Unraveling the Swine Genome: Implications for Human Health

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Abstract

The pig was first used in biomedical research in ancient Greece and over the past few decades has quickly grown into an important biomedical research tool. Pigs have genetic and physiological traits similar to humans, which make them one of the most useful and versatile animal models. Owing to these similarities, data generated from porcine models are more likely to lead to viable human treatments than those from murine work. In addition, the similarity in size and physiology to humans allows pigs to be used for many experimental approaches not feasible in mice. Research areas that employ pigs range from neonatal development to translational models for cancer therapy. Increasing numbers of porcine models are being developed since the release of the swine genome sequence, and the development of additional porcine genomic and epigenetic resources will further their use in biomedical research.

CREATING THE BUILDING BLOCKS: GENOMICS, TRANSGENICS, AND CLONING

The release of its genome sequence has provided a critical component for the development and broad acceptance of the pig as a biomedical model (**http://www.ensembl.org/Sus_scrofa/Info/Index**) (1, 2). Key building blocks for full use of the pig as a biomedical model are now in place: the completed genome sequence, the ability to produce transgenic animals, and the ability to replicate the model through somatic cell cloning (1, 3). The emergence of genetic information and the development of the necessary tools to target genetic manipulations, in combination with the ability to clone pigs, provide an innovative approach to validating and creating new and highly relevant animal models. These building blocks have stimulated the development of genomic postulates for evaluating animal models and the significance of the pig (4), including

1) isolating and propagating the gene from the animal,

2) characterizing (manipulating) the gene in vitro,

3) reintroducing the putative gene (creating a transgenic animal) to test causality, and

4) demonstrating the causal relationship through the induced phenotype.

This article was developed to provide background on the need for relevant animal models and to address each of the aspects of the genomic postulates. Owing to the considerable physiological (5) and genomic similarities between pigs and humans (6), the pig provides a uniquely relevant animal model for human disease. In addition, a recent search using the Computer Retrieval of Information on Scientific Projects (CRISP) (1999–2003) has indicated that the National Institutes of Health—and through it more than 20 institutes and centers—has provided more than 2,400 separate grants that featured the pig. Thus, a broad foundation supporting the pig as a model in biomedical research already exists from which to build future programs. There is also growing interest within the biomedical community with respect to the use of pigs in bioengineering, imaging, and behavioral studies.

SWINE AS A MODEL IN THE PRE-GENOMIC ERA

The Animal Model Concept: Criteria for Relevance and Validation

Animal models have played a central role over the centuries in scientific investigations of human disease, etiology, disease progression, and treatment strategies. Historically, animal models of human disease were deemed relevant only if they were useful in recapitulating disease pathogenesis and assisting in the development of approaches for intervention or therapy (7). Ultimately, researchers recognized that animal models need to reliably mimic the normal anatomy and physiology of human organs and tissues of interest, as well as to accurately reflect the morphological and biochemical aspects of disease pathogenesis (4).

The use of animals in scientific research is an ancient practice (**Table 1**). As early as the time of Erasistratus of Alexandra (302–258 BC), pigs were used to elucidate the function of the circulatory and respiratory systems (8). Although the Catholic Church prohibited work on human cadavers, Galen, a Greek surgeon, physician, and philosopher (AD 129–c. 200), who was considered the founder of experimental physiology, dissected apes and pigs to better understand the circulatory, respiratory, and nervous systems (9). Galen mistakenly assumed that everything he learned from those animals directly correlated to humans. Today, we understand that animal models are not 100% correlated with humans, and some animal models correlate better than others. In the seventeenth century, an English physician, William Harvey (1578–1657), used small animal,

Table 1 Timeline of pigs in biomedical science[a]

Date	Event
302–258 BC	Erasistratus: studied the circulatory system of pigs
129–200 AD	Galen: studied the respiratory and nervous system of pigs
1578–1657	William Harvey: established principles of circulation
1865	Claude Bernard: conducted first experimental research on animals
1915–1949	Porcine thyroid extract used to treat hyperthyroidism
1921–1982	Porcine insulin used to treat diabetes
1930–Present	Porcine heparin only FDA-approved source
1960s	Thyrotropin releasing factor and luteinizing hormone releasing factor isolated from porcine hypothalami (Nobel Prize 1978)
1969	First porcine aortic valve implanted
1981	First transgenic mouse (microinjection)
1982	Genentech introduced recombinant human insulin
1986	First transgenic pig (microinjection)
1989	Pig generated by transfer of embryonic cell (blastomere) nucleus into enucleated oocyte
1991	Tao injected cultured fetal fibroblast cells into enucleated oocytes
1996	Dolly born; first clone from adult somatic cell nuclear transfer
1997	Retinitis pigmentosa (Rhodopsin, mutant P347L)
2000	Pigs cloned by nuclear transfer from adult somatic cells
2001	Huntington's disease porcine model
2002	Porcine sequencing initiative white paper submitted
2003	Pig Project Sequencing initiated
	The Encyclopedia of DNA Elements (ENCODE)
2004	Pilot human epigenome project published
2008	Cystic fibrosis porcine model
2009	Porcine 60k single nucleotide polymorphism chip
2010	Huntington's disease porcine model 2
2012	Pig genome sequence published
	Neonatal pig brain MRI atlas online (**http://pigmri.illinois.edu/**)
	Retinitis pigmentosa porcine model (Rhodopsin, mutant P23H)
	A porcine model of familial adenomatous polyposis

(*Continued*)

Table 1 (*Continued*)

Date	Event
2013	Genome-edited pigs born (TALEN and ZNF injected into zygote)
2014	Inducible P53/Kras porcine cancer model
	Formation of the International Swine Methylome Consortium

[a]The pig has been has played a major role in biomedical studies for centuries. This timeline includes major landmark accomplishments from the earliest investigations in ancient Greece to the current day.

sheep, and pig studies to accurately describe how blood was pumped in a circular course around the body by the heart, thus establishing the principles of circulation. The idea of inducing disease in model animals was first suggested by Claude Bernard in 1865, when he published the *Introduction to Experimental Medicine* (10). It was the first time animal models were used for experimental research, rather than for visualization and understanding of biological systems (4). In the twentieth century, cardiopulmonary resuscitation as well as immunological and organ transplantation techniques were developed using animal models. Additionally, spontaneous animal diseases were studied. Technological advances now allow researchers to induce disease in animals through various surgical, genetic, or chemical methodologies.

Advantages and Disadvantages of Animal Models

The use of animals in biomedical research has traditionally and primarily been conducted in mice and other rodents. However, larger animals, such as nonhuman primates, dogs, cats, and pigs, have also been used. The rodents' small size creates a challenge for their use as human disease models that employ surgical or imaging methodologies, whereas nonhuman primates are expensive and additionally raise ethical concerns. Dogs and cats have also been used, but these models, when not used as spontaneous clinically presented models, also raise ethical concerns owing to their use as companion animals. Over recent years, pigs have emerged as an important biomedical model due to their anatomical, genetic, and physiological similarities with humans, as well as their broad availability, short generation interval, large litter size, and the fact that they are a food source (4).

Since pigs are also similar in body mass to humans, they are ideal models for tissue engineering, imaging (11), surgery, chemotherapy, and radiation studies that cannot be tested accurately in small animals (12). There is a vast amount of research on the genetic and environmental interactions associated with complex polygenic physiological traits in pigs, which makes them a relevant model for studies in obesity, female health, cardiovascular disease, nutritional studies, and communicable disease (5). In addition, there are many established cell lines from a variety of swine tissues used for a wide range of in vitro studies.

