Review Article

Clinical lung xenotransplantation – what donor genetic modifications may be necessary?

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Abstract: Barriers to successful lung xenotransplantation appear to be even greater than for other organs. This difficulty may be related to several macro anatomic factors, such as the uniquely fragile lung parenchyma and associated blood supply that results in heightened vulnerability of graft function to segmental or lobar airway flooding caused by loss of vascular integrity (also applicable to allotransplants). There are also micro-anatomic considerations, such as the presence of large numbers of resident inflammatory cells, such as pulmonary intravascular macrophages and natural killer (NK) T cells, and the high levels of von Willebrand factor (vWF) associated with the microvasculature. We have considered what developments would be necessary to allow successful clinical lung xenotransplantation. We suggest this will only be achieved by multiple genetic modifications of the organ-source pig, in particular to render the vasculature resistant to thrombosis. The major problems that require to be overcome are multiple and include (i) the innate immune response (antibody, complement, donor pulmonary and recipient macrophages, monocytes, neutrophils, and NK cells), (ii) the adaptive immune response (T and B cells), (iii) coagulation dysregulation, and (iv) an inflammatory response (e.g., TNF-α, IL-6, HMGB1, C-reactive protein). We propose that the genetic manipulation required to provide normal thromboregulation alone may include the introduction of genes for human thrombomodulin/endothelial protein C-receptor, and/or tissue factor pathway inhibitor, and/or CD39/CD73; the problem of pig vWF may also need to be addressed. It would appear that exploration of every available therapeutic path will be required if lung xenotransplantation is to be successful. To initiate a clinical trial of lung xenotransplantation, even as a bridge to allotransplantation (with a realistic possibility of survival long enough for a human lung allograft to be obtained), significant advances and much experimental work will be required. Nevertheless, with the steadily increasing developments in techniques of genetic engineering of pigs, we are optimistic that the goal of successful clinical lung xenotransplantation can be achieved within the foreseeable future. The optimistic view would be that if experimental pig lung xenotransplantation could be successfully managed, it is likely that clinical application of this and all other forms of xenotransplantation would become more feasible.

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Introduction

Many patients with end-stage lung disease (e.g., idiopathic pulmonary hypertension or that associated with congenital heart disease, interstitial pulmonary fibrosis, cystic fibrosis, sarcoidosis, emphysema, and those unfortunate individuals with destruction of the large airways) might benefit significantly from lung transplantation in terms of better quality of life and longer survival. Successful lung xenotransplantation, using pig lungs, could circumvent the huge barriers to access created by the limited number of lungs from deceased human donors that are available each year.

However, it is well-known that the barriers to successful lung xenotransplantation appear to be even greater than those of other organs, for example, heart, kidney, where there is still as yet no clinical applicability despite substantial progress over the past decade [1–24]. This may be related to several anatomic factors, such as the uniquely fragile structure of the lung parenchyma and associated blood supply that results in heightened vulnerability of organ function to segmental or lobar airway flooding caused by loss of vascular integrity, which is also pertinent to acute respiratory distress syndrome (ARDS) or non-cardiac pulmonary edema. These factors are compounded by micro-anatomic considerations, such as the presence of large numbers of resident inflammatory cells, such as pulmonary intravascular macrophages and natural killer (NK) cells [15,18,19,25], and the high levels of von Willebrand factor (vWF) associated with the microvasculature. These clearly are also important issues in human allotransplantation. Physiologic differences in characteristics of the pulmonary vascular endothelium due to rheology, expression of adhesion molecules, or nitric oxide or prostanoid metabolism [19,25–27] and susceptibility of the lung vasculature to increased resistance sufficient to precipitate right heart failure and low cardiac output are other possible contributors to the lung's particular vulnerability to vascular injury and thrombosis. All of these mechanisms can be implicated in ARDS, ischemia-reperfusion injury, and vascular injury after allotransplantation and are substantially compounded by cross-species molecular incompatibilities in the xenograft context.

Xenotransplantation of the lungs, therefore, presents possibly the greatest challenge to those of us in this field of research. The optimistic view would be that if experimental pig lung xenotransplantation could be successfully managed, it is likely that clinical application of this and all other forms of xenotransplantation would become more feasible.

