The Legacy of Tom Starzl Is Alive and Well in Transplantation Today[1]

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Introduction

It is an honor to celebrate the life and career of Dr. Thomas Starzl with the American Philosophical Society. As Dr. Clyde Barker so elegantly summarized in his talk, "Tom Starzl and the Evolution of Transplantation,"[2] Dr. Starzl was a larger-than-life figure whose allure drew young surgeons to the field of liver transplantation and spurred them to careers as surgeon-scientists. Dr. Starzl's impact still lives on today through those he inspired and by the influence of his body of work. To illustrate the enduring nature of his scientific contributions, I will review three areas of active transplant research that have the potential to radically advance the field, which connect with foundational work by Dr. Starzl. The compendium of Dr. Starzl's work, with over 2,200 scientific papers, 300 books and chapters, 1,300 presentations, and hundreds of trainees, far exceeds even the most prolific efforts of other contributors to transplantation; corroborating his preeminence in the field, in 1999 he was identified as the most cited scientist in all of clinical medicine.[3] It was a relatively simple task to find robust threads of his early investigations in the advances of today because his work permeates nearly every aspect of the field.

The areas of work on which I will focus seek to address the two primary challenges facing the field of transplantation—the need for toxic lifelong immunosuppression to prevent allograft rejection, and an inadequate organ supply to treat all those who would benefit from organ replacement. These two problems confer untold morbidity and mortality to those who await lifesaving organs as well as to those who

1 Read 9 November 2017.

2 Dr. Clyde Barker's paper was presented at the American Philosophical Society General Meeting on 9 November 2017 and is published in this issue as a biographical memoir.

3 Thomas E. Starzl's UPMC web page, https://www.starzl.pitt.edu/.

receive transplants. Fortunately, innovative treatment approaches that are tantalizingly close at hand will allow transplant without chronic immunosuppression, and novel preservation technology and xenografts modified by powerful new gene editing protocols will dramatically expand the organ supply.

Chimerism-Based Transplantation Tolerance

Advances in immunosuppression have been integral to improving transplant outcomes as evidenced by a strong correlation over the last three decades among the development of more potent, more specific, and less morbid agents with improved recipient and graft survival (Meier-Kriesche et al. 2004). For example, with modern immunosuppression regimens, one-year survival for low-risk deceased donor kidney recipients is greater than 97 percent with rejection rates of only about 5 percent. Despite these advances, the current agents still carry risk of significant morbidity and mortality, including increased risk of CV disease, infection, and malignancy. In liver and lung recipients, the most effective antirejection agents of the day, calcineurin inhibitors, manifest significant renal toxicity leading to a 10–20 percent incidence of renal failure requiring dialysis by 10 years post-transplant. Strategies that allow successful transplantation without chronic immunosuppression would represent a tremendous advance for transplant patients.

In 1992 Dr. Starzl made the fascinating observation that donor organ–derived cells can be detected in the tissue of recipients for many years after the transplant, a phenomenon termed *microchimerism* (Starzl et al. 1992; Starzl, Demetris et al. 1993). Dr. Starzl's revelation that there is a bidirectional interaction between host and donor lymphoid systems provides a powerful conceptual framework for ongoing progress. Moreover, this work is intimately interwoven with the Nobel Prize investigations of Sir Peter Medawar, widely hailed as "the father of transplantation." Medawar recognized that the acceptance of skin grafts between fraternal cattle twins was a consequence of hematopoietic chimerism derived in utero from a shared placental circulation unique to cattle twins. In now-classic work, he applied this knowledge to demonstrate that a state of a long-lived lymphoid chimerism could be achieved by inoculation of neonatal rodents with a foreign strain's lymphocytes (Billingham, Brent, and Medawar 1953). The resulting donor strain chimerism led to reeducation of the host immune system such that grafts from the same donor strain were then recognized as "self" and accepted indefinitely without any immunosuppression. These results led Starzl to predict in 1962 that development

of lymphoid chimerism would be the key to developing clinical transplantation tolerance, a hypothesis not actualized until 40 years later.[4]

