicating absence of coagulation, and scores of 1–3 indicating mild, 4–8 moderate, and 9–12 strong activation of coagulation.

Statistical analyses

Statistical analysis was performed by using the software GraphPad InStat 3.01 (GraphPad Software, San Diego, CA). The proportion of grafts undergoing EGF vs. DXR was compared by using Fisher's exact test. Biochemical data and semi-quantitative scores are presented as mean $\pm$ standard deviation. Variables that were normally distributed were assessed with a one-way analysis of variance and Student's t-test. Variables that were not normally distributed were assessed with Mann-Whitney test. A p value of <0.05 was considered statistically significant.

RESULTS

Incidence of EGF

When no cobra venom factor or IS was administered to a baboon that had received a GalTKO pig heart (n = 4), EGF developed in 100% of cases (Table 1). When cobra venom factor and IS were administered, EGF occurred in 20% of hearts (2/10) and 43% of kidneys (3/7), giving an overall incidence of 29% (5/17) (p<0.05). With the same or similar regimen, in the presence of a GalTKO.hCPRP graft, the incidence of EGF was reduced to 7% (1/14) (Table 1). EGF was characterized by typical macroscopic and histological features of antibody-mediated rejection with interstitial hemorrhage, edema and thrombosis (Figure 1A). There was no difference (p=0.7) in pre-transplant CH50 values between GalTKO and

GalTKO.hCPRP groups. Pre-transplantation administration of cobra venom factor decreased complement CH50 activity by $>93\pm6.5\%$.

Effect of anti-nonGal antibody and complement activation on graft survival

There was no difference in baboon pre-transplant anti-nonGal IgM (Figure 2A) and IgG (not shown) antibody binding to vascular GalTKO endothelial cells between the GalTKO and GalTKO.hCPRP groups, irrespective of the development of EGF or DXR. In biopsies of xenografts taken 30min after reperfusion, baboon anti-nonGal IgM and IgG were equally present on the graft vascular endothelium in cases that developed EGF (IHC score, IgM: 2.4 ± 0.7 ; IgG: 2.2 ± 0.5) or DXR (IgM: 2.2 ± 0.7 ; IgG: 2.3 ± 0.5) (Figure 1B). We concluded that anti-nonGal antibody deposition was a factor in the development of both EGF and DXR, but did not differ in intensity in association with the kinetics of graft failure.

EGF was associated with increased complement deposition on the vascular endothelium of the graft on biopsy 30 min after graft reperfusion. When EGF occurred in GalTKO grafts, the average score of complement deposition was 1.4 ± 0.4 , which was not substantially different from the score in the single GalTKO.hCPRP graft that underwent EGF (2.0). However, complement deposition was less intense in GalTKO organs that did not have EGF (0.6 ± 0.4 , $p<0.01$ vs. EGF). There was no difference in initial complement deposition between GalTKO (0.6 ± 0.4) and GalTKO.hCPRP (0.7 ± 0.9) organs when DXR was subsequently observed. When comparing all EGF vs. all DXR organs, reduced initial complement deposition was significantly associated with DXR ($p<0.05$) (Figure 1B and 2B).

Relation between coagulation activation and graft survival

There was significantly greater activation of platelets (determined by plasma β -thromboglobulin) when EGF developed than when DXR occurred (mean 406 vs. 147 UI/ml; $p<0.01$) (Figure 3A). There was no difference in the development of TAT formation in recipients in which EGF occurred (409 ± 692 $\mu\text{g/ml}$) compared to those in which DXR occurred (869 ± 2195) (not shown). Driven primarily by platelet activation parameters, the overall cumulative coagulation score was significantly greater in baboons with EGF than in those which developed DXR (mean score 9.3 vs 3.6; $p<0.0005$) (Figure 3B). However, when DXR developed, there appeared no difference in initial coagulation scores between recipients of GalTKO and GalTKO.hCPRP grafts.

DISCUSSION

The study reported here represents the largest series to date comparing outcomes associated with GalTKO and GalTKO.hCPRP pig organs in the baboon model. We found that hCPRP expression is associated with reduced initial complement activation in the graft, as expected, and with a reduced incidence of EGF.

The transgenic expression of a hCPRP in pigs as a means to reduce hyperacute rejection of a pig organ transplanted into a primate was first suggested by Dalmaso [15] and, independently, by White [16] and their respective colleagues. The first hCPRP-transgenic pigs expressed CD55 (decay-accelerating factor) [17], which proved a major advance over wild-type pigs as sources of organs for xenotransplantation in nonhuman primates [18].

