Sustained function of genetically modified porcine lungs in an ex vivo model of pulmonary xenotransplantation

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BACKGROUND: Xenotransplantation could provide a solution to the donor shortage that is currently the major barrier to solid-organ transplantation. The ability to breed pigs with multiple genetic modifications provides a unique opportunity to explore the immunologic challenges of pulmonary xenotransplantation.

METHODS: Explanted lungs from wild-type and 3 groups of genetically modified pigs were studied: (i) α1,3-galactosyltransferase gene knockout (GTKO); (ii) GTKO pigs expressing the human complementary regulatory proteins CD55 and CD59 (GTKO/CD55-59); and (iii) GTKO pigs expressing both CD55-59 and CD39 (GTKO/CD55-59/CD39). The physiologic, immunologic and histologic properties of porcine lungs were evaluated on an ex vivo rig after perfusion with human blood.

RESULTS: Lungs from genetically modified pigs demonstrated stable pulmonary vascular resistance and better oxygenation of the perfusate, and survived longer than wild-type lungs. Physiologic function was inversely correlated with the degree of platelet sequestration into the xenograft. Despite superior physiologic profiles, lungs from genetically modified pigs still showed evidence of intravascular thrombosis and coagulopathy after perfusion with human blood.

CONCLUSIONS: The ability to breed pigs with multiple genetic modifications, and to evaluate lung physiology and histology in real-time on an ex vivo rig, represent significant advances toward better understanding the challenges inherent to pulmonary xenotransplantation.

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gene knockout (GTKO) pigs was a significant step toward the clinical applicability of xenotransplantation, but did not completely mitigate against the downstream effects of complement and coagulation activation that result in thrombotic microangiopathy and consumptive coagulopathy. This is not surprising given the presence of non-Gal antigens and non-antibody-mediated processes that activate complement and coagulation pathways.

Extending previous work, in this study we took GTKO pigs that were genetically modified to specifically protect against complement and coagulation pathway incompatibilities. CD55 regulates the middle stages of the complement activation pathway at the level of the C3 and C5 convertases. CD59 regulates the terminal stage, formation of the membrane attack complex, and thus “mops-up” complement activation that escapes the CD55 checkpoint. CD39 is a cell surface molecule that converts adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP), which is then converted to adenosine by CD73. The net effect is removal of the pro-inflammatory signal ATP and the platelet agonist ADP, and generation of adenosine, which has anti-inflammatory effects.

For this study of pulmonary xenotransplantation we used an ex vivo perfusion rig as our experimental platform. This model has the advantage of reduced cost, the ability to perfuse the pig lung with human blood and, importantly, the ability to continuously interrogate xenograft function at a physiologic, biochemical and histologic level. We present preliminary results using an ex vivo technique demonstrating improved graft function from porcine lungs explanted from genetically modified pigs when perfused with human blood.

Methods

Generation of transgenic pigs

GTKO pigs were generated by homologous recombination and cloning by nuclear transfer, as previously described. These pigs lack expression of the Gal epitope on all organs and tissues but are otherwise phenotypically normal. Transgenic pigs coexpressing the human complement regulators CD55 and CD59 were generated by conventional oocyte microinjection. Transgenic pigs expressing human CD39 were produced by transfection of pig fetal fibroblasts followed by cloning using stable transfectants as the nuclear transfer donors. GTKO pigs were mated with CD55-59 and CD55-59/CD39 transgenic pigs producing GTKO/CD55-59 and GTKO/CD55-59/CD39 pigs that were available for this study. Representative staining of the Gal epitope and CD55 in experimental pigs is shown in Figure 1. The tissue expression of CD59 in transgenic CD55-59 pigs has been demonstrated elsewhere. Use of the pigs was approved by the animal ethics committee of St Vincent’s Hospital, Melbourne.

Surgical technique

For each experiment, lungs were obtained from cross-bred Large White/Landrace pigs weighing 30 to 35 kg. The donor pig was anesthetized, intubated, paralyzed and ventilated (10 ml/kg room air at 20 breaths/min through a volume cycle ventilator). After intravenous injection of 10,000 U heparin, a mid-line sternotomy was performed. The right ventricle and left atrium were opened and the pulmonary artery cannulated via the right ventricle. Ventricular fibrillation was induced by electrical stimulation using a needle electrode. Lung preservation was achieved by flush cooling the pulmonary artery with Perfadex solution (Vitrolife, Sweden). The left atrial appendage was incised for venting. The catheter was then removed and the heart–lung block excised.