We have considered what developments would be necessary to allow successful clinical lung xenotransplantation. Our initial major conclusion is that this will only be achieved by multiple genetic modifications of the organ-source pig, in particular to render the vasculature more compatible and resistant to thrombosis. Because it may be difficult and risky to administer long-term drug therapy, for example, potent anticoagulants and anti-platelet agents, we believe that pharmacologic systemic therapies are unlikely to make the major contribution. These approaches, however, may prove to be of additional therapeutic value, particularly if their use can be limited to brief intervals, as at the time of xenograft implantation. Nevertheless, such agents will be useful in possibly determining the mechanisms involved in pig lung graft failure, for example, the coagulation factors that may be playing a major role.

If a few specific and central coagulation pathway targets can be identified, and neutralized, the information obtained will indicate what genetic modifications would be required in the pig. To some extent, because of the rapidity of the pathologic events that take place when the pig lung is perfused with human blood, pig lung xenotransplantation might provide a sensitive early-warning model to predict how genetic manipulations or exogenous agents might improve survival of pig kidney or heart xenotransplantation. However, although some genetic modifications may be important for all organs, others may need to be organ-specific.

Present status of lung xenotransplantation

This topic has been reviewed by others [17,28] and, briefly, by us [29,30], will only be summarized here. Ex vivo perfusion of the pig lung with human blood has proved a valuable method of assessing the acute effect of any therapeutic measure on pig lung function [31–33] (reviewed in Cantu et al. [17]). When survival for longer than a few hours is anticipated, orthotopic lung xenotransplantation in non-human primates has been used [7,17,34]. The longest survival of a pig lung after transplantation into a non-human primate has been < 5 days [19], although survival with the recipient totally dependent on the graft has been shorter.

This situation is in marked contrast to survival of pig hearts and kidneys, where heterotopic (non-life-supporting) heart grafts have functioned for periods of up to 6–8 months [35–38], and life-supporting orthotopic heart and kidney grafts have supported the recipient's life for < 2 months [39–41] and 2–3 months [42,43], respectively. Lung xenograft

performance is more comparable to survival of pig liver grafts, which has not as of yet reached 10 days [44–46].

As with other pig vascularized organs, the pig lung can be injured by antibody binding and complement activation at the endothelial cell interface [9,32,33,47,48]. The lung also appears particularly susceptible to the effects of coagulation dysregulation [49,50]. Indeed, because of the multiple pathologic processes taking place, immediate or early pig lung graft failure has been termed "hyperacute pulmonary xenograft dysfunction" rather than "hyperacute pulmonary xenograft rejection [17]." Despite depletion of antibody and/or complement, and the use of lungs from genetically modified pigs (e.g., GTKO, CD46, CD55), lungs fail rapidly, with elevated pulmonary vascular resistance and massive pulmonary edema [2,17,30,32,33]. Thrombotic injury occurs, and the associated profound systemic hypotension (which requires inotropic support to maintain recipient hemodynamics) and consumptive coagulopathy remain major problems [13,14,24,51].

Apart from the problems associated with the xenotransplantation of any pig organ, as alluded to earlier, there appear to be some hurdles specific to the inflammatory responses to the lung vasculature and to thrombotic reactions within the pulmonary venous system and at the level of alveolar capillaries. For example, unlike cardiac and renal xenografts, the vasculature of the xenografted lung releases large quantities of vWF [51]. When human vWF binds to GP1b on human platelets, platelet activation and adhesion occur, but only if the platelets are subjected to shear stress [52–54]. In contrast, pig vWF binds to human or non-human primate GP1b on quiescent platelets, leading to platelet aggregation even in the absence of shear stress [14,55–57]. Primate xenoantibodies also bind to carbohydrate epitopes on the vWF and possibly to associated heparan sulfates released from pig lungs and do not remain bound to the lung vascular endothelium [10]. The vWF-xenoantibody complex has an enhanced capacity to aggregate human and other primate platelets. When lungs from vWF-deficient pigs have been utilized, graft failure has been more typical of the hyperacute rejection seen after heart or kidney xenotransplantation; antibody has been deposited along the graft endothelium and has been accompanied by complement activation, with associated interstitial hemorrhage and edema [35,58]. These observations suggest that vWF plays a major role in the pathogenesis of pulmonary xenograft failure.

The major problems that require to be overcome are therefore multiple and include (i) the innate immune response (antibody, complement, macrophages, monocytes, neutrophils, and NK cells), (ii) the adaptive immune response (T and B cells), (iii) coagulation dysregulation, and (iv) an inflammatory response (e.g., TNF-α, IL-6, HMGB1 [59], C-reactive protein). It would appear that exploration of every available therapeutic path will be required if lung xenotransplantation is to be successful.