Expression of another hCPRP, e.g., CD46, was associated with similarly encouraging results [19].

Although GalTKO pigs were first suggested in 1993 [20], they first became available 10 years later based on interval advances in genetic engineering and cloning technologies [21]. The GalTKO genetic modification extended graft survival over wild-type pig organs [3–6]. No hyperacute rejection or EGF was reported in these initial studies, but these studies specifically selected recipients baboons with low anti-pig antibody titers.

Although our groups each also independently found that hyperacute rejection was uncommon in association with GalTKO organs, EGF was not. As we report here, and as previously described in abstract form in 2010 [22], when GalTKO.hCPRP pigs became available to us, the expression of a hCPRP significantly reduced the incidence of EGF of GalTKO xenografts. Similarly, McGregor and his colleagues demonstrated a significant benefit from the combination of GalTKO with hCPRP expression using a different line of genetically-modified pigs [8].

The present retrospective multi-center study allowed us to explore the relative contribution of several factors that might be associated with EGF and/or DXR based on mechanistic considerations. Our data suggest that the pre-transplant level of anti-nonGal antibody and its binding to the graft do not account for differences in graft behavior. In contrast, cumulative indices reflecting activation of the complement and coagulation cascades, both systemically and in the graft, correlate with graft behavior, and appear to implicate both mechanisms in the etiology of GalTKO xenograft injury.

The extent of binding of anti-nonGal IgM and IgG to the graft probably plays a role in initiation of both EGF and DXR [23], but did not differ in relation to the speed of graft failure, i.e., EGF or DXR. Interestingly, pre-transplant anti-nonGal IgM levels in baboons from this study were generally higher than in a normal human serum pool (MFI = 35 ± 15 , dotted box in Figure 2A) and higher than in specific pathogen-free baboons [24, 25]. In contrast, early xenografts performed with GalTKO organs employed baboons selected for low anti-nonGal antibody levels [3].

We found that the transgenic expression of a hCPRP on the vascular endothelium of a GalTKO pig (i) reduced the incidence of EGF and associated complement deposition, (ii) complement deposition and platelet activation correlated with early GalTKO organ failure, and (iii) the expression of a hCPRP on the graft reduced EGF, but did not prevent coagulation activation.

These data suggest that additional transgenic expression of proteins that regulate the coagulation cascade may be helpful, and could prove necessary, to attenuate dysregulated coagulation, and delay or prevent DXR, whether DXR is triggered primarily by anti-nonGal antibody and complement or by the several molecular incompatibilities that have been identified between pig and primate coagulation cascades. In support of this hypothesis, GalTKO.hCPRP pigs expressing one of several human ‘anti-thrombotic’ genes, including thrombomodulin [26, 27], CD39 [28], and endothelial protein C receptor [29], are now

becoming available, and appear to be consistently associated with extended pig heart [30], kidney [31], and lung [32–34] graft survival.

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ABBREVIATIONS

Gal	galatose- α 1,3-galactose
GalTKO	α 1,3-galactosyltransferase gene-knockout
hCPRP	human complement pathway-regulatory protein
DXR	delayed xenograft rejection (graft failure >3 days)
EGF	early graft failure (<3 days)
IS	immunosuppressive therapy
TAT	thrombin-antithrombin

References

1. Ekser B, Ezzelarab M, Hara H, et al. Clinical xenotransplantation – the next medical revolution? *Lancet*. 2012; 379(9816):672–683. [PubMed: 22019026]
2. Good AH, Cooper DK, Malcom AJ, et al. Identification of carbohydrate structures that bind human antiporcine antibodies: implications for discordant xenografting in man. *Transplant Proc*. 1992; 24:559–562. [PubMed: 1566430]
3. Kuwaki K, Tseng YL, Dor FJ, et al. Heart transplantation in baboons using α 1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med*. 2005; 11:29–31. [PubMed: 15619628]
4. Tseng YL, Kuwaki K, Dor FJ, et al. α 1,3-galactosyltransferase gene-knockout pig heart transplantation in baboons with survival approaching six months. *Transplantation*. 2005; 80:1493–1500. [PubMed: 16340796]
5. Chen G, Qian H, Starzl T, et al. Acute rejection is associated with antibodies to non-Galantigens in baboons using Gal-knockout pig kidneys. *Nat Med*. 2005; 11:1295–8. [PubMed: 16311604]
6. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of α 1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 2005; 11:32–34. [PubMed: 15619627]
7. Hara H, Long C, Lin YJ, et al. In vitro investigation of pig cells for resistance to human antibody-mediated rejection. *Transplant Int*. 2008; 21:1163–1174.
8. McGregor CG, Ricci D, Miyagi N, et al. Human CD55 expression blocks hyperacute rejection and restricts complement activation in Gal knockout cardiac xenografts. *Transplantation*. 2012; 93:686–692. [PubMed: 22391577]