Surgical configuration of the ex vivo rig

The ex vivo lung evaluation model used in this study was similar to that initially described by Steen et al., and essentially consists of a centrifugal pump, a membrane deoxygenator and a ventilator (Figure 2). For the first 5 experiments (wild-type, n = 2; GTKO/CD55-59, n = 1; and GTKO/CD55-59/CD39, n = 2) the explanted heart remained in situ and was used to anchor the cannulae. The pulmonary artery was perfused by a cannula, introduced via the right ventricle with its tip sitting in the right atrium (Figure 2). The left atrial appendage was incised for venting. The catheter was then removed and the heart–lung block excised.

Figure 1 Representative staining of porcine lung tissue. (A) Gal staining in wild-type and GTKO pig lungs. (B) Human CD55 and isotype control staining in CD55-59 transgenic pig lung.
Establishing porcine lungs on the ex vivo rig

After appropriate de-airing, the initial 200 ml of blood perfusate flush was discarded and the extracorporeal circuit connected at a low flow rate. The perfusate, and thus the lungs, were gently warmed and then ventilated (10 ml/kg, positive end-expiratory pressure [PEEP] 5 cm H₂O, fraction of inspired oxygen [FI O₂] 50%, respiratory rate 8 breaths/min) once the lung temperature reached 32°C. The initial extracorporeal perfusate comprised 2 liters of Steen solution (Vitrolife, Sweden), trometamol buffer 20 ml (1.25 g/ml) and meropenem 500 mg. Steen solution is a buffered extracellular solution with added albumin to optimize colloid osmotic pressure. The perfusate was maintained at a physiologic pH (7.4) by addition of the buffer. Prior to each experiment, 60 minutes was required to establish the porcine lungs on the rig. During this period the temperature of the lungs and perfusate flow was steadily increased and the lungs were ventilated (Table 1). Addition of human blood to the perfusate occurred once the porcine lungs demonstrated stable physiologic function (absence of increased pulmonary vascular resistance, left atrial pressure 3 to 5 mm Hg and adequate ventilation at low airway pressure). Left atrial and pulmonary vein pressure was measured as a surrogate marker for satisfactory pulmonary venous drainage of the ex vivo circuit. Oxygen, carbon dioxide and nitrogen were supplied to the membrane oxygenator at flow rates that were adjusted so that the blood entering the lungs mimicked that of normal venous blood. Institutional ethics approval was granted for use of human blood.

**Study design**

Initial experiments were performed to demonstrate stable physiologic function of the lungs over a 6-hour time period of continuous perfusion with Steen solution. Having demonstrated that the ex vivo rig could sustain stable lung function over many hours, subsequent studies assessed porcine lung function when perfused with human blood. To minimize variability between experiments, fresh human blood was sourced from the same 3 healthy volunteers (all blood group A with stable anti-Gal antibody titers) for each experiment. The hematocrit was maintained at >20% with the addition of approximately 600 ml of human blood. Ten separate experiments were performed using wild-type (n = 3), GTKO (n = 2), GTKO/CD55-59 (n = 3) and GTKO/CD55-59/CD39 (n = 2) pigs, with the investigators running each experiment blinded to the genetic background of each pig.

**Study outcome variables**

The perfusion and ventilatory performance of the swine xenograft was evaluated longitudinally (measuring pulmonary artery pressure, pulmonary vascular resistance and left atrial partial pressure of oxygen [PO₂]), as previously described. The arteriovenous oxygen difference was calculated as: \( \Delta \text{AVO}_2 = (1.34/\text{C} \times \text{Hb}/\text{C} \times S_{\text{a}r} - (1.34/\text{C} \times \text{Hb}/\text{C} \times S_{\text{v}e}), \) with Hb indicating hemoglobin and S is the arterial (S̄a) or venous (S̄ve) oxygen saturation. Pulmonary vascular resistance was calculated by dividing the pulmonary artery pressure by the measured perfusate flow (mm Hg/liter/min). The study end-point for perfusion was development of overwhelming pulmonary edema ascending into the trachea, preventing mechanical ventilation. The study end-point for ventilation was defined as an arteriovenous difference in PO₂ < 50 mm Hg. If pulmonary function remained stable, all experiments were terminated 4 hours after commencement of perfusion with human blood, at which point the lungs were

