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THERAPEUTIC ISSUES IN THE TREATMENT OF VASCULARIZED XENOTRANSPLANTS USING GAL-KNOCKOUT DONORS IN NONHUMAN PRIMATES

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Abstract

Purpose of review—Solid organ xenotransplantation could be the future of transplantation, but improved outcomes are required in experimental models before clinical trials are justified. This review summarizes recent advances in solid organ xenotransplantation using organs from α 1,3-galactosyltransferase gene-knockout (GTKO) pigs (with or without other genetic modifications) and novel therapeutic approaches.

Recent findings—Work on the development of genetically-engineered pigs has been considerable during the past few years, with many research institutes reporting the outcomes of research. Multiple gene modifications on a GTKO background have been reported, and the results of transplantation using organs from these pigs have been published. Progress, however, has been variable, and several obstacles, e.g., coagulation dysregulation, have been identified. Heterotopic pig heart xenotransplantation has been associated with graft survival exceeding 8 months, but kidney graft survival has not improved significantly.

Summary—The availability of GTKO pigs with additional genetic modifications aimed towards expression of multiple complement-regulatory proteins and/or human thromboregulatory genes, combined with novel immunosuppressive regimens, e.g., the inclusion of B cell-depleting agents, should improve pig organ survival in the near future.

Keywords

α 1,3-galactosyltransferase gene-knockout; complement-regulatory proteins; organs; xenotransplantation

INTRODUCTION

The identification of Gal α 1,3Gal (Gal) as the major target antigen for human and nonhuman primate (NHP) anti-pig antibodies and the production of α 1,3-galactosyltransferase gene-knockout (GTKO) pigs [1] have helped to resolve the problem of hyperacute rejection.

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However acute humoral xenograft rejection, chronic rejection, and other barriers to success, e.g., coagulation dysregulation in the form of thrombotic microangiopathy and/or consumptive coagulopathy, need to be addressed if graft survival is to be significantly prolonged.

The initial studies in which kidneys [2] and hearts [3] from GTKO pigs were transplanted into NHPs had encouraging results, but used immunosuppressive regimens that are unlikely to be used clinically. New strategies should be aimed towards (i) extending xenograft survival, (ii) minimizing the side-effects of the immunosuppressive agents, and (iii) enabling clinical trials to be considered.

Extensive reviews of xenotransplantation in pig-to-NHP models have been reported [4*,5]. The current review summarizes the most recent results of solid organ xenotransplantation using GTKO pig organs (with or without additional genetic modifications) in the pig-to-NHP model in combination with novel therapeutic approaches.

HEART XENOTRANSPLANTATION (Table 1) [6-13]

Experimental heart xenotransplantation can be performed using three different techniques: (i) heterotopic intra-abdominal model, (ii) heterotopic intra-thoracic model, and (iii) orthotopic model.

Heterotopic intra-abdominal model

Mohiuddin et al achieved graft survival of >8 months using hearts from GTKO pigs expressing a human complement-regulatory protein (CRP), CD46 [11**]. Median graft survival was increased from 10 to 60 days by the addition of a B cell-depleting agent (anti-CD20mAb). However, their study was not without significant complications associated with surgical technique, immunosuppressive regimen, and other aspects of recipient management [14,15].

Byrne and colleagues performed 11 transplants using GTKO or GTKO/CD55 pig hearts [8]. Despite T and B cell depletion and splenectomy, median survivals were not prolonged in comparison to other reports in the literature. However, expression of CD55 reduced complement deposition in the graft. Similarly, Azimzadeh et al transplanted GTKO or GTKO/CRP (CD46 or CD55) pig hearts into baboons, and demonstrated that complement deposition and early graft failure were significantly reduced by CRP expression [6*].

Ezzelarab et al demonstrated that costimulation blockade with an anti-CD154mAb prolonged graft survival, but early or late xenograft failure was associated with activation of the innate immune system [9*]. Consumptive coagulopathy occurred in 6 of 9 recipients. Using the same model and immunosuppression as Ezzelarab et al [9*], Burdorf et al concluded that a high pre-transplantation level of anti-nonGal antibodies limited the efficacy of costimulation blockade [7]. There is clearly a need for alternative or adjunct therapies to control the humoral response.

