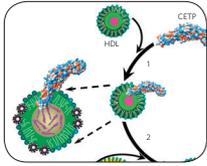


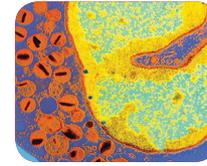
IN this section



CETP inhibitors raise doubts over 'good' HDL p5



FDA decrees GM salmon fit for dinner plates p7



Severe asthma gets first biologic in decades p10

Xenotransplantation makes a comeback

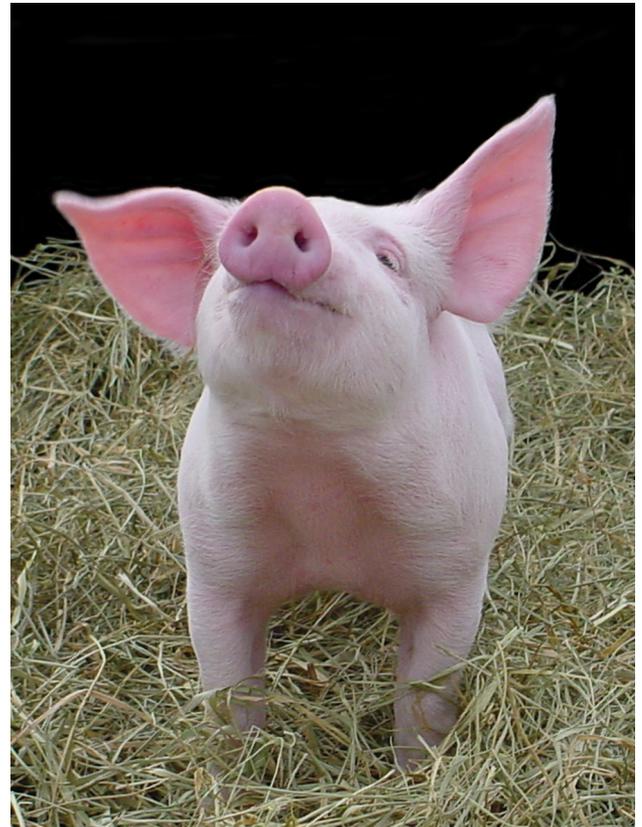
A newly announced genome-editing experiment—the largest documented to date—is the latest in a series of advances reinvigorating the field of xenotransplantation. In October, Harvard University geneticist George Church and colleagues at Boston-based startup eGenesis described (*Science* 350, 1101–1104, 2015) the use of CRISPR-Cas9 to disrupt all 62 genomic copies of porcine endogenous retrovirus (PERV) in cultured pig kidney epithelial cells. That feat, which dwarfs the greatest number of simultaneous DNA changes ever recorded using genome editing, removes one potential hurdle facing use of pig xenotransplants in human patients—zoonosis. But eGenesis is not the only company actively exploring the commercial prospects of xenotransplantation; Silver Spring, Maryland-based United Therapeutics has been quietly building infrastructure and partnerships—most notably with La Jolla-based Synthetic Genomics—to bring xenotransplantation closer to reality.

The recent *Science* paper addresses a widely perceived risk that has never been documented. “Nobody’s ever shown that PERVs could actually cause a disease in a human,” points out David Sachs, director of the Transplantation Biology Research Center Laboratories at the Massachusetts General Hospital in Boston. Yet according to Geoff MacKay, interim CEO at eGenesis, who in the 1990s was vice president of transplant immunology at Novartis, it was a major consideration leading the Basel-based pharma and other companies that had been bullish on xenotransplantation to drop those programs.

At the time, MacKay says, everyone viewed immune rejection as the main barrier to using nonhuman organs for transplants. But companies expected to overcome this problem, including Novartis, which earmarked \$1 billion for the effort. Other firms pursuing xenotransplantation were Edinburgh, UK-based PPL Therapeutics, the company behind Dolly, the first cloned sheep, and Cambridge, Massachusetts-based Genzyme now owned by Paris-based pharma Sanofi. When the complication of PERVs became apparent, this represented one hurdle too many, MacKay says.

Today, interest is once again waxing thanks both to a better understanding of organ rejection and to the use of genome editing tools, such as CRISPR-Cas9, to manipulate pig genes encoding proteins that trigger immune recognition. Though regulators have yet to set definitive rules for how long xenotransplants must survive before clinical trials can begin, Muhammad Mohiuddin, chief of the transplantation section at the National Heart, Lung, and Blood Institute (NHLBI), notes that a 1999 document (http://www.fda.gov/ohrms/dockets/ac/99/other/6_3-4mtg.doc) from the US Food and Drug Administration suggested a threshold of 90% survival at 60 days and 50% survival at 90 days for “clinical cardiac bridge transplantation,” a kind of temporary transplant designed to sustain a recipient until a suitable human donor can be found. Already, that threshold is being put to the test. Mohiuddin and his colleagues at NHLBI, working with Blacksburg, Virginia-based biotech Revivacor—then a spin-out from UK’s PPL Therapeutics, now a subsidiary of United Therapeutics—have kept a heart xenograft alive for 945 days, albeit not in a life-sustaining capacity (*J. Thorac. Cardiovasc. Surg.* 148, 1106–1014, 2014). And researchers at Emory University have maintained a life-sustaining kidney xenograft in a baboon for 227 days.

Expanding the pool of organs for transplantation remains a major challenge. According to data from the Organ Procurement Transplant Network (<http://www.organdonor.gov/about/data.html>), 123,851 individuals were on



Revivacor was one of the first to genetically engineer pigs as an alternative tissue source for transplantation.

transplant waiting lists in the US in 2014, but only 29,532 transplants were performed. And that gap is widening. Even when human organs are available, recipients will have to spend the rest of their lives on immunosuppressant therapies, exposing them to such comorbidities as cancer, diabetes and osteoporosis.

Xenotransplantation, says A. Joseph Tector, chief medical officer at the Indiana University Health Transplant Institute, promises a nearly limitless supply of organs. But the body swiftly attacks a porcine organ placed in a human (or other primate). The main issue is the immune response (hyperacute, acute and chronic rejection by antibody-mediated and cell-mediated responses) to a transplanted animal organ, although other incompatibilities including uncontrolled complement activation and

blood coagulation abnormalities also play a role in rejection.

The immune system's attack is mainly directed at one sugar, the alpha-1,3-galactose (α -gal) on cell surfaces. At least in theory, eliminating this sugar on the transplanted organ could reduce the need for immunosuppressant drugs. In the early 2000s, several teams—one led by Sachs (*PNAS* **101**, 7335–7340, 2004), another by David Ayares at Revivicor (*Nat. Biotechnol.* **20**, 251–255, 2002) and another by Randall Prather at the University of Missouri (*Science* **295**, 1089–1092, 2002)—generated pigs with knockouts in the gene encoding α -1,3-galactosyltransferase by somatic cell nuclear transfer (SCNT) of engineered pig fibroblasts. Sachs later showed that transplants from such animals elicited reduced immune responses, surviving nearly three months in baboons (*Nat. Med.* **11**, 32–34, 2005).

Today, in addition to eliminating α -gal, researchers are attempting to 'humanize' pig organs by making other changes. In 2014, Mohiuddin, working with Revivicor's α -gal knockout pigs, introduced two key modifications: transgenic expression of the human CD46 complement-regulatory protein and human thrombomodulin to block rapid platelet coagulation and internal bleeding. Combined with an immunosuppressive regime that included antibodies to eliminate or block B cells (anti-CD20) and a costimulatory pathway (anti-CD40), the team kept a pig heart alive in a baboon for over 2.5 years, albeit not in a life-sustaining capacity. They have now expressed six human genes in a pig heart, he says, and still more genes are on his wish list. (Table 1)

Like many in the field, Mohiuddin's xenotransplantation strategy balances two goals: creating the least immunogenic pig organ possible and using the least immunosuppression to deliver it. The problem is, it takes a lot of genetic work to identify the right combination of variables.

That's where genome editing comes in. Making a transgenic pig isn't like modifying a mouse, Tector notes. "The problem with engineering pigs is nobody has been able to isolate pig embryonic stem cells. So, you have to engineer somatic cells." To date, xenotransplant donors have largely been created by conventional gene targeting via homologous recombination and somatic cell nuclear transfer (SCNT). But it can take 36 months to create a pig homozygous for a single gene alteration, Tector says, and seven or eight years to hit two. Furthermore, xenotransplantation studies are extraordinarily expensive, running \$200,000 to \$300,000 apiece, according to MacKay.