| Table 1 Establishment of Porcine Lungs on the Ex Vivo Rig |
|-----------------|-------|-------|-------|-------|-------|-------|
| Perfusion time (min) |
| 0 | 10 | 20 | 30 | 40 | 50 | 60 |
| Perfusion temp (°C) | 25 | 27 | 29 | 31 | 33 | 35 | 37 |
| Flow (liters/min) | 0.3 | 0.5 | 0.7 | 1.2 | 1.7 | 2.2 | 2.5 |
| Minute ventilation (liters/min) | — | — | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
sent for histologic evaluation. With the lung established on the rig, lung tissue and blood samples were collected at 0.5, 15, 30, 60, 120, 180 and 240 minutes after perfusion with human blood.

### Analysis of tissue biopsies

Depending on which genetically modified pig was being studied, the donor swine lung was evaluated for the relevant expression of Gal, CD55-CD59 and CD39. Features suggestive of acute lung injury were assessed in each lung biopsy and a quantitative score of injury was assigned by a histopathologist blinded to the details of the pig used in each experiment (Table 2). Immunohistochemistry was performed on frozen sections to assess for complement and coagulation activation (monoclonal antibodies against IgG, IgM, C3, C5b-9, CD61, von Willebrand factor and fibrinogen [all from DakoCytomation, Glostrup, Denmark], and C4d [Biomedica Medizinprodukte GmbH & Co. KG, Vienna, Austria]). Lung tissue from an autologous experiment (pig lung perfused with pig blood) was used as a negative control in all staining experiments.

### Assessment of perfusate

Once the lung block was established on the rig, perfusate samples were obtained prior to introduction of human blood and at serial time-points afterward. The cellular composition with respect to the fraction of neutrophils, lymphocytes, monocytes, macrophages and platelets was determined at each time-point. As a marker of complement activation, the levels of soluble C3a were serially assessed.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4 (AMPL. Software, Turramurra, Australia). Numerical data were analyzed using parametric statistical methods and are presented as the mean and standard error of the mean. Repeated 1-way analysis of variance (ANOVA) was used to assess differences in data collected from the 4 types of porcine lung studied (wild-type, GTKO, GTKO/CD55-59, GTKO/CD55-59/CD39). If \( p < 0.05 \), post hoc 2-group comparisons were performed with Tukey’s range test. Comparison of 2 variables was assessed with chi-square analysis, and the odds ratio (OR) and 95% confidence interval (CI) were calculated and presented. Two-tailed \( p \)-values were calculated using an unpaired \( t \)-test. Statistical significance was defined as \( p < 0.05 \).

### Results

#### Establishing the ex vivo rig

For the 10 experiments, the mean time from instillation of pneumoplegia to lung explant and ice preservation was \( 20 \pm 2 \) minutes. Cannulation to the ex vivo rig and initial perfusion with Steen solution took a further \( 88 \pm 2 \) minutes, after which it took \( 74 \pm 4 \) minutes to establish stable physiologic ventilator and perfusion function. Wild-type porcine lungs demonstrated stable physiologic function for 6 hours while being continuously perfused with Steen solution (data not shown).

#### Lung survival and performance

Wild-type lungs failed rapidly (\( \Delta \text{AVO}_2 < 50 \text{ mm Hg} \)) after addition of human blood, surviving only \( 21 \pm 4 \) minutes. In comparison, all but one of the genetically modified lungs demonstrated adequate oxygenation (\( \Delta \text{AVO}_2 > 50 \text{ mm Hg} \)) at 240 minutes (pre-determined study end) (\( p < 0.0001 \)). One of the GTKO/CD55-59 lungs showed inadequate oxygenation (\( \Delta \text{AVO}_2 < 50 \text{ mm Hg} \)) at 190 minutes. The best gas exchange achieved after addition of human blood, as assessed by the \( \Delta \text{AVO}_2 \), was significantly higher in the genetically modified porcine lungs (mean \( \Delta \text{AVO}_2 131 \pm 13 \text{ mm Hg} \)) compared with the wild-type lungs (\( \Delta \text{AVO}_2 84 \pm 7 \text{ mm Hg} \)) (Figure 3A) (\( p < 0.05 \)). Baseline pulmonary vascular resistance (PVR) for all lungs (5.3 \( \times 10^9 \) cells/liter reducing to 3.3 \( \times 10^9 \) cells/liter), whereas there was a significantly smaller 16% decrease seen with the GTKO/CD55-59 and