Brenner et al transplanted hearts from GTKO, GTKO/CD46, or GTKO/HO-1 (heme oxygenase-1) pigs. There was no difference in median survival in relation to the various genotypes of the organ-source pig, but extended survival was achieved when immunoadsorption was carried out [10].

The identification of major anti-nonGal antibodies and development of strategies to reduce the corresponding antigens on the pig [16] may lead to prolonged survival.

Heterotopic intra-thoracic model

Bauer et al [12] employed the original Losman and Barnard technique of intra-thoracic heterotopic heart transplantation [17]. This would be a clinically-applicable approach because, in view of the continuing presence of the recipient's native heart, graft failure would not necessarily result in death of the patient [12]. They reported two cases using GTKO/CD46 pig hearts in baboons, and incorporated pre-transplantation immunoadsorption of anti-pig antibody in the recipient. The first recipient died within hours from air embolism and brain damage, while the second graft functioned for 50 days.

Orthotopic model

McGregor et al [13**,18] reported orthotopic heart transplantation of GTKO/CD55 pig hearts transplanted into baboons using a clinically-applicable immunosuppressive regimen (Table 1). Longest survival was 57 days (with good cardiac function), which is the longest reported to date, and median survival was 28.5 days.

KIDNEY XENOTRANSPLANTATION (Table 2) [6,7,9, 19-25]

In a non-immunosuppressed pig-to-monkey model, an Australian group showed that the combination of GTKO with expression of CD55/CD59/HT (H-transferase) improved renal xenograft survival and delayed or prevented the development of consumptive coagulopathy [22].

Under the Xenome Project of the European Union, pigs with multiple genetic modifications (GTKO/CD55/CD39/HT) were transplanted into NHPs. Cozzi et al in cynomolgus monkeys [19*] and Le Bas-Bernardet et al in baboons [21] reported similar outcomes using different clinically-applicable immunosuppressive regimens. In the Cozzi et al experience [19*], multiple genetic modifications were not associated with prolonged recipient survival, though preliminary coagulation data were consistent with a lower extent of coagulopathy when compared with previous studies using pigs expressing CD55 on a wild-type background. They drew attention to the presence of CD20⁺ infiltrating cells in the majority of the grafts, which correlates with Mohiuddin's experience that the addition of a B cell-depleting agent to the immunosuppressive regimen is beneficial.

The longest survivals of baboons with life-saving pig kidney grafts were reported by the Boston group in their thymokidney transplantation models [20,25]. Greisemer et al [20] performed seven GTKO pig-to-baboon thymokidney transplants. Median survival was 49 days, with the longest survival being 83 days. This baboon was the only one that received whole body radiation, and appears to have been reported previously [2]. Several immunosuppressive regimens, including different T cell-depleting (anti-thymocyte globulin or monoclonal mouse anti-human CD2b antibody) and B cell-depleting (anti-CD20 monoclonal antibody) agents were tested. The kidney grafts showed no signs of cellular infiltration or deposition of IgG, and no grafts were lost from rejection. In the same thymokidney model, the same group reported that high levels of preformed cytotoxic anti-nonGal antibody did not induce hyperacute rejection, but were associated with early graft loss [25], correlating with the observations of Burdorf et al [7].

With the goal of inducing transplantation tolerance, the Boston group performed GTKO pig-to-baboon renal xenotransplants using an extensive immunosuppressive regimen combined with GTKO pig bone marrow administration [23]. Two baboons received kidney transplants 2 or 17 days after bone marrow administration. One baboon died from renal failure (day 8) and the other developed thrombocytopenia and consumptive coagulopathy, and expired with pulmonary edema after 24 days.

Burdorf et al reported kidney transplants in baboons from GTKO, GTKO/CD55, and GTKO/CD46 pigs [7]. Various immunosuppressive regimens included T cell-depletion and costimulation blockade. Prolonged survival was achieved with GTKO/CD55 and GTKO/CD46 pigs when compared to GTKO pigs. They observed the same outcomes as in their heart xenotransplantation model in that a high pre-transplant level of anti-nonGal antibodies was associated with a substantial incidence of early graft failure. This antibody response has been reviewed by others [16,26]

Ezzelarab et al reported that the GTKO genetic modification alone with depletion of complement activity in the recipient by cobra venom factor was not enough to prevent acute humoral xenograft rejection and/or consumptive coagulopathy [9*]. Lin et al studied the role of tissue factor expression on circulating platelets and peripheral blood mononuclear cells (PBMCs) following either GTKO or GTKO/CD46 pig kidney transplantation in baboons [24**]. Tissue factor was detected on platelets on post-transplant day 1, but was not detected on PBMCs until consumptive coagulopathy was beginning to develop. Graft histopathology showed fibrin deposition and platelet aggregation (n=6), but with only minor features indicating a humoral immune response, and no macrophage, B or T cell infiltration. Prevention of recipient platelet activation will be critical for successful pig kidney transplantation.