Currently available genetically engineered pigs

An increasing number of genetically engineered pigs are becoming available worldwide (Table 1). However, at present, very few genetically engineered pigs express more than two or three modifications, and it will take some time before pigs with multiple genetic modifications are available in sufficient numbers to test in ex vivo and in vivo lung xenotransplantation models.

Hearts from α1,3-galactosyltransferase gene-knockout (GTKO) pigs transgenic for expression of CD46 and/or CD55 appear largely resistant to hyperacute rejection in non-human primates and therefore could be the basis for further genetic

Table 1. Genetically modified pigs currently available for xenotransplantation research[a]

Gal antigen deletion or ``masking''
  α1,3-galactosyltransferase gene-knockout (GTKO)
  Human H-transferase gene expression (expression of blood type O antigen)
  Endo-beta-galactosidase C (reduction in Gal antigen expression)
Complement regulation by human complement-regulatory gene expression
  CD46 (membrane cofactor protein)
  CD55 (decay-accelerating factor)
  CD59 (protectin or membrane inhibitor of reactive lysis)
Anticoagulation and anti-inflammatory gene expression or deletion
  Human tissue factor pathway inhibitor (TFPI)
  Human thrombomodulin
  Human CD39 (ectonucleoside triphosphate diphosphohydrolase-1)
  von Willebrand factor (vWF)-deficient (natural mutant)
Suppression of cellular immune response by gene expression or downregulation
  Porcine CTLA4-Ig (Cytotoxic T-Lymphocyte Antigen 4 or CD152)
  LEA29Y (Inhibition of the B7/CD28 co-stimulatory pathway of T-cell activation)
  CIITA-DN (MHC class II transactivator-knockdown, resulting in swine leukocyte antigen class II knockdown)
  Human TRAIL (tumor necrosis factor-alpha-related apoptosis-inducing ligand)
  HLA-E/human β2-microglobulin (inhibits human natural killer cell cytotoxicity)
  Human CD47 (for species-specific CD47-signal regulatory protein-alpha natural interaction on macrophages)
  Human FAS ligand (CD95L)
  Human GnT-III (N-acetylglucosaminyltransferase III) gene
Anticoagulation, anti-inflammatory, and anti-apoptotic gene expression
  Human A20 (tumor necrosis factor-alpha-induced protein 3)
  Human heme oxygenase-1 (HO-1)
  Human TNFRI-Fc (tumor necrosis factor-alpha receptor I-Fc)
Prevention of porcine endogenous retrovirus (PERV) activation
  PERV siRNA

[a]Modified from Cooper DKC[208].
Pigs with combinations of genetic modification, for example, GTKO with added transgenes are available.

Immunomodulatory
• hCD59
• CIITA-DN
• NeuGc-KO
• hHLA-E/G

Anti-inflammatory & Anti-apoptotic
• hHO-1
• hA20

GTKO.hCRP

Thromboregulatory
• pvWF-KO/hvVWF-KI

Thromboregulatory & Anti-inflammatory
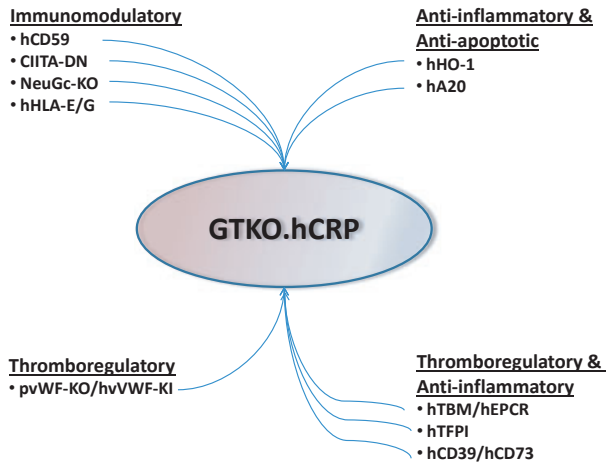• hTBM/hEPCR
• hTFPI
• hCD39/hCD73

Fig. 1. Genetic manipulations of pigs that are likely to be required to provide lungs for successful clinical xenotransplantation. hCRP = transgenic expression of one or more human complement-regulatory proteins, for example, CD46 +/− CD55.

manipulation (Fig. 1). When combined with potent immunosuppressive therapy, they also go a long way to resist classical acute humoral rejection, and acute cellular rejection can also be prevented or significantly delayed.

However, the currently available genetically engineered pigs whose organs have been transplanted into non-human primates do not fully prevent the activity of innate immune cells, including NK cells, macrophages, monocytes, and neutrophils [58], and neither do these genetic modifications prevent the consumptive coagulopathy that develops [60].