The pig is proving to be a robust cancer model because porcine cells are quite resistant to transformation, requiring multiple genetic changes, just as in human cells. In addition, many parallels of cancer biology are conserved at a molecular level, and frequently occurring mutations in human cancers also induce tumorigenesis in porcine cells (13). In addition, compared with rodents, the pig metabolizes drugs and can develop tumors of a size similar to those in humans (14, 15). Because of this, the pig provides an ideal system for preclinical studies of imaging, preclinical drug screening, and many interventional therapies like hyperthermia, radiation, or photodynamic therapy (16).

Pigs also provide atherosclerosis, myocardial infarction, and general cardiovascular models (17). Understanding of the genetic and physiological basis of cardiovascular diseases has come from small animal models, such as mice. However, these mouse models often fail to develop the complex characteristics of cardiovascular diseases associated with human disease. In contrast to those in mice, the anatomy and physiology of the normal cardiovascular system in swine closely resemble those of humans, and as noted by McKenzie and coworkers (18), the coronary vasculature of the pig heart is nearly identical to that of humans in terms of anatomical distribution, reactivity, and blood flow.

There is a need for models of metabolic and gastrointestinal diseases, such as obesity and inflammatory bowel disease, and although the pig's gastrointestinal (GI) tract differs anatomically from that of humans, the physiology of its digestive processes provides a robust model for human digestive diseases. The pig is emerging rapidly as a biomedical model for the study of energy metabolism and obesity in humans for many reasons, including the lack of postnatal brown fat, similar metabolism, comparable organ sizes, and omnivorous diets (19). Likewise, the similar anatomy of the urinary system and function of the kidneys make it a relevant model (20).

The use of pigs in neuroscience research has increased during the past decade. Researchers have recognized the pig's potential as an experimental model for human brain and cognitive development, as well as environmentally induced developmental alterations (21). The pig is also an increasingly popular laboratory animal for transgenic manipulations of neural genes (22). The pig brain resembles the human brain more in anatomy, growth, and development than do the brains of commonly used small laboratory animals. In addition, the pig brain is large enough that imaging techniques can identify cortical and subcortical structures. A web-based MRI pig brain atlas of the neonatal pig (**http://pigmri.illinois.edu**) has been developed recently at the University of Illinois (23). This is an open resource available to everyone and covers domestic breed piglets.

Historical Perspectives and Implications of Porcine Models for Human Health

Humans have long benefited from biomedical research performed by using products generated from the pig (**Table 1**). Pigs are raised in large numbers for agricultural purposes, thus making large quantities of experimental tissues readily available from abattoirs. This availability has allowed swine tissues to be used as the source of many therapeutic biomedical products with widespread use throughout the world. These include peptides, proteins, glycoproteins, enzymes, and tissues.

Insulin, for example, was discovered in 1921, and by 1923 commercial quantities were being produced by Eli Lilly. Initially, both bovine and porcine insulin were used; however, porcine insulin is more similar to the human protein than that from the bovine (1 versus 3 amino acid differences) (24) and less immunogenic. Thus, porcine insulin became the treatment of choice until 1982, when Genentech produced the first recombinant human insulin, an event that triggered the phaseout of the porcine-derived product. Over those 60 years, millions of diabetics worldwide were treated using porcine insulin.

Insulin has not been the only porcine biomedical product. Although porcine thyroxin was isolated in 1915 (25) and its chemical structure determined in 1926 (24) and synthesized by 1927 (26), it was a long time before the process was commercially viable. So until 1949, tablets of desiccated porcine thyroid extract remained the treatment for hypothyroidism. Initially, 3 tons of pig thyroid glands yielded only 33 g of thyroxin. This product is still available; however, it is not often the treatment of choice (27).

Another early identified and widely used porcine biological is heparin, which acts as an anticoagulant, blocking the blood clotting cascade. Heparin was first isolated from porcine intestines in the 1930s and today is still the only Federal Drug Administration–approved source of heparin.

Heparin is a glycoprotein, and synthesis of this type of molecule has not yet been successful. Currently, the global use of this product is 100 metric tons, or 1.5 billion doses yearly. Production of this quantity consumes tissue from 700 million pigs yearly (27).

In the 1960s, porcine hypothalami were used for Nobel Prize–winning work that identified, isolated, and determined the structure of the first brain hormones. It was commonly believed that hormones in the brain were secreted to control pituitary function, and so a race to identify these hormones ensued. Roger Guilleman (using sheep tissue) and Andrew Schally (using pig tissue) worked independently to isolate both thyrotropin releasing factor and luteinizing hormone releasing hormone. In 1978, Guilleman and Schally shared the Nobel Prize for Medicine for this work, along with Rosalyn Yallow for her development of the radioimmunoassay (28, 29).

In addition to the extracted biologicals, porcine tissues are used routinely for replacement heart valves and patches. The bioprosthetic heart valves, first introduced in the 1960s, are made of porcine or bovine pericardium preserved with glutaraldehyde. Patients receiving these valves do not require anticoagulation therapy. However, these glutaraldehyde-treated implants do pose some risk of deterioration in young patients, which can necessitate additional replacements. The failed valves show evidence of inflammation, frequently observed in xenotransplantation rejection. In the developed world, 275,000 valve replacements are performed annually in elderly patients. However, there are 15 million patients with rheumatic heart disease worldwide that could benefit from cardiac valve replacement surgery. The production of genetically engineered pigs, such as α1,3-galactosyltransferase gene-knockout pigs, that do not express this protein would provide a potential source of non- or less-immunogenic valves to meet worldwide demand (30). Several porcine biological graft materials are also currently available and include dermal, pericardial, and submucosal products used in hernia repair and to reinforce body wall defects and incision lines. Extensive studies are now determining which tissue source and physical, enzymatic, or chemical processes provide the most reliable products (31).

Implications in Pharmacology and Toxicology

Pigs have also been very useful in the fields of pharmacology and toxicology. Several studies that have employed an advanced, in vivo, multisampling-site pig model for studies of drug transport and metabolism have been published (32, 33). Studies of general toxicology have been performed using oral, cutaneous, parenteral, and inhalation routes in the minipig. For reproductive toxicology studies as well as safety pharmacology, the minipig offers numerous advantages over rodents and the commonly used non-rodent dog model (34). Pigs have been used to study the effects of exposure to alcohol, tobacco, feed additives, and environmental pollutants.

Use as Surgical Models

Over the past 20 years, pigs have replaced dogs as the general surgical model in the international arena for both training and research. Pigs are used to train surgeons, develop new techniques, and test devices, and as tissue donors for humans. A range of procedures, including general surgery, laparoscopy and endoscopy, transplantation, trauma procedures, implantation devices, and transplantations, are performed on pigs (35).

With the great shortage of human organs and tissues for transplantation, the pig has long been an animal of interest for liver, kidney, pancreas, or islet and heart transplantations (20). Although the size and function of these tissues are comparable to those in humans, one limitation for

xenotransplantation is the potential for cross-species infection. Although no invasive disease was reported, porcine cytomegalovirus has been detected in the tissues of a nonhuman primate recipient following a transplant (36). In contrast to exogenous infectious agents, such as cytomegalovirus, which theoretically can be excluded from the organ-source pigs, porcine endogenous retroviruses are another concern (37–39).