Over the last six decades, a concerted effort has been directed at understanding the processes of tolerance to self-antigens and applying this knowledge to gaining acceptance to foreign cells and grafts. What has been learned is now guiding development of new approaches to secure transplant survival without immunosuppression. One of the most promising lines of experimentation combines a bone marrow transplant with a kidney transplant from the same donor to gain transient or permanent hematopoietic chimerism and secure tolerance to the kidney. The first reported success at intentional clinical chimerism-induced allogeneic tolerance to solid organs was by the Massachusetts General Hospital (MGH) team in 2008 in the *New England Journal of Medicine* (Kawai et al. 2008). In the published experience to date, this line of study has gained stable transplantation tolerance in 7 of 10 patients treated with the protocol. The MGH protocol entails an intensive conditioning regimen the week prior to transplant to facilitate engraftment of the donor marrow. The dependence on pre-transplant treatment currently limits application of this strategy to live donor kidney transplants. The conditioning consists of agents to deplete B and T lymphocytes and thymus irradiation. Donor bone marrow and the kidney transplant are performed concurrently. The patient then remains on limited conventional immunosuppression for about nine months and is then weaned off over one to two months.

The outcome of the first 10 patients (Figure 1) shows kidney function over time (creatinine levels) and the point of immunosuppression discontinuation. That 7 of 10 patients have experienced long-term (more than five years) drug-free survival qualifies this as a powerful, precedent-establishing result. The first patient in this series is a particularly compelling case. This patient had a prior kidney transplant as a teenager with conventional immunosuppression treatment. Unfortunately, she suffered miserably from complications of the antirejection medications, the most problematic being warts on the bottom of her feet from papilloma virus, induced by immunosuppression. The warts were so severe she could not walk; therefore, in full consultation with her physicians, she elected to discontinue immunosuppression and allow the first transplant to reject, requiring resumption of dialysis. When she heard rumors of the coming MGH tolerance trial, she promptly volunteered to be the inaugural subject. She is now 15 years post her second kidney transplant with normal kidney function despite being off all immunosuppression for more than 14 years. She has