What pig genetic modifications will be necessary?

Immunomodulatory

Antibody and complement
In view of the lung's susceptibility to immune and non-immune injury, in addition to GTKO, the expression in the pig of more than one human complement-regulatory protein, for example, CD46, CD55, and CD59, may prove beneficial [34,61–66], even if just to increase the overall level of complement-regulatory proteins (Fig. 1).

NonGal antigens
For clinical lung xenotransplantation (as opposed to experimental pig lung transplantation in non-human primates), the current evidence suggests that the expression of N-glycolylneuraminic acid (NeuGc) on the pig vascular endothelium will be detrimental [67]. The absence of expression of NeuGc in CMP-N-acetylneuraminic acid hydrox-

ylase gene-knockout (NeuGc-KO) pigs will reduce anti-nonGal antibody binding, and its numerous sequelae [68–73].

The nature of other nonGal antigen targets remains elusive [74–80], although genomic and proteomic studies have identified some potential targets of anti-nonGal antibody [76,77]. It remains to be determined whether these are expressed in the lung, whether NeuGc decorates these proteins, and how GTKO impacts the high mannose or other epitopes. It is probably unrealistic, even with modern genetic engineering, to alter all or even most of the targets of anti-nonGal antibodies. However, it may be necessary and practical to pursue the most immunogenic antigens and determine the protein or carbohydrate bound by the antibody. To that end, genetic modifications could be made that may affect numerous proteins and carbohydrates that bear those epitopes. These strategies should be designed to remove the dominant antigens while preserving anti-thrombotic properties of the associated vascular proteoglycans, such as heparan sulfate.

Natural killer and related innate immune cells
Although attention has been drawn to the fact that the magnitude of the role of NK and NKT cells in xenograft rejection remains uncertain and that it may be controlled by exogenous immunosuppressive therapy [81], we suggest that, in the case of lung xenotransplantation, genetic engineering directed toward inhibiting NK cell activity may prove valuable [82–86]. The expression of HLA-E and/or G and/or Cw3, therefore, is likely to inhibit NK cell function, though, perhaps not completely across species barriers [87–93]. Pigs transgenic for overexpression of human HLA-E have been produced in combination (through breeding) with GTKO/hCD46 genetics (E. Wolf, personal communication).

The adaptive (T cell) immune response
Any genetic manipulations that allow reduction in the exogenous immunosuppressive therapy required to control the primate adaptive immune response to the pig lung will be invaluable. The evidence is that pigs with MHC class II transactivator-knockdown (CIITA-DN) will reduce the adaptive T-cell response [94]. Expression of CTLA4-Ig would also be beneficial in neutralizing the adaptive response, but pigs that express CTLA4-Ig ubiquitously have been shown not to be viable [95], and therefore, expression would be required selectively on the endothelial cells. As it may prove difficult to obtain the optimal level of immunosuppression by the endogenous expression

of CTLA4-Ig, with a risk of over-immunosuppression rendering the pig liable to infectious complications [95], it may be wiser to administer exogenous CTLA4-Ig, if this is required for a limited time.

The problem of pig pulmonary macrophages

Pig lungs contain both alveolar and intravascular macrophages. The latter comprise a significant percentage (estimated at about 16%) of the endothelial surface of the microvasculature [96–98]. Pig macrophages, like hepatic Kupffer cells, can remove debris, microparticles, and primate platelets from the circulation and are also major sources of inflammatory cytokines (e.g., TNF-α, IL-1, IL-6), metabolites (e.g., thromboxane), and procoagulant factors (e.g., tissue factor, PAI-1) [8,25,99,100]. Pig macrophages, including those resident in the lung, recognize and may phagocytose human platelets and blood cells because they are identified as foreign bodies [101]. This may be due in part to species incompatibility of the signal regulatory protein-alpha (SIRP-α) expressed on macrophages that enables the cell to identify "self" from "non-self" and control phagocytosis (Fig. 2). The "self"-confirming ligand of SIRP-α, CD47, is expressed on platelets and blood cells as well as on most other tissues.

When human SIRP-α recognizes human CD47 (e.g., on human platelets), human macrophages are not activated and phagocytosis of CD47-expressing cells does not occur [102–105]. While it is known

that human SIRP-α can bind pig CD47, it is not known whether pig SIRP-α can recognize human CD47 [106]. To prevent phagocytosis of human platelets by pig macrophages, it might be necessary to express human SIRP-α on pig macrophages (Fig. 2). It is also feasible to impact these proinflammatory pathways at another site, such as in the context of CD39-mediated scavenging of extracellular nucleotides given the associated requirements in this process for chemotaxis, phagocytosis, and platelet activation, which are all dependent upon purinergic signaling [107,108].

There are several factors, however, that may complicate human SIRP-α expression. Cross-species binding of human SIRP-α to pig CD47 has a significantly higher binding affinity than to human CD47 yet does not produce an inhibitory tyrosine phosphorylation signal on SIRP-α [103,106]. Therefore, expression of human SIRP-α may compete with endogenous pig SIRP-α for binding to available pig and human CD47. Interestingly, studies with recombinant SIRP-α suggest that while human red blood cells are bound by SIRP-α, in contrast, human mesenchymal stem cells shown to express human CD47 are not bound [106]. This difference may be explained by altered binding affinity at or near the highly polymorphic domain 1 of human SIRP-α [109]. Thus, expression of human SIRP-α with appropriate affinity for CD47 ligand may be critical to inhibiting platelet phagocytosis.

Pre-transplant depletion of pig macrophages, for example, by clodronate liposomes, has been shown to be beneficial, with decreased thromboxane production, platelet sequestration, C3a levels, and TNF-α release, inhibition of rise in pulmonary vascular resistance, and prolonged graft function [22,25,110–112]. However, this approach may have potential deleterious effects on tolerance induction by impacting other antigen-presenting cells, as noted in renal xenotransplant models, that may preclude widespread application.

In addition, it has been shown that anti-GP1b Fab reduces activation and sequestration of human platelets in a xenogeneic pig lung perfusion model [113–115]. Depletion of macrophages in the pig before lung harvesting may resolve the problems associated with them, but it is uncertain whether they will be replaced by regenerating pig macrophages already resident in the graft, in which case the original problem may recur, or by human macrophages.

The problem of recipient macrophages

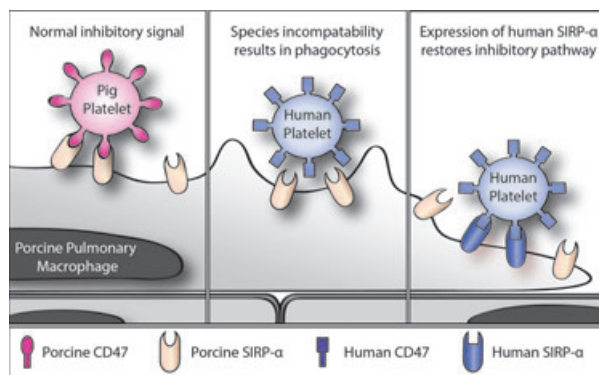The primate recipient macrophages are important innate immune cells involved in pig graft rejection.

Fig. 2. Schematic representation of CD47-signal regulatory protein-alpha (SIRP-α) interaction in relation to natural expression of SIRP-α on pig pulmonary macrophages. Left: In the organ-source pig, there is a normal inhibitory signal of porcine CD47 (in this example expressed on platelets) that is recognized by porcine SIRP-α. Center: After pig lung xenotransplantation, there might be a lack of recognition of human CD47 by porcine SIRP-α, resulting in phagocytosis of human platelets. (Some binding between SIRP-α and CD47 occurs, but there is a deficiency in signaling that prevents signal transduction, the cause of which is uncertain.) Right: Transgenic expression of human SIRP-α on pig pulmonary macrophages would result in recognition of human CD47 on human platelets, thus inhibiting phagocytosis.

The SIRP-α/CD47 interaction described earlier is also responsible for regulating pig graft rejection by primate recipient macrophages, whatever their location.

Expression of pig CD47 on pig platelets and cells does not inhibit the activation of human macrophages [103]. In the case of pig-to-human lung xenotransplantation (although, of course, phagocytosis of the pig organ does not occur), the absence of the inhibitory effect of the expression of human CD47 on the pig tissues may lead to activation of the recipient (primate) macrophages (Fig. 3). This may result in increased inflammatory responses with the production of TNF-α and other cytokines by the human macrophages.