### Differential blood cell counts within the perfusate

The total white cell count in the perfusate decreased over the course of all experiments. Compared with baseline values, there was a mean 45% decrease in white cell count in the experiments involving the wild-type and GTKO porcine lungs (5.3 \( \pm 0.8 \times 10^9 \) cells/liter reducing to 3.3 \( \pm 0.5 \times 10^9 \) cells/liter), whereas there was a significantly smaller 16% decrease seen with the GTKO/CD55-59 and
GTKO/CD55-59/CD39 lungs (5.2 ± 0.3 × 10⁹ cells/liter reducing to 4.4 ± 0.4 × 10⁹ cells/liter; p = 0.02). Notably, neutrophils were preferentially lost from the perfusate with >85% of the neutrophils sequestered into the lung within 5 minutes of the start of each experiment. Change in platelet count within the perfusate varied between experiments. Compared with baseline values, the platelet count remained relatively stable in the experiments involving the GTKO, GTKO/CD55-59 and GTKO/CD55-59/CD39 porcine lungs; however, with the wild-type lungs, graft dysfunction was associated with a drop in platelet count in all experiments (Figure 4A). The degree of platelet sequestration within the xenograft 30 minutes after addition of human blood to the perfusate was significantly correlated with lung oxygenation (r = 0.8, p < 0.001) (Figure 4B).

**Histology**

Baseline experiments demonstrated that porcine wild-type lungs on the ex vivo rig maintained normal histologic appearances after prolonged perfusion with either autologous blood (Figure 5A) or Steen solution (Figure 5B). Perfusion of wild-type lungs with human blood was associated with rapid organ failure. Gross macroscopic appearances included widespread pulmonary hemorrhage and pulmonary edema (Figure 5C). Histologic features associated with lung failure included blood vessel congestion, accumulation of neutrophils in alveolar walls, intra-alveolar and interlobular hemorrhage, fibrin thrombus formation and hemorrhagic infarction (Figure 5D). Similar to the wild-type lungs, the lungs from the GTKO pigs showed evidence of blood vessel congestion, intra-alveolar hemorrhage and fibrin along the alveolar walls, and visceral pleura within 15 minutes of perfusion with human blood.

Correspondingly, at this early time-point, the composite lung injury score was higher for both the wild-type (score of 25 out of 72) and GTKO (30/72) compared with the GTKO/ CD55-59 (14/72) and GTKO/CD55-59/CD39 lungs (14/72) (repeated measures ANOVA, p = 0.03). Despite stable physiologic function and normal macroscopic appearances in the GTKO/CD55-59 and GTKO/CD55-59/CD39 lungs, there was still evidence of blood vessel congestion and intra-alveolar hemorrhage at study end.

**Immunohistochemistry**

At baseline, immunofluorescence for C5b-9, CD61, von Willebrand factor (vWF), IgG and IgM was negative, although positive staining for fibrin was seen in all lungs along the visceral pleural surface, and extended to the alveolar walls after addition of human blood. A comparative analysis was performed for each xenograft 1 hour (or at experiment termination for rapidly failing lungs) after addition of human blood. In all experiments, positive staining for CD61, a marker for platelet glycoprotein IIIa, and vWF was seen in the alveolar walls. Positive staining for IgG and C5b-9 was only seen in the wild-type experiments, whereas staining for IgM was occasionally seen but was not consistently different between treatment groups.

**Complement activation**

Levels of C3a measured in the perfusate 30 minutes (or at study end if earlier) after addition of human blood were significantly higher in the wild-type experiments compared with those involving the genetically modified porcine lungs (1,057 ± 483 ng/ml vs 436 ± 58 ng/ml; p < 0.05). There
was no significant difference in C3a levels when the various genetically modified porcine groups were analyzed.