4 Thomas E. Starzl's UPMC web page, https://www.starzl.pitt.edu/.

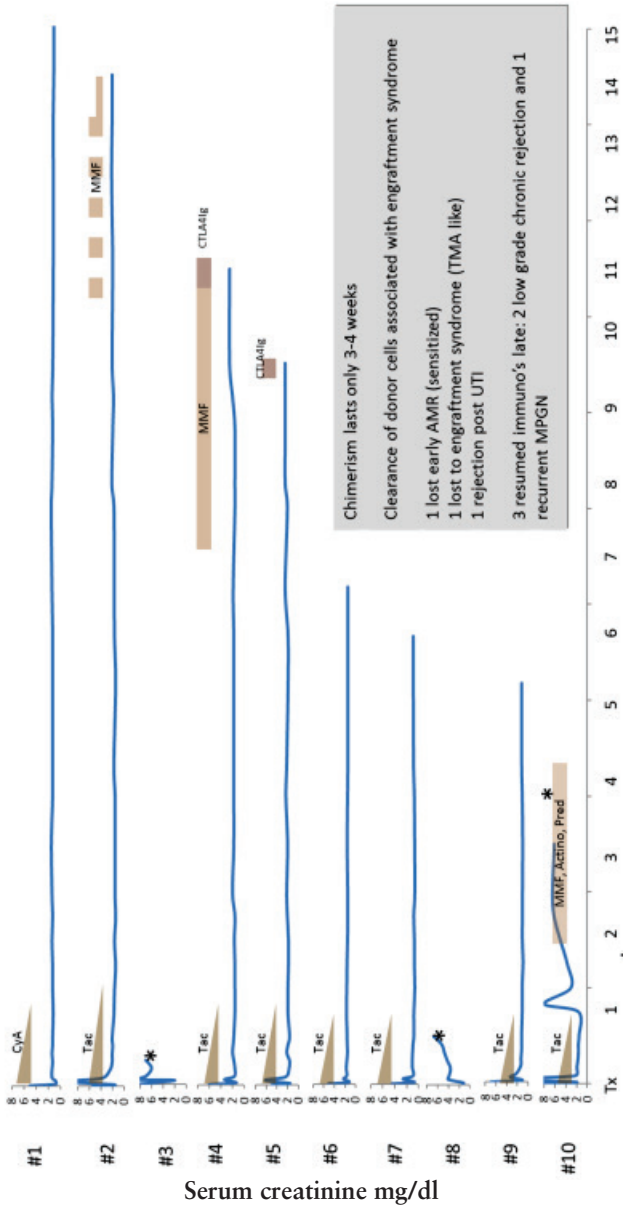

FIGURE 1. The first successful regimen of clinical transplantation tolerance combining renal transplant with donor bone marrow. Successful experience with the first 10 combined live donor bone marrow and kidney transplantation at MGH. The graph shows renal function over time by creatinine levels (blue lines) and the immunosuppression duration (triangles). Note that the first patient has now been immunosuppression-free for more than 14 years with normal function. The gray box details aspects of the treatment as well as the cause of graft loss in three patients, and the reason for resumption of immunosuppression late in their course in another three. Data updated from Kawai et al. (2008) and provided with permission.

returned to a normal life and is running marathons. She is unencumbered by daily medications and frequent blood checks, and is a strong advocate for tolerance.

As might be expected with the first iteration of any complex and bold innovation, there were unanticipated outcomes, and this was evident in this initial series. One case of antibody-mediated graft injury caused a graft loss, and graft endothelial injury by a poorly understood process termed *engraftment syndrome* was common in participants and so severe in one patient that the graft was irreversibly injured. Both issues have been addressed by subsequent protocol modifications. Another interesting aspect of this regimen is that the donor cells persist in the circulation for only three to four weeks, yet tolerance was durable, lasting for years. A plausible explanation for this mechanistic paradox is that donor microchimerism, as detailed by Starzl, is augmented by the infusion of donor bone marrow.

There are several other groups now very active in this area of research with their own permutations of the bone marrow–kidney approach. Perhaps the most interesting variant comes from the team at Northwestern University who have devised a protocol that promotes full chimerism with near-complete and long-term replacement of the host's immune system with cells of donor origin, a result with both advantages and disadvantages (Leventhal et al. 2013). On the plus side, full chimerism may more reliably secure durable tolerance; a downside, though, is that full chimerism incurs increased risk of the donor cells attacking the host, a process known as *graft versus host disease*. This occurs not infrequently in bone marrow transplant recipients, and in some cases, can be life-threatening.

A critical next step to allow broader application of these chimerism-based regimens is their refinement to permit use with deceased donor kidney transplants as well as other organs such as heart, lung, and liver, for which the recipients are typically too sick and the operations too complex for such intensive conditioning at the time of transplant. Members of the MGH team have recently devised such an approach termed *delayed tolerance* in which the recipient's conditioning and donor marrow infusion are held until a few months post-transplant. Importantly, this regimen has proven highly effective in nonhuman primate studies and is planned for human testing beginning in 2019 (Figure 2). From a mechanistic perspective, the delayed regimen is attractive in avoiding inflammation early post-transplant that might impede tolerance development. On the other hand, the few-month delay might permit donor reactive memory T cells to be generated before conditioning that could thwart tolerance development.