Development of Micro- and Minipig Breeds for Human Health

Throughout the history of its domestication, the pig has been exposed to a range of selective pressures from environmental factors, such as climate, pathogens, nutritional resources, and husbandry practices, and the planned selection for unique traits related to metabolism, fecundity, or meat production. These manipulations created a variety of distinct phenotypes relevant to current and future human research (1, 40). Breeding of miniature lines began when the popularity of porcine models increased and smaller, more manageable pigs with lower food and space requirements became necessary. Globally, several minipig strains have been developed through selective breeding practices rather than transgenic techniques. Some groups have used feral miniature breeds, such as the Yucatan, Westran, Lanyu, and Vietnamese potbellied pig, whereas others crossbred domestics with minipigs. Continued selection of some of these mini breeds has led to the development of lines of micropigs, such as the Micro Yucatan. Multiple companies breed them specifically for biomedical research, under conventional or specific pathogen–free conditions. The history of mini- and micropig breeds is covered in chapter 1 of *The Minipig in Biomedical Research* (41). The pig will continue to grow as the biomedical model of choice for bioengineering, experimental surgery, and zoonosis research related to the emergence of new diseases, such as swine influenza. As pig models have continued to grow in popularity compared to dog models, the pig has become the most common large laboratory animal species (4).

SWINE AS A MODEL IN THE POST-GENOMIC ERA

Rationale for the Pig Genome Project: Informing Human Health

The porcine sequencing initiative paper (28) was submitted to the National Human Genome Research Institute in 2002 to articulate the utility and value of knowing the porcine genomic sequence. The proposal set the stage for the worldwide collaborative effort to develop and publish a high-quality draft genome sequence for a female domestic Duroc pig (2). The pig genome was sequenced, under the auspices of the Swine Genome Sequencing Consortium (28, 42), by using a hybrid approach to combine hierarchical shotgun sequencing of BAC clones and whole-genome shotgun sequencing (42). In addition, the pig mitochondrial genome sequence (43, 44) has provided support for pig models of mitochondrial diseases.

Analysis of the pig genome has allowed the identification of natural mutations, which increase the potential for additional pig biomedical models. One hundred and twelve (45) positions have been observed where the porcine protein has the same amino acid that is implicated in a human disease. These include genes implicated in multifactorial diseases, such as obesity, diabetes, Parkinson's, and Alzheimer's disease (2). In addition, the sequencing of the genomes of 48 individual pigs has revealed 32,548 nonsynonymous SNPs, 6 known to be associated with human disease and 11 that have been linked to human disease phenotypes. Identification of these porcine variants allows further study of these diseases in a suitable pig model.

SWINE MODELS OF MULTIGENETIC DISEASES

Application of GWAS in Comparative Genomic Approaches

Genome-wide association studies (GWAS) search the genome for SNPs occurring more frequently in subjects with a particular disease than in individuals without the disease. Through use of the Illumina PorcineSNP60 BeadChip, GWAS can detect genetic variants and genomic regions associated with diverse phenotypes. GWAS was used to identify genomic regions controlling eating behavior in pigs, and comparative mapping approaches were used to map similar genomic regions to the human genome (46). In a study to identify genomic regions influencing eating behaviors in humans, researchers identified SNPs on porcine chromosome 1 associated with daily feed intake in pigs. These SNPs were located within quantitative trait loci (QTLs) that were homologous with human chromosome 6q23-24, a region that significantly influences obesity in humans (47).

Model for Cardiovascular Research

Cardiovascular diseases are the leading cause of death worldwide, with an estimated 20 million deaths in 2005 accounting for 30% of deaths worldwide (48). Atherosclerosis is the major component of cardiovascular disease. Atherosclerosis is characterized by thickening of the arterial wall resulting from the buildup of lipids, cholesterol, macrophages, calcium, and cellular waste products. The thickened vessel walls cause a significantly diminished blood flow through the affected artery. Pigs prove excellent models for human atherosclerosis owing to their similar development of spontaneous atherosclerosis as they age (49). Moreover, pigs and humans share SNPs in genes affecting atherosclerosis. For instance, an arginine-to-cysteine mutation in the low-density lipoprotein receptor, a predictor for atherosclerosis in humans, contributes to hypercholesterolemia in pigs (50). Apolipoprotein E4 (ApoE4) is associated with increased risk of atherosclerosis in humans. Jensen et al. (17) demonstrated that cloned, genetically defined ApoE4 pigs fed high-fat, high-cholesterol diets had increased total and low-density lipoprotein cholesterol plasma levels within 60 days, thus providing a model in which the earliest stage in atherosclerosis can be induced and mitigated. The D374Y mutation in the human proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene is known to cause hypercholesterolemia and eventually atherosclerosis. Recently, Al-Mashhadi and coworkers (51) developed transgenic Yucatan minipigs that express D374Y-PCSK9. This genetic model exhibits severe hypercholesterolemia and human-like progressive atherosclerotic lesions on high-fat, high-cholesterol diets. Additionally, the Göttingen minipig is amenable to current invasive and noninvasive imaging techniques to evaluate the effects of new therapies and devices used during preclinical assessment of cardiovascular disease (52).

Developing a Pig Model for Obesity

Obesity is a polygenic disorder defined by an accumulation of fat stores in adipocytes and is associated with inflammation in adipose tissue (53). The Ossabaw pig breed has been used as a model for the study of metabolic syndrome, a term describing the co-occurrence of abdominal obesity, insulin resistance, impaired glucose intolerance, hypertension, and increased low- and high-density lipoprotein levels (54). The Ossabaw pig develops all the pathological aspects of metabolic syndrome when fed a high-calorie atherogenic diet (55). The tripling of childhood obesity since 1980 has stimulated the creation of an increasing number of pig models for childhood obesity (56). Porcine childhood (the interval between weaning and puberty) lasts approximately 22 weeks, allowing sufficient time for dietary approaches and intervention strategies (57). Chronic feeding of

a high-energy, low-protein diet to prepubertal pigs leads to increased adiposity, insulin resistance, low-density lipoprotein hypercholesterolemia, and hepatobiliary disorder, further demonstrating the utility of the pig as a potential model for childhood obesity (57).

Helicobacter pylori

Helicobacter pylori is the leading cause of peptic ulcer disease and gastric adenocarcinoma—the second leading cause of cancer-related deaths in infected individuals (58). Although *H. pylori* does not naturally inhabit the pig's stomach, *H. pylori* infection can be established consistently in gnotobiotic and conventional pigs with induced lesions (59), demonstrating that pigs are a useful model of human *H. pylori* infection. Recently, Kronsteiner and associates (60) inoculated pigs with two *H. pylori* strains and observed Th1 cell responses characterized by increased $CD4^+$ $Tbet^+$ cells and elevated gamma (γ) interferon mRNA in peripheral blood mononuclear cells. Based on their findings, they concluded that their novel pig model of infection closely mimicked the human gastric pathology, providing opportunities to better understand the effector and regulatory responses involved in human *H. pylori* infections.