Expression of human CD47 in the pig graft should inhibit human macrophage activity through its inhibitory effect on human SIRP-α (Fig. 3). But will transgenic expression of human CD47 in the same pig create problems for the pig? If pig CD47 also remains expressed in the pig, then this should continue to inhibit pig SIRP-α on pig macrophages, and no phagocytosis of the pig's own cells and

Fig. 3. Schematic representation of CD47-signal regulatory protein-alpha (SIRP-α) interaction in relation to natural expression of SIRP-α on human macrophages. Left: After transplantation of an unmodified pig lung into a human, the expression of pig CD47 on the endothelial cells of the pulmonary blood vessels will not be recognized by human SIRP-α-expressing macrophages, which will therefore not be inhibited but will become activated; inflammatory cytokines will be produced and graft injury will occur. Right: When a lung from a pig transgenic for human CD47 is transplanted, the human SIRP-α-expressing macrophages will recognize the pig tissues as "self," and activation will be inhibited; cytokine production and graft injury will not occur.

platelets should occur, although this remains uncertain. It is promising, however, that Tena et al. [116] recently announced not only several fetuses expressing human CD47, but the birth of a human CD47-expressing pig. The health and survival of this pig is currently being studied.

The generation of human CD47-expressing pigs and the recent production of viable human SIRP-α-expressing mice indicate a potential that CD47/SIRP-α-signaling can be manipulated and still produce a viable pig that should exhibit less aberrant xenogeneic macrophage activity. It may be necessary to employ a method of inducible expression of human CD47, so that expression only occurs at the time of organ transplantation [117,118]. An alternative approach that warrants consideration is transgenic expression of both human SIRP-α and human CD47 in the pig (Dr. J. Estrada, personal communication); this might prevent phagocytosis of pig platelets and cells in the organ-source pig but should enable a human CD47-expressing lung to be transplanted into the human recipient. However, these models remain speculative.

What complicates this already complex topic further is that there is evidence that expression of CD47 on a cell is associated with an inflammatory state and an increase in vasoconstriction and pulmonary vascular resistance [119,120]. This is related to the role of CD47 as a receptor for thrombospondin-1 [121–122], which limits the angiogenic and vasodilator activities of nitric oxide. Complicating the role of CD47 is that thrombospondin-1 regulates T-cell survival via CD47 [123,124]. Of further relevance to lung xenotransplantation is the observation that thrombospondin-1-null mice are resistant to platelet aggregation by thrombin [125]. Any increase in CD47 expression (e.g., by transgenic expression of human CD47 in the pig) may therefore prove detrimental to the outcome of the graft, particularly a lung xenograft. With the many roles CD47 appears to play, knockout of the gene for CD47 would probably result in a non-viable pig. In summary, in regard to solid organ xenotransplantation, the problem of SIRP-α/CD47 remains unresolved and the exact approach to take is uncertain.

With the exception caused by doubt about what to do with regard to SIRP-α/CD47, the modifications mentioned earlier aimed at immunomodulation may be essential if successful clinical lung xenotransplantation is to be achieved (Fig. 1).

However, there may be other modifications that will provide additional or alternative benefits. For example, the expression of tumor necrosis
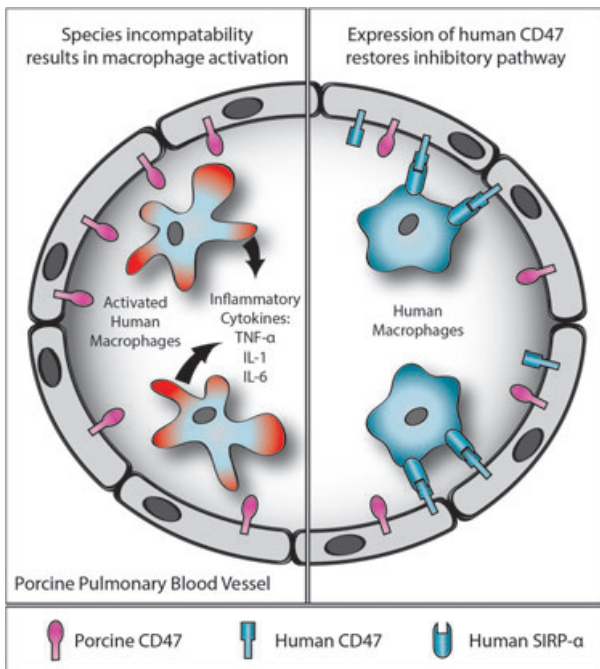
factor (TNF)-α-related apoptosis-inducing ligand (TRAIL) should have some effect on the innate immune response [126]. GTKO/CD46/TRAIL pigs are now available [127]. When available, programmed cell death ligand-1 (PDL-1) pigs may provide additional protection against the adaptive immune cellular responses [128].