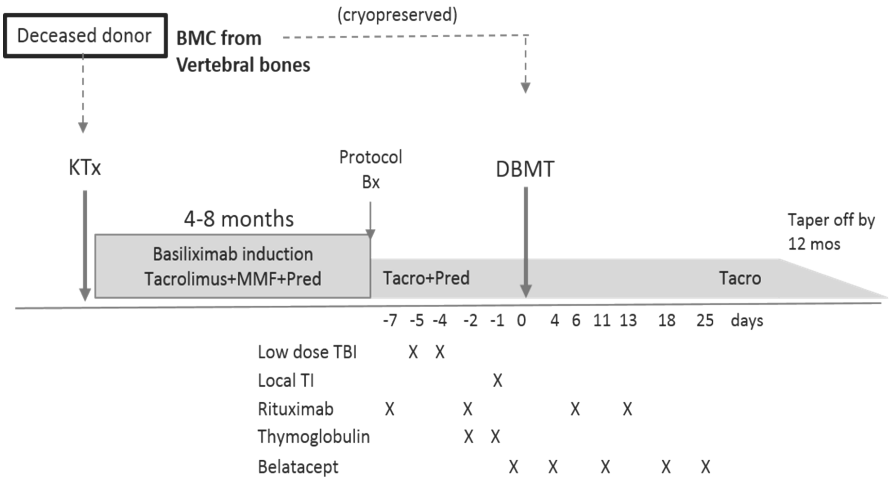

FIGURE 2. Planned regimen for induction of delayed clinical tolerance. To allow application of tolerance to deceased donor recipients of kidney and potentially other organs, a delayed induction of tolerance is planned. Patients will receive a transplant under conventional immunosuppression and after four to eight months will undergo conditioning and donor bone marrow infusion with the goal of immunosuppression within the following year. From Kawai et al. (unpublished), with permission.

The studies described here show great promise for broad clinical application and have been a tremendous stimulus to the field. Numerous tolerance trials are now under way, many using derivations of the work described above that rely on chimerism. Hopefully, a safe and widely applicable tolerance protocol will become available soon.

Increasing the Organ Supply by Physiologic Organ Preservation

The greatest impediment to transplantation realizing its full potential in saving the lives of patients with irreversible organ failure is an inadequate supply of organs to treat all those who could benefit. More than 120,000 Americans currently await transplant but only ~34,000 transplants were performed in 2017 and, tragically, about 8,000 Americans died each year while waiting.[5] These statistics grossly underestimate the potential need for organs for all those who could derive benefit from transplantation since the field has set restrictions on who is a transplant candidate in order to optimize utility of the limited organ

5 "Organ, Eye, and Tissue Donation Statistics," Donate Life America, accessed November 9, 2017, https://www.donatelife.net/statistics/.