Spontaneous Melanoma

Melanoma is a malignant tumor of melanocytes, cells that produce the dark pigment melanin responsible for skin color. Human cutaneous malignant melanoma accounts for 75.2% of skin cancer–related deaths (61). Pigs and humans have similar hair follicle and blood vessel patterns in the skin, and pig skin is structurally similar to human skin with regard to epidermal thickness and dermal-epidermal thickness ratios (62). Cutaneous malignant melanoma of the Sinclair minipig is an inherited malignancy with many of the histopathological characteristics of human melanoma (63), representing an excellent model for understanding human melanoma development. The Melanoblastoma-bearing Libechov minipig breed (64) was developed purposefully to support research of cutaneous melanomas. This breed has furthered the understanding of tumor development, helping to determine the genetic basis of melanoma and the genes involved in the incidence of spontaneous cutaneous melanoma (65). QTLs associated with cutaneous melanoma have been identified in pigs, and comparative mapping has revealed that some of these QTLs are homologous to human regions 1p36, 3p25, 9p21, 9q21, and 16q24, which are involved in human melanoma by having putative candidate genes for melanoma (66).

GENOMIC CONSTRUCTION OF HUMAN DISEASES AND MODELS

Enabling Technologies: Transgenesis and Somatic Cell Cloning

The 1985 landmark discovery by Brinster and associates (67) demonstrated the first permanent integration of a gene by microinjection of DNA into one-cell porcine embryos. They also developed a specific method to permit visualization of pronuclei and nuclei of pig embryos (68). Prather and coworkers (69) reported in 1989 that a piglet could be generated from the transfer of a blastomere nucleus to an enucleated oocyte. Two years later, Tao and associates (70) reported the successful development of pig embryos reconstructed by microinjection of cultured fetal fibroblast cells into enucleated oocytes. This was followed by the work of Polejaeva and coworkers (71), who showed the successful production of cloned piglets from a cultured adult somatic cell population using a new nuclear transfer procedure. Lai & Prather (72) reported step-by-step the nuclear transfer

procedure in pigs. This body of work has been the basis for many other swine genetic engineering studies over the past decade (3, 4).

The number of genetically modified pigs produced in the past 15 years has grown exponentially as techniques have been standardized and become globally accessible to more researchers (73). The availability of the pig genome sequence provides an important resource for improving strategies to generate genetically engineered biomedical models (2). Transgenic animals are now commonly used worldwide as models of human disease. This technology provides a true in vivo environment for evaluating the mechanisms by which gene expression is modulated during development, adulthood, and disease states (3, 4). Recently, RNA interference and somatic cell nuclear transfer (SCNT) technologies have been used to attenuate the expression of specific genes in swine tissues (74). Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) comprise a new, powerful class of tools that are redefining the boundaries of biological research (75). Recently, it has been demonstrated that both TALEN and ZFN injected directly into pig zygotes can produce live genome-edited pigs (76). These embryonic and genome editing techniques will allow the development of a wide range of promising porcine models for translational medicine.

Stem Cells and Regenerative Medicine

Bone regeneration studies have revealed that porcine adipose-derived stem cells derived from various donor sites, including subcutaneous interscapular sites and buccal fat pads, have osteogenic capability (77). Porcine and human bones are similar in morphology, healing rate, mineral density, and composition. A recent study showed that application of porcine adipose-derived stem cells accelerated healing of a noncritical defect in the ramus of the pig mandible (78). The generation and application of porcine induced pluripotent stem cells (piPSCs) may enable the testing for safety and efficacy of therapies in the field of human regenerative medicine (79). piPSCs can be generated with a single transfection of a CAG-driven polycistronic plasmid expressing *POU5F1, SOX2, KLF4,* and *C-MYC* (80). Ezashi and coworkers (81) emphasize that we should not assume that the recipes used to maintain human and mouse embryonic stem cells, especially the growth factors, are necessarily optimal for piPSCs from a pig. Thus, further studies and reagent development can enhance the usefulness of this new technology.

Tissue Engineering

Tissue-engineered cartilage obtained by combining scaffolds and cells has been introduced into research and clinical practice for the treatment of articular cartilage defects. Chondrocytes isolated from swine articular cartilage have been used with success (82). While many in vitro studies are based on porcine chondrocytes derived from abundantly available hybrid pigs, minipigs also have been used. Minipig chondrocytes expressed *COL2A1*, *COL2A2*, *SOX9*, and *ITGB1* at a higher level than hybrid pig chondrocytes (83).

Xenotransplantation

A severe shortage of organs and tissues for transplantation has stimulated increased consideration of pigs as a potential solution, particularly with the recent ability to genetically modify pigs to overcome acute rejection (84). Transgenic strategies have also been developed to reduce the potential risk of infections by endogenous porcine retrovirus (85).

The most significant advances to date have been the production of pigs expressing a human complement-regulatory protein (86) and knockout α1,3-galactosyltransferase pigs (87). Genetic engineering of pigs to prevent the coagulation dysfunction that occurs between a pig organ graft and the recipient primate may be achieved by the expression of thrombomodulin, tissue factor pathway inhibitor, CD39, or other genes expressed in the pig vascular endothelium (88). A future challenge will be to combine the most important and efficient genetic modifications into multitransgenic pigs for clinical xenotransplantation (85). For example, the development of piPSCs from GALT knockout tissue would provide an excellent cell source for complex genetic manipulations (89).

Drug Metabolism and Novel Drug Targets

Drug metabolizing enzymes (DMEs) play central roles in the metabolism, elimination, and detoxification of xenobiotics introduced into the body (**Figure 1**). Analysis of the pig genome has revealed high homology between porcine and human genes, including genes associated with drug metabolism. The characterization of porcine drug metabolism genes and the genes involved in regulating drug metabolism can provide insights into human drug metabolic diseases and individual variability of responses toward a drug.

Most of the tissues and organs express diverse and various DMEs, including phase I and phase II metabolizing enzymes and phase III transporters. These DMEs can be present in abundance at the basal level, or expression can be induced after exposure to drugs (90). Phase I metabolism includes oxidation, reduction, hydrolysis, and hydration. Enzymes catalyzing these reactions are found in virtually all tissues, especially in the hepato-intestinal axis (91). Phase I DMEs consist primarily of the cytochrome P450 (CYP) superfamily and are found abundantly in the liver, GI tract, lung, and kidney (92). In humans, five *CYP* gene families, *CYP1, CYP2, CYP3, CYP4,* and *CYP7,* are believed to play crucial roles in hepatic and extrahepatic metabolism and elimination of xenobiotics and drugs (93). The products of phase I metabolism are generally more polar and more readily excreted than the parent compounds and are often substrates for phase II enzymes (91). The pig is an appropriate animal model for the investigation of drug disposition, as the transporters and CYP enzymes are very similar to those in humans (94). The CYPs constitute the major enzyme family capable of catalyzing the oxidative biotransformation of most drugs and other lipophilic xenobiotics and are of particular relevance for clinical pharmacology. Several of these CYP subfamilies have been characterized for the pig and minipig (34). They include the main liver enzyme of drug metabolism (CYP3A) in comparable amounts and activity levels to humans (94). In addition, the porcine pregnane X receptor protein regulates CYP3A, which metabolizes almost half of the prescription drugs in humans, and has higher homology to that of humans than the mouse gene product (15, 95). That makes the pig a better model than the mouse to determine if a compound is toxic to humans. For these reasons, pigs are considered an ideal model for evaluating the safety of pharmaceuticals and biopharmaceuticals (96).