Thromboregulatory

The imbalance between human "coagulation" factors and pig "anticoagulation" factors is both quantitative and qualitative. The already thrombophilic xenograft vasculature is further compromised once endothelial injury occurs as a result of the primate immune response, with acquired loss of cell-surface anticoagulant molecules resulting in a pro-thrombotic environment [129]. Despite long-term inhibition of the humoral response, in GTKO pig-to-baboon heart transplants, thrombotic microangiopathy ultimately develops in all grafts [35]. Therefore, protection from immune injury will help toward adequate thromboregulation and balanced coagulation-anticoagulation.

However, it is unlikely that this protection will be absolute, particularly in the environment of the pig lung, and so genetic manipulations to redress the thromboregulatory imbalance are likely to be essential. Once again, multiple pathways of coagulation may need to be interrupted or neutralized if coagulation dysregulation is to be overcome [57,130,131].

Pig thrombomodulin/endothelial protein C-receptor
Pig thrombomodulin is a poor activator of human thrombin, resulting in a low production of activated human protein C [132–135]. Pigs expressing human thrombomodulin have recently been produced [136–138]. Current evidence is that human thrombomodulin expression in pigs will go some way to reduce the coagulation dysfunction that is a major problem in xenotransplantation [139–141], but expression of human endothelial protein C-receptor (EPCR) may also be required for maximal effect [84,133,142–145]. Whether a genetically modified pig expressing both of these human genes will be viable is uncertain, but healthy pigs with constitutive expression of human EPCR have recently been produced (D. Ayares, personal communication).

Tissue factor pathway inhibitor (TFPI)
Even though there is evidence that pig TFPI may regulate human tissue factor pathways [146], additional expression of human TFPI may provide benefit [147–149]. However, it is likely that this will

need to be expressed selectively on the endothelial cells of the vasculature, rather than ubiquitously at high levels in the pig (as previous attempts suggest constitutive-expressing TFPI-transgenic pigs are not viable [D. Ayares, unpublished]).

An alternative to expression of human TFPI may be expression of the anti-thrombin, protein, hirudin [148–150], or reduction in the expression of tissue factor in the pig, which could be achieved with siRNA technology or targeted, regulated suppression of expression [151–153].

CD39/CD73 and purinergic pathways
Expression of human CD39 (ecto-nucleoside triphosphate diphosphohydrolase [E-NTPDase], which is responsible for breakdown of ATP to ADP and then to AMP) and/or CD73 (ecto-5″-nucleotidase [E5′N], which is involved in the hydrolysis of extracellular AMP to adenosine) should prove beneficial for their thromboregulatory and anti-inflammatory effects (see below) and will also have some immunomodulatory impact [154–166]. Expression of human CD39 alone should have a beneficial thromboregulatory effect and may be all that is required, but it may also be necessary to express human CD73, which may enhance this effect on the adenosine pathway. Further, there are data suggesting that CD73 overexpression may be beneficial in sepsis [167]. As with thrombomodulin/EPCR, the expression of both human CD39 and human CD73 at high constitutive levels in the pig might result in untoward effects.

Pig vWF
As outlined earlier, a major problem is the expression of pig vWF, and therefore, it is almost certain that a human vWF knock-in pig will be required (in which human vWF, or certain specific human domains) replaces pig vWF. (Of note, the longest pig lung graft survival in a non-human primate recorded to date involved the transplantation of a lung from a vWF-deficient pig that had been depleted of pulmonary intravascular macrophages [19]). However, due to the large size of the vWF locus (180 kb, 52 exons) and complexity of this gene, the development of a humanized vWF knock-in pig is likely to prove a major challenge to those involved in the genetic engineering of pigs and may be difficult to achieve. Selective manipulation of individual GP1b binding sites on pig vWF, to "humanize" them, may be easier to accomplish and is predicted to decrease non-physiologic interaction of pig vWF with human GP1b.

Another concern in this approach relates to potentially different levels of sialic acid expressed

by platelet vWF receptors, specifically the GPIbα subunit expressed by platelets and endothelium, to those in the recipient primate. These are important considerations in that relative desialylation levels may promote platelet clearance in both lung and liver xenotransplantation. Such potential incompatibilities may be further impacted by GTKO genetic manipulations in a deleterious manner.

Anti-inflammatory

There is increasing evidence that an inflammatory response does not resolve after pig organ xenotransplantation in non-human primates (M. Ezzelarab, unpublished). Furthermore, there is also increasing evidence of interaction between the coagulation–anticoagulation system and an inflammatory response and, indeed, between these two responses and the innate immune system [144,145, 168,169].