**Discussion**

In this series of preliminary experiments on ex vivo performance of porcine lungs after addition of human blood, we have demonstrated that genetic modification of the donor results in superior graft function compared with wild-type lungs. Specifically, we were able to demonstrate superior oxygenation of the perfusate and reduced pulmonary vascular resistance when human blood was perfused through GTKO, GTKO/CD55-59 and GTKO/CD55-59/CD39 lungs compared with wild-type porcine lungs. Although there was a trend suggesting that xenograft performance increased in line with the greater number of genetic modifications present, further experiments would need to be performed to confirm these observations.

A number of pulmonary xenotransplant studies have confirmed that the absence of the Gal epitope protects against hyperacute rejection. Likewise, an overall increase in the expression of complement-regulatory proteins CD55 and CD59 (and CD46) protects against complement-mediated damage to the pulmonary xenograft. A recent study looking at single genetic modifications, and using an ex vivo model similar to our own, demonstrated that GTKO porcine lungs survived longer when perfused with human blood compared with lungs overexpressing complement-regulatory proteins. In the current study, the overexpression of CD55-59 on a GTKO background resulted in similar xenograft performance to that seen with the single GTKO modification. Of note, positive staining for C5b-9, the complement membrane attack complex, was only seen in the wild-type lungs, and there were no significant differences in the immunohistologic changes relating to complement activation between the GTKO and GTKO/CD55-59 porcine lungs. These findings suggest that the Gal epitope as a target for anti-Gal antibodies is a major driver of complement-mediated hyperacute rejection.

Characteristic pathohistologic changes consistent with hyperacute rejection developed rapidly in the wild-type lungs and their appearance was associated with almost immediate graft failure. Although pulmonary function was significantly prolonged in the genetically modified lungs, at study end, histologic analysis demonstrated pathologic changes consistent with intravascular thrombosis, platelet deposition and coagulation. However, the histologic changes were less overt in the GTKO/CD55-59/CD39 lungs compared with the other groups, suggesting that the overexpression of CD39 may result in greater protection against the injuries inherent to pulmonary xenotransplantation. In keeping with the histologic changes, GTKO/CD55-59/CD39 showed superior graft performance when perfused with fresh human blood.

Human CD39 aids in the breakdown of ATP to ADP and then to AMP, and is postulated to have both thromboregulatory and anti-inflammatory effects. The observation that there was reduced platelet sequestration from the perfusate into the GTKO/CD55-59/CD39 xenograft suggests that the overexpression of CD39 may be reducing platelet activation, albeit not completely given that platelet deposition was observed in all treatment groups during the course of each experiment. The importance of thromboregulation and minimizing platelet activation and deposition within the lung is highlighted by the inverse correlation between platelet sequestration and oxygenation of the perfusate (ΔAV oxygenation).

The thromboregulatory benefit of CD39 may be enhanced by the co-expression of CD73, which further enhances the hydrolysis of extracellular AMP to adenosine. Thrombophilia within the graft vasculature remains a significant barrier to the enduring success of xenotransplantation, and strategies to achieve thromboregulation may need to include novel anti-thrombotic and anti-platelet
vWF was detected by immunofluorescence in all study groups, and clearly remains a therapeutic target to further improve xenograft performance. Porcine vWF, unlike the human equivalent, does not require shear stress or platelet activation as a precursor to activation. To date, the longest survival of a porcine–primate pulmonary xenotransplant has been achieved using lungs transplanted from a vWF-deficient pig that was also depleted of pulmonary vascular macrophages.

The ex vivo rig as a tool for assessing lung function was first pioneered by Steen et al. and has become an important prelude to assessing xenograft function before consideration of large animal xenotransplantation. This model has the advantage of reduced cost, the ability to perfuse the pig lung with human as opposed to baboon blood, and importantly the ability to continuously interrogate xenograft function at a physiologic, biochemical and histologic levels. Of note, the ex vivo assessment of lungs has now entered the clinical interface of human lung transplantation, with ex vivo lung perfusion (EVLP) increasingly being used to assess the suitability of marginal donor lungs prior to their eventual transplant into a suitable recipient. As further genetic modifications are introduced, ex vivo assessment becomes an essential preliminary evaluation tool of the porcine lung that is performed prior to subsequent non-human primate transplantation and, potentially, eventual human transplantation.

In conclusion, on a GTKO background, we have been able to introduce a number of further genetic modifications aimed to protect porcine lungs from hyperacute rejection, a significant barrier to the clinical applicability of pulmonary xenotransplantation.

**Disclosure statement**

The authors have no conflicts of interest to disclose.

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