Phase II metabolism involves conjugation with endogenous hydrophilic compounds to increase polarity and water solubility, thereby increasing excretion in the bile and urine, resulting in a detoxification effect. Phase III transporters play crucial roles in drug absorption, distribution, and excretion. They include P-glycoprotein, multidrug resistance–associated protein, organic anion transporting polypeptide 2, and ABC transporters. They are expressed in many tissues, including liver, intestine, kidney, and brain. Genetically modified animal models are important for understanding the pathogenesis of human disease and developing therapeutic strategies and are also essential in developing new drugs (97).

Regulating the expression of various drug metabolism enzymes can affect metabolism, pharmacokinetics, drug-drug interactions, and their ability to protect the human body against

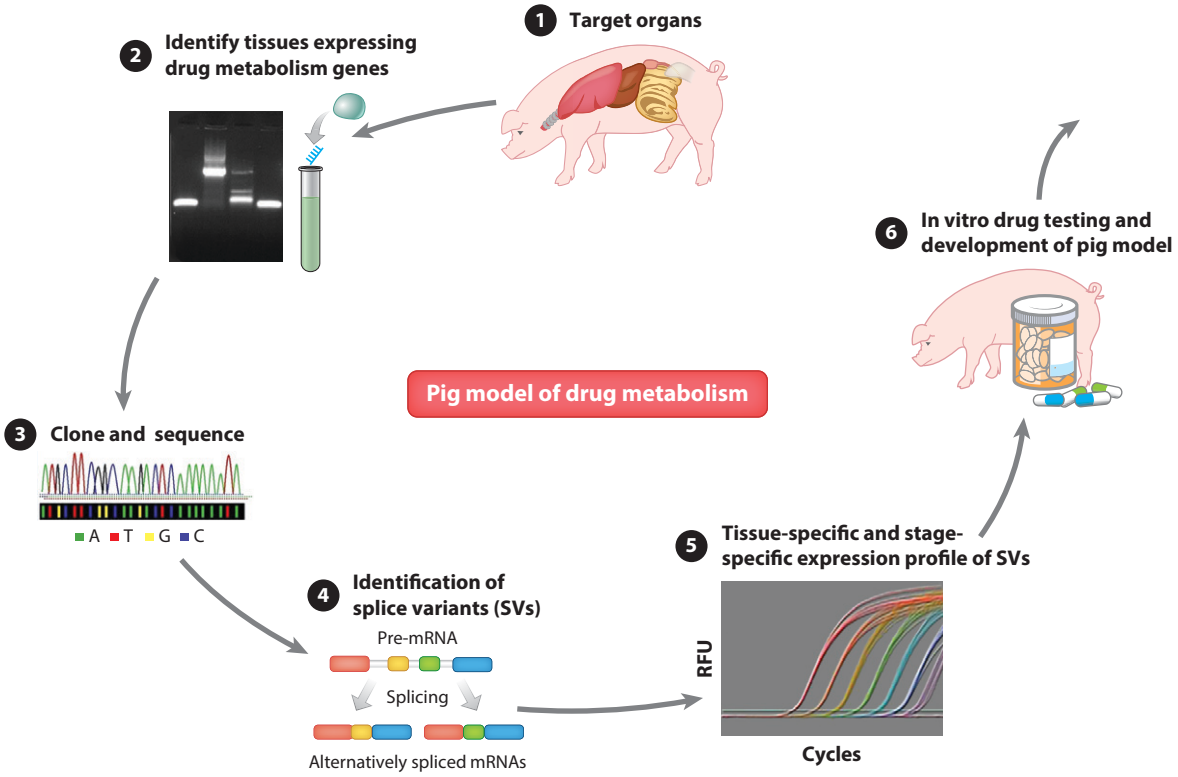

Figure 1

Assessing drug metabolism in a porcine model. To properly assess drug metabolism using a porcine model, biomedically relevant tissues are targeted and gene expression related to drug metabolism is determined (❶,❷). Relevant genes are then cloned and sequenced (❸). Splice variants are identified, followed by assessment of tissue-specific and stage-specific expression (❹,❺). In vitro drug testing with monitored expression of these metabolism genes and their splice variants is determined, ultimately leading to the development of a pig model (❻) to screen new drugs that will more accurately and efficiently validate drug metabolism and toxicity observed in human clinical trials.

exposure to environmental xenobiotics (91). Different nuclear receptors, including orphan nuclear receptors, play a crucial role in the metabolism and clearance of drugs and xenobiotics introduced into the body (98). The characterization of porcine pregnane X receptor (99) and farnesoid X receptor (95) has contributed to the development of a porcine model of human drug metabolic diseases. The tissue- and stage-specific expression of the drug metabolism enzymes in pigs and their comparison to humans will be of great interest. **Figure 1** provides an overview to examine the expression of enzymes and splice variants involved in drug metabolism and its regulation in pigs. Gene expression is then followed by in vitro drug testing to develop a swine model of drug metabolism and testing that could be used in drug development screening.

Pig Models of Monogenic Diseases

Monogenic diseases result from modifications in a single gene. Though relatively rare, they affect millions of people worldwide and are responsible for a heavy loss of life. The most common monogenic diseases include cystic fibrosis (CF), Huntington's disease (HD), thalassaemia, sickle

cell anemia, haemophilia, and Tay-Sachs disease. Porcine models of several of these diseases have been developed and are described below.

Cystic fibrosis. One validated monogenic pig model is a CF model (100). This autosomal-recessive genetic disorder is caused by a mutation in the gene encoding the CF transmembrane conductance regulator (CFTR) anion (101). Although it affects multiple organs, morbidity and mortality occur primarily owing to lung disease (101). *CFTR* genes in porcine fibroblasts can be disrupted by homologous recombination and the cells used as nuclear donors in SCNT to produce $CFTR^{-/-}$ pigs (100, 102). CFTR knockout pigs exhibit CF symptoms similar to those observed in CF patients (103). Newborn CFTR knockout piglets manifest symptoms of CF similar to those in humans, such as meconium ileus, pancreatic destruction, early focal biliary cirrhosis, and gall bladder abnormalities (100, 104), in contrast to CFTR knockout mice, which do not develop CF symptoms (105).

Huntington's disease. HD is an autosomal-dominant neurodegenerative disease caused by the accumulation of misfolded huntingtin protein that carries an expanded polyglutamine (polyQ) in its N terminus (106). The disease is characterized by progressive degeneration of neurons leading to cognitive decline, movement disorder, and psychiatric problems (107). The first transgenic pig model for HD by microinjection did not demonstrate any HD phenotypes (108). A second line of transgenic HD pigs express the human N-terminal (208 amino acids) mutant huntington polypeptide with an expanded 105 polyQ tract (109) and develop a clinically relevant HD phenotype characterized by dyskinesia, chorea-like movement, and typical apoptotic neurons with DNA fragmentation in the brain.

Retinitis pigmentosa. Retinitis pigmentosa is an inherited retinal degenerative disease in which patients develop a lack of peripheral vision owing to the loss of rod photoreceptors. A transgenic pig model that expresses a mutated rhodopsin gene (Pro347Leu) has been developed to study retinitis pigmentosa (110). In 2012, Ross et al. (45) developed a minipig model expressing the human rhodopsin mutation P23H, which is the most common form of the autosomal-dominant disease.

Developing Cancer Models

Over the years, many porcine models of cancer have been developed (**Table 2**). These range from using the pig to test surgical and ultrasound ablation protocols to the spontaneous melanoma model to transgenic models now being developed. The first genetically modified models showed that porcine fibroblasts could be transformed with four to six gene alterations. Adam and colleagues (14) showed that these cells were tumorigenic. The cells formed colonies in soft agar, tumors in immunodeficient mice and the donor animal. Although tumor growth in autologous recipients occurred only when the pigs were immune suppressed, this work provided the first method of inducing tumors in a large animal. The resultant tumors in the pigs grew to very large sizes, ideal for preclinical applications (4). This model also can be exploited to generate many different tumor types useful for preclinical studies.

Several transgenic pig models have been reported recently. The project design for one of these models is described in **Figure 2**. These transgenic onco-pigs were engineered to contain oncogenic $KRAS^{G12D}$ and dominant-negative $p53^{R167H}$ downstream of a LoxP-polyA (STOP)-LoxP sequence (LSL) and CAG promoter. This design allows for tissue- and time-specific oncogene expression when recombination is triggered by Cre-recombinase. In vitro experiments showed

Table 2 Porcine tumor models

Strategies	Site	Objective	References
Phantom tumors (injection of agrose, cellulose, and glycerol)	Liver	Study of ultrasound thermal ablation	112
Phantom tumors (injection of liquid plastic)	Kidney	Kidney tumors for the development of laparoscopic nephrectomy	113
Chemical carcinogen tumor induction	Different sites	Study of tumors	114
Inherited mutations	Melanoma	Characterization of cutaneous melanomas of varying severity, including highly invasive and metastatic lesions in minipigs	115
Inherited mutations	Melanoma	Characterization of melanoma lesions that share many histopathological and clinical features with human melanoma, yet eventually spontaneously regress in minipigs	116
Inherited mutations	Skin lesions	Characterization of a variety of skin lesions in early life of Sinclair pigs that spontaneously regress	117
Inherited mutations	Skin lesions	Study molecular aspects of tumor regression in Sinclair pigs	13
Autologous transplantation of primary porcine cells expressing oncogenes	Various sites	Development of a genetically malleable porcine tumor model	14
Pigs carrying the v-Ha-ras oncogene	No phenotype was observed	Study of different human tumors	118
Constitutive expression of human G112 transcriptional activator in keratinocytes	Basal cell carcinoma	Basal cell carcinoma–like lesions developed without gross tumor development	119
Gene-targeted *BRCA1* inactivation	No animals survived beyond 18 days; the causes of prenatal mortality remain unclear	Breast cancer model development	120
Make truncating mutations in the APC gene	Colon and rectal cancer	Studies of familial adenomatous polyposis	121
Generation of pigs with an inducible oncogene TP53	No in vivo studies	Studies of different kinds of cancer	122
Transgenic pigs with inducible expression of oncogenes $KRAS^{G12D}$ and dominant-negative $TP53^{R167H}$	Various sites	Development of an inducible (spatial and temporal) model of cancer	Unpublished data

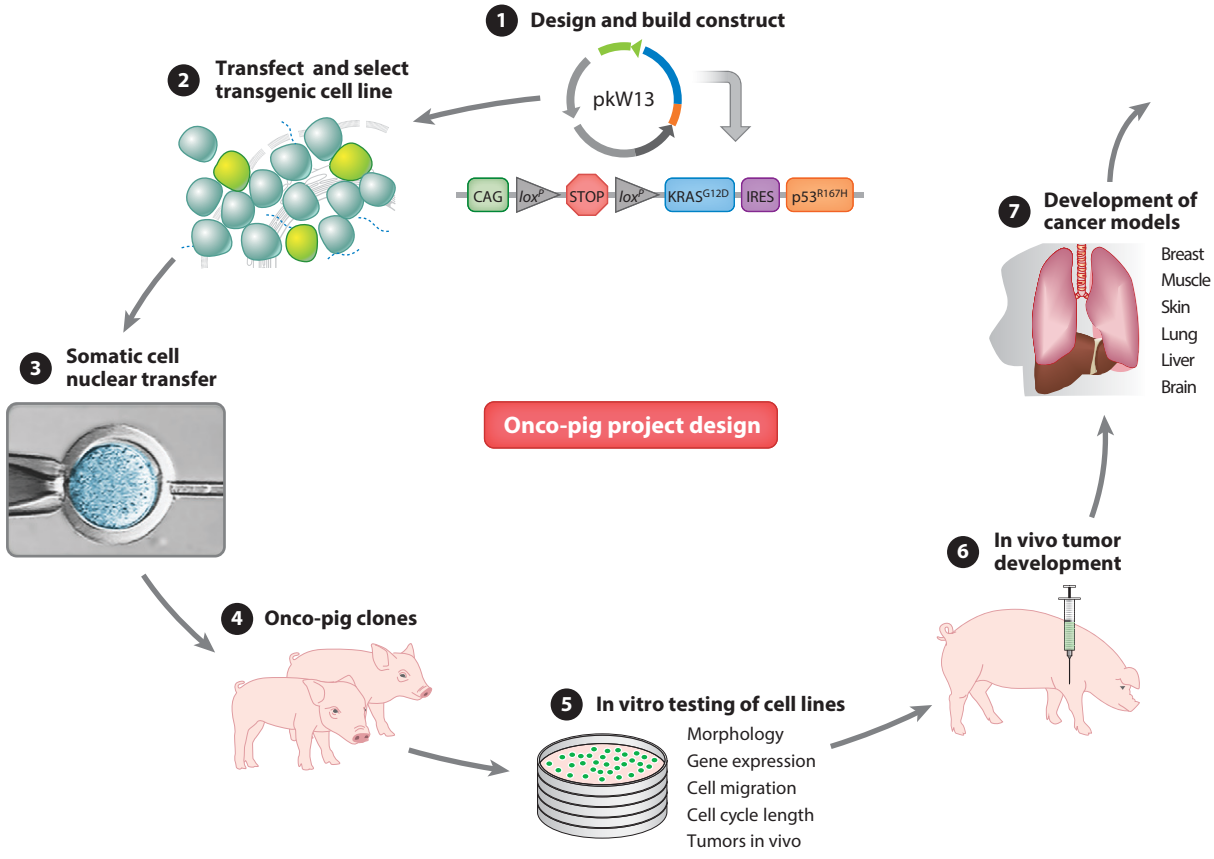

Figure 2

Development of the onco-pig project. To develop a robust inducible porcine cancer model for a variety of tumor types, a construct containing mutated KRAS and P53 genes was produced. This construct was designed to suppress the expression of the mutant KRAS and P53 genes via a Lox-Stop-Lox trigger system until recombination is induced ❶. Porcine embryonic fibroblasts were transfected with this construct ❷, transgenic cell colonies were selected, somatic cell nuclear transfer ❸ was performed, and embryos were transferred into recipient pigs. Once born ❹, transgenic fibroblast cell lines generated from the clones were used for validation of the system, including routine assays of cell transformation and tumor development in immunocompromised mice ❺. Tumor induction in pigs and development of porcine models of cancer will lead to development of various cancer models (❻,❼).

that oncogene expression was induced following induced recombination. This expression quickly altered the phenotypic characteristics indicative of oncogenesis, such as cell migration rates, cell proliferation, colony formation in soft agar, and tumor development in immunodeficient mice. In vivo work will include tumor development in various tissues and characterization of various porcine cancer models (111).