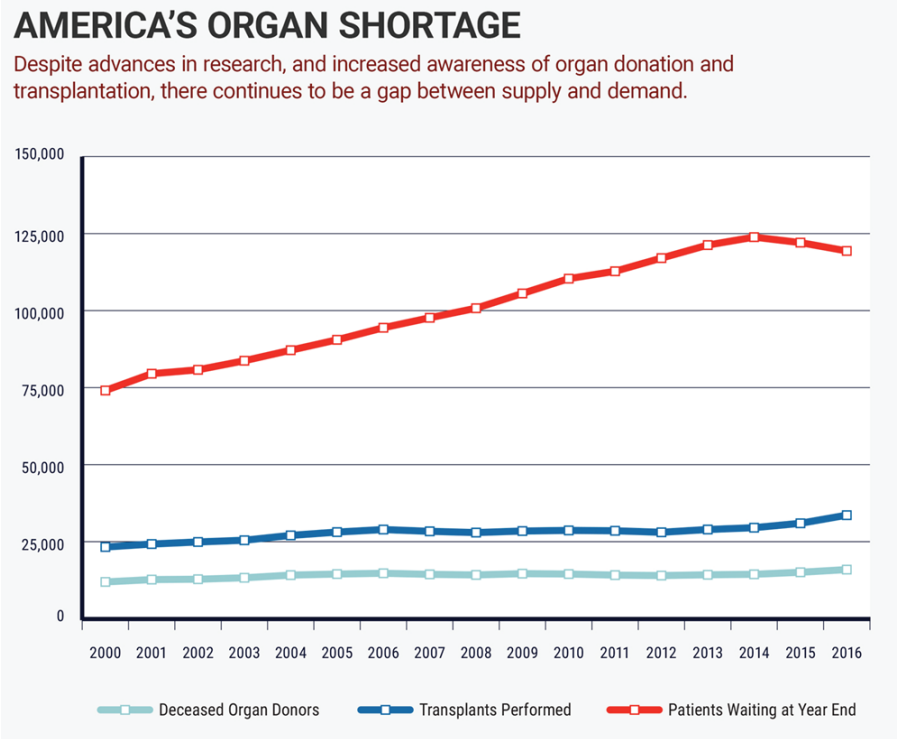

FIGURE 3. The transplant organ shortage. A disparity between the number of organs available and the number of actual donors exists for each of the commonly transplanted organs. Data from the Organ Procurement and Transplantation Network (OPTN) shows the collective data for all organs. More than ~120,000 patients await transplantation and only 30,000 organs were transplanted last year in 2017. Based on OPTN data as of November 9, 2017.

supply. In addition to those who die waiting, patients often experience a wait that exceeds seven years before gaining access to a kidney transplant in many areas in the United States.

This frequently shown graph depicts the disparity between the number of organs available and the number of patients awaiting transplant (Figure 3). For many years, we referred to the "ever-growing disparity or organ supply and demand," but for the first time, in 2015 and 2016, there was an increase in the number of donors and a slight narrowing of the gap between need and availability. Unfortunately, this is largely attributable to the opioid epidemic which will hopefully be short-lived.

A new preservation approach shows promise to make more organs available by allowing better assessment of transplant suitability of the more than 5,000 organs currently being discarded after recovery

(Stewart et al. 2017). The standard for organ preservation for the last 30–40 years has been to store organs recovered from a deceased donor in a preservation solution on ice to slow organ metabolism (Southard and Belzer 1995). In fact, Starzl was the first to use cooled fluids to chill the excised organs and the first to carry out organ recovery by cold perfusing the organs in situ.[6] For livers, this approach provides safe and reliable storage for up to 12 hours, though most centers generally aim to limit the period of cold ischemia to 6–8 hours, especially for marginal liver grafts. However, it is well established that cold storage does not completely arrest the metabolic activity of the organ (Guibert et al. 2011). Thus, the liver quality progressively deteriorates as storage time increases. A relevant marker of this decline is the energy or ATP stores of the graft that are slowly but inexorably consumed as it sits on ice. As ATP falls, membrane gradients are lost and cells swell and suffer injury that eventually becomes irreversible. For organs that have been exposed to warm ischemia during recovery, such as organs from donors where life support is withdrawn and the recovery surgery must await cessation of cardiac activity (donation after circulatory death [DCD]), we have found that ATP is >90 percent exhausted within about 30 minutes of warm ischemia. During cold storage at 4°C, ATP levels reach the same degree of depletion after about 12 hours (J. F. Markmann, unpublished data).

A potential solution to this problem is found in use of novel perfusion devices currently in various stages of clinical trial assessment. Normothermic ex vivo organ perfusion maintains the stored organs under physiological conditions, including ~37°C, and by perfusion with oxygenated blood and nutrients. For liver, three commercial devices are currently in early phase clinical trials in the United States. It should be noted that the possibility that organs could be maintained viable ex vivo is not new, and Dr. Starzl was among the first to oxygenate organs, using a hyperbaric approach in the late 1960s (Marchioro et al. 1963; Starzl et al. 1984). Oxygen is an essential component to supporting normal metabolism ex vivo and likely the key factor in the resurgence of interest in ex vivo organ perfusion. In addition, the early attempts in the 1980s used devices that were large and cumbersome, making widespread application impractical. After a few decades of technological advances the devices are sleek, compact, portable, and sophisticated, allowing precise control of temperature, flow, and delivery of nutrients, oxygen, and vasodilators as needed. Figure 4 shows an example of perhaps the most sophisticated device

