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Genetically Modified Pigs for Medicine and Agriculture

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Abstract

The ability to genetically modify pigs has enabled scientists to create pigs that are beneficial to humans in ways that were previously unimaginable. Improvements in the methods to make genetic modifications have opened up the possibilities of introducing transgenes, knock-outs and knock-ins with precision. The benefits to medicine include the production of pharmaceuticals, the provision of organs for xenotransplantation into humans, and the development of models of human diseases. The benefits to agriculture include resistance to disease, altering the carcass composition such that it is healthier to consume, improving the pig's resistance to heat stress, and protecting the environment. Additional types of genetic modifications will likely provide animals with characteristics that will benefit humans in currently unimagined ways.

Introduction: the pig as a model for human medicine

In our review we will provide a justification for using pigs as models for medicine, and then provide a brief overview of the procedures for creating genetically modified pigs. Then we will review the genetic modifications that have been published in pig and discuss their applications to medicine and agriculture.

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Abbreviations: ALA, alpha linolenic acid; AVR, acute vascular rejection; CFTR, cystic fibrosis transmembrane conductance regulator; CVD, cardiovascular disease; DAF, decay-accelerating factor; DHA, docosahexaenoic acid; DPA, Docosapentaenoic acid ; DXR, delayed xenograft rejection; EPA, Eicosapentaenoic acid; GGTA1, alpha-1,3-galactosyltransferase gene; HAR, hyperacute rejection; LA, linoleic acid; MAP, multipotent adult progenitor; MCP, membrane cofactor protein; NHP, non-human primate; NTPDase-I, nucleoside triphosphate diphosphohydrolase I; PUFAs, polyunsaturated fatty acids; SCNT, somatic cell nuclear transfer; SLA, swine leukocyte antigen; SMGT, sperm mediated gene transfer; TFPI, tissue factor pathway inhibitor; VLDL, very low density lipoprotein.

Swine have become important in biomedical research as they are excellent models for cardiovascular disease (Turk and Laughlin, 2004), atherosclerosis (Ishii *et al*., 2006), cutaneous pharmacology (Herkenne *et al*., 2006), wound repair (Graham *et al*., 2000), cancer (Du *et al*., 2007), diabetes (Dyson *et al*., 2006), ophthalmological studies (Shatos *et al*., 2004) and toxicology research, lipoprotein metabolism, and pathobiology of intestinal transport, injury and repair, as well as being considered as a potential source of organs for xenotransplantation (Lai *et al*., 2002b). Reviewers at the National Institutes of Health (NIH) consider pigs to be a very important model for the human condition as evidenced by the fact that for the past 6 years extramural support of research on pigs has been well over $100,000,000 (NIH Office of the Director): that compares with the entire United States Department of Agriculture competitive grants program via the NRI at ~$180,000,000. The NIH considers the pig to be so important that it has helped establish the National Swine Resource and Research Center (http://nsrrc.missouri.edu/), at the University of Missouri-Columbia, to serve as a genetic resource for the biomedical community. While the pig offers many similarities to the human system and condition, they offer other advantages as a research model. They have a short generation interval, short gestation (114 days), and multiple offspring. Thus for domestic species they can reproduce rapidly and provide an ongoing source of research animals. A limiting factor for basic research to proceed in any species is access to a sequenced genome. Without a sequenced genome that particular species will become a second class-species. Fortunately funds have been secured to complete the swine genome (http://www.csrees.usda.gov/newsroom/news/2006news/swine.html) and the first draft is expected to be completed soon (www.piggenome.org). Furthermore, the genome of the pig is also quite similar to the human, as a phylogenetic approach using swine genome sequence data shows that the pig genome is 3x closer to the human than is the mouse (Wernersson *et al*., 2005). Finally, there are some inbred pig strains available.

Methods to make genetically modified pigs

Genetic modification of domestic animals can now be accomplished by a variety of methods (Robl *et al*., 2007). Genetic modification in mammals was initially via pronuclear injection. In essence, a DNA construct was injected directly into one of the pronuclei of a zygote (Brinster, 1981). This technique was initially established in mice, but later applied to a variety of mammals (Wall *et al*., 1997). The next technique is that of oocyte transduction. Mature oocytes arrested at metaphase II of meiosis are first isolated. Then a replication incompetent retrovirus is injected between the zona pellucida and the oocyte plasma membrane. After an opportunity is provided for the retrovirus to infect the oocyte and integrate into the chromosomes, then the oocyte is fertilized. This procedure is highly efficient as integration occurs only if the nuclear envelop is absent; and the oocyte is arrested in metaphase II of meiosis. Oocyte transduction was applied to cattle (Chan *et al*., 1998) and then to the pig (Cabot *et al*., 2001). The third technology for creating transgenic pigs is that of sperm-mediated gene transfer (SMGT). In SMGT the DNA construct of interest is mixed with the sperm and then used for in vitro fertilization or for insemination (Lavitrano *et al*., 2006). This is also a highly efficient process.

With each of the techniques listed above there is little control over the integration of the construct. It is not possible to predetermine how many copies of the gene become

integrated, nor into which chromosome or chromosomes they are integrated. To facilitate targeted integration either a more efficient procedure must be developed or homologous recombination must be performed followed by selection of the cells with the specific modification desired. In mice this latter technique can be performed on embryonic stem cells. The few cells with the correct modification can be expanded and then used to make chimeras with wild type host blastocyst stage embryos (Doetschman *et al*., 1987). Unfortunately the ES cell technology to make genetic modification has only been fully developed for the mouse and a true ES cell (one that can go germline) has yet to be identified in any other species. At least two additional options exist for the creation of animals with a targeted modification. The first is that of genetically modifying a male germ cell, followed by transfer of those germ cells to a male that has had his germ cells depleted. The newly developing sperm would then carry the transgene and be capable of producing transgenic offspring. While this technology is still in development, in the foreseeable future transgenic pigs will be made via germ cell modification (Honaramooz *et al*., 2007). The final method of making a targeted modification is that of modifying a somatic cell followed by NT to clone the animal (Lai *et al*., 2002b). Currently the only method available for making targeted modifications, knock-ins or knock-outs, is via cloning from a modified somatic cell.

The cloning technology in pigs was first developed by using blastomere stage nuclei as donor cells (Prather *et al*., 1989) (*Figure 1*). The thought at the time was that complex reprogramming of a somatic cell could not be completed by the cytoplasm of the oocyte. It was not until many years later that adult cells were first used to successfully create offspring by nuclear transfer (Polejaeva *et al*., 2000) (*Figure 1B*). One year later came the first report of genetic modification followed by nuclear transfer and cloning (Park *et al*., 2001a) (*Figure 1C*).

The use of somatic cells for genetic modification and as donors for NT presents certain challenges. The foremost is the limited lifespan of most somatic cells. In general, fetal-derived fibroblast cells begin to undergo senescence after about 30 population doublings. Thus, if one is to make a genetic modification to such cells, the selection and expansion of the clones with the desired genotype must be completed quickly prior to senescence. Fortunately, progress is being made in the production of clones from stem cells, i.e. cells that can be grown for extended periods of time prior to differentiating or undergoing senescence. There are now at least two examples of cloned pigs from stem cell lines. The two types of stem cell donors include mesenchymal stem cells (Jin *et al*., 2007) and skin-derived stem cells (Hao *et al*., 2008).

In addition to the advancements in cloning from stem cell lines, additional advancements in the technology for making the genetic modification are appearing. One technique with much promise is that of using zinc finger nucleases to make the modification (Mani *et al*., 2005; Morton *et al*., 2006; Porteus, 2006). This can be a highly efficient process, and if used in combination with stem cells as donors for cloning, it may result in a practical method of genetically modifying swine.

Control of gene expression

A large number of factors determine if a transgene is expressed or not. Generally the focus is upon the construct design and getting the desired promoter, enhancer and

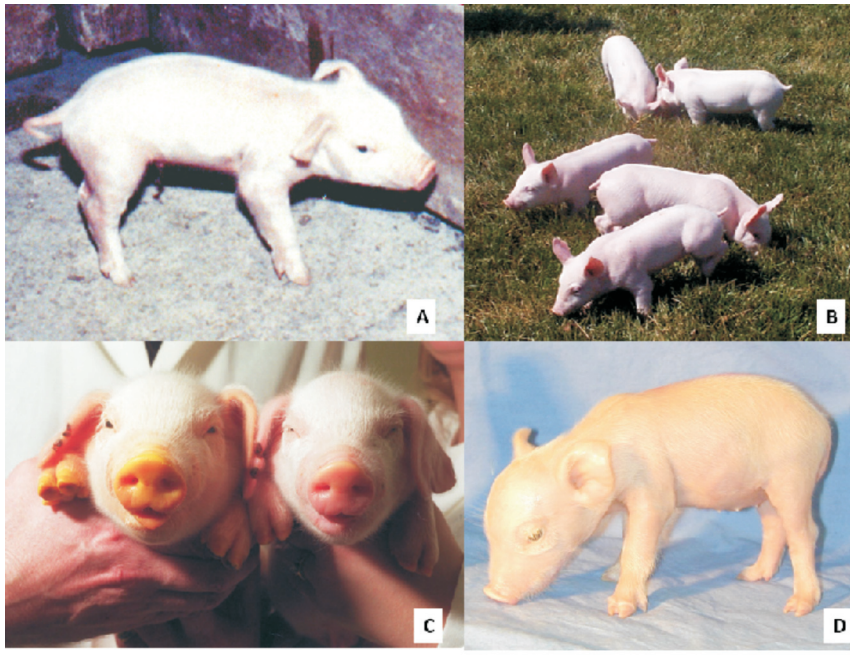

Figure 1. Pigs that resulted from advancements in cloning and genetic modification technology. A. The first cloned pig derived from a blastomere nucleus as a donor (Prather *et al*., 1989). B. The first pigs cloned from somatic cell donor nuclei (Millie, Alexa, Crista, Carrell, and Dotcom) (Polejaeva *et al*., 2000). C. The first transgenic pigs resulting from nuclear transfer (Park *et al*., 2001a). The pig on the left carries the transgene for the enhanced green fluorescent protein, while the pig on the right is a wild type under normal lighting. D. The first pig with a gene knocked out, GGTA1 (Lai *et al*., 2002a).

other control elements into the design. The goal of this review is not to describe all the factors that control transgene expression as there are many reviews that provide that information (Conese *et al*., 2007; Kwaks and Otte, 2006; van Gaal *et al*., 2006; Venter, 2007; Vilaboa *et al*., 2005; Vilaboa and Voellmy, 2006; Xiao *et al*., 2007), but rather to focus on one example of being able to exogenously control gene expression. The first attempt in farm animals to create a tight regulation of expression was in pigs. Here an autoregulative tetracycline-responsive bicistronic expression cassette designed to result in ubiquitous expression of human regulators of complement activation was introduced into the pig genome (Kues *et al*., 2006). Unexpectedly the cassette was silenced by DNA methylation, except in muscle fibers. However when the animals were crossed, resulting in two cassettes in a single animal, the cassette was demethylated and reactivated. Much is still to be learned from attempts at making domestic animals with genes that can be tightly controlled.

Medical reasons to genetically modify pigs

XENOTRANSPLANTATION

Due to similarities in physiology, and size, pigs have been considered a source of organs for transplantation to humans. Pigs have short gestation time and large litter size,

and they can be raised in pathogen free conditions. Compared to non-human primates (NHPs), pigs have less chance to transmit infectious diseases to human, and have fewer ethical issues as organ donors. Unfortunately, when a pig organ or cells are transferred to a primate the cells are immediately lysed. This is due to the presence of pre-formed antibodies in the primate that recognize a specific cell surface carbohydrate; a terminal α-1,3-galactose epitope on the cell surface. The gene in the pig that is responsible is called α-1,3-galactosyltransferase (GGTA1). Because this gene is not functional in humans and Old World monkeys, they developed a very high titer of anti-gal antibodies in their body. It has been reported that, in humans, up to 1% of the total circulating IgG is anti-α1,3Gal natural antibody (Galili *et al*., 1985). The pre-formed antibodies immediately recognize the α-1,3Gal epitope, which then activate the complement system and result in hyperacute rejection (HAR) of the transplanted cells or organs/ tissues from pigs. To overcome this major obstacle in xenotransplantation, a number of strategies have been employed to reduce or eliminate α-1,3 Gal induced HAR. These methods include overexpression of α-2,3-sialyltransferase or α1,2-fucosyltransferase in pig cells to compete with α1,3GT; treatment of pig organs with α-galactosidase to remove surface α-1,3Gal epitope (Costa *et al*., 2002; Miyagawa *et al*., 2001); expression of complement inhibitor genes, such as human decay-accelerating factor (DAF), CD59 or CD46 (membrane cofactor protein (MCP) in transgenic pig organs to suppress the complement reaction (Cozzi, 1995; Fodor *et al*., 1994; Loveland *et al*., 2004; McKenzie *et al*., 2003; Rosengard, 1995); and temporary depletion of natural anti α-1,3Gal antibody from recipients prior to and after transplantation (Ghanekar *et al*., 2001; Zhong *et al*., 2003). However, all these methods only partially removed the α1,3 Gal from the surface of the xenografts, or temporarily removed the anti-α-1,3 gal antibodies from recipients. The anti-α-1,3 gal antibodies will come back as soon as the treatment stops, and the residual α1,3 Gal molecules on pig cells were still sufficient to activate the complement cascade and cause destruction of the grafts (Galili, 2001; Joziasse and Oriol, 1999). Another possible solution to this problem of HAR was to disrupt or knock-out the gene i.e. GGTA1, that is responsible for production of α-1,3 Gal epitopes (*Figure 1D*). Once that was accomplished (Dai *et al*., 2002; Lai *et al*., 2002b) and homozygous knock-out pigs produced (Kolber-Simonds *et al*., 2004; Phelps *et al*., 2003), then the organs could be transplanted into baboons. These xenotransplantation experiments showed that if GGTA1 is knocked out the life of the kidney and hearts could be extended without the HAR (Chen *et al*., 2005; Kuwaki *et al*., 2005; Tseng *et al*., 2005; Yamada *et al*., 2005).

With a possible solution to the HAR, the focus has moved to the delayed xenograft rejection (DXR) or acute vascular rejection (AVR). The exact cause of AVR is still unknown, but may be due to antibodies against non-α-1,3Gal xeno-antigens, molecular incompatibilities of coagulation regulation between pig and NHPs, and innate cellular responses from NK cells and macrophages. Many proposals have been made to deal with AVR. Most of them are to make transgenic GGTA1 KO pigs with human genes which have specific functions. Some examples are: using human DAF, CD46 or CD59 to inhibit complement activity (Cozzi and White, 1995; Hinchliffe et al., 1998; van den Berg et al., 2000); using anti-coagulate gene tissue factor pathway inhibitor (TFPI) and CD39, or nucleoside triphosphate diphosphohydrolase I (NTPDase-1) to suppress micro thrombosis (Chen et al., 1999; Dwyer et al., 2004); using HLA-G or HLA-E to inhibit NK cells (Crew, 2007; Lilienfeld et al., 2007; Wang et al., 2004);

using hemoxygenase-1 (HO-1), A20 and death decoy receptor (TR6/DcR3) genes (Akamatsu et al., 2004; Daniel et al., 2004; Shi et al., 2003) to protect endothelial cells from inflammatory damage and apoptosis, as well as using CTLA4Ig to inhibit T cells activation (Wekerle et al., 2002). Other proposals include removing potential rejection-related pig genes such as SLA, Intercellular adhesion molecular-1 (ICAM-1) or co-stimulatory molecules by directly knocking out those pig's genes or using siRNA technology to inhibit their expression. To date, several such transgenic pigs have been generated either on a wild-type pig background or GGTA1 knock-out background. GGTA1 knock-out pigs with a human DAF have been produced and available in National Swine Resource and Research Center (www.nsrrc.missouri.edu). GGTA1 knock-out pigs with human α-1,2-fucosyltransferase gene to modify the carbohydrate has been reported (Ramsoondar et al., 2003). One group has added the human leukocyte antigen in transgenic pigs (Tu et al., 2000). A human tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) transgenic pig showed the transgenic pig lymphocytes could induce apoptosis of human lymphoid cells (Klose et al., 2005). Transgenic pigs with human CD39 have been generated to prevent the formation of thrombosis in transplanted organs (Dwyer et al., 2007). Petersen et al recently reported the production of hHO-1/hDAF transgenic pigs by nuclear transfer. Kidneys from hHO-1/DAF transgenic pigs survived ex vivo perfusion for 240 min with AB-pooled human blood and exhibited no indication for xenogenic activation of the human coagulation system. On the contrary, kidneys from wild-type pigs only lasted for 60 min in ex vivo perfusion (Petersen et al., 2008). The same group also reported birth of seven healthy piglets carrying human A20 gene driven by a CMV-β-actin promoter (Oropeza et al., 2008). Many other transgenic pigs on the GGTA1 knock-out background are under development and we expect the results will be published in the near future. Successful xenotransplantation will likely require multiple modifications to overcome both innate immunity as well as the acquired immunity that will be generated against the molecules on the surface of the pig organ.

PHARMACEUTICALS

An interesting application of pharmaceutical production in pigs is the production of human hemoglobin in the blood of pigs (Sharma *et al*., 1994). It is thought that isolation of the human hemoglobin from the blood of the transgenic pig may provide a source for treating trauma patients. It may seem surprising, but pigs have also been used to produce pharmaceuticals in their milk. While pigs are not usually considered a dairy breed of livestock, they have been used to produce Protein C, an in-activator of coagulation factors Va and VIIIa (Van Cott *et al*., 2001), as well as human coagulation factor VIII and IX (Lindsay *et al*., 2004; Paleyanda *et al*., 1997) in their milk. Interestingly the pig mammary epithelial cells are unique among livestock in making the complex post-translational modifications needed for FIX and FVIII biological activity (Van Cott *et al*., 2004).

MODELS OF HUMAN DISEASE

Pigs have also been genetically modified in attempts to create better models of human disease. Six examples will be discussed below.

Retinitis Pigmentosa

The first model of human disease to be discussed is a line of pigs that has a mutated version of the rhodoposin gene that results in pigs getting retinitis pigmentosa (RP). This model (Banin *et al*., 1999; Petters *et al*., 1997) has a Pro347Leu mutation. These animals develop early and severe rod loss, likely resulting in subsequent degeneration of the cones in a pattern that mimics rod and cone loss in humans. The size and physiology of the pig eye is, again, similar to humans and thus these pigs will clearly be very valuable for preclinical studies (Ghosh *et al*., 2007).

Cardiovascular Disease:Fat-1

Polyunsaturated fatty acids (PUFAs) are fatty acids with 18 or more carbon atoms and two or more double bonds. There are two major groups of PUFAs, omega-6 (n-6) and omega-3 (n-3), depending on the position of the double bond nearest the methyl end (the ω carbon) of the fatty acid. Both n-3 and n-6 PUFAs are significant structural components of the phospholipids membranes of tissues throughout the body and n-3 PUFAs are especially rich in the retina and brain in which DHA constitutes over 36% of total fatty acids (DeFilippis and Sperling, 2006; Lin *et al*., 1993; Simopoulos, 2001; Simopoulos, 2003; Simopoulos, 2006). Mammals lack the desaturases necessary to synthesize both LA (n-6) and ALA (n-3). Furthermore, the n-3 and n-6 PUFAs are not inter-convertible in mammalian cells because mammals also lack the enzyme, omega-3 fatty acid desaturase. Therefore, LA and ALA and their elongation and desaturation products are essential fatty acids (EFAs) to mammals, and they must be acquired from the diet (Simopoulos, 2001; Simopoulos, 2003; Simopoulos, 2006). Over the past 100-150 years, there has been an enormous increase in the consumption of n-6 PUFAs due to the increased intake of vegetable oils from corn, sunflower seeds, cottonseed, and soybeans, which all contain high levels of n-6 PUFAs but very little n-3 PUFAs. In addition, intake of n-3 PUFAs is much lower today because of the decrease in fish consumption and the industrial production of animal feeds rich in grains containing n-6 fatty acids, leading to production of meat and eggs rich in n-6, but poor in n-3 PUFAs (Simopoulos, 1998; Simopoulos, 2001; Simopoulos, 2003; Simopoulos, 2006). Today, in Western diets, the ratio of n-6 to n-3 PUFAs ranges from 10-20:1 instead of the traditional range of 1-2:1. It has been proposed that the high n-6/n-3 PUFA ratio may contribute to the high prevalence of many modern human diseases such as CVD, diabetes, obesity, cancer and depression. (Simopoulos, 2002a; Simopoulos, 2002b; Simopoulos, 2003; Simopoulos, 2006).

The potential anti-atherosclerotic effects of omega-3 PUFAs has been well recognized and studied in last 30 years after Bang *et al* first reported in 1976 that the low mortality from CVD among the Greenland Eskimos whose diet was mainly n-3 PUFA-rich marine vertebrates (Bang *et al*., 1976). The seminal finding in Eskimos was confirmed and extended in Western populations (Ascherio *et al*., 1995; Dolecek, 1992; von Schacky, 1987; von Schacky, 2000; von Schacky, 2004) as well as in large animal experiments including swine (Kim *et al*., 1993; Kim *et al*., 1991; Weiner *et al*., 1986) and nonhuman primates (Davis *et al*., 1987; Harker *et al*., 1993; Parks *et al*., 1990a; Parks *et al*., 1990b). Many studies have shown that n-3 PUFAs have broad beneficial effects on many factors at all stages of atherogenesis. These major effects include: 1) lowering triacylglycerol, cholesterol, and VLDL concentration; 2) reducing platelet

aggregation and thrombosis; 3) reducing the production of inflammatory markers such as CRP, IL-6, E-selectin, ICAM-1, VCAM-1IL-1β and TNF-α; 4) down-regulating PDGF in quiescent and activated mononuclear cells; and 5) inhibiting ventricular arrhythmias (De Caterina *et al*., 2004; Mori and Beilin, 2004; Simopoulos, 2001; Simopoulos, 2003; von Schacky, 2000; von Schacky, 2004).

An n-3 fatty acid desaturase gene, fat-1, which converts n-6 PUFAs to n-3 PUFAs, was cloned from *Caenorhabditis elegans* (roundworm) by Dr. Browse's group in 1997 (Spychalla *et al*., 1997). Fat-1 transgenic mice have been generated (Kang *et al*., 2004) and several studies have shown high n-3 PUFAs and balanced n-6/n-3 ratio in fat-1 mice have significantly inhibitory effect on colitis (Hudert *et al*., 2006), chemically induced acute hepatitis (Schmocker *et al*., 2007), prostate cancer (Berquin *et al*., 2007), and melanoma (Xia *et al*., 2006). We have introduced the humanized version of the fat-1 gene into pigs by somatic cells nuclear transfer (Lai *et al*., 2006; Li *et al*., 2006). The fat-1 pigs not only have a 3 fold increase n-3 PUFAs, but also reduce their n-6 PUFAs by 25% since the FAT-1 protein effectively converts n-6 PUFAs into n-3 PUFAs in those pigs. As a result, the n-6/n-3 ratio has been reduced 5-fold (from 8.52 to 1.69) compared to the control pigs. We have also found that all the tissues tested from the fat-1 transgenic pigs (heart, muscle, liver, kidney, spleen, brain, skin and tongue) showed much lower n-6/n-3 ratios than control pigs, indicating that the fat-1 gene is expressed well in most tissues (Lai *et al*., 2006; Li *et al*., 2006). We have successfully produced four litters of F1 fat-1 transgenic piglets from the founder boars by natural breeding with wild-type gilts. The n-3 PUFAs concentration and n-6/n-3 ratio in tails from all F1 fat-1 piglets are very similar to those in tail samples from their fathers.

The fat-1 transgenic pig is an excellent unique large animal model to study the preventive effects against atherosclerosis and the underlying mechanisms by n-3 PUFAs and balanced n-6/n-3 ratio. First, pigs have very similar physiology and lipid metabolism to humans, and pigs are a useful model for the evaluation of atherosclerosis from the perspective that lesions develop spontaneously, their circulatory system and localization of lesions are similar to humans, and the lesions are responsive to dietary intervention by exhibiting regression after prolonged periods (Fan and Watanabe, 2000; Kim *et al*., 1991; Rowsell *et al*., 1965; Turk and Laughlin, 2004; Weiner *et al*., 1985); Second, the fat-1 transgenic pigs have significantly high n-3 PUFAs (especially EPA and DPA) concentration and an ideal n-6/n-3 ratio (~2:1). The balanced n-6/n-3 ratio is critical to reduce inflammation, and such a low n-6/n-3 ratio (~2:1) is very difficult to achieve by feeding pigs with fish oil, which only increases n-3 PUFAs but will not reduce n-6 PUFAs in pigs; Third, it will be a well-controlled, single-factor experiment because all pigs in both experimental and control groups will be fed with the exactly same diet. It will exclude the potential interference from other fish oil components, such as cholesterol, chemical contaminants or other fatty acids, and extra energy. Fourth, the influence of genetic background to the results will be minimal if the same sex, non-transgenic littermates are used as controls.

Diabetes

Diabetes is a disease that is becoming more of a problem in the high fat diet and sedentary lifestyle of North Americans. Interestingly, there is one natural model of

type 2 diabetes: the Ossabaw Island pig. When these pigs are active they remain lean, but when they become sedentary and on a high fat diet they have a propensity to develop type 2 diabetes (Boullion *et al*., 2003; Dyson *et al*., 2006). Recently a pig has been genetically modified to create a model of type 2 diabetes. This group used a lentiviral vector to deliver a dominant negative glucose-dependent insulinotropic polypeptide receptor under the control of the rat *Ins2* promoter. These animals had a reduced insulin release and higher glucose levels as compared to non-transgenic littermates as measured in response to an oral glucose tolerance test (Renner *et al*., 2008). Genetically modified animals like these will be very useful for the study of diabetes.

Huntington's Disease

Huntington's Disease is associated with a CAG repeat in a gene called 'huntington'. As the length of the trinucleotide repeat increases the onset occurs at an earlier age (Macdonald *et al*., 1993; Rosenblatt *et al*., 2001). Huntington's Disease is inherited as an autosomal dominant disease that gives rise to progressive neural cell death associated with choreic movements and dementia. In an attempt to make a better model to study Huntington's Disease, the pig Huntington gene was cloned from a miniature pig and combined with a rat neuron-specific enolase promoter and injected into the pronuclei of pig zygotes. After embryo transfer five transgenic pigs were produced (Uchida *et al*., 2001).

Cystic Fibrosis

Cystic fibrosis is the most common genetic disease in adolescents in North America. The disease is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This aberrant protein results in less movement of chloride ions across the membrane. When this mutation is introduced into mice they do not exhibit all of the expected phenotypes. While they do have gastrointestinal problems, they do not exhibit any airway disease (Grubb and Gabriel, 1997) either because mice have a compensatory mechanism that rescues pulmonary function, or the lifespan of the mouse is insufficient to develop the disease. Thus there is no model to study cystic fibrosis-induced lung disease except for the affected children. Since the pig lung shares many physiological properties with humans, Michael Welsh's laboratory at the University of Iowa and our group (RSP) at Missouri moved forward to knockout the CFTR gene in the pig. In addition we have created a version that has a deletion of the 508th amino acid (phenyalanine). This is the most common mutation in North American Caucasians. Heterogygote animals with the knockout mutation have been created (Rogers *et al*., 2008) and have successfully transmitted the mutation to both male and female offspring. When these animals reach sexual maturity and reproduce it should result in homozygous null animals that hopefully will exhibit a lung disease phenotype.

Alzheimer's Disease

Recently a modification has been introduced to generate pigs that may exhibit symptoms of Alzheimer's Disease. This was accomplished by inserting a mutated

version of the "Swedish mutation" of the amyloid precursor protein gene (Kragh *et al*., 2008). While it is not yet known if the animals will develop the disease it represents a first step into this important area of human medicine.

CELL TRACKING

Molecular markers which permit identification of cells within a mixed population have proven to be immensely useful for a variety of studies. A number of lines of pigs transgenic for the enhanced green (Cabot *et al*., 2001; Park *et al*., 2001b), red, blue (Webster *et al*., 2005) and orange (Matsunari *et al*., 2008) versions of these fluorescent proteins have been made. The utility of these cells can be illustrated with a few examples. The first is a study where Multipotent Adult Progenitor (MAP) cells were isolated from the blood of a mature female pig. It was possible to differentiate these MAPs into a variety of cell types, including hollow endothelial tubules, astrocytes and glial cells, osteoblasts, adipocytes, and smooth muscle (Price *et al*., 2006). In addition, the ability of these cells to differentiate into neuronal cells and to establish connections to endogenous neurons after injection into rat brains could be easily tracked since they were fluorescent.

Another example began in 2002 when Dr. Mike Young (Harvard) and Henry Klassen (UC-Irvine) suggested using the eGFP expressing pig eye for a tracking study of ocular regeneration; and as it turned out this collaboration involved additional groups from Lund in Sweden, Children's Hospital of Orange County in California, and Copenhagen University in Denmark. The project begins with an individual from Harvard or UC-Irvine flying into Columbia to collect fetal pig eyes. The eyes are carried back to Harvard or UC-Irvine and retinal progenitor cells are cultured and expanded. These cells are then transported to Copenhagen where the group uses a recipient pig that has induced retinal damage. The eGFP retinal progenitor cells are then transplanted into the damaged eye. After up to 10 weeks the eyes are harvested and transported to Lund where sections are cut and the contribution of eGFP expressing cells to the different cell types of the retina are determined (Klassen *et al*., 2004; Shatos *et al*., 2004). The retinal progenitor cells appear to migrate to the area of damage, integrate into the different layers of the retina, express retinal-specific markers and morphologically appear to differentiate into rods and cones. Again, identification of the cells that were transplanted was facilitated by using retinal progenitor cells that were genetically marked with the eGFP.

HUMAN/PIG HYBRID ORGANS

In an attempt to produce human hepatocytes that can be transferred to humans that have impaired liver function two lines of pigs have been made that have a liver-specific promoter (albumin or alpha-fetoprotein) driving a suicide gene (thymidine kinase or cytosine deaminase) (Beschorner *et al*., 2003a; Beschorner *et al*., 2003b). The goal here would be to transfer human hepatocytes to a pre-immune transgenic fetal pig. After the pig is born and reaches adult size, the pig would be treated with the appropriate precursor (ganciclovir or 5-fluorocytosine, respectively). The precursor would be taken up into all the cells in the transgenic/chimeric pig, but only in the pig hepatocytes

would the precursor be converted into a toxic analog and destroy those cells. The surviving human hepatocytes would regenerate and might be harvested for transfer into humans that need hepatocytes. While this process is still under development it is showing promise (Beschorner *et al*., 2007).

BIOARTIFICIAL LIVER SUPPORT

One group has attempted to develop a bioartificial liver support system to treat patients with severe liver failure. Here a human albumin gene was introduced into a pig (Naruse *et al*., 2005). The gene was expressed in the pig liver. The authors suggest that pig livers producing major human hepatic proteins would be ideal to minimize xenogenic protein influx. Further development and characterization of pigs with a similar modification is warrented. Levy *et al* reported two successful extracorporeal hepatic supports with transgenic (hDAF/hCD59) porcine livers used as a bridge to human liver transplantation (Levy *et al*., 2000).

Agricultural reasons to genetically modify pigs

A number of genes have been inserted in pigs to either enhance growth and production traits, or to alter the product so that it is more environmentally safe, leaner or healthier for human consumption. These additions include the genes for growth hormone (GH1) (Hammer, 1985) , insulin-like growth factor 1 (IGF1) (Pursel *et al*., 1999), phytase (Golovan, 2001), B-cell CLL/lymphoma 2 (BCL2) (Guthrie *et al*., 2005), delta 12 fatty acid desaturase (Saeki *et al*., 2004), omega-3 desaturase (hfat-1) (Lai *et al*., 2006), and bovine alpha-lactalbumin (LALBA) (Bleck, 1998). Some of the modifications may improve productivity of the animal (GH1, IGF1), while others are intended to alter the composition of the carcass so that it may be more healthy for the animal, and for humans to consume (delta 12 fatty acid desaturase, hfat-1). One modification was envisioned to increase the number of viable eggs that a female would produce (BCL2), another to increase the weaning weight of piglets (LALBA), and another was to help reduce pollution caused by elimination of inorganic phosphorous (phytase). Other modifications can be envisioned that may make the animals more or less susceptible to disease, or more tolerant to heat stress. While none of these modifications have been approved to enter the human food chain, the above modifications show that many of these ideas can be reduced to practice and result in a possible economic or health benefit to the producer and consumer.

Conclusion

In conclusion, genetic modification in pigs is now possible with a precision that was not previously possible. Genes can be added randomly or in precise locations resulting in genetic additions, modifications, knock-in or knockouts. These modifications can change the pig so that it is a better model of human disease, that it produces pharmaceuticals, or that the health of the animal or consumer is improved. Since many transgenic or knockout rodents models are not ideal models for human disease, more and more transgenic/knockout pigs will be in demand as human disease models

for the study of mechanisms and testing of new drugs and treatments. Successful modification of pigs for xenotransplantation will provide unlimited supply of organs and tissues to alleviate the current organ shortage crisis. We expect that therapeutical human proteins from pigs will soon be used to treat human diseases and, one day, heathier pork and bacon from environmentally-friendly pigs will be accepted on the dining room table. The biggest limitation in creating genetically modified pigs to benefit mankind is our imagination.

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