DISSECTING NATURE AND NURTURE: MODELING LIFESTYLE DISEASES

Early Life Environmental Insult Models

Swine models have been used to study the associations between a variety of pre- and postnatal environmental insults on health and development, shedding light on the importance of both nature

and nurture during human development, because the intrauterine environment can have significant effects on development and health later in life. Administration of β-hydroxy-β-methylbutyrate during the last two weeks of pregnancy results in offspring with improved skeletal system development and bone mass acquisition at six months of age (123), whereas prenatal vitamin A deficiency has been shown to reduce immune responses to rotavirus vaccinations in piglets (124). This study could help explain the reduced protective efficacy of human vaccines in developing countries, where vitamin A deficiency is common.

In addition to prenatal nutritional deficiencies, physical insults can have a significant effect on human health and development. Porcine models of human esophageal obstruction provide evidence for the importance of fetal fluid swallowing for growth and GI tract development in the final trimester (125). This model provides an alternative to human studies that would be difficult owing to ethical concerns. These studies support the fetal programming hypothesis, which suggests adaptations made by the fetus during environmental insult can permanently change the function and structure of the body in adult life (126).

Gastrointestinal Microbiome

There is growing evidence from human and pig studies to suggest that alterations in GI microbial community structure, which is shaped by delivery mode (natural versus cesarean) and early life environment, can have significant effects on human health and immune system development (127, 128). Pig models of preterm birth provide insights into the importance of diet and nutritional intake on intestinal health and maturation, as well as bacterial overgrowth and the risk of developing GI disorders, such as necrotizing enterocolitis (129). In addition to diet, the physical environment is also an important aspect of healthy human development, as demonstrated by studies in which pigs housed inside harbor different GI mucosa–adherent microbiota compared with those housed outside, resulting in altered GI-specific gene responses, including increased expression of type I interferon genes, MHC class I, and several chemokines (130). Altered systemic immune responses to the respiratory pathogen *Mycoplasma hyopneumoniae* have also been reported in pigs exposed to different microbial environments early in life (128), and reduced allergic responses have been demonstrated in pigs supplemented with the probiotic *Lactobacillus rhamnosus* HN001 early in life (131). These pig models suggest that early life environmental insults can significantly alter microbiome composition and subsequent development of the human immune system early in life and that more thought needs to be given to how early life environment can be optimized to improve human health status in adulthood.

Nutritional Deficiencies

Although it is important for healthy development, GI microbiome composition cannot fully explain the mechanisms by which early life environmental insults affect human health. Nutritional deficiencies are a major issue in children, but the long-term effects of these early life deficiencies are not well known. Porcine neonatal calcium deficiency is associated with reduced bone flexural strength, mineral density, and mesenchymal stem cell activity, suggesting the potential for long-term effects on bone integrity through mesenchymal stem cell programming (132). Early life nutritional deficiencies may also affect cognitive development in humans, as studies assessing hippocampal-dependent learning in pigs (21) show reduced spatial learning and memory in iron-deficient piglets compared with controls (133). In addition, similar reductions in cognitive development, as well as increased microglial activation in the hippocampus and inflammatory gene expression in multiple brain regions, have been reported in piglets infected with porcine

reproductive and respiratory syndrome virus at seven days of age (134). This study is of particular interest, as little is known about the effects of peripheral infection on human brain development and cognitive behavior, despite infectious disease being the most common cause of illness in children.

Epigenetics in Development and Health

The risk of developing many adult onset diseases has been linked to early life environmental insults, as well as environmental exposures and nutrition in adulthood. Epigenetic mechanisms, including DNA methylation, represent a link between genetics and environment, being responsible for establishing changes in gene transcription in response to environmental exposures that persist long after removal from said environment. Therefore, it should be no surprise that we find alterations in DNA methylation and other epigenetic marks in many human diseases where the risk of disease development is linked to environmental exposures, such as obesity, diabetes, neurodegenerative disorders, cardiovascular disease, and cancer. Many studies suggest that global DNA methylation reprogramming during early embryogenesis is important for normal development and that these epigenetic patterns can be altered throughout pre- and postnatal development (135, 136). Indeed, aberrant methylation patterns have been linked to the development, prognosis, and potential diagnosis of many of these human diseases in human and mouse studies. In a porcine model of intrauterine malnutrition, both maternal protein excess and deficiency throughout pregnancy resulted in global DNA hypomethylation in the liver, as well as altered methylation and gene expression of DNA methyltransferases in hepatic and skeletal muscle, and genes involved in chromosome compaction and fetal growth in the liver of offspring up to 188 days of age (137). Likewise, increased paternal intake of methylating micronutrients results in increased shoulder percentage, lower fat levels, and altered lipid metabolism and metabolic pathway gene expression in liver and muscle, and altered methylation patterns in the liver of F2 pigs (138). These aberrant epigenetic marks emphasize the importance of maternal environment in healthy human development, providing a possible link between the prenatal environment, phenotype, and disease risk in adulthood. Although human and mouse studies have started to characterize aberrant methylation patterns in diseases such as Alzheimer's disease, cardiovascular disease, diabetes, inflammatory bowel disease, and cancer, little work has been done to date to assess these patterns in porcine models of these diseases.

Development of the Porcine Epigenome

Owing to the physiological and metabolic similarities of pigs and humans, pigs are an excellent model to define how environmental signals such as food, smoking, alcohol, and stress affect humans and contribute to chronic diseases, such as diabetes, cardiovascular disease, and cancer. Although there are an increasing number of studies on epigenetic patterns in humans and mice, few studies to date have focused on epigenetic patterns in pigs, the majority of which are limited to a small number of tissues and techniques with limited resolution, making inferring site-specific methylation patterns impossible. Global methylation analysis has identified differentially methylated regions across multiple porcine tissues, including liver, spleen, lung, kidney, and stomach (139). Differentially methylated regions associated with differentially expressed genes involved in lipid metabolism and regulation of immune-related cytokines have been reported in porcine superficial and deep backfat (140). These results shed light on the functional and metabolic differences between subcutaneous adipose tissue compartments, which are related to multiple human obesity-related metabolic and cardiovascular diseases. In addition, carcinogens such as

cadmium have been shown to alter global DNA methylation patterns by decreasing the expression of genes involved in the maintenance of DNA methylation patterns in porcine cell lines, highlighting a potential epigenetic mechanism for cancer progression in humans (141).

Epigenetic resources have been developed in humans through the human ENCODE project, which has used targeted approaches such as reduced-representation bisulfite sequencing (RRBS) to catalog DNA methylation patterns in 82 human tissue samples and cell lines, as well as the genomic location of histone modifications in 22 cell types (142). The Human Epigenome Project is also under way, with the goal of providing genome-wide DNA methylation patterns of all human genes in all major tissues. A project that has a pilot study assessing DNA methylation patterns of the human major histocompatibility complex (143) has successfully analyzed DNA methylation patterns on human chromosomes 6, 20, and 22 from multiple cell types of interest to the biomedical community (144).

