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Genetic Manipulation in Pigs

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Abstract

Purpose of Review—Recent developments in the field of genetic engineering have made it possible to add, delete or exchange genes from one species to another. This technology has special relevance to the field of xenotransplantation, in which the elimination of a species-specific disparity could make the difference between success or failure of an organ transplant. This review focuses on developments in both the techniques and applications of genetically modified animals.

Recent Findings—Advances have been made using existing techniques for genetic modifications of swine and in the development of new, emerging technologies, including enzymatic engineering and the use of siRNA. Applications of the modified animals have provided evidence that genetically modified swine have the potential to overcome both physiologic and immunologic barriers that have previously impeded this field. Use of GalT-KO animals as donors have shown marked improvements in xenograft survivals.

Summary—Techniques for genetic engineering of swine have been directed toward avoiding naturally existing cellular and antibody responses to species-specific antigens. Organs from genetically engineered animals have enjoyed markedly improved survivals in non-human primates, especially in protocols directed toward the induction of tolerance, presumably by avoiding immunization to new antigens.

Keywords

Transplantation; xenotransplantation; genetic engineering; knock-out; xenograft

INTRODUCTION

One of the major advantages of the pig as a potential xenograft donor is the ability of the genome of these animals to be modified by modern techniques of genetic engineering. The breeding characteristics of these animals, in particular their relatively short gestation time and large litter size, combined with the availability of techniques for oocyte manipulation and artificial insemination, have made it possible to carry out both addition of genes (i.e. transgenic animals) and elimination of gene expression (i.e. knockout animals) for selected genes involved in xenograft rejection. Some of the first transgenic animals were produced for this purpose just over a decade ago [1;2] while knockout animals for alpha-1,3-galactosyl transferase [3–5] have become available over the past five years. We intend to review here both the techniques available for producing genetic modifications of swine and some of the most recent data using such modified animals in the field of xenotransplantation.

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TECHNIQUES FOR GENETIC MODIFICATIONS OF SWINE

Techniques for genetic modification are crucial for xenotransplantation research. Major advances have been made since the first transgenic large animals, including transgenic pigs were obtained through pronuclear microinjection [6], mainly by use of assisted reproductive technologies [7]. These advances made possible the first inactivation (knock-out) of a gene in a large animal model by combining genetic engineering on cultured somatic cells with somatic cell nuclear transfer [8;9].

Pronuclear Microinjection

Pronuclear microinjection is now seldom used, having been largely replaced by more efficient and less expensive techniques. Klose et al [10] used microinjection to insert the tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) into pigs; ubiquitous expression levels were detected in 3 lines without side effects. Martin et al [11] developed transgenic pigs that express the human T cell inhibitory molecule hCTLA4-Ig, under the control of the neuron-specific enolase promoter. Expression was found in various areas of the brain in these transgenic pigs, including the mesencephalon, hippocampus and cortex. Kues et al [12] also generated transgenic pigs by microinjecting an autoregulatory, tetracycline-responsive, bicistronic expression cassette (NTA). The expression pattern correlated inversely with the methylation status of the NTA transcription start sites, indicating epigenetic silencing of one of the NTA cassettes.

Sperm-Mediated Gene Transfer (SMGT)

Recently, a technique to produce multitransgenic pigs has been optimized, incubating genes for three fluorescent proteins with spermatozoa, which were subsequently used for insemination. Seven of the 18 piglets born were reported to be transgenic by PCR analysis [13]. The benefits of this technique, compared to pronuclear microinjection, are efficiency, low cost and ease of use. Nevertheless, the insertion is random and the transgene can be rearranged, thus affecting the expression levels. The expression of the transgene long-term remains controversial [14]. SMGT has also been used with a nonviral episomal vector [15] that can offer several advantages, including the prevention of insertional mutagenesis and position effects on expression. Twelve of 18 fetuses that were recovered retained the episomal reporter gene, and 9 expressed it. ICSI-mediated gene transfer (ICSI-MGT), a variant of SMGT, in combination with somatic cell nuclear transfer (SCNT) [16] produced transgenic pigs by co-incubating sperm with the bicistronic vector hALB-GFP and microinjecting the spermatozoa directly into the ooplasm. After the transfer of 702 embryos into 5 gilts, 2 out of 35 fetuses recovered were transgenic. Using somatic cells from the transgenic pigs for nuclear transfer, 767 embryos were re-transferred to 5 recipients, resulting in 6 live transgenic piglets. This is not more efficient than the two techniques used separately, however it can be suitable when a large construct (ranging from -100 to more than -1000 kb, i.e. YAC, BAC, microchromosome) has to be transfected [17].

Viral-Mediated Transgenesis

Lentiviral gene transfer is extremely efficient, with 80–100% of the animals being born transgenic after oocyte or embryo infection [18], or somatic cell culture infection. Lentiviruses have been used in a variety of experiments to transduce cells with various transgenes. These experiments include siRNA knock-down for stem cells and for somatic cells, and nuclear transfer (see below) for generating desired modifications. Lentiviruses are unlikely to be used in clinical applications due to the risks associated with multiple integration. Such risks include oncogene activation, insertional mutagenesis and silencing of lentiviral sequences [19] by methylation and high frequency of mosaicism in founder animals.

Another class of viruses used was the adeno-associated virus (AAV). Recently, Rogers et al produced a CFTR-null pig using AAV-mediated gene targeting and SCNT [20]. These authors generated a pig with both null (knock-out) and $\Delta F508$ (knock-in) modifications. Gene targeting using the AAV approach has resulted in a very efficient strategy for obtaining knock-out of the CFTR gene that is not expressed in fibroblasts.

Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT) has become the leading tool for generating animals from genetically engineered somatic cells. SCNT works better in pigs than in other large animals [21;22]. A recent innovation to make the technique more user-friendly is the zona-free system. Zona removal may be performed after enucleation for zona-free fusion, activation and culture [21;23], or before enucleation for handmade cloning [24;25]. Major limitations are represented by the lack of embryonic stem cell (ESC) technologies. Somatic cells are being used, but they have a limited lifespan, thus restricting the time the cells can be cultured *in vitro* for genetic manipulation. For xenotransplantation purposes, the use of miniature pigs is more attractive for a number of reasons. However, these breeds do not carry the same reproductive efficiency as commercial breeds, and they are not as readily available. SCNT efficiency in miniature pigs, using miniature sow recipients, is very low. When miniature pig embryos are transferred to commercial sows, the outcome is improved but not to the level of the commercial breeds [26;27]. Using a combination of SCNT with heterozygous cells for alphaGal, and crossbreeding of the resulting animals, a commercial line of pig homozygous for the knockout of the Gal epitope has been generated with a low level of inbreeding [28] that is beneficial for reproductive efficiency.

EMERGING TECHNOLOGIES

Several new technologies are becoming available that may be of great benefit in the future to the construction of genetically modified large animals.

Enzymatic Engineering

Transposons, also called “jumping genes,” are class II mobile genetic elements; they are small segments of DNA able to move from one DNA to another using transposition mediated by enzymes (transposases). DNA “cut and paste” transposons have been used for precise and efficient delivery of DNA expression cassettes in vertebrate cells. In a recent study, it was shown that co-transfection of PEGE cells with Sleeping Beauty (SB), Passport (PP) Tol2 and piggyBac (PB), with their corresponding transposase expression constructs, resulted respectively in 13.5, 5, 21 and 28-fold increases over transfection without transposase [29]. In addition to increasing the efficiency of integration, transposase-mediated transgenesis precisely integrates a single copy of the transposon into one or more locations in the genome, avoiding the integration of G/C rich prokaryotic elements of the vector and avoiding transgene concatemerization that can cause shutdown of gene expression. Other powerful tools in genome modifications are represented by Cre and FLP recombinases that catalyze a conservative DNA recombination event between two short recombinase recognition sites (RRS), loxP and FRT. This can permit deletion or inversion of the DNA between two RRs, depending on their orientation [29]. Also in lentiviral (LV) mediated transgenesis, the use of some drugs like cytokines or proteasome inhibitors can increase LV gene transfer [30;31]. Santoni de Sio has shown that human hematopoietic stem cells (HSCs) can be transduced to high efficiency by a short exposure to LVs in the presence of SCF, TPO, IL-6 and Flt3L. Moreover, it was shown that the proteasome restricts LV transduction in HSCs and that using the reversible peptide-aldehyde proteasome inhibitor MG132 and the peptide-boronate inhibitor PS-341 during the LV-GFP transduction period, there is a substantial drug-dose dependent increase in the frequency of transgene expressing cells and in their mean fluorescence intensity. Zinc finger

nucleases (ZFNs) show promise in improving the efficiency of gene targeting by introducing DNA double-strand breaks in target genes, which then stimulate the cell's endogenous HR machinery. Many studies have been developed in human and mouse cells [32;33]. A strategy to speed multitransgenic pig production is represented by recent adaptation of the 2A system from foot and mouth disease virus (FMDV) to mammalian transgenic technology [34;35]. In this system the open reading frame (ORF) consists of multiple individual cDNAs separated by sequences encoding 2A and furin cleavage sites. A single complex mRNA is produced and translated into a single polypeptide that is cleaved into individual exogenous proteins at the 2A sites.

siRNA

In cells transfected with siRNA vectors, targeted mRNAs are degraded by endonuclease activity and the amount of protein translated is reduced by over 95%, thus resulting in a significant knock-down and is an alternative approach to achieving more complex and difficult Knock-outs (KO). This technique is particularly useful when more than one copy of the endogenous gene is present and the usual KO approach is not feasible. This is indeed the case of pathogens like porcine endogenous retrovirus (PERV); for example "knock-down" of PERV expression has been shown in transgenic pigs expressing siRNA corresponding to the viral pol2 sequence [36]. On the other hand, recently an alternative strategy to reduce PERV transmission was proposed [37], using APOBEC3G, which is a single-strand DNA cytosine deaminase that can inhibit many retrovirus elements. It engages assembling retrovirus particles, accesses the RNA genome containing virus core and upon reverse transcription, deaminates cDNA cytosines to uracils, inactivating the coding capacity of the virus.

Control of Transgene Expression

Most transgenic animals carry transgenes expressed in all tissues. Under specific circumstances, and to avoid possible side effects due to undesired transgene expression in some tissues, it may be necessary to have a promoter that is inducible, or that is tissue-specific. Although this technology has been well-established in the mouse, information is only recently becoming available in the pig [12].

XENOTRANSPLANTATION USING TRANSGENIC ANIMALS

The use of genetically modified animals in transplant research has necessarily lagged behind the technological advances that have been made in producing such animals. However, the effects of these modifications are already becoming apparent:

Organ Transplants

The first transgenic modifications of swine reported involved introduction of human complement-regulatory proteins (CRPs) as transgenes into outbred swine. The three CRPs most extensively studied have been hDAF [1], CD46 [38] and CD59 [39]. While early studies using these transgenic animals showed clear advantages in terms of inhibition of complement-mediated graft destruction, none of these modifications was capable on its own of preventing eventual humoral rejection [40]. Subsequent studies have attempted to combine the use of these transgenic animals with additional modifications or treatments designed to further reduce antibody or complement-mediated activity. For example, Lam et al have reported the effect of chronic complement inhibition by SCR1 (soluble complement receptor type 1, TP10) to the treatment regimen for renal transplantation of hDAF pig kidneys into cynomolgus monkeys [41], but without significant improvement in xenograft survival. McGregor and colleagues reported a median survival of 96 days for pig hearts from CD46 transgenic pigs transplanted heterotopically in baboons using Rituximab and Thymoglobulin as induction therapy along with a standard immunosuppressive regimen including tacrolimus, sirolimus steroids and an

inhibitor of anti-Gal antibodies [42]. Shimizu and colleagues reported on a series of pig-to-baboon renal xenografts using kidneys from hDAF pigs and treating with extracorporeal absorption of anti-Gal antibodies, additional inhibition of complement with cobra venom factor (CVF) and strong T cell immunosuppression [43]. Nevertheless, humoral xenograft rejection occurred, characterized by both antibody deposition and thrombotic microangiopathy.

The Shimizu study points out that even though dysregulated coagulation is part of the rejection picture, immune-mediated damage probably initiates the rejection process. This hypothesis has been substantiated by studies from Byrne et al [44], who showed that increasing immunosuppression rather than increasing anticoagulation was capable of extending cardiac xenograft survival of CD46 transgenic pig hearts in baboons.

While, until the recent availability of GalT-KO pigs, anti-Gal antibodies were considered to be the major cause of humoral rejection in pig-to-primate xenotransplantation, there were already some studies indicating that anti-non-Gal antibodies might also play a role. One such study was reported by Chen et al [45], who demonstrated that life-supporting renal xenografts from hDAF transgenic pigs into baboons, in which anti-Gal antibodies were chronically neutralized by soluble Gal-containing polymers, anti-non-Gal antibodies still led to acute humoral rejections [45].

Cellular Studies

Because of the difficulty of experiments involving organ xenografts *in vivo*, a number of studies have been reported using cellular parameters of xenograft rejection to examine the effects of transgenic modifications. Forte and colleagues have demonstrated that NK cytotoxicity by human NK cells toward porcine lymphoblastoid and endothelial cells could be inhibited markedly by expression of HLA-E on the cell surface of these porcine cells [46;47]. While it is not clear what role NK cells play in direct rejection of xenograft organs, they clearly could be important in rejection of cellular transplants, such as those involved in the induction of tolerance through mixed chimerism [40]. Also using *in vitro* assays, Diaz-Roman et al [48] have provided evidence that some of the lack of protection from complement-mediated lysis by hDAF *in vivo* in non-human primates could be due to a difference between the complement of humans versus that of non-human primates. Manzi et al [49] have shown that transfection of a pig endothelial cell line with a truncated form of human SCR1 is capable of protecting these cells from complement-mediated lysis by human natural antibodies. However, transgenic pigs expressing SCR1 have not yet been tested.

Islet Xenotransplantation

Although studies from Hering and colleagues [50] have suggested that Gal is not a problematic antigen on adult porcine islets, non-Gal antigens may indeed still play a role. Kamoda et al [51] have demonstrated that islets from transgenic pigs expressing N-acetylglucosaminyltransferase-III (GnT-III) enjoy prolonged survival after transplantation into cynomolgus monkeys.

GalT-KO XENOTRANSPLANTATION STUDIES

Studies using the new Gal-knockout (GalT-KO) organs as xenografts to non-human primates have been carried out in several laboratories with very divergent results, depending on the treatment regimens utilized.

Pig-to-Primate Organ Xenotransplantation Using Chronic Immunosuppression

Chen and colleagues [52] found little advantage in the use of GalT-KO pig kidneys over previous transgenic kidneys when transplanting into baboons using two potent

immunosuppressive regimens. Because they found marked evidence for elevated anti-non-Gal antibodies at the time of rejection, they concluded that antibodies to non-Gal antigens may present a major barrier to xenotransplantation, even using GalT-KO pigs. Kuwaki et al [53] reported improvement in the survival of heterotopically transplanted pig hearts in baboons when GalT-KO pigs were substituted for hDAF pigs as the donors. However, although one animal in these studies showed a survival of a GalT-KO heterotopic heart for 179 days [53], even this survival was not markedly greater than the maximum achieved in the same model using hDAF grafts, the longest of which survived 139 days [54]. In both of these studies, the rejection observed showed evidence for initiation by antibodies, suggesting that even with T cell immunosuppression, there was sufficient T cell help to produce T cell-dependent antibody responses to non-Gal antigens. Tseng et al [55] analyzed the same animals and suggested that anticoagulation with aspirin may have been a major factor in delaying the onset and progression of thrombotic microangiopathy in the longest surviving heterotopic hearts. However, Shimizu et al [56] demonstrated that these thrombotic lesions were always accompanied by evidence for both T cell and antibody activity, making it unclear whether the thrombotic complications were primary or secondary to immune reactivity in these immunosuppressed animals.

Approaches Directed Toward Tolerance Induction

In contrast to results obtained using immunosuppressive drug regimens, Yamada and colleagues have demonstrated prolonged survival of life-supporting vascularized renal xenografts when vascularized thymic tissue was co-transplanted at the same time as the kidney [57]. While hDAF kidneys had never survived greater than 34 days, GalT-KO kidneys transplanted simultaneously with vascularized thymus survived over 80 days, and showed no evidence for development of new cellular or antibody-mediated immune destruction. Indeed, the longest surviving kidneys were still functioning and showed relatively normal gross and histologic appearance up to the time that the animals expired or had to be euthanized for other reasons.

CONCLUSIONS

As evidenced from this brief review, techniques for genetic engineering of swine have already produced several genetically modified strains of animals of value for xenotransplantation. Considering the large number of techniques being developed for this purpose and the continuing need for ways of prolonging survival of xenotransplanted organs, it is likely that new transgenic and knock-out animals will become available in the near future. Theoretically, it should be possible to add, remove or exchange any gene identified as important for xenograft rejection. In addition, one could conceive of specifically modified animals that would be ideal donors for individual organs. For example, since EPO is produced by the kidney, it might be of benefit to use transgenic pigs expressing human EPO instead of porcine EPO for renal transplants, while liver transplants might require an exchange of a limited number of proteins or enzymes that might be found to have species-specific incompatibilities. The use of inbred swine as the stock into which transgenes or knock-outs are introduced [58] would make it possible to make these changes without modifying the inbred background of the swine. The authors of this review are indeed attempting at present to utilize this strategy for genetic engineering of miniature swine as part of the European Union's "Xenome" project (www.xenome.eu).

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