6 Thomas E. Starzl's UPMC web page, https://www.starzl.pitt.edu/.

FIGURE 4. Ex vivo organ perfusion to improve and increase the organ supply. An example of a liver perfusion device is shown (Transmedics Liver Organ Care System). This device, perhaps the most sophisticated under clinical trial, permits perfusion over a range of temperatures, allows precise control of perfusion rates and pressures, and is fully portable with battery supply to last 12 hours. It has three infusion ports to allow addition of nutrients, bile salts, and vasoactive drugs to alter vascular resistance. Bile production as well as metabolic activity is readily monitored. There is also a detachable remote control panel that allows for monitoring and adjustments of organ perfusion while separate from the device during transit.

currently being trialed for liver perfusion, the Transmedics Liver Organ Care System.

Lung, heart, liver, and kidney perfusion devices are currently each at different phases of clinical trial assessment. The lung is the most advanced with two devices now FDA-approved for clinical use in the United States. The data from the Toronto team who has pioneered the lung studies suggests a 30 percent increase in organ utilization using ex vivo perfusion (Cypel et al. 2011). Illustrative of the benefit of lung perfusion are donor lungs compromised by pulmonary edema, a common condition in organ donors. Traditionally, such organs were declined for transplant because of the risk of poor post-transplant performance. Using ex vivo perfusion, the lungs can be "dried out" on the pump, function improved, and suitability for transplant reliably assessed. These organs are now commonly transplanted.

Similarly, ex vivo liver perfusion has the potential to make more livers available for transplant. Even just converting the period of cold storage to warm-physiologic storage will be of benefit by improving

the condition of injured organs. Ex vivo perfusion also will allow a longer preservation period, facilitating elective recipient procedures which are known to have fewer technical mishaps and complications. This advance also will be most welcomed by transplant surgeons. Most important, there are many organs that are currently discarded because their quality cannot be fully discerned at the time of donation. For example, if offered a liver that a surgeon subjectively estimates will have a 20 percent risk of failure, most surgeons would likely decline use of the organ except for a most desperate recipient. This means that four out of five livers so classified are discarded unnecessarily. Placing the liver on the pump and observing its function will allow a more objective evaluation, resulting in more imperfect organs being successfully transplanted.

While on the pump, a wealth of information about the function of the donor liver can be gleaned, including bile production, oxygen consumption, and markers of injury. Clearance of lactate from the perfusate is a key marker of adequate oxygen delivery and absence of anaerobic metabolism. Albumin and urea production are also readily measured as are blood flow and vascular resistance. Given these attributes it seems almost inevitable that ex vivo liver perfusion will rapidly become standard for all but the most perfect organs, and this innovation alone could add 1,000 livers to the transplant pool.

While this would save many lives, there may be even more powerful opportunities using these devices in the future. Ex vivo perfusion is a disruptive and enabling technology that brings possibilities that are just now being conceived. The technology may permit repair of defective organs, such as the livers that are now discarded due to steatosis or fatty liver disease, a condition of growing prevalence due to the obesity epidemic. Early data suggests that fat can be reduced or eliminated from a liver while on the pump, potentially rending these organs suitable for transplant.

However, even more exciting is the possibility that liver regeneration can be induced ex vivo. The remarkable ability of the liver to regenerate allows a liver to be split into two functional segments, permitting live donor and split deceased donor liver transplant. Despite this opportunity, live donors constitute only about 3 percent of all livers transplanted each year. The comparatively low utilization of live donors is because adult-to-adult live donor transplants generally require 60 percent of the donor liver to support a normal-sized adult recipient, an operation with a mortality risk of 1/1000 and a >30 percent complication rate. Removal of a smaller and anatomically simpler segment of only 25 percent of the donor liver might be a tenfold safer operation, more similar in risk to donating a kidney.