9. Cooper DK, Hara H, Ezzelarab M, et al. The potential of genetically-engineered pigs in providing an alternative source of organs and cells for transplantation. *J Biomed Res.* 2013; 27:249–53. [PubMed: 23885264]
10. Burdorf L, Stoddard T, Zhang T, et al. Expression of human CD46 modulates inflammation associated with GalTKO lung xenograft injury. *Am J Transplant.* 2014; 14:1084–95. [PubMed: 24698431]
11. Le Bas-bernardet S, Tillou X, Branchereau J, et al. C1-inhibitor and plasma exchange do not prolong the survival of multi-transgenic GalT-KO pig kidney xenografts in baboons. *Am J Transplant.* 2015; 15:358–70. [PubMed: 25612490]
12. Ezzelarab M, Garcia B, Azimzadeh A, et al. The innate immune response and activation of coagulation in α 1,3-galactosyltransferase gene-knockout xenograft recipients. *Transplantation.* 2009; 87:805–812. [PubMed: 19300181]
13. Azimzadeh AM, Byrne GW, Ezzelarab M, et al. Development of a consensus protocol to quantify primate anti-non-Gal xenoreactive antibodies using pig aortic endothelial cells. *Xenotransplantation.* 2014; 21:555–66. [PubMed: 25176173]
14. Wu G, Pfeiffer S, Schröder C, et al. Coagulation cascade activation triggers early failure of pig hearts expressing human complement regulatory genes. *Xenotransplantation.* 2007; 14:34–47. [PubMed: 17214703]
15. Dalmaso AP, Vercellotti GM, Platt JL, Bach FH. Inhibition of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor. Potential for prevention of xenograft hyperacute rejection. *Transplantation.* 1991; 52:530–533. [PubMed: 1716798]
16. White DJ, Oglesby T, Liszewski MK, et al. Expression of human decay accelerating factor or membrane cofactor protein genes on mouse cells inhibits lysis by human complement. *Transpl Int.* 1992; 5:648–650.
17. Cozzi E, White DJG. The generation of transgenic pigs as potential organ donors for humans. *Nat Med.* 1995; 1:964–969. [PubMed: 7585226]
18. Cozzi E, Vial C, Ostlie D, et al. Maintenance triple immunosuppression with cyclosporin A, mycophenolate sodium and steroids allows prolonged survival of primate recipients of hDAF porcine renal xenografts. *Xenotransplantation.* 2003; 10:300–310. [PubMed: 12795679]
19. McGregor CG, Davies WR, Oi K, et al. Cardiac xenotransplantation: recent preclinical progress with 3-month median survival. *J Thorac Cardiovasc Surg.* 2005; 130:844–851. [PubMed: 16153938]
20. Cooper DKC, Koren E, Oriol R. Genetically engineered pigs. *Lancet.* 1993; 342:682–683. [PubMed: 8103167]
21. Phelps CJ, Koike C, Vaught TD, et al. Production of α 1,3-galactosyltransferase-deficient pigs. *Science.* 2003; 299:411–414. [PubMed: 12493821]
22. Azimzadeh AM, Kelishadi S, Ezzelarab M, et al. Early graft failure of GTKO pig organs in baboons is reduced by hCPRP expression. *Am J Transplant.* 2010; 10(Supplement 4):186. Abstract #499.
23. Gollackner B, Goh SK, Qawi I, et al. Acute vascular rejection of xenografts: roles of natural and elicited xenoreactive antibodies in activation of vascular endothelial cells and induction of procoagulant activity. *Transplantation.* 2004; 77:1735–1741. [PubMed: 15201675]
24. Mohiuddin MM, Corcoran PC, Singh AK, et al. B-cell depletion extends the survival of GTKO.hCD46Tg pig heart xenografts in baboons for up to 8 months. *Am J Transplant.* 2012; 12:763–71. [PubMed: 22070772]
25. Zhou H, Iwase H, Wolf RF, et al. Are there advantages in the use of specific pathogen-free baboons in pig organ xenotransplantation models? *Xenotransplantation.* 2014; 21:287–290. [PubMed: 24547993]
26. Yazaki S, Iwamoto M, Onishi A, et al. Production of cloned pigs expressing human thrombomodulin in endothelial cells. *Xenotransplantation.* 2012; 19:82–91. [PubMed: 22497510]
27. Wuensch A, Baehr A, Bongoni AK, et al. Regulatory sequences of the porcine THBD gene facilitate endothelial-specific expression of bioactive human thrombomodulin in single- and multitransgenic pigs. *Transplantation.* 2014; 97:138–47. [PubMed: 24150517]

28. Wheeler DG, Joseph ME, Mahamud SD, et al. Transgenic swine: expression of human CD39 protects against myocardial injury. *J Mol Cell Cardiol.* 2012; 52:958–61. [PubMed: 22269791]
29. Harris DG, Quinn KJ, French BM, et al. Meta-analysis of the independent and cumulative effects of multiple genetic modifications on pig lung xenograft performance during ex vivo perfusion with human blood. *Xenotransplantation.* 2014; commentary 2015. doi: 10.1111/xen.12149doi: 10.1111/xen.12159
30. Mohiuddin MM, Singh AK, Corcoran PC, et al. Genetically engineered pigs and target-specific immunomodulation provide significant graft survival and hope for clinical cardiac xenotransplantation. *J Thorac Cardiovasc Surg.* 2014; 148:1106–13. discussion 1113–4. [PubMed: 24998698]
31. Iwase H, Liu H, Wijkstrom M, et al. Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. Submitted.
32. Burdorf, L.; Zhang, T.; Stoddard, T., et al. Pilot evaluation of GalTKO.hCD39 lungs in a xenogeneic pig lung perfusion model. International Xenotransplantation Association Meeting (IXA); Venice, Italy. October 2009;
33. Burdorf L, Rybak E, Zhang T, et al. Human EPCR Expression in GalTKO.hCD46 Lungs Extends Survival Time and Lowers PVR in a Xenogenic Lung Perfusion Model. *J Heart Lung Transplant.* 2013; 32:S137.
34. Harris DG, Gao Z, Sievert EP, et al. Transgenic Human Thrombomodulin Expression Reduces Xenogeneic Thrombosis: a Promising Means of Reducing Pig Lung Xenograft Thrombotic Injury. *J Heart Lung Transplant.* 2014; 33:S108.

Figure 1A

Figure 1B

Figure 1. Macroscopic and microscopic analysis of xenografts

(A) Macroscopic appearances of rejected pig organs [left], and microscopic appearances (trichrome staining [middle] and hematoxylin and eosin staining [right]) are shown for representative heart and kidney xenografts with EGF, and hematoxylin and eosin staining for representative heart and kidney 30min biopsies with DXR [far right].

(B) Immunofluorescence for tissue deposition of IgM, IgG, C3, C5b-9, CD41 and fibrin from representative biopsies for each group.

Figure 2A

Figure 2B

Figure 2. Role of antibody and complement

(A) Baboon anti-nonGal IgM levels were measured by flow cytometry on pig aortic endothelial cells and data expressed as median fluorescence intensity (MFI) ($n = 35$). Pre-transplant anti-nonGal antibody levels did not predict graft outcome. Anti-nonGal antibody deposition was associated with both EGF and DXR. The dotted box represents anti-nonGal IgM levels in a pool of normal human serum.

(B) Complement activation in the graft was quantified using a semi-quantitative score as defined in Methods ($n = 22$). Complement deposition (at 30 min) was significantly associated with EGF.

Each dot represents an individual xenograft; the horizontal line represents the median.

Figure 3A

Figure 3B

Figure 3. Activation of platelets and coagulation

(A) Platelet activation (determined by plasma levels of baboon β -thromboglobulin) was significantly increased when EGF developed (n = 30).

(B) Coagulation activation in the graft and blood was quantified using a semi-quantitative score as defined in Methods (n = 32). EGF of GalTKO pig xenografts was associated with a

higher cumulative coagulation score, but coagulation was not decreased by expression of hCPRP.

Each dot represents an individual xenograft; the horizontal line represents the median.

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Table 1
Incidence of early graft failure (EGF, <3 days) in baboons receiving heart or kidney grafts from GalTKO or GalTKO.hCPRP pigs

<u>Therapy</u>	<u>GalTKO</u>		<u>GalTKO.hCPRP</u>		<u>Total</u>
	<u>Heart</u>	<u>Kidney</u>	<u>Heart</u>	<u>Kidney</u>	
No CVF/IS	4/4 (100%)	ND	ND	ND	4/4 (100%)
CVF/IS	2/10 (20%)	3/7 (43%)	0/9 (0%)	1/5 (20%)	5/17(29%) vs. 1/14(7%)

CVF = cobra venom factor therapy; IS = immunosuppressive therapy; ND = not done