Table 1 Immunological genes as candidates for engineered pig xenotransplants

Immune response	Rejection mechanism	Targets
Innate	Complement	Expression of human complement regulators CD55, CD46 and CD59
	Macrophages	Expression of human 'don't eat me' antigen CD47 and human signal regulatory protein alpha (SIRPa)
	Natural killer cells	Expression of human leukocyte antigen G (HLA-G) and HLA-E/beta2 microglobulin
Adaptive	T cells	Class II, MHC transactivator dominant negative (CIITA-DN), MHC class I knockout, cytotoxic T lymphocyte antigen (CTLA) 4-IgG Fc expression
	B cells	α -1,3 galactosyltransferase knockout and CMP-N-acetylneuraminic hydroxylase knockout.

CMP, Cytidine monophospho

Now the increased speed and ease of CRISPR-Cas9 enables multiplexing. Tector's team took under six months to eliminate simultaneously all seven major histocompatibility complex (MHC) class I genes in pigs (*J. Immunol.* **193**, 5751–5757, 2014). Church's team, led by eGenesis CSO Luhan Yang, was able to streamline the process of knocking out PERV genes by identifying highly conserved sequences among the PERV genes in the pig cell line they were using. Then, by judicious design of only two guide RNA templates, they created clones in which all 62 PERV genes were disrupted. In co-cultivation experiments, they showed that such clones did not transmit virus to human cells.

eGenesis—a company co-founded by Church and Yang to advance and commercialize CRISPR-based xenotransplantation—is using the technology to knock out and knock in genes for regulating coagulation, complement, major histocompatibility and other immune functions in porcine fibroblasts that are then transferred to embryos by SCNT. According to MacKay, the company's 'Pig 2.0,' still in development, will contain 16 genetic modifications (in addition to the PERV knockouts)—a degree of genetic alteration previously unheard of in the field. "That level of multiplexicity, to be able to do all of this at once, is unique to CRISPR-Cas9," MacKay says.

He says the company's goal is to mine the literature, as well as the expertise of its yet-to-be-announced scientific advisory board, to identify the best gene targets for what he calls pig version 3.0 and beyond. The company currently is raising series-A financing to fund its preclinical studies through 2018, MacKay says.

eGenesis's top priority is kidney transplantation, MacKay says, with additional interest in islet and/or pancreatic transplants and other solid organ applications. The company's first primate xenotransplantation experiment—which will be outsourced to an undisclosed partner until the company

has enough space of its own to handle the animals—is scheduled for 2016.

Another target for xenotransplantation is the lungs, due to their extreme fragility and susceptibility to damage. Indeed, says David Cooper, professor of surgery at the Thomas E. Starzl Transplantation Institute at the University of Pittsburgh, lung transplantation studies typically measure in hours or days. For United Therapeutics' CEO Martine Rothblatt (also the founder of Sirius XM), it's personal; Rothblatt has a daughter with an incurable lung disease, pulmonary arterial hypertension. In addition to acquiring Revivicor, United Therapeutics has embarked on a xenotransplantation research collaboration with Synthetic Genomics, a company founded by J. Craig Venter. "Lungs represent the greatest need and the greatest challenge [in organ transplantation]," says Sean Stevens, head of the mammalian synthetic biology program at Synthetic Genomics.

Synthetic Genomics will be taking a multi-pronged approach to these problems, Stevens says. The company will leverage its expertise in transcriptomics and bioinformatics to track the gene-expression changes that occur during lung xenotransplantation to identify the mechanisms and pathways involved in both the donor and the host; use "proprietary site-specific integration and genome-editing technologies" to introduce genetic changes in pig fibroblasts; and exploit its DNA synthesis technology to "rapidly engineer multi-gene changes in a single manipulation of the genome." The company will also be developing "in vitro assays and models to prioritize the modifications we ultimately will implement," Stevens says, including induced pluripotent stem cell-based systems and organoids.

Overall, the xenofield seems on the comeback trail. "Everybody is cautiously optimistic, but nobody can put their finger on a time-frame," Stevens says. Counters his chief technology officer, Todd Peterson, "Probably not going to be 10 years, though."

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