Unfortunately, 25 percent of a liver is generally too little liver mass to support an adult recipient. However, if the small segment of liver mass could be expanded ex vivo to a size capable of supporting adult recipients, the approach could easily supply enough livers to eliminate the liver organ shortage. Thus, inducing a controlled and regulated regeneration ex vivo holds great interest.

Many of these novel strategies are likely to require an extended period on the pump ex vivo. While the safe duration of perfusion has not yet been established, some recent case reports from Europe indicate successful transplant can still be achieved after 24 hours of liver storage on a perfusion machine (Watson et al. 2017). In experiments using porcine livers, ex vivo perfusion has been sustained for up to four days with maintenance of apparently normal physiologic function. This advance will facilitate many novel areas of investigation in the future.

Xenotransplantation

"That xenotransplantation is the future of transplantation, and always will be" poignantly captures the inability of xenotransplantation to meet the lofty expectations set decades ago. Despite pervasive skepticism, recent advances in gene editing radically increase the potential for success. Not surprisingly, Dr. Starzl was responsible for perhaps the boldest xenotransplant effort yet reported. In the 1960s, he and his team at the University of Pittsburgh transplanted six baboon kidneys and three chimpanzee livers to human recipients and showed that their rejection was not immediate and that their function could sustain the recipients (Starzl et al. 1964; Starzl and Putnam 1969). With development of more effective immunosuppression, Dr. Starzl tried again, transplanting two baboon livers to human recipients. Equipoise here was based on the expected resistance of the baboon organ to hepatitis B virus, which infected the recipient and was a contraindication to the human organ transplant. The experience was a partial success with survival of the first graft for 70 days (Starzl, Fung et al. 1993; Starzl et al. 1994). The experiment was informative, highlighting the possibility of physiological incompatibility occurring with xenogeneic protein interactions. For example, the recipient manifested a uric acid level many fold below the human norm, but a level normal in baboons. Ultimately, it was conceded that baboon donors, while attractive in their phylogenetic proximity to humans, would be logistically difficult to use due to slow breeding, and because their use is ethically objectionable to the majority of society, causing a shift toward use of porcine donors.

Unlike nonhuman primates, use of pig donors are acceptable to most Americans, and pigs offer organs of correct size and can be readily

bred in great numbers. In the late 1990s, serious consideration of pig organ transplants was prompted by genetic modification of pigs to alter or eliminate endothelial cell antigens targeted by human antibody and by addition of human complement regulatory proteins to overcome species incompatibilities in the complement cascade (McCurry et al. 1995). The potential for an unlimited organ supply encouraged a huge investment by Big Pharma based on the potential of nascent transgene technology. However, this push was also accompanied by a scholarly public debate with concerns about moving forward and calls for a moratorium to halt this line of work (Bach and Fineberg 1998). The primary fear was the possibility of pigs transmitting a lethal infection (specifically, porcine endogenous retroviruses [PERVs] that are present in all pig strains) that could cause a pandemic in humans. The pharma initiative to design pigs compatible with humans was ultimately aborted due to slow progress and concern for a xenozoonotic catastrophe (Butler 1998).

During the ensuing decades of work, progress has been slow but steady, and pig xenograft survival in nonhuman primates remains modest. For lungs, survival has been limited to less than two weeks; liver one month (Shah et al. 2017); kidney 410 days (Iwase et al. 2017); and heart greater than two years survival in a heterotopic position (Mohiuddin et al. 2014). Survival in a life-supporting or orthotopic position has been markedly less. In addition, this longer-term success in heart and kidney has been achieved with immunosuppression not readily applicable to human transplantation, though the result still represents an important step forward. In work by the MGH team, pig liver graft survival sustaining the health of a baboon for just shy of a month suggests that the physiologic incompatibilities are not so severe as to lethally corrupt life-supporting biologic pathways, and may even allow consideration of a pig liver being used as a temporary bridge to transplant in the not-too-distant future.

