BUILDING STRATEGIES FOR PORCINE CANCER MODELS

EDITED BY: Tiago Collares, Fabiana K. Seixas, Laurie Rund and Lawrence B. Schook

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BUILDING STRATEGIES FOR PORCINE CANCER MODELS

Topic Editors:
Tiago Collares, Federal University of Pelotas, Brazil
Fabiana K. Seixas, Federal University of Pelotas, Brazil
Laurie Rund, University of Illinois at Urbana-Champaign, United States
Lawrence B. Schook, University of Illinois at Chicago, United States

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The eBook *Building Strategies for Porcine Cancer Models* presents a series of articles demonstrating the state-of-the-art developments in pig models for cancer research. Renowned researchers dedicated to the reproduction, genomic and biological engineering of the pig model for biomedicine contribute to this special research area. Although advances in these areas are occurring at surprising speeds, they are still far from realizing all the potential benefits that this biological model could provide to science. The current biomedical models may limit the frontier of knowledge in the cancer research.

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Editorial: Building Strategies for Porcine Cancer Models

Tiago Collares[1]*, *Fabiana K. Seixas*[1], *Laurie A. Rund*[2] and *Lawrence B. Schook*[2,3]

[1] Laboratory of Cancer Biotechnology, Biotechnology Postgraduate Program, Technology Development Center, Federal University of Pelotas, Pelotas, Brazil, [2] Animal Science, University of Illinois at Urbana-Champaign, Champaign, IL, United States, [3] Department of Radiology, University of Illinois at Chicago, Chicago, IL, United States

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Editorial on the Research Topic

Building Strategies for Porcine Cancer Models

INTRODUCTION

Pigs are proven models for biomedical studies due to their anatomical and physiological similarities with humans. Pig models have increasingly been validated for mimicking human diseases (Prather, 2013). The sequencing of the swine genome has demonstrated genetic similarities with humans (Groenen et al., 2012).

These considerations led Schook et al. (2015) to develop a transgenic swine model of cancer—the oncopig cancer model (OCM). In addition, recent transcription profile studies of the oncopig's soft tissue sarcoma cells demonstrated altered TP53 signaling, activation of Wnt signaling, and epigenetic reprogramming—all transcriptional features found in human soft tissue sarcoma tumors (Schachtschneider et al., 2017a).

In addition to sarcomas, other cancers have been developed to date using the OCM platform: hepatocellular carcinoma (HCC) and pancreatic cancer (PA) (Schachtschneider et al., 2017c). Oncopig HCC acquired histopathological characteristics similar to human HCC [arginase expression and alpha-fetoprotein (AFP) secretion] and formed tumors after autologous injection (Schachtschneider et al., 2017b). In addition, human HCC transcriptional characteristics were also detected in porcine HCC (Schachtschneider et al., 2017b). In a review recently published by Segatto et al. pigs were proposed as a complementary platform for the discovery of new therapies against cancer through phenotypic screening of compounds, due to the metabolic, physiological, and genetic similarities of pigs with humans (Segatto et al., 2017).

On the research topic "Building strategies for porcine cancer models," seven papers have been published, with contributions from 40 authors from different institutions around the world, with a focus on molecular and cellular approaches for the development of porcine cancer models.

Watson et al. have reviewed the limitations of rodent models of cancer through a comparison of knockout mouse models to human patients. The authors have highlighted the advantages of using the swine as the biomedical model for cancer research, reviewing special aspects from the swine genome sequence and potential homologies to the human genome. They present the advantages of targeted gene editing using custom endonucleases—specifically TALENs and CRISPRs—and transposon systems, to make novel pig models of cancer with broad preclinical applications.

Schook et al. have discussed genetic modification technologies successfully used to produce porcine biomedical models, in particular the Cre-Lox system as well as the major advances and perspectives of the CRISPR/Cas9 system, highlighting its capacity to induce mutations at a chosen time and space, a characteristic that is especially important when creating genetic models of cancer. Recent advancements in porcine tumor modeling and genome editing will bring porcine models to the forefront of translational cancer research.

Duran-Struuck et al. have reviewed the limitations in using rodents to model human diseases, including the large differences in size, anatomy, physiology, drug metabolism, chromosome

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Edited and reviewed by:
Geoff Clark,
University of Louisville, United States

***Correspondence:**
Tiago Collares
collares.t@gmail.com

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structures, and genetics. The authors have highlighted an exciting perspective from their experience with myeloid and lymphoid tumors in major histocompatibility complex characterized miniature swine and future approaches regarding the development of a large animal transplantable tumor model. This work covered the incidence of chronic myeloid leukemias in swine, highlighting the importance of genetic studies of these tumors that can provide a new platform for the development of novel human therapeutics for genetically similar human tumors. Moreover, they discussed the potential of swine models to study post-transplant lymphoproliferative disease (PTLD), since immunosuppressed swine present several characteristics that closely resemble human PTLD. The authors reported their attempt to establish an immortal cell line that could induce PTLD when inoculated into the same inbred line animals.

Gutierrez et al. have presented the potential applications and advantages of using pigs, particularly minipigs, as indispensable large animal models in fundamental and clinical research, including the development of therapeutics for inherited and chronic disorders including cancers. The authors have reviewed examples of naturally occurring conditions in pigs that closely mimic those affecting humans (like malignant spontaneously regressing melanomas, dwarf phenotype, and ventricular septal defect) as well as examples of induced swine models of diseases (for type I diabetes, obesity and metabolic syndromes, and liver cancer models) and established engineered pig models (for cystic fibrosis, heart arrhythmias, xenotransplants, and several types of cancer).

Overgaard et al. have shared original research regarding the use of pigs as a large animal model for cancer vaccine development. Their work demonstrated that the pig model is highly appropriate for addressing the questions related to optimal adjuvant composition and vaccine formulations. The authors investigated whether it is possible for pigs to generate immune responses to cancer antigens RhoC and IDO, using three different adjuvants (CAF09, CASAC, or ISA 51 VG). The results showed that all adjuvants tested were capable of generating some cytotoxic T lymphocytes (CTL) response to the cancer antigens following peptide immunization. These findings support the further use of the pig as a large animal model for vaccine development against human cancer.

Clark et al. have demonstrated that BRCA1 inactivation in pig cells promotes transformation and thus serves as a model for human cancers. The authors established an immortalized porcine breast cell line and stably inactivated BRCA1 using miRNA. The cell line developed the characteristics of breast cancer stem cells and exhibited a transformed phenotype. These results validate the concept of using pigs as a model to study BRCA1 defects in breast cancer and establish the first porcine breast tumor cell line.

Bourneuf has reviewed melanoma genetics, discussing some of the most common mutations found in this type of tumor in humans. Examples of melanoma animal models have also been discussed, with emphasis on the porcine MeLiM model. This work suggests that the spontaneous tumor progression and regression occurring in these models could shed light on the interplay between endogenous retroviruses, melanomagenesis, and adaptive immune response.

CONCLUSION

Combined, the studies on this research topic have demonstrated that pigs are proven useful models for cancer studies including in (1) the development of genetically engineered pigs by using different technologies like TALENs, CRISPRs, transposons, and the Cre-Lox system; and (2) models for myeloid, lymphoid, breast, and melanoma cancers. Thus, the porcine genome sequence coupled with somatic cell cloning has led to the development of innovative porcine cancer models to support translational cancer research.

AUTHOR CONTRIBUTIONS

TC and FS are responsible for organizing the materials and writing this editorial. LR and LS are responsible for proofreading this editorial.

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PERSPECTIVE
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Efficacy of the porcine species in biomedical research

Karina Gutierrez[1], Naomi Dicks[1], Werner G. Glanzner[1], Luis B. Agellon[2] and Vilceu Bordignon[1]**

[1] Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, QC, Canada, [2] School of Dietetics and Human Nutrition, McGill University, Sainte-Anne-de-Bellevue, QC, Canada

Since domestication, pigs have been used extensively in agriculture and kept as companion animals. More recently they have been used in biomedical research, given they share many physiological and anatomical similarities with humans. Recent technological advances in assisted reproduction, somatic cell cloning, stem cell culture, genome editing, and transgenesis now enable the creation of unique porcine models of human diseases. Here, we highlight the potential applications and advantages of using pigs, particularly minipigs, as indispensable large animal models in fundamental and clinical research, including the development of therapeutics for inherited and chronic disorders, and cancers.

Keywords: Large animal models, biomedical research, swine, pigs, minipigs, clones, transgenics

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Edited by:
Tiago Collares, Federal University of Pelotas, Brazil

Reviewed by:
Thomas John, Ludwig Institute for Cancer Research, Australia
Howard Donninger, University of Louisville, USA

****Correspondence:***
Vilceu Bordignon, Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, QC H9X 3V9, Canada
vilceu.bordignon@mcgill.ca;
Luis B. Agellon, School of Dietetics and Human Nutrition, McGill University, Sainte-Anne-de-Bellevue, QC H9X 3V9, Canada
luis.agellon@mcgill.ca

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Introduction

The first evidence of swine domestication dates back to approximately 7000–9000 years ago (Jones, 1998; McGlone and Pond, 2003; Köhn, 2011; Larson et al., 2011; **Figure 1A**). China and Europe have been, since domestication, the pig-breeding centers dictating the profile of the pig breeds (Jones, 1998; Amills et al., 2001). The reason for domestication was to provide meat as a source of food protein, which stimulated pig selection and farming (Jones, 1998; Köhn, 2011). Studies have been conducted using genome-wide genotyping and genetic variability to trace the migration, selection, and improvement from ancient wild species to modern swine (Giuffra et al., 2000; Bosse et al., 2014a,b). It is generally accepted that the majority of all modern breeds are derived from the Eurasian wild boar (European and Asian wild boars; Porter, 1993; Bosse et al., 2014b). Although pig selection started just after domestication, it has only been since the mid-20th century that performance has been used as the main tool in the animal selection process (Safranski, 2008). More recently, molecular biology technologies, genome-wide association studies, and next-generation sequencing have been applied to enhance the selection process of domesticated pig breeds (e.g., Duroc, Landrace, Pietrain, Yorkshire, etc.) to further improve traits of high economic value such as feed conversion, meat quality, growth, precocious puberty, and prolificity (Sahana et al., 2013; Tart et al., 2013; Jiang et al., 2014; Sanchez et al., 2014).

The variety of modern pig breeds available today (Buchanan and Stalder, 2011), are a product of human intervention since domestication, but especially during the last century (**Figure 1A**). Besides breeds specialized for food production, smaller sized breeds (miniature- and micro-pigs) with certain characteristics such as obedience, friendly nature, and cognitive ability have also been selected for the purpose of companion animals. In addition, their use in biomedical research has been increasing considerably in the last years (**Figure 1B**).

Compared with other animals used in research (e.g., mice, rats, rabbits, and dogs), domestic farm pigs are much larger (>300 kg adult size), therefore, requiring more space and feed, and

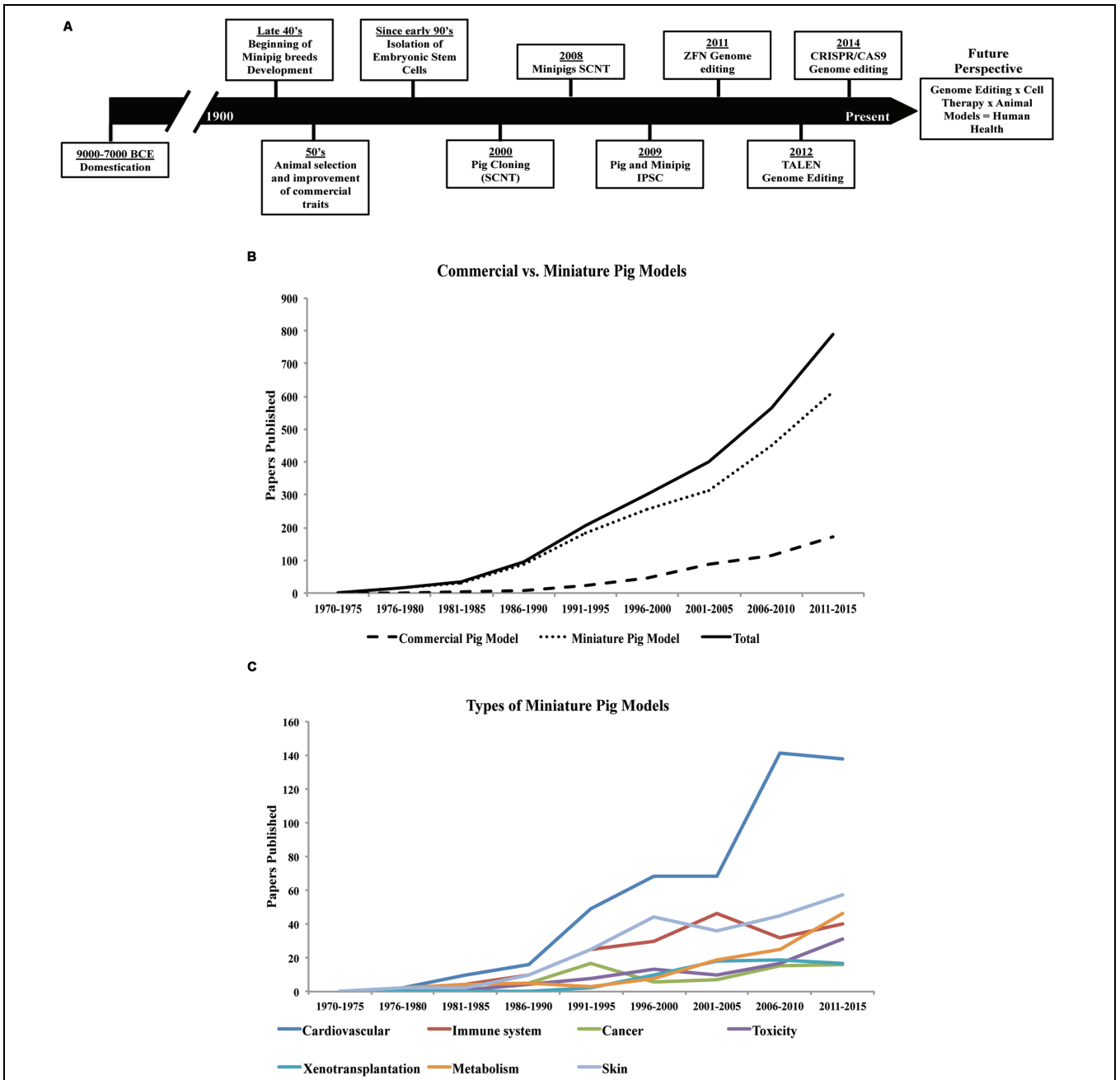

FIGURE 1 | History of pigs in agriculture and research since domestication. (A) Timeline, significant events and application of different technologies in the selection and breeding of the porcine species (Jones, 1998; Onishi et al., 2000; Polejaeva et al., 2000; McGlone and Pond, 2003; Brevini et al., 2007a,b; Estrada et al., 2008; Safranski, 2008; Wakai et al., 2008; Esteban et al., 2009; Ezashi et al., 2009; Hauschild et al., 2011; Köhn, 2011; Larson et al., 2011; Carlson et al., 2012; Hai et al., 2014; Whitworth et al., 2014). **(B)** Use of the porcine species in research, and **(C)** application of minipig models in a variety of studies (based on articles indexed by PubMed, from 1970 to the present date).

making them harder to handle. Mini- or micro-pigs are hence more desirable for research use. The adult sizes vary among breeds, reaching around 20–30 kg for a Panepinto micropig to 100 kg for a Munich minipig (Köhn, 2011). Although many minipig breeds are a product of crossbreeding, some breeds, like the Yucatan pigs, are naturally occurring stocks (Panepinto, 1996; Köhn, 2011). Since the late 1940s, minipigs have been further developed specifically for biomedical research purposes (England and Panepinto, 1986; Köhn, 2011).

There are now several minipig breeds available for use in research (Panepinto, 1996). The main breeds developed in the USA are Yucatan, Sinclair (also known as Minnesota or Hormel miniature pig), Hanford, NIH minipig and Panepinto miniature pig. The minipig breeds developed in Europe

are Göttingen, Munich, Berlin, Mini-Lewe, Czech-Republic, Vietnamese potbellied and Mini-Sib. In Asia, the breeds include Ohmini, Clawn, Lee Sung, and Chinese minipigs. The Göttingen and Yucatan breeds are the most commonly used minipigs in research, although there is no apparent clear reason for preference. Unlike the Yucatan, a natural breed, the Göttingen minipig was developed specifically for research use. Other breeds are used only by specific research groups, thus limiting their widespread availability in research. Nevertheless, the interest in the use of pigs in biomedical research has been rising over the last 40–45 years (**Figure 1B**).

Use of Pigs in Biomedical Research

Biomedical research is broad, spanning studies on underlying disease mechanisms to the evaluation of safety and effectiveness of preventative measures, diagnostic tests, and therapies. Most animal studies in recent times have used the murine species due to their small size, fast reproductive cycles and short lifespan. In addition, the availability of murine embryonic stem cells, fully annotated genome, and facile tools for targeted genetic manipulation have all contributed to the elucidation of gene functions and disease pathophysiology. However, in many cases, mouse models do not adequately represent features of human disorders (Seok et al., 2013). In this regard, animals that better represent human pathophysiology are required. Pigs and humans share many similarities such as size, physiology, anatomy, metabolic profile, and longer lifespan (Panepinto, 1996; Spurlock and Gabler, 2008; Kuzmuk and Schook, 2011; Swindle et al., 2012). For example, pig skin is structurally similar to human skin regarding thickness and spacing between hair follicles, making it useful for studies on wound healing and burn lesions (Sullivan et al., 2001). Pigs also share anatomical and physiological similarities with respect to the renal system, making them valuable for pharmacological studies (Dalgaard, 2014; Huppertz et al., 2015). Pigs can also be useful in the study of nutrient absorption and intestinal transport, as well as the pathogenesis of gastrointestinal diseases (Sangild et al., 2014). All these characteristics contribute to the development of superior models of human conditions (Kuzmuk and Schook, 2011).

The choice between outbred or inbred strains can have a significant impact on research outcomes (Festing, 2014). While, outbred strains may be better suited for quantitative trait loci studies, experiments addressing mechanistic aspects would benefit from the use of inbred strains (Chia et al., 2005). Some minipig breeds are already established for specific applications due to their unique characteristics (**Table 1**). Pigs have also been used for testing new therapies, devices, and efficacy and safety of new drugs prior to human trials. For instance, a novel endovascular chemotherapy filter, designed to reduce circulatory drug excess *in vitro*, was successfully tested in pigs (Patel et al., 2014). As well, a new method for pediatric liver transplantation was validated using pigs (Leal et al., 2015). Regarding pharmacokinetic and cytotoxic tests, pigs have been used for testing topical skin formulations (Mitra et al., 2015), and are considered a better choice compared to dogs for the study of drugs that are metabolized by the aldehyde oxidase (AOX1), *N*-acetyltransferase (NAT1 or NAT2) or cytochrome (CYP2C9-like) enzymes (Dalgaard, 2014).

In general, there is low incidence of naturally occurring pathologies described in pigs. The reason for this is twofold. First, human intervention by way of selective breeding has eliminated genes that increased disease susceptibility. Second, the majority of the domestic farm pigs are slaughtered at a young age ($<$ 6 months old), precluding the detection of late onset diseases such as cancer. On the other hand, Vietnamese potbellied minipigs raised as companion animals do reach old ages. Indeed, a retrospective study found a variety of neoplasms with widespread metastases in these pigs of advanced age ($\sim$11 years; Newman and Rohrbach, 2012). The most common malignances found included hepatic and intestinal carcinomas, and uterine and ovarian smooth muscle tumors (Newman and Rohrbach, 2012).

Occurrence of malignant spontaneously regressing melanomas has been described in Sinclair minipigs (Millikan et al., 1974; Oxenhandler et al., 1979). Selective interbreeding, by removing animals with red coat color that do not develop the lesions, increased the frequency of tumor formation in these selected minipigs (Millikan et al., 1974). The tumors appear from birth and culminate in skin depigmentation after tumor regression showing a phenotype similar to human vitiligo (Millikan et al., 1974). Studies conducted in these minipigs have shown decreased telomerase activity during melanoma regression (Pathak et al., 2000), which has also been observed by inhibiting telomerase activity in human melanoma cells (Burchett et al., 2014). Therefore, these minipigs may represent a useful model to study malignant melanomas because the tumors appear spontaneously and then either regress or grow progressively and metastasize similarly to human melanomas (Oxenhandler et al., 1979).

Another example of a naturally occurring condition in pigs is the dwarf phenotype, caused by a single amino acid change in the α1 chain of type X collagen (Nielsen et al., 2000). The *COL10A1* gene, which encodes type X collagen, is expressed in hypertrophic chondrocytes during endochondral ossification. In humans, an amino acid variation in the same position of the type X collagen protein has been shown to be the cause of Schmid metaphyseal chondrodysplasia (SMCD), a mild skeletal disorder associated with dwarfism (Warman et al., 1993). Since mice lacking type X collagen do not develop abnormalities in long bone development (Rosati et al., 1994), pigs represent a better animal model of human SMCD.

Another naturally occurring disease observed in Yucatan minipigs mimics human ventricular septal defect (VSD; Swindle et al., 1990). The VSD in pigs can be observed in fetal stages similar to the congenital anomaly in humans, and can be used for the study of new methods of diagnosis or therapies (Swindle et al., 1990; Amin et al., 2006).

Despite a number of natural occurring pig phenotypes that resemble human diseases, for most of human pathologies it is difficult to find representative animal models in nature. Thus, manipulation of diet, use of drugs and/or surgeries has been necessary to generate appropriate models. For example, minipig models for Type I diabetes were induced

TABLE 1 | Characteristics and applications of minipig breeds for the study of human conditions.

Parameter	Yucatan	Gottingen	Hanford	Sinclair/Minnesota
Adult body size (kg)	70–83	~45	80–95	55–70
Average litter size	6	6.5	6.7	7.2
Age to puberty (months)	4–6	3–5	4–6	4–6
Genetic background	Purebred	Outbred	Outbred	Outbred
Cloning	somatic cell nuclear transfer (SCNT; Estrada et al., 2008)	SCNT (Wakai et al., 2008)	Information not available	SCNT (Do et al., 2012)
Transgenics	Homologous recombination *BRCA1* (breast cancer susceptibility gene 1) – gene knockout by rAAV – model for breast cancer (Luo et al., 2011, 2012)* Introduction of missense mutation via rAAV – *TP53* gene – cancer cells (Sieren et al., 2014) Introduction of nonsense mutation via rAAV – *SCN5A* gene – cardiac arrhythmia (Park et al., 2015)	Homologous recombination *BRCA1* (breast cancer susceptibility gene 1) – gene knockout by rAAV – model for breast cancer (Luo et al., 2012)† rAAV vectors encoding GFP (Kornum et al., 2010)	Information not available	ZFN – mono and biallelic knockout pigs – *CMAH* gene – xenoantigen involved in the rejection phenomenon (Kwon et al., 2013) TALEN – biallelic modified pigs – *RAG2* gene – immune system (Lee et al., 2014)
Applications	Wound healing (Eggleston et al., 2000) Cardiovascular model for ventricular septal defect (VSD; Swindle et al., 1990) Metabolic Disorder (Phillips et al., 1982)	Toxicity Studies (Bollen and Ellegaard, 1997; van Mierlo et al., 2013) Skin pharmacokinetics tests (Mitra et al., 2015) Metabolic Syndrome (Johansen et al., 2001) Neurodegenerative disease – Parkinson Model (Bjarkam et al., 2008) Obesity (Christoffersen et al., 2013) Heart failure (Schuleri et al., 2008)	Dermal studies – toxicology (Leigh et al., 2012) Wound healing (Reger et al., 1999) Surgery training (Purohit et al., 1993) Tests of new therapies in tissue regeneration (Van Dyke et al., 2015)	Oncology (malignant spontaneously regression melanoma; Oxenhandler et al., 1979) Dermatology – skin depigmentation (Millikan et al., 1974) Models of human alcoholism (Dexter et al., 1976) Pediatric hypothyroidism (Tank et al., 2013)

*The animals died 18 days after birth.
†Cloned animals were not yet born at the time of publication.

via administration of streptozotocin or alloxan to selectively destroy insulin-producing cells (Phillips et al., 1980; Larsen et al., 2002). High-energy diets in young minipigs lead to the development of obesity and metabolic syndromes, with increased visceral fat deposition, glucose intolerance, decreased insulin sensitivity, and higher levels of blood cholesterol and triglycerides, which progress to Type 2 Diabetes mellitus (Xi et al., 2004; Neeb et al., 2010; Koopmans and Schuurman, 2015). Other chemicals have been used to induce cellular dysregulation and damage in pigs including the administration of *N*-nitrosodiethylamine to produce a liver cancer model (Li et al., 2006).

Use of Engineered Pigs in Biomedical Research

Genetically modified animals have been instrumental in advancing our understanding of gene function and significance of inappropriate gene expression in metabolic malfunction in mammals. Genome editing holds great promise in generating these models, and has already permitted the rapid development of new pig models of several human diseases (Rogers et al., 2008; Prather et al., 2013; Hai et al., 2014; Dicks, 2015).

The cystic fibrosis (CF) model is an example of genetically engineered pigs created by targeted inactivation of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Rogers et al., 2008). The resulting pigs exhibit clinical features and disease progression consistent with those observed in CF infants. In contrast, inactivation of the *CFTR* gene in mice did not produce the comorbidities typically observed in human CF patients (Snouwaert et al., 1992).

Advanced reproductive technologies, such as somatic cell nuclear transfer (SCNT), can now be routinely applied to large animal species, including minipigs. Minipigs of different breeds have been cloned from different cell types, including genetically modified cells (Estrada et al., 2008; Kurome et al., 2008; Wakai et al., 2008; Zhao et al., 2009). In addition SCNT offers the possibility of creating isogenic and immunocompatible animals from the same cell line. Importantly, models of severe disorders can be generated from engineered cultured cells without the need of breeding sick animals. The sequencing of the pig

genome is another key development in the production of gene-modified pigs in the post-genomic era (Schook et al., 2015a). Genome editing techniques, including zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered, regularly interspaced, short palindromic repeats (CRISPR) together with CRISPR associated (Cas) nucleases (CRISPR/Cas), now allow the precise manipulation of gene sequences in germ, embryonic and somatic cells (Hauschild et al., 2011; Carlson et al., 2012; Cong et al., 2013; Hai et al., 2014; Whitworth et al., 2014; Dicks, 2015). Among these methods, the CRISPR/Cas9 system is emerging as the method of choice because it permits gene editing to be accomplished in only one step by injecting both the specific guide RNAs and endonuclease into zygotes (Hai et al., 2014; Whitworth et al., 2014).

Another example of human disease that has the potential to be studied in genetically engineered pigs is heart arrhythmias (Park et al., 2015). Mutations in the *SCN5A* gene, which encodes a subunit of the cardiac sodium channel $Na_v1.5$ required for excitability and conduction in the myocardium, were found in patients with Bruguda syndrome (Hedley et al., 2009). $SCN5A^{E558X/+}$ engineered Yucatan minipigs with reduced expression of the sodium channel $Na_v1.5$ have been created and these animals exhibit conduction abnormalities and susceptibility to ventricular arrhythmias (Park et al., 2015). There has also been considerable interest in genetically modified pig strains suitable for xenotransplantation. Most research into the development of appropriate xenotransplantation strains focused on addressing hyperacute rejection, which is initiated rapidly and involves preformed natural human antibodies and the complement system (Cooper et al., 2002). This has been possible by targeting cell surface antigens such as α-1,3 galactosyltransferase (Miyagawa et al., 2001; Lai et al., 2002; Phelps et al., 2003; Takahagi et al., 2005) or complement regulatory proteins such as human decay accelerating factor (Murakami et al., 2002). The pigs made deficient of α-1,3 galactosyltransferase have contributed to the reduction of immunogenicity of donor tissue/organs (Phelps et al., 2003). Transgenic pigs expressing antibodies against cytotoxic T-cell lymphocyte antigen receptor, a cell-mediated immune response suppressor, were also developed (Phelps et al., 2009).

A pig model for the human familial adenomatous polyposis was generated by inactivation of the adenomatous polyposis coli (*APC*) gene (Flisikowska et al., 2012). Mice lacking the *APC* gene exhibit non-metastatic neoplasias only in the small intestine (Su et al., 1992). However, the pig model of colon and rectal cancer reproduces the human features of the disease, which includes the development of polyps spread along the whole large bowel in young animals. A candidate gene for the development of breast and ovarian cancer models is the breast cancer-associated gene 1 (*BRCA1*), which has been manipulated in both Yucatan and Göttingen cells, but lines of modified minipigs remain to be produced (Luo et al., 2011, 2012). The *TP53* gene, which encodes the tumor suppressor protein p53 and is the most commonly observed suppressed gene in human tumors, was found to be mutated in Li-Fraumeni patients having increased risk to develop multiple types of cancers (Gonzalez et al., 2009). Suppression of p53 in mesenchymal stem cells derived from pig bone marrow exhibits chemoresistance *in vitro* (Leuchs et al., 2012). Mutation of *TP53* gene in Yucatan minipigs resulted in development of lymphomas and osteogenic tumors (Sieren et al., 2014). More recently, a new engineered pig strain termed "oncopig" was developed, which promises inducible formation of a wide variety of cancers that are potentially novel platforms for research and therapeutics development (Schook et al., 2015b). These examples illustrate the potential of genetically engineered pigs as robust models for the study of human pathologies that are not well represented in small laboratory animal species.

Improving the Usefulness of Pigs in Biomedical Research

Rodents have been the choice animal model for basic research, but are not always suitable for translational research due to marked differences in size, lifespan as well as metabolic, anatomical, and physiological discrepancies. On the other hand, the pig is more closely related to humans in terms of these parameters (Swindle et al., 2012) and, therefore, is better suited for recapitulation of human diseases. Indeed, the use of the pig in translational research is increasingly gaining acceptance (**Figure 1C**). Dogs and non-human primates have traditionally been used for this purpose, but rising ethical concerns have reduced their favor and increased demand for alternatives (Swindle et al., 2012). The number of peer-reviewed papers describing the use of pigs as biomedical models has risen eightfold over the past 30 years (**Figure 1B**). Already, