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Genetically engineered livestock for biomedical models

Christopher S. Rogers

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Abstract To commemorate Transgenic Animal Research Conference X, this review summarizes the recent progress in developing genetically engineered livestock species as biomedical models. The first of these conferences was held in 1997, which turned out to be a watershed year for the field, with two significant events occurring. One was the publication of the first transgenic livestock animal disease model, a pig with retinitis pigmentosa. Before that, the use of livestock species in biomedical research had been limited to wild-type animals or disease models that had been induced or were naturally occurring. The second event was the report of Dolly, a cloned sheep produced by somatic cell nuclear transfer. Cloning subsequently became an essential part of the process for most of the models developed in the last 18 years and is stilled used prominently today. This review is intended to highlight the biomedical modeling achievements that followed those key events, many of which were first reported at one of the previous nine Transgenic Animal Research Conferences. Also discussed are the practical challenges of utilizing livestock disease models now that the technical hurdles of model development have been largely overcome.

C. S. Rogers (✉)
Exemplar Genetics, 2500 Crosspark Rd., Ste E126, Coralville, IA 52241, USA
e-mail: chris.rogers@exemplargenetics.com

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Introduction

The first genetically engineered (GE) livestock animals were published in 1985, but it took another 12 years before this technology would be successfully utilized to model a human disease (Hammer et al. 1985; Petters et al. 1997). Early methods were limited to randomly integrated transgenes, primarily via pronuclear injection of DNA or virus into an early embryo followed by transfer to a surrogate mother. With the development of somatic cell nuclear transfer (SCNT), transgenesis was predominantly moved to primary cells, where selection methods and molecular characterization of transgene integration and copy number made for a more efficient process. SCNT also made it possible to begin considering gene-targeted models. Due to the lack of established embryonic stem cells from livestock species, the methods used to make gene-targeted mice were not applicable (Piedrahita 2000). However, if a somatic cell could be gene-targeted, it could be combined with SCNT to generate animals with a desired disease mutation.

The first gene to be targeted in a livestock animal was ovine alpha 1 (I) procollagen (*COL1A1*) in 2000, with the purpose of directing the specific integration of an alpha 1-antitrypsin cDNA (McCreath et al. 2000).

Additional gene targeting followed in other species, but the efficiencies were extremely low—prohibitively so in many cases—and typically limited to highly expressed genes (Dai et al. 2002; Lai et al. 2002). The use of recombinant adeno-associated virus (rAAV) to deliver gene targeting constructs provided a breakthrough, increasing gene targeting efficiencies to levels that allowed even genes with low or no expression to be readily targeted, selected, and screened (Hickey et al. 2011; Luo et al. 2012; Rogers et al. 2008b). Improvements in cell type isolation, culture conditions, targeting construct delivery methods, and targeting construct design are robust and efficient enough now that almost any simple modification (deletion, disruption, or small insertion) is possible in livestock species. With recently developed gene editing platforms, including TALENs and the CRISPR/Cas9 system (see Tan et al. in this issue), even more diverse modifications of the genomes of livestock species will be seen in the future (Tan et al. 2013).

Diseases models

This review is intended to demonstrate the breadth of GE large animal models developed over the last two decades, and not to provide full detail of the production methods or characterization. Readers are encouraged to seek out the primary literature for further details.

Livestock species are obvious choices for many biotechnology applications. Cows, goats, and sheep are frequently engineered to produce secreted proteins in milk or blood (see Bertolini et al. in this issue). Pigs and sheep have been pursued as possible organ donors for humans due to their size and similar anatomy (see Niemann and Petersen in this issue). However, genetically engineered mice have been the most-used species for biomedical applications, mainly being used to understand a gene/protein function or to model a human disease. While they have been excellent for elucidating gene pathways in a mammalian setting, they have been less successful at recapitulating human disease phenotypes and predicting drug efficacies. The reasons are numerous and disease-specific, but are largely attributable to differences in anatomy, physiology, genetics, lifespan, and size. Even in cases where rodent models do manifest key phenotypic aspects of human disease, they pose limitations as preclinical models, particularly for purposes of medical imaging technology, drug development, and surgical intervention.

Several livestock species have been utilized as alternatives to rodent models, however pigs have dominated the biomedical space. This is primarily due to their reproductive advantages; pigs can have large litters multiple times per year. Wild-type pigs, goats, and sheep have long been used in research due to similarities to humans with regard to physiology and anatomy. Their size makes them particularly useful for (1) utilizing clinical imaging equipment designed for humans, (2) developing patient-sized medical devices, such as stents and pacemakers, (3) developing and refining surgical procedures, and (4) providing access to large tissue samples and opportunities to collect samples in a longitudinal manner. Several diseases have been induced in these wild-type animals, such as balloon injury-induced atherosclerosis or streptozotocin-induced diabetes (Marshall 1979; Steele et al. 1985). Furthermore, naturally occurring mutations in livestock species have been studied as disease models, including hypercholesterolemia and several neurological disorders (Jolly et al. 1992; Karageorgos et al. 2011; Prescott et al. 1991; Rapacz et al. 1986). However, the ability to engineer the genome of livestock animals has broadened their utility and enabled new studies into disease mechanisms and therapeutic interventions that were not previously possible.

Since 1997, many genetically engineered models of human disease have been generated in livestock species. Longer times for production, gestation, and sexual maturation, as well as the logistical challenges of studying large animals mean that many of these models are still works in progress and that limited data are currently available. This review will focus on the animals for which phenotypic characterization and applied studies have been reported (see Table 1 for a list). It is important to note that many other models are in process, but limited space prevented the inclusion of an exhaustive list.

Cystic fibrosis

An excellent example of the impact an improved GE livestock biomedical model can have on a research

Table 1 Summary of GE livestock models of human diseases

Human disease	Species	Gene/modification type	Technical approach	Reference
Cystic fibrosis				
	Domestic pig	CFTR-null	rAAV and SCNT	Rogers et al. (2008b, c)
	Domestic pig	CFTR-null	Electroporation and SCNT	Klymiuk et al. (2012)
	Domestic pig	CFTR-F508del	rAAV and SCNT	Rogers et al. (2008b)
Diabetes				
	Domestic pig	GIPR dominant-negative human transgene	Embryo injection with lentivirus	Renner et al. (2010)
	Domestic pig	INS-C96Y porcine transgene	Electroporation and SCNT	Renner et al. (2013)
	Domestic pig	HNF-1α dominant-negative human transgene	ICSI and SCNT	Umeyama et al. (2009)
Cardiovascular disease				
	Yucatan miniature pig	PCSK9 dominant-negative human transgene	SB transposition and SCNT	Al-Mashhadi et al. (2013)
	Yucatan miniature pig	LDLR-null	rAAV and SCNT	Davis et al. (2014)
Cardiac arrhythmia				
	Yucatan miniature pig	SCN5A-E558X	rAAV and SCNT	Park et al. (2015)
	Goat	TGF-β1 human transgene	Electroporation and SCNT	Polejaeva (2013)
Cancer				
	Yucatan miniature pig	TP53-R167H	rAAV and SCNT	Sieren et al. (2014)
	Domestic pig	TP53-R167H	Electroporation and SCNT	Leuchs et al. (2012)
	Domestic pig	APC1061 and APC1311	Electroporation and SCNT	Flisikowska et al. (2012)
	Yucatan miniature pig	BRCA1-null	rAAV and SCNT	Luo et al. (2011)
	Goat	KRAS-G12D human transgene	Electroporation	Gong et al. (2014)
	Domestic pig	KRAS-G12D	Electroporation and SCNT	Li et al. (2015)
Huntington's disease				
	Sheep	HTT-CAG73 full-length human transgene	Pronuclear injection	Jacobsen et al. (2010)
	Tibetan miniature pig	HTT-CAG105 partial human transgene	Electroporation and SCNT	Yang et al. (2010)
	Libĕchov miniature pig	HTT-CAG145 full-length human transgene	Pronuclear injection	Baxa et al. (2013)
Alzheimer's disease				
	Göttingen miniature pig	APP695sw human transgene	Lipofection and SCNT	Kragh et al. (2009)
	Göttingen miniature pig	PSEN1M1461 human transgene	Lipofection, RMCE, and SCNT	Jakobsen et al. (2013)
Parkinson's disease				
	Banna and Bama miniature pig	PARK2- and PINK1-null	CRISPR/Cas9 and SCNT	Zhou et al. (2015)
	Domestic pig	PARK7-null	TALEN	Yao et al. (2014)

Table 1 continued

Human disease	Species	Gene/modification type	Technical approach	Reference
Spinal muscular atrophy				
	Domestic pig	SMN-null	Electroporation and SCNT	Lorson et al. (2011)
Amyotrophic lateral sclerosis				
	Tibetan miniature pig	SOD1-G93A human transgene	Electroporation and SCNT	Yang et al. (2014)
	Yucatan miniature pig	SOD1-G93A human transgene	Electroporation and SCNT	Chieppa et al. (2014)
Ataxia-telangiectasia				
	Minnesota miniature pig	ATM-null	Electroporation and SCNT	Kim et al. (2014)
	Yucatan miniature pig	ATM-null	rAAV and SCNT	Beraldi et al. (2015)
Duchenne muscular dystrophy				
	Domestic pig	DMDex52del	Electroporation and SCNT	Klymiuk et al. (2013)
Polycystic kidney disease				
	Chinese experimental miniature pig	PKD1-null	ZFN and SCNT	He et al. (2015)
Hemophilia A				
	Domestic pig	FVIII-null	Electroporation and SCNT	Kashiwakura et al. (2012)
Retinitis pigmentosa				
	Domestic pig	RHO-P347L porcine transgene	Pronuclear injection	Petters et al. (1997)
	NIH miniature pig	RHO-P23H human transgene	Electroporation and SCNT	Ross et al. (2012)
Stargardt-like macular dystrophy				
	Domestic pig	ELOVL4-5bpdel and ELOVL-Y270X transgene	Pronuclear injection	Sommer et al. (2011)
Cone rod dystrophy				
	Domestic pig	GUCY2D dominant negative human transgene	Embryo injection with lentivirus	Kostic et al. (2013)
Hereditary tyrosinemia type I				
	Domestic pig	FAH-null	rAAV and SCNT	Hickey et al. (2014)
Albinism				
	Banna and Bama miniature pig	TYR-null	CRISPR/Cas9 and SCNT	Zhou et al. (2015)
Immunodeficiency				
	Domestic pig	IL2RG-null	Electroporation and SCNT	Suzuki et al. (2012)
	Domestic pig	IL2RG-null	ZFN and SCNT	Watanabe et al. (2013)
	Minnesota miniature pig	RAG2-null	TALEN and SCNT	Lee et al. (2014)
	Bama miniature pig	RAG1/RAG2-null	TALEN and SCNT	Huang et al. (2014)

rAAV recombinant adeno-associated virus, *SCNT* somatic cell nuclear transfer, *ICSI* intracytoplasmic sperm injection, *SB* sleeping beauty, *RMCE* recombination-mediated cassette exchange, *CRISPR* clustered regularly interspaced short palindromic repeats, *TALEN* transcription activator-like effector nuclease, *ZFN* zinc-finger nuclease. Gene symbols are defined in text

field is found with cystic fibrosis (CF). CF is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR*) (Welsh et al. 2001). The CFTR protein transports chloride and bicarbonate across epithelia in the lung, intestine, pancreas, sweat gland, and other sites. CFTR also regulates the activity of a variety of other membrane transport processes. The most common CF-associated mutation is the deletion of Phe508 (F508del), which causes CFTR misprocessing and degradation by the proteosome instead of normal trafficking to the surface of the epithelial membrane.

CF is a multi-system disease, however airway infection and inflammation cause most of the morbidity and mortality. As the disease progresses, bacteria permanently colonize the airways, and patients develop persistent respiratory symptoms, progressive airway inflammation, and most die of respiratory failure. How this is caused by mutation of CFTR has been difficult to establish. To better understand the origins of the disease, several CF mouse strains carrying null and missense mutations were developed (Grubb and Boucher 1999). However, during their limited lifespan, CF mice fail to develop the lung or pancreatic disease typically found in humans. A better model was needed.

In addition to the general advantages outlined above, pigs were selected as a potential model of CF because of key similarities to humans with regard to lung anatomy and physiology, respiratory immune system responses, and their use as models of bacterial and viral pneumonias (Rogers et al. 2008a). Combining gene targeting and SCNT, two different mutations were made in the porcine *CFTR* gene, a null mutation and the common F508del (Rogers et al. 2008b). Published in 2008, these pigs were the first gene-targeted large animal models of a human disease (Rogers et al. 2008c). Another CF pig model has since been reported (Klymiuk et al. 2012). Initial studies revealed CF pigs develop all of the key clinical manifestations seen in patients, including lung disease. Since that time, the CF pig model has been used to address several fundamental questions about disease pathogenesis (Stoltz et al. 2015). For example, Stoltz and colleagues demonstrated that newborn piglets have a host-defense defect, allowing early bacterial infection in the absence of inflammation, thus addressing a long-standing "chicken and egg" paradox (Stoltz et al. 2010). This host-defense defect was found, in part, to be caused by reduced pH of airway surface liquid, thus inhibiting various antimicrobial peptides that are normally found there (Pezzulo et al. 2012). It was also discovered that mucous of CF pigs fails to detach from submucosal glands in the airway, thus impeding normal clearance of pathogens from the lung (Hoegger et al. 2014). Because of studies like these, the porcine CF model has led to an improved understanding of this human disease and provided an opportunity to develop more informed therapeutic interventions. The CF story also serves as an excellent example of the true potential of a GE livestock disease model.

Diabetes mellitus

Diabetes mellitus (or diabetes) refers to a series of disorders that share a common feature of increased blood glucose levels over time. The cause of this hyperglycemia can be categorized in four classes or types. Type 1 diabetes mellitus results from the inability of the pancreas to produce sufficient insulin due to destruction of pancreatic beta cells and represents $\sim$10 % of cases. Type 2 diabetes mellitus represents the majority ($\sim$90 %) of cases and is caused by insulin resistance and the eventual failure to produce insulin. The third class represents various types of diabetes mellitus, often caused by specific mutations, and the fourth class is gestational diabetes. In total, diabetes mellitus affects almost 400 million people worldwide and is expected to grow as a human health problem.

Pigs offer several specific advantages for modeling human diabetes mellitus, especially with regard to pancreatic anatomy and physiology, glucose metabolism, and insulin regulation. These and other key attributes are described in an excellent review of the use of pigs for diabetes research by Wolf et al. (2014). To date, three different GE porcine models have been developed.

In an effort to develop a model of a pre-diabetic condition, Renner and colleagues generated transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor (*GIPR*) (Renner et al. 2010). Referred to as $GIPR^{dn}$, this mutant receptor has normal GIP-binding activity, however the signal transduction capacity has been abolished. Thus, $GIPR^{dn}$ competes with the endogenous wild-type

receptor, lessening the insulinotropic action of GIP. The $GIPR^{dn}$ pigs develop normally, but show an age-dependent decline in glucose control that results from impaired insulin secretion. This model also demonstrates a concomitant reduction in pancreatic beta-cell mass. The $GIPR^{dn}$ pig has been useful for identifying biomarkers and for pre-clinical studies of therapeutics for Type II diabetes (Renner et al. 2012; Streckel et al. 2015).

A porcine model of permanent neonatal diabetes (PNDM) has also been developed (Renner et al. 2013). Mutations in the human insulin (*INS*) gene cause insulin-deficient, permanent diabetes, typically in the neonatal period. Transgenic pigs expressing porcine INS^{C94Y} (orthologous to the human INS^{C96Y}) are believed to produce a mutant insulin protein that is misfolded, induces endoplasmic reticulum stress, and triggers beta-cell apoptosis. The INS^{C94Y} transgenic pigs are hyperglycemic in the perinatal period, develop cataracts, and demonstrate reduced body weight and beta-cell mass in an age-dependent manner. Longer-term studies may be required to observe renal and neuronal phenotypes. The authors highlight the fact that INS^{C94Y} transgenic pigs may offer unique opportunities to study gene- and cell-based therapies, as well as advance islet transplantation experiments.

Finally, a third transgenic pig has been developed to model maturity onset diabetes of the young 3 (MODY3) (Umeyama et al. 2009). In humans, this disease is caused by mutations in hepatocyte nuclear factor 1α (*HNF-1α*). Umeyama and colleagues developed pigs expressing a dominant negative human *HNF-1α* cDNA. The resulting pigs possessed elevated glucose and evidence of abnormal islets.

Cardiovascular disease

Cardiovascular disease, or atherosclerosis, is the leading cause of death in the world. The disease is caused by the accumulation of low-density lipoprotein (LDL) in the subendothelial matrix of blood vessel walls, with this buildup being proportional to the amount of circulating LDL. This results in plaque formation, arterial obstruction, and diminished blood flow to organs. If these plaques rupture, it can lead to occlusive thrombosis and ultimately myocardial infarction or stroke. In humans, mutations in several cholesterol metabolism-related genes, including LDL receptor (*LDLR*) and proprotein convertase subtilisin/kexin type 9 (*PCSK9*), cause familial hypercholesterolemia (FH), which is associated with severe atherosclerosis and early death (Brautbar et al. 2015; Goldstein and Brown 2015).

Pigs have been a popular choice for studying cardiovascular disease. Like humans, when a diet consisting of high saturated fat and/or cholesterol is consumed, pigs develop elevated LDL and subsequent atherosclerosis (Dixon et al. 1999). Furthermore, a line of pigs with a naturally occurring LDLR mutation has been studied as a model of FH (Prescott et al. 1991; Rapacz et al. 1986).

The first reported GE model of cardiovascular disease focused on PCSK9. Normally, PCSK9 regulates the membrane expression of the LDL receptor by targeting it for lysosomal degradation. A gain-of-function mutation (D374Y) has been identified in a subset of FH patients that leads to a higher rate of LDL receptor degradation, thus reducing the overall number of receptors present at the cell membrane (Horton et al. 2009). Al-Mashhadi et al. developed a Yucatan miniature pig with liver-specific expression of a human *D374Y-PCSK9* transgene (Al-Mashhadi et al. 2013). As expected, these pigs have fewer LDL receptors, reduced LDL clearance, hypercholesterolemia, and spontaneous development of atherosclerotic plaques. They also show the phenotype can be accelerated by using a high fat, high cholesterol diet. In addition to recapitulating the important features of human FH, the authors also demonstrate one of the true strengths of large animal species by utilizing clinical imaging technology (positron emission tomography and computed tomography) developed for humans.

Using a gene targeting strategy, our group developed a Yucatan miniature pig with a targeted disruption of the endogenous *LDLR* (Davis et al. 2014). Since *LDLR*+/− pigs have one unaffected allele, they present with mild hypercholesterolemia, however the *LDLR*−/− pigs are unable to clear LDL, and thus have more severely elevated cholesterol levels and develop human-like atherosclerosis. These phenotypes can be further accelerated and exacerbated with a western-style diet, providing a versatile model of human cardiovascular disease.

Cardiac arrhythmia

The beating of the heart is governed by a coordinated propagation of electrical activity through cardiac muscle, which is mediated through several families of ion channels. Sodium, potassium, and calcium channels generate the cardiac action potential, and mutations in these channels cause a wide range of disorders, or channelopathies, by disrupting the normal rhythm of heart (i.e. arrhythmias) (George 2014). These disorders have been studied in mice, but the vast differences between mouse and human hearts, including size and heart rate, have limited their utility (Nerbonne 2014). Working in the Yucatan miniature pig, our team introduced a loss-of-function mutation in the endogenous *SCN5A* (Park et al. 2015). This gene encodes the alpha subunit of the sodium channel $Na_V1.5$ and is a site of mutation for many cardiac arrhythmias. The *SCN5A* mutant pigs exhibited numerous electrocardiogram abnormalities and a phenotype consistent with progressive cardiac conduction defects. In addition to being useful for drug and device development, the *SCN5A* model demonstrates that pigs may be a good species in which to model other ion channelopathies.

Cardiac electrical activity can also be perturbed by damage to heart tissue. This is the basis for a recently described goat model of atrial fibrillation. Polejaeva and colleagues introduced a transgene designed to overexpress human TGF-β1 in the atria of the caprine heart (Polejaeva 2013). A similar approach in mice induced atrial fibrosis, which is thought to increase the susceptibility to atrial fibrillation (Verheule et al. 2004). However, the mouse model has limited translational applicability. Atrial fibrillation is the most common clinically significant cardiac rhythm disturbance in humans and is associated with a significantly increased risk of stroke (Mickelsen et al. 2005). It is hoped that the goat can provide a better model.

Cancer

Cancer is a group of diseases resulting from uncontrolled cell growth, which can affect numerous cell types and organs at all stages of life. Despite an ever-growing understanding of the environmental risk factors, genetic contributions, and pathogenic mechanisms, the diagnoses and treatments for cancer remain inadequate.

The first GE large animal with confirmed cancer was a Yucatan miniature pig with mutant tumor protein p53 (Sieren et al. 2014). *TP53* (encoding p53) is the most commonly mutated gene in human cancers (Levine and Oren 2009). Known as the "guardian of the genome", p53 is a transcription factor that regulates critical cell functions including cell-cycle arrest and apoptosis. The loss of proper p53 function predisposes cells to unregulated growth, tumor formation, and metastasis. Patients with germline *TP53* mutations have Li-Fraumeni syndrome and are subject to multiple cancer types (Nichols et al. 2001). Sieren and colleagues introduced a mutation frequently seen in human cancers (R167H; orthologous to human R175H) into the endogenous *TP53* allele (Sieren et al. 2014). Characterization of tumor development in homozygotes ($TP53^{R167H/R167H}$) and heterozygotes ($TP53^{R167H/+}$) was enabled by the use of clinical imaging technology. $TP53^{R167H/R167H}$ pigs developed a variety of cancers within the first year and a half of life, including lymphoma, osteogenic tumors (osteosarcoma), and Wilms tumor (nephroblastoma). In addition to successfully detecting the solid tumors, the imaging technology was used to guide tissue harvest for histopathological validation and molecular genetic analyses. As seen with the human mutant p53, the porcine p53-R167H protein failed to mediate protective checkpoint responses in cells and promoted significant chromosomal instability in tumors from $TP53^{R167H/R167H}$ pigs. No solid tumors were detected in the $TP53^{R167H/+}$ pigs during 2.5 years of observation, which may make them well suited for testing cooperative effects of secondary oncogene activation or tumor suppressor gene loss. Another group has also reported the lack of tumor development in similarly designed *TP53* heterozygote pigs (Leuchs et al. 2012).

Familial adenomatous polyposis (FAP) is an inherited disorder that predisposes patients to colorectal cancers (Fodde et al. 2001). FAP is caused by germline mutations in the adenomatous polyposis coli (*APC*) gene and is inherited in an autosomal dominant fashion. Patients with FAP develop benign polyps in the colon early in life, and if not removed, the polyps will become malignant. Using the domestic pig as a model, Flisikowska and colleagues introduced two different FAP-associated nonsense mutations into the endogenous *APC* gene, APC^{1061} and APC^{1311} (Flisikowska

et al. 2012). The human ortholog of APC^{1061} is associated with a less severe, later-onset disease, while the ortholog of APC^{1311} has an early onset, severe phenotype. Both genotypes were evaluated at 1 year of age with the assistance of human endoscopic imaging equipment. As expected, the later onset-associated $APC^{1061/+}$ pig had no polyps, however the $APC^{1311/+}$ pig did possess numerous lesions and polyps throughout the colon and rectum. Histopathology confirmed pre-cancerous lesions similar to those seen in humans, showing great promise for future cancer development and utility as a model for human FAP.

Other common cancer-related genes have been targeted in livestock species, and while no cancer development has been reported in these animals as of yet, it is important to note their creation and potential availability for crossing with existing, as well as new, models. For example, several groups, including our own, have generated pigs with inducible *KRAS* mutations that could be studied alone or in conjunction with mutant *TP53* pigs (Li et al. 2015). The breast cancer associated gene 1 (*BRCA1*), which in addition to breast cancer also plays a role in ovarian cancer, has also been targeted in pigs (Luo et al. 2011). Finally, in an effort to develop a caprine model of cancer, goats with an inducible *KRAS* mutation are being developed, though cancer development has yet to be reported (Gong et al. 2014).

Huntington's disease

Huntington's disease (HD) is caused by autosomal dominant inheritance of an expanded trinucleotide repeat (in this case CAG, encoding glutamine) in the huntingtin (*HTT*) gene (Johnson and Davidson 2010). As with other trinucleotide repeat disorders, the severity of disease is proportional to the number of CAG repeats. In humans, the presence of less than 28 repeats is considered normal, but more than 40 is pathogenic. A juvenile-onset form of HD is seen when more than 60 CAG repeats are present.

Sheep with a transgene consisting of the full-length human *HTT* cDNA carrying 73 CAGs and driven by the human *HTT* promoter have been developed (Jacobsen et al. 2010). These sheep are confirmed to express mutant huntingtin and develop aggregates and inclusions in the brain, however they have yet to display clinical signs of the disease in their first 5 years of life (Morton and Howland 2013).

Two groups have produced transgenic porcine HD models. Yang and colleagues reported the creation of pigs with a transgene consisting of an N-terminal fragment of human huntingtin with 105 CAG repeats (Yang et al. 2010). Pigs with the highest transgene expression died within hours or days of birth. The one pig that survived had the lowest levels of mutant huntingtin. It is unclear if the severe and rapid phenotype experienced was the result of general transgene toxicity, SCNT effects, or related to the *HTT* genotype. Baxa et al. produced two different transgenic pigs, one with an N-terminal fragment of human huntingtin and the other with the full-length protein, each with 145 CAG repeats (Baxa et al. 2013). Mutant huntingtin expression has been confirmed in these pigs, but no overt HD phenotype has been observed in the first 3 years.

It is important to note that the lack of phenotypic presentation in the HD sheep and pig models is not necessarily unexpected. HD is a slowly progressing disease, and it is possible these models will require more time to fully manifest disease. Also, each of the HD models discussed thus far has been designed to express an exogenous mutant transgene on the background of two wild-type *HTT* alleles. It is possible the expanded CAG repeats would be more appropriate if expressed from an endogenous allele. Our lab has recently developed a porcine model of HD by using homologous recombination to expand the normal CAG tract to 85 repeats (data not published). It is still too early to know whether this approach will yield a more useful model.

Alzheimer's disease

Alzheimer's disease (AD) is a chronic neurodegenerative disorder associated with loss of memory, confusion, and dementia. Two transgenic porcine models of AD have been developed that express mutations associated with familial AD. In the first example, Göttingen miniature pigs were engineered with a single copy of a transgene encoding the neuronal splice variant of the amyloid precursor protein (*APP*) gene referred to as APP695, which contains a two amino acid mutation commonly referred to as the

Swedish mutation (APP695sw) (Kragh et al. 2009). In humans, this mutation leads to disease as early as 45 years of age. Pigs expressing APP695sw are being monitored for clinical signs, including memory deficits, but have yet to demonstrate a disease phenotype by 2 years of age (the latest time reported so far) (Sondergaard et al. 2012). This same group has also generated a transgenic Göttingen miniature pig expressing a mutant form of presenilin (PSEN1M1461), however no phenotypic data has been published (Jakobsen et al. 2013).

Parkinson's disease

Parkinson's disease (PD) results from the progressive loss of dopamine-producing cells in the brain, causing tremors, rigidity, slowed movement, and postural instability. Two groups have recently generated gene-edited pigs that may serve as models of PD. In the first, TALENs were used to generate DJ-1-deficient pigs by disrupting the *PARK7* gene, which is also associated with earlier onset PD (Yao et al. 2014). Unfortunately, all of the pigs died within the first 2 days of life due to apparent defects associated with SCNT. The second group used the CRISPR/Cas9 system to make double knockouts of early-onset PD-associated genes parkin RBR E3 ubiquitin protein ligase (*PARK2*) and PTEN-induced putative kinase 1 (*PINK1*) (Zhou et al. 2015). Preliminary studies showed that both proteins are absent from the brains of *PARK2*−/−/*PINK1*−/− pigs, however no phenotypic symptoms have been observed in the first 7 months of life (the latest time reported so far).

Spinal muscular atrophy

Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease caused by mutations in the *SMN1* gene, which leads to progressive muscle wasting and impaired mobility. Humans also have an *SMN2* gene, which is nearly identical to *SMN1*, but a sequence variation causes most of the *SMN2*'s transcripts to yield a truncated protein that is rapidly degraded. Approximately 10-20 % of *SMN2* transcripts are normal however, thus providing for a nominal amount of normal SMN protein, otherwise the SMN1 deficiency would be embryonic lethal. The variable amount of full-length *SMN2* transcripts also accounts for the variability of disease severity.

Lorson et al. are developing a porcine model of SMA (Lorson et al. 2011). However, because pigs lack an *SMN2* gene, the complicated genetic scenario seen in humans must be replicated in order to produce viable pigs. This is being achieved in a three-step manner. First, one *SMN1* allele is being disrupted by homologous recombination. Second, the full human *SMN2* is being added as a transgene to the *SMN1*± background. Finally, breeding is being used to get the *SMN1* allele to homozygosity (Prather et al. 2013).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive disease characterized by the loss of motor neurons, leading to muscle weakness, atrophy, paralysis, and death (Boillee et al. 2006). Most ALS cases are sporadic, however ~10 % are believed to be genetic. One commonly mutated gene in familial ALS is superoxide dismutase 1 (*SOD1*) (Rosen et al. 1993).

Two groups have generated transgenic pigs expressing the G93A mutant of human *SOD1* (Chieppa et al. 2014; Yang et al. 2014). Yang et al. provide phenotypic characterization of an initial cohort of $hSOD1^{G93A}$ pigs. They describe an age-dependent development of clinical manifestations, including motor neuron degeneration that leads to hind limb motor defects. The $hSOD1^{G93A}$ pigs also demonstrate cellular and biochemical phenotypes that are seen in humans, but not in similar murine models. Many of the phenotypic developments appear to be transgene dose-dependent, so further refinement of the model will likely be necessary. Longer studies with a larger number of animals will be required to understand the true utility of the porcine models of ALS, but the early data are promising.

Ataxia-telangiectasia

Ataxia telangiectasia (AT) is a progressive, multisystem disorder caused by recessive mutations in the AT-mutated (*ATM*) gene (Lavin 2008). AT is characterized by cerebellar degeneration in children leading to motor impairment, dysarthria, and oculocutaneous

telangiectasia. The disease includes additional manifestations including immune disorders, increased susceptibly to cancer, and respiratory infections.

Porcine AT models are being produced by at least two different groups. Kim et al. used homologous recombination to disrupt endogenous *ATM* and have reported the generation of heterozygote animals (Kim et al. 2014). Our team is also developing an AT model. Initial studies with *ATM−/−* pigs found early cerebellar lesions including loss of Purkinje cells and altered cytoarchitecture suggesting a developmental etiology for AT. Also, *ATM−/−* pigs develop motor deficit phenotypes similar to humans (Beraldi et al. 2015).

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disease caused by mutations in the X-linked gene, *DMD,* which encodes the protein, dystrophin (Wallace and McNally 2009). DMD patients experience progressive weakness and degeneration in skeletal muscle, including the diaphragm, cardiac muscle, as well as some smooth muscle.

A dystrophin-deficient porcine model was developed by Klymiuk et al. by deleting exon 52 of the endogenous *DMD* in domestic pigs (Klymiuk et al. 2013). Exon 52, as well as neighboring exons, is frequently deleted in humans and is a site of intense interest for targeting several gene-based therapies (Touznik et al. 2014). Therefore, this DMD model could have broad application for understanding pathophysiology as well as developing various therapeutic approaches. These pigs develop many of the clinical manifestations of the human disease including elevated creatine kinase, dystrophic skeletal muscle, impaired mobility, and progressive muscle weakness, however the phenotype appears to be much more rapid and severe. Many DMD pigs died shortly after birth, and maximum lifespan is reported to be 3 months. Our lab has developed a similar model in Yucatan miniature pigs, and our experience is similar, though some pigs have lived as long as 6–7 months (data not published).

Autosomal dominant polycystic kidney disease

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common, potentially fatal genetic disorders in humans and is primarily characterized by the growth of cysts within the kidney, which eventually destroys renal function and commonly leads to renal failure (Igarashi and Somlo 2002). Mutations in one of two genes, *PKD1* or *PKD2*, are the cause of ADPKD. The *PKD1* and *PKD2* genes encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively. Mutations in *PKD1* are the most common, accounting for 85 % of all ADPKD cases.

A mono-allelic knockout of porcine *PKD1* was recently published (He et al. 2015). Renal cysts were detected as early as 3-months of age and grew slowly over the course of the 24-month study. This slow progression is similar to what is seen in humans, and may provide an appropriate setting for studying therapeutic interventions. About half of ADPKD patients eventually require dialysis or renal transplantation. ADPKD also affects other organs, and manifestations include the development of ductal cysts in the liver and pancreas, hypertension, vascular aneurysms, cardiac hypertrophy, and valve defects. Besides noting the presence of hepatic cysts in *PKD1* mutant pigs, this study did not investigate the other renal and extrarenal features of the human disease; therefore additional characterization will be required.

Hemophilia A

Hemophilia A is an X-linked bleeding disorder caused by mutations in the coagulation factor VIII (*FVIII*) gene (Bolton-Maggs and Pasi 2003). Patients experience excessive bleeding in the brain, joints, and muscles, which can be life threatening. A porcine model of hemophilia A has been developed by disrupting the endogenous *FVIII* gene (Kashiwakura et al. 2012). Resulting pigs experienced severe bleeding at birth, and 3 of 4 piglets were dead within 3 days. A fourth piglet continued to experience bleeding events, but was effectively treated with human factor VIII infusions. However, that piglet eventually developed inhibiting antibodies against the factor VIII treatment and died. The hemophilia A pig will be extremely challenging to maintain, but does have the potential to serve as an effective model of the disease.

Retinitis pigmentosa

Retinitis pigmentosa (RP) is a degenerative retinal disease that leads to loss of vision (Daiger et al. 2014).

The disease begins with the loss of rod photoreceptors early in life, followed by the slowly progressive loss of cone photoreceptors in the central retina. Clinically, this manifests initially as night blindness, with subsequent loss of peripheral vision and ultimately central vision resulting in total blindness. RP is a heterogeneous disease, both genetically and phenotypically. Mutations in over 50 genes have been shown to cause RP.

Because of the many similarities between the human and porcine eye, including the ratio of rod and cone photoreceptors ratios as well as size, two different GE pigs have been developed to model RP. Both are transgenic pigs expressing different RP-associated rhodopsin (*RHO*) mutants. The first was developed in a domestic pig line by Petters and colleagues and contains the full porcine *RHO* with a P347L mutation (Petters et al. 1997). As mentioned before, this pig was the first GE large animal model of a human disease. Phenotypic characterization revealed many similarities to the human disease, including early loss of rod photoreceptors and a progressive loss of cones. This pig has been useful in better understanding the early mechanistic steps of disease pathogenesis (Li et al. 1998). A second RP pig was developed for the purpose of developing cell-based therapies. Working in the NIH miniature pig, Ross et al. inserted a transgene carrying the full human *RHO* gene with a P23H mutation, which is the most common mutation in autosomal dominant RP (Ross et al. 2012). These pigs also developed the phenotypic characteristics of human RP. In addition to being a smaller and more manageable breed of pigs, the NIH miniature pig is inbred and has a defined major histocompatibility complex haplotype. This will allow the P23H pig to be used for pre-clinical testing of cell-based therapies with less likelihood of immunological rejection.

Stargardt-like macular dystrophy

One weakness of modeling retinal diseases in rodents is their lack of a macula, a specialized region of the human retina responsible for visual acuity. While pigs do not have a formal macula, they do possess a comparable region called the area centralis (Chandler et al. 1999). Due to this fact, Sommer et al. hypothesized that the pig would yield an improved model of Stargardt-like macular dystrophy type 3 (STGD3), an autosomal dominant form of macular degeneration with a juvenile onset (Sommer et al. 2011). Utilizing a transgenic strategy, pigs expressing mutant elongation of very long chain fatty acids-4 (*ELOVL4*) were generated. Resulting pigs demonstrated protein mislocalization, photoreceptor loss, and abnormal retinographic data, suggesting this model may provide useful insight into pathogenic mechanism of STGD3. Furthermore, this model could open the door to other models of macular degeneration, which is the leading cause of blindness in older adults (Stone et al. 2001).

Cone rod dystrophy

A third porcine model of a retinal disease has been produced for the purpose of studying cone rod dystrophies (CRDs) (Kostic et al. 2013). CRDs are inherited retinal dystrophies that result from the loss of cones, followed by the loss of rods (in contrast to RP, described above) (Hamel 2007). The transgenic expression of a dominant-negative guanylate cyclase 2D (*GUCY2D*) mutation resulted in diminished retinal function and abnormal retinal histology. These findings were accompanied by progressive visual impairment over the duration of the study. Further study of this model may lead to a better understanding of cone dystrophies and provide a platform in which to test therapeutic interventions.

Hereditary tyrosinemia type 1

Hereditary tyrosinemia type 1 (HT1) is a metabolic liver disease caused by the lack of the enzyme, fumarylacetoacetate hydrolase (*FAH*). In humans, the disease presents in the first months of life and is fatal if not treated. Hickey and colleagues developed a FAH-deficient pig via homologous recombination; however unlike humans, FAH deficiency results in embryonic lethality (Hickey et al. 2014). Live *FAH*−/− pigs were produced after administering NTBC to pregnant sows (NTBC is a drug that blocks an upstream enzymatic step, preventing the accumulation of toxic metabolites including fumarylacetoacetic acid) and were phenotypically normal at birth. Removal of NTBC treatment leads to acute liver failure and resembles some aspects of the human HT1 phenotype at that point. The authors

suggest that the *FAH−/−* pig may also serve as a unique tool for testing in utero gene therapy, as well as for organ complementation and regenerative medicine applications.

Albinism

Mutations in the gene encoding tyrosinase (*TYR*) cause oculocutaneous albinism type 1, a metabolic disorder characterized by the lack of melanin production in the hair, skin, and eyes (Oetting 2000). Targeting the *TYR* gene in mice is a popular choice for testing and optimizing new gene GE techniques due to the rapid phenotypic read-out. CRISPR/Cas9 was used to generate bi-allelic *TYR−/−* pigs, which expressed the expected albinism phenotype (Zhou et al. 2015).

Immunodeficiency

Severe combined immunodeficiency (SCID) defines a group of genetic disorders in which defects in the T- and B cell systems severely impair the immune system (Rivers and Gaspar 2015). Two SCID types have recently been modeled in pigs, X-linked SCID and Omenn syndrome. By mutating the X-linked interleukin-2 receptor gamma chain (*IL2RG*), several groups have generated pigs that recapitulate the human disease, including a small or non-existent thymus, impaired T- and NK-cell production, and susceptibility to pathogens (Suzuki et al. 2012; Watanabe et al. 2013). Models of Omenn syndrome have been produced by disrupting porcine recombination activating genes 1 (*RAG1*) and/or 2 (*RAG2*) (Huang et al. 2014; Lee et al. 2014). By preventing V(D)J recombination, these pigs lack functional B-and T-cells and fail to thrive. While these pigs do model human SCIDs, their primary purpose is for regenerative medicine and xenotransplantation studies, which is reviewed by Niemann and Petersen in this issue.

Conclusion

The GE livestock biomedical model field has come a long way in its first 18 years. GE pig, sheep, and goat models have been featured in (and on the covers of) the top scientific journals and in the lay press. They have reinvigorated disease-focused research communities by providing improved research tools and attracting new funding. GE livestock researchers have been on, if not a bit ahead of, the cutting edge for new gene-based and reproductive technologies. The field now finds itself with the reality that most of the technical hurdles between what can be *imagined* and what can be *made* have been overcome. So what will be the next major challenge?

Perhaps the biggest hurdle facing GE livestock disease models is acceptance. Whether it comes from researchers and institutions more accustomed to rodent models, funding agencies balking at larger expenses and longer timelines, or preclinical end-users who are hesitant to change the status quo, large animal models like those described here are often met with significant resistance. Because of the greater costs and efforts required to utilize these models, the expectations for their usefulness are correspondingly (and sometimes unrealistically) high. The current paradigm will be hard to shift. How might this be accomplished? The burden will be on model developers to make the models that *need* to be made, not just the ones that *can* be made. This will require multidisciplinary groups with sufficient human disease perspective, clinical veterinary knowledge, and a clear plan for meaningful model design, characterization, and validation. The costs of developing a better disease model in a livestock species are high, but every stakeholder understands the fact that the costs of not having one are even higher. The successful models will have to show their worth by revealing new disease mechanisms, improved predictive efficacies, better drug and device safeties, and ultimately, by contributing to improved human health. Only a few models have been around long enough to even begin to be judged by these criteria, but if those are any indication, the acceptance hurdles can eventually be overcome, just as the technical ones were.

As we celebrate Transgenic Animal Research Conferences I through X, it is also exciting to look ahead to the biomedical-related progress that will be presented at the meetings between now and XX. This is likely to include successful applications of GE livestock animals in regenerative medicine, gene and cell based therapies, and a few advances we have not even considered yet. Livestock animal species have the potential to fill a significant portion of the gap between the currently used animal models and

humans. If they are to live up to this potential, those attending the next ten Transgenic Animal Research Conferences are likely to have a front row seat to the process.

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