LIVER XENOTRANSPLANTATION (Table 3)

The first and only xenotransplantations using GTKO pig livers came from the Pittsburgh group [27**], who reported 10 liver xenotransplants from GTKO or GTKO/CD46 pigs using a clinically-applicable immunosuppressive regimen. The importance of size-matching between pig liver and recipient baboon was emphasized. The narrow abdomen of baboons necessitated the use of livers from pigs that were almost 60% smaller in weight than the recipient baboon. Six of 10 baboons survived for 4-7 days. No obvious difference was observed between GTKO and GTKO/CD46 pig livers. In all cases, liver function was adequate, as evidenced by tests of detoxification, complement activity, coagulation parameters, and production of pig proteins, including coagulation factors [28*]. Pig coagulation factors and proteins appeared to function adequately in baboons, although interspecies compatibility of such proteins remains to be confirmed.

Survival beyond 7 days was prevented by a profound thrombocytopenia that developed within one hour after reperfusion of the graft, ultimately resulting in spontaneous hemorrhage at various sites [27**]. The authors postulated that the thrombocytopenia was associated with the expression of tissue factor on platelets after contact with the pig endothelium, resulting in platelet and platelet-PBMC aggregation and deposition of aggregates in the liver graft, although phagocytosis of the platelets by pig Kupffer cells or hepatocytes could not be ruled out.

The Pittsburgh study was primarily undertaken to determine whether the pig liver could be used as a bridge to allotransplantation in patients with fulminant liver failure. Bridging options, and inclusion and exclusion criteria have been discussed [29*]. The immediate development of thrombocytopenia needs to be prevented before a clinical trial would be justified.

COAGULATION DYSREGULATION

Three review papers discussed this topic [30*, 31*,32] and are reviewed elsewhere in this issue by Cowan et al [33]. The need for pigs expressing one or more human 'thromboregulatory' genes, such as tissue factor pathway inhibitor (TFPI) or human thrombomodulin, is crucial. Two attempts using CD39 pig kidneys have been reported

(Table 2), but adequate expression of CD39 was doubtful [19*,21]. The effect of expression of a thromboregulatory gene or genes therefore remains untested. There are several pharmacologic agents that would likely prove beneficial, e.g., heparin, aspirin, ketorolac, prostacyclin (Tables 1–3), but which interventions will be beneficial, yet effective and safe, also remains uncertain [30*,32].

The discrepancies between the results reported by various groups with regard to the development of coagulopathy cannot be fully explained, though a number of factors may be playing roles, e.g., differences in the source of the pigs, the immunosuppressive regimen, and the organ transplanted. The literature suggests that the development of consumptive coagulopathy is less common or delayed when intensive immunosuppressive therapy is administered [34], though the exact mechanism of this effect remains uncertain. It is well-known that the costimulation blockade agent, anti-CD154mAb, is associated with thromboembolic events [35–37], and it is possible that this may be a factor in some of the reported studies, though thrombotic microangiopathy develops in NHPs receiving conventional immunosuppressive therapy [34]. Our experience of transplanting GTKO pig organs does not indicate to us that the GTKO genetic modification increases the ‘thrombogenicity’ of the organ graft. However, Knosalla et al have reported that there is a clear heterogeneity between renal and cardiac xenograft endothelium, which may account for the observed increased incidence of consumptive coagulopathy after pig kidney xenotransplantation [38*].

COMPLEMENT REGULATION

Miyagawa et al published a valuable review on complement regulation in the GTKO era [39**]. After GTKO pig organ xenotransplantation, complement is activated by (i) the classical pathway, by the interaction of anti-nonGal antibodies and nonGal antigens, and by ischemic injury, (ii) the alternative pathway, especially after pig islet transplantation, and (iii) the lectin pathway. The complement system represents an important recognition and effector mechanism of acute humoral xenograft rejection. The various CRPs, e.g., CD35, CD46, CD55, CD59, C1-inhibitor, regulate complement activation at different points in the complement cascade. Therefore, it will be preferable to express multiple human CRP genes in GTKO pigs rather than rely on a single gene.

Cowan and D’Apice emphasized the importance of anti-nonGal antibodies in the humoral response to a GTKO pig organ [32]. Without complete suppression of production of these antibodies, there is progressive activation and injury of the graft endothelium, resulting in thrombotic microangiopathy, with subsequent graft loss [31**].

THE POTENTIAL ROLE OF CYTOTHERAPY

Cytherapy (e.g., the infusion of bone marrow, T regulatory cells, or mesenchymal stem cells [MSCs]) to the recipient of a solid organ xenograft could be beneficial [23]. In their quest for microchimerism, Greisemer et al administered GTKO bone marrow to baboons [23]. Of the 4 baboons, 2 received pig kidney transplants, with no significant prolongation of graft survival compared to that in baboons that did not receive bone marrow (summarized above).

Although there are several different subtypes of T regulatory cells, CD4⁺CD25⁺Foxp3⁺ T cells are the most characterized and have been suggested as an immunosuppressive strategy in xenotransplantation [40*,41*,42]. Although *in vitro* studies show expanded T regulatory cells inhibit the baboon cellular response to pig PBMC, the effect on prolongation of xenograft survival needs confirmation in NHP studies [43]. In contrast to cadaveric

allotransplantation, the organ donor is known prior to xenotransplantation, which should facilitate therapy with T regulatory cells.

The report of successful treatment of severe graft-versus-host disease after clinical bone marrow transplantation by MSCs created substantial interest [44]. MSCs prohibit the proliferation of T cells, inhibit dendritic cell maturation, and induce T regulatory cells, and hence may prove a potential therapy for solid organ transplantation. Ezzelarab et al discussed the potential of (organ) donor-specific genetically-modified pig MSCs [45].

FUTURE DIRECTIONS

Five years after the first results of GTKO pig-to-NHP organ transplantation were reported, the addition to GTKO of one or more human CRPs has been associated with better outcomes. The expression of multiple CRPs is probably preferable. Anti-thrombotic gene expression is required to reduce coagulation dysregulation. Table 4 [46-54] summarizes recently generated genetically-engineered pigs. In this regard, human thrombomodulin-expressing pigs have been produced [55], but not yet tested *in vivo* [52*].

Pigs with potential resistance to the human cellular response are also becoming available. Stimulatory and inhibitory receptor interactions in xenotransplantation have been reviewed elsewhere [56]. The production of human Fas ligand-expressing pigs, which may prevent human CD8⁺ and natural killer cell cytotoxicity, has recently been reported [48*]. With the aim of protecting against injury by natural killer cells, Weiss et al produced HLA-E/human beta2-microglobulin-expressing pigs [47]. Pigs expressing CTLA4-Ig have been produced so successfully that the level of CTLA4-Ig in the blood was several times higher than the therapeutic level [46*]; these pigs demonstrated features of immunoincompetence, e.g., infections, and required euthanasia. MHC class II transactivator (CIITA) gene knock-down pigs have been generated (*Dai Y et al, unpublished*). *In vitro* testing of the human CD4⁺ T cell response to cells from CIITA pigs indicated a much diminished human T cell response, suggesting that organs and cells from these pigs should be significantly protected against the human/NHP cellular immune response [49*].

Other pigs that have become available include human HO-1-expressing pigs [51] and human A20-expressing pigs [50]. These will hopefully provide some anti-apoptotic, anti-inflammatory, and cell protective effects. Porcine endogenous retrovirus siRNA-expressing transgenic pigs, aimed at preventing the activation and propagation of porcine endogenous retrovirus, have been produced [53*,54].

Newer immunosuppressive and adjunctive agents, e.g., anticoagulants, may also be beneficial. Alemtuzumab (anti-CD52mAb) combined with mycophenolate mofetil depleted and maintained the number of CD4⁺ cells at <25% for at least one year in cynomolgus monkeys [57*], and therefore may have a role in xenotransplantation.

Conclusion

In summary, the increasing number of genetic modifications being made to GTKO pigs [58] and more effective immunosuppressive, anticoagulant, and anti-inflammatory regimens provide the expectation of improved experimental results that may eventually lead to the initiation of clinical trials.

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ABBREVIATIONS

CIITA	class II transactivator
CRP	complement-regulatory protein
Gal	Gal α 1,3Gal
GTKO	α 1,3-galactosyltransferase gene-knockout
mAb	monoclonal antibody
MSC	mesenchymal stem cells
NHP	nonhuman primate
PBMC	peripheral blood mononuclear cells
TFPI	tissue factor pathway inhibitor

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Outcomes of heart xenotransplantation in GTKO pig-to-NHP models between January 2009 and October 2010

Table 1

Author (Year) [Ref#]	Type	Pigs	Recipi- ents	N.	Survival (days)	Mean (Median) Survival (days)	Immunosuppression	Anti- coagulant	Outcome
Azimzadeh (2009) [6]	H1A	GTKO	Baboon	14	n.a	n.a	None (n=4), ATG+anti-CD154+CVF+MMF+Cs (+/-CTLA4-Ig)	Heparin, Aspirin	Early graft failure, complement deposition
	H1A	GTKO/CRP	Baboon	9	n.a	n.a	None (n=1), ATG+anti-CD154+CVF+MMF+Cs (+/-CTLA4-Ig)	Heparin, Aspirin	CRP (either CD46 or CD55). Reduced incidence of early graft failure, minimal complement deposition
Burdorf (2009) [7]	H1A	GTKO	Baboon	3	<1,<1, 6	3 (1)	ATG+anti-CD154+CVF+MMF+Cs (n=2) + CTLA4-Ig (n=1)	n.a.	n.a.
	H1A	GTKO/CD46	Baboon	6	2,8,10,10,1 2,28	12 (10)	ATG+anti-CD154+CVF+MMF+Cs (n=4) + CTLA4-Ig (n=2)	n.a.	n.a.
Byrne (2009) [8]	H1A	GTKO	Baboon	6	n.a.	n.a (21)	Splenectomy+Rituximab+ATG+TAC+RAPA	n.a.	HAR in 90 min (n= 1), AHXR, chronic vascular antibody deposition, complement deposition
	H1A	GTKO/CD55	Baboon	5	n.a.	n.a (28)	Splenectomy+Rituximab+ATG+TAC+RAPA	n.a.	No complement deposition, chronic vascular antibody deposition
Ezzelarab (2009) [9]	H1A	GTKO	Baboon	9	2,5h,1,6,6, 7,12,12,35, 56	15 (7)	None (n=2), CVF only (n=1), CVF+ATG+Leflunomide (n=1), low-dose anti-CD154+CTLA4-Ig+MMF (n=1), ATG+CVF+anti-CD154+MMF+Cs (n=4)	Heparin, Ketorolac, PGI2	No accommodation, AHXR, CC, TM, stenosis at anastomoses, ischemic myopathy
Brenner (2010) [10]	H1A	GTKO	Baboon	2	8,8	8 (8)	Rituximab+TAC+RAPA+Cs+FcyRIIb	n.a.	No rejection. Died due to ileus
	H1A	GTKO/CD46	Baboon	3	7,17,29	18 (17)	Rituximab+TAC+RAPA+Cs+FcyRIIb+ATG +/- immunoadsorption	n.a.	Mild-moderate rejection, sepsis, left ventricular thrombus
	H1A	GTKO/HO-1	Baboon	2	8,13	11 (11)	Rituximab+TAC+RAPA+Cs+FcyRIIb +/- ATG	n.a.	Humoral rejection, cellular rejection, graft failure
Mohiuddin (2010) [11]	H1A	GTKO/CD46	Baboon	22	n.a. 179, >195 ongoing	n.a -(10, n=6) (60, n=14)	None (n= 2), ATG+MMF+CVF+Cs+anti-CD154 (n=6) ATG+MMF+CVF+Cs+anti-CD154 +Rituximab (n=14)	Heparin, Aspirin, Ketorolac	HAR when no IS. Several focal complications related to heterotopic xenografting (abdominal adhesions, bleeding, intestinal obstruction)
Bauer (2010) [12]	H1T	GTKO/CD46	Baboon	2	<1, 50	26 (26)	Immunoadsorption pretransplantation, Rituximab+TAC+RAPA+MMF+Cs	n.a.	Died from cerebral air-embolism (10h). Poor myocardial perfusion due to ventricular fibrillation
McGregor (2009) [13]	OHT	GTKO/CD55	Baboon	6	2,14,23,34, 40,57	28 (29)	ATG+TAC+RAPA+Cs	n.a.	Rejection (extensive data are n.a)

AHXR= acute humoral xenograft rejection, ATG= antithymocyte globulin, CC= consumptive coagulopathy, CRP= complement-regulatory protein, Cs= corticosteroids, CVF= cobra venom factor, HAR= hyperacute rejection, HIA= heterotopic intra-abdominal, HIT= heterotopic intra-thoracic, IS= immunosuppression, MMF= mycophenolate mofetil, n.a= not available, OHT= orthotopic, PGI2= prostacycline, RAPA= rapamycin, TAC= tacrolimus, TM= thrombotic microangiopathy.

Table 2

Outcomes of kidney xenotransplantation in GTKO pig-to-NHP models between January 2009 and October 2010

Author (Year) [Ref#]	Pigs	Recipi-ents	N.	Survival (days)	Mean (Median) Survival (days)	Immunosuppression	Anti-coagulant	Outcome
Azinzadeh (2009) [6]	GTKO	Baboon	7	n.a	n.a	None (n=1), ATG+anti-CD154+CVF+MMF+Cs (+/- CTLA4-Ig)	Heparin, Aspirin	Early graft failure, complement deposition
	GTKO/CRP	Baboon	5	n.a	n.a	ATG+anti-CD154+CVF+MMF+Cs (+/- CTLA4-Ig)	Heparin, Aspirin	CRP (either CD46 or CD55), reduced early graft failure incidence, minimal complement deposition
Burdorf (2009) [7]	GTKO	Baboon	3	<1,5,7	4 (5)	ATG+anti-CD154+CVF+MMF+Cs (+/- CTLA4-Ig)	n.a.	n.a.
	GTKO/CD55	Baboon	3	<1,12,12	8 (12)	ATG+anti-CD154+CVF+MMF+Cs	n.a.	n.a.
	GTKO/CD46	Baboon	2	5,12	9 (9)	ATG+anti-CD154+CVF+MMF+Cs (+/- CTLA4-Ig)	n.a.	n.a.
Ezzelarab (2009) [9]	GTKO	Baboon	3	2, 3, 5	3 (3)	CVF only (n=1), low-dose anti-CD154+CTLA4-Ig+MMF (n=1), ATG+CVF +anti-CD154+MMF+Cs(n=1)	Heparin, Ketorolac, PGI2	AHXR, CC, renal artery thrombosis
Cozzi E (2009) [19]	GTKO/CD55/CD59/CD39/HT	Cyno	6	8 to 22	16 (16)	CyP+CSA+MMF+Cs	n.a.	Kidney failure, abdominal bleeding (n=1), AHXR
Greisemer (2009) [20]	GTKO	Baboon	7	18,28,40,49,57,81,83	51 (49)	(a) Thymokidney+Rituximab+ATG+LoCD2b + anti-CD154+TAC+MMF (n=4), (a) - LoCD2b (n=2), (a) - TAC + WBI (n=1)	n.a.	Died from drug reaction, invasive CMV infection, AML, pleural effusion from proteinuria, ARDS,
Le Bas-Bernardet (2009) [21]	GTKO/CD55/CD59/CD39/HT	Baboon	5	4,4,12,13,1 4	13 (13) (ISed)	None (n=2) or CyP+TAC+MMF+Cl inhibitor	n.a.	AHXR
Salvaris (2009) [22]	GTKO/CD55/CD59/HT	Baboon	6	<2h,2,3,3,4, 5	3 (3)	None	None	HAR, CC
Greisemer (2010) [23]	GTKO	Baboon	2	8,24	16 (16)	Splenectomy+WBI+Thymus Irriditation+ ATG+LoCD2b+TAC+GTKO BM (+CVF in one case)	Heparin, PGI2	Renal failure, pulmonary edema, gross enlargement of the kidney, hemorrhage and thrombi
Lin (2010) [24]	GTKO	Baboon	1	7	7	ATG+anti-CD154+CVF+MMF+Cs	Heparin, Ketorolac, PGI2	CC
	GTKO/CD46	Baboon	6	2,4,9,10,10, 16	9 (10)	None (n=1), ATG+anti-CD154+MMF (n=4), ATG+anti-CD154+MMF+Cs (n=1)	Heparin, Ketorolac, PGI2	Fluid overload, CC
Nishimura (2010) [25]	GTKO	Baboon	4	16,16,17,50	25 (17)	Thymokidney+ATG+Rituximab+anti-CD154 +low dose TAC+MMF	n.a.	Sudden death (thrombi in vessels), AHXR

AMI= acute myocardial infarction, ARDS= acute respiratory distress syndrome, BM= bone marrow, CMV= cytomegalovirus, CSA= cyclosporine, cyno= cynomolgus monkey, CyP= cyclophosphamide, LoCD2b= mouse anti-human CD2b antibody, WBI= whole body irradiation. Other abbreviations as for Table 1.

Table 3
Outcomes of liver xenotransplantation in GTKO pig-to-NHP models between January 2009 and October 2010

Author (Year) [Ref#]	Type	Pigs	Recipients	N.	Survival (days)	Mean (Median) Survival (days)	Immunosuppression	Anticoagulant	Outcome
Ekser 2010 [27]	Orthotopic	GTKO	Baboon	2	<1, 6	4 (4)	ATG+TAC+MMF+Cs	PGI2	Donor vs recipient size-mismatch (n=1), death or euthanasia due to hemorrhage in abdomen and lungs
	Orthotopic	GTKO/CD46	Baboon	8	<1, <1, 1, 4, 5, 6, 6, 7	4 (5)	ATG or CyP+TAC+MMF+Cs, CL (to 2 donors), CVF (to one recipient)	Heparin (in two cases), Ketorolac (in 4 cases), PGI2	PNF on CL treatment, Donor vs recipient size-mismatch (n=1), very high TAC levels, death or euthanasia due to hemorrhage in abdomen and lungs

CL= clodronate liposomes, PNF= primary non-function. Other abbreviations as for Tables 1 and 2.

Table 4

Recent generation of genetically-engineered pigs

Author (Year) [Ref#]	Genetically engineered Pigs	Site of Expression	Function	Mechanism of Action
<i>For prevention of immune injury</i>				
PHELPS (2009) [46]	Porcine CTLA4-Ig	High, ubiquitous	Costimulation blockade and decreased T cell response	Porcine CTLA4-Ig binds to pig CD80 or CD86 and prevents direct response (Pigs - susceptible to opportunistic infection)
WEISS (2009) [47]	HLA-E/human β 2-microglobulin	Endothelium	protection against human anti-pig NK cells	Interacts with inhibitory receptor CD94/NKG2A on human NK cells
CHOI (2010) [48]	Human FAS ligand	Membrane bound - metalloproteinase resistant	Prevents Human CD8+ and NK cell cytotoxicity	FAS-FAS ligand-induced apoptosis
HARA (2010) [49]	CIITA-DN	not available	Immuno protection	Inhibition of SLA Class II expression
OROPEZA (2010) [50]	Human A20	Skeletal muscles, heart and PAECs	Anti-inflammatory and anti-apoptotic	Protects against TNF α -mediated apoptosis, blocks NF- κ B and caspases
PETERSON (2010) [51]	Human hemeoxygenase-1	not available	Cytoprotective	Anti-apoptotic and cell protective
<i>For prevention of coagulopathy</i>				
PETERSON (2009) [52]	Human thrombomodulin (h TM) in hCD59/CD55 background	Kidney>heart>> lungs> liver	Anticoagulant/Anti inflammatory activity	Binds human thrombin and activates protein C (anticoagulation)
<i>For prevention of PERV</i>				
DIECKHOFF (2008) [53] RAMSOONDAAR (2009) [54]	Transgenic PERV siRNA expression	Ubiquitous	Prevention of propagation of PERV	RNA interference of <i>gag</i> and <i>pol</i> PERV genes

CIITA-DN= Major histocompatibility complex Class II TransActivator gene knockdown, NF- κ B= nuclear factor kappa-light-chain-enhancer of activated B cell, NK= natural killer, PAEC= porcine aortic endothelial cell, PERV= porcine endogenous retrovirus, SLA= swine leukocyte antigen, TNF= tumor necrosis factor,