Despite the gradually improving duration of pig graft survival in nonhuman primates, a variety of barriers persist. The elimination of the target of preformed human antibodies toward the major endothelial cell pig antigen was an important step, but once this was eliminated other antigens were then revealed. Equally important are incompatibilities in coagulation and complement pathways between pigs and humans. Efforts in the 1990s began to address this problem by introducing human complement regulatory proteins into the porcine germline to gain expression in the pig endothelium (McCurry et al. 1995). These too yielded only incremental progress. Interestingly, the most commonly encountered barrier in human allotransplantation—cell-mediated

immunity—has not yet been identified as a major problem in xenotransplantation. This may be a consequence of the fact that grafts have been so rapidly destroyed by humoral and innate immunity that cellular immunity didn't have time to manifest. Alternatively, current immunosuppression regimens effective at blocking T cell function in allotransplantation are also effective for xenotransplantation.

Perhaps the most prohibitive barrier, though, remains fear of human infection by PERVs. It is known that PERVs can infect human cells in vitro, though these studies have been conducted primarily with immortalized cell lines, and it remains unclear to what extent primary cells can be infected. Thus, while the precise risk of PERVs to humans remains unknown, the elimination of this potential risk would be a reassuring advance.

The reason there is now such hope for pig xenotransplantation is the availability of powerful new gene editing technology such as CRISPR-Cas9 that dramatically increases the precision and efficiency of genetic modification. CRISPR-Cas9 is based on a naturally existing bacterial defense system against viral infections that include DNA cutting enzymes (Cas9) and RNA segments or guide RNAs. These bacterial components can be adapted to target specific eukaryotic genome sites of interest to add or delete activity of specific genes (Jinek et al. 2012; Mali et al. 2013; Cong et al. 2013).

The approach used to generate modified pigs is to first modify a pig fibroblast cell line in vitro to disrupt all the PERV genes (Yang et al. 2015). The cell line is then used as a source of genetic material by recovery and transfer of nuclei to porcine embryos that are implanted in a surrogate sow. Using the CRISPR-Cas9 technology, George Church and Luhan Yang from eGenesis, a startup company, recently reported successful targeting and disruption of all 62 copies of PERVs in the pig genome. These pigs have now been successfully raised to maturity (Niu et al. 2017). The prior record for simultaneous genome modification was three to four, thus highlighting the power of this new methodology.

As with any gene editing attempt, there exists the possibility that mistargeting could cause unintended changes in other genes—so-called off-target effects. In contrast to embryo editing, or human in vivo editing, an advantage of porcine donor editing is that the genome of starting nuclei of the pig can be carefully sequenced to eradicate those with dangerous off-target edits. In addition, the lifespan of the pig donor provides an opportunity to assess for mutation and carcinogenesis before organs are recovered. In many ways, the CRISPR system is ideally suited to re-engineering pigs as donors.

While full success is unlikely to be achieved with the first iteration of genetic modification, the strength of the approach lies in the ability to make sequential alterations as the biology of the failure of the first set is understood. Importantly, survival of pig organs with conventional immunosuppression would represent a major advance by providing unlimited availability of organs. Ultimately, though, an unlimited supply of non-immunogenic organs should be possible, for which immunosuppression is not required.

Transplantation: The Future

Transplantation has been described as one of the medical miracles of the 20th century (Morris 2004). While certainly true, the statement belies the extraordinary untapped potential that, once harnessed, will dwarf the impact of transplantation during the prior century. Starzl's vision for areas like tolerance, organ preservation, and xenotransplantation will undoubtedly be a big part of 21st-century transplantation, where organ replacement will be safer, more widespread, and more effective at saving the lives of those with end-stage organ failure.

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