Following xenotransplantation, an inflammatory state may be more injurious to the fragile, highly vascularized lung than to organs such as the heart, as exemplified by the lung acting as the first and often principal site of injury in ARDS, as well as in the systemic inflammatory response syndrome (SIRS) and sepsis. Multiple anti-inflammatory genes may therefore be required for successful pig lung xenotransplantation. These are likely to include CD39 (+/− CD73), thrombomodulin (+/− EPCR) [140] (see above), and heme oxygenase-1 [170–172] (+/− A20) [172,173], all of which we believe may be required. Expression of several of these genes, for example, heme oxygenase-1 or A20, is likely to have the added advantage of reducing apoptosis of pig cells. Alternative or additional modifications that may need to be considered include expression of the TNF-α receptor, bcl, Lnk, and/or TRAIL [126,127,174,175].

Comment

The possible genetic modifications that may be required are summarized in Fig. 1, which illustrates the steep road ahead if we are to establish pig lung xenotransplantation in the clinical arena. Many of these genetic modifications, however, will almost certainly be beneficial if clinical heart, kidney, or liver xenotransplantation is the goal, and therefore, efforts made to genetically modify pigs will not only impact lung xenotransplantation. Success with the xenotransplantation of these other organs is likely to be achieved with fewer genetic manipulations, and progression to the clinic is likely to be earlier than envisaged for the lung.

The infusion of mesenchymal stem cells (of human or genetically engineered pig origin [176,177]) may provide immunomodulatory and anti-inflammatory effects, and ex vivo expansion of recipient T-regulatory cells (potentially CD4$^+$ CD39$^+$ populations) may also contribute to suppression of the immune response [178–183].

Unfortunately, there is no suitable in vitro model of lung xenotransplantation that could provide much valuable information, and therefore, we will need to persist with ex vivo pig lung perfusions with human blood and associated in vitro assays. This model is preferable to the more expensive and time-consuming in vivo orthotopic lung transplantation in baboons. However, a demonstration at intervals that the graft can support the life of the recipient is essential to confirm that real progress toward the clinic is being made. Whenever gas transfer and other physiologic parameters of graft function remain essentially normal for >6 h in the ex vivo lung perfusion model, evaluation of the translational promise of the approach by life-supporting orthotopic lung xenotransplantation in non-human primates should be carried out.

Although the barriers to clinical lung xenotransplantation are significant, they are certainly not insurmountable. However, if sufficient progress is to be made to initiate a clinical trial within the foreseeable future, novel approaches to speed up the process of genetic engineering of pigs will likely be essential. Steps are already underway to enable multiple genes to be expressed in the pig simultaneously [184–187], and these newer techniques may allow the rapid production of pigs with multiple genetic manipulations.

Even if existing techniques are not entirely successful, the technology of genetic engineering is steadily improving and new techniques are being introduced, such as zinc finger nucleases [188–194], transcription activator-like effector nucleases (TALENS) [195], meganucleases [196], sleeping beauty transposons [197], and the use of artificial chromosomes [198], which may lead to greater efficiency [199]. Interspecific blastocyst complementation and the in vivo generation of organs derived from xenogeneic donor pluripotent stem cells is in its infancy but may also have potential [200].

The ability to direct genes to specific tissues within the pig's body, for example, islets, neuronal cells, or endothelial cells, has already been achieved with islet-specific gene expression, using an insulin promoter [201–203], and neuron-specific gene expression, using an enolase promoter [204–206]. Gene expression in the endothelial cells alone

should overcome at least some of the complications of ubiquitous expression of a transgene [137,207]. The ability to switch an inducible transgene on or off should also prove beneficial [117,118]. A transgene that is found to be detrimental to the life of the pig when expressed ubiquitously or extensively in the source animal may prove entirely safe when expressed only in a single organ or in a specific cell type, for example, endothelium, after transplantation into the recipient. Such an approach may also facilitate repopulation of a xenograft with host progenitor cells and the development of a fully functional chimeric organ rendered fully tolerant to host immune responses.

Conclusions

To initiate a clinical trial of lung xenotransplantation, even as a bridge to allotransplantation (with a realistic possibility of survival long enough for a human lung allograft to be obtained), significant advances and much experimental work will be required. We suggest that the problems are much more likely to be overcome by genetic engineering of the pig than by drug administration to the recipient. We are optimistic that the goal of successful clinical lung xenotransplantation can be achieved within the foreseeable future. However, it will require the allocation of significant resources (manpower, facilities, and funding) if success is to be assured. Work aimed at achieving this goal will inevitably be immensely beneficial to the development of pigs that are suitable as sources of the other major organs.

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Disclosure of conflict of interest

David Ayares and Carol Phelps are employees of Revivicor Inc. No other author has a conflict of interest.

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