To fully utilize the pig as a relevant model for human diseases, there is a need to increase available porcine genomic resources, including a DNA methylation map, as the evidence for the role of epigenetic modifications in human development and disease continues to grow. **Figure 3** highlights the protocol by which production of a swine methylome map could be achieved using a combination of RRBS and RNA-seq to target CpG sites of interest and determine potential biological significance. A porcine methylome map of multiple biomedically relevant tissues and time points would allow us to use methylation and gene expression patterns to better understand the similarities and differences in developmental stages between humans and pigs, as well as the importance of these patterns in normal development. In addition, it would allow better understanding of how well porcine disease models reflect human disease at the molecular level. These insights could increase the potential to detect, diagnose, and treat specific diseases and cancer types in a model organism with high physiological and genetic similarities to humans.

Implication for Human Health and Development

Multiple studies on a variety of cancer types have revealed differential levels of DNA methylation, histone acetylation, and histone methylation in tumors that correlate with tumor morphology, biological subtype, and patient outcome, suggesting an important role of epigenetic modifications in cancer progression and diagnosis (145, 146). As knowledge of the links between epigenetic patterns, health, and human diseases increases, the need increases for epigenetic resources. Those resources will further the study of these patterns in biomedically relevant model organisms like pigs. In addition, recent studies looking at the colocalization of multiple epigenetic marks, such as DNA methylation and histone modifications between humans, pigs, and mice, have shown conserved combinations of epigenetic marks across species (147). These results suggest that interspecies comparisons can distinguish functionally relevant combinations of epigenetic marks across species, making a porcine epigenome a valuable tool for determining the importance of these marks in human health and development.

FUTURE CONSIDERATIONS AND OPPORTUNITIES

The porcine genome provides the foundation to develop novel animal models to validate human conditions and to support clinical trials to expedite drugs, devices, and diagnostics. Clearly, further refinement of the pig genome will be critical to fully exploiting the physiological characteristics of the pig to develop quantitative trait nucleotides (QTNs) causative for human diseases. Also essential will be the development of innovative bioinformatics tools that are linked to

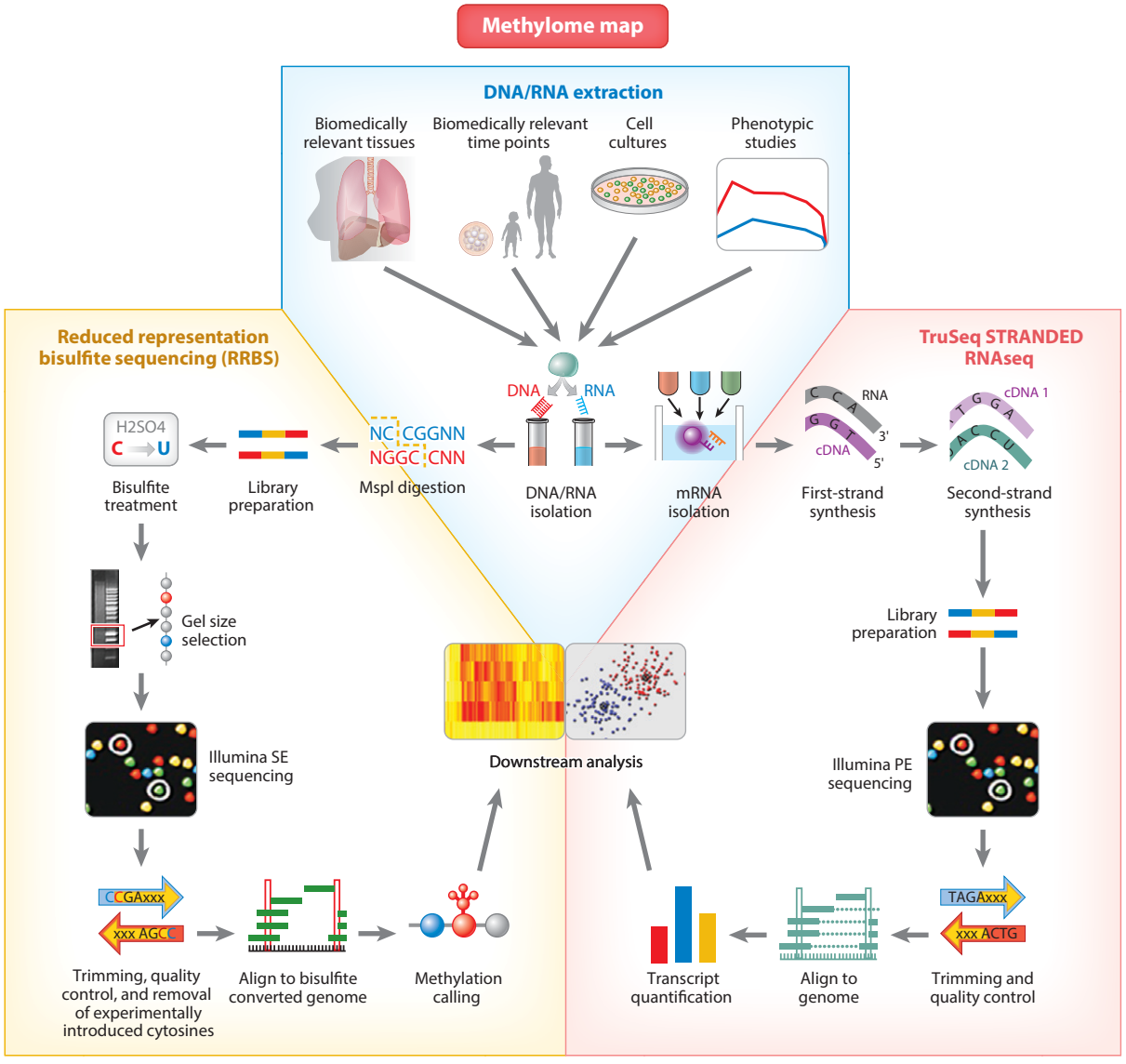

Figure 3

Guidelines for production of the porcine methylome map. Production of the porcine methylome map begins with the extraction of DNA and RNA from biomedically relevant tissues at relevant time points (*blue*). DNA is prepared for reduced-representation bisulfite sequencing by digestion with MspI, followed by library preparation and bisulfite treatment. Treated libraries are size selected by gel electrophoresis, and single end (SE) sequencing is carried out. Sequence data is processed by adaptor trimming, removal of low-quality bases, and experimentally introduced cytosines. Processed reads are then aligned to a bisulfite-converted version of the genome, after which methylation calls are made by comparing the number of methylated and unmethylated reads at each site and determining the ratio of methylated to total reads (*yellow*). RNA is prepared for TruSeq Stranded RNAseq by isolating mRNA from total RNA and performing first-strand synthesis (*red*). Second-strand synthesis is performed using dUTP in place of dTTP, limiting second-strand elongation during sequencing. Illumina library preparation is performed on the cDNA, followed by paired end (PE) sequencing. Adaptor trimming and removal of low-quality bases is performed, followed by alignment to the genome and transcript quantification. The methylation calls and expression data are used to determine the methylation patterns associated with each tissue, time point, and phenotype, as well as the effects on gene expression.

emerging new genetic modeling tools. The opportunities for validating existing models and rapidly testing QTN-based hypotheses are endless.

Historically, the pig has served biomedical research as an invaluable model. The postgenome period will accelerate its utility in clinical and translational research. Essential for fullest applicability of the minipig breeds, owing to their size and housing needs, will be their further development via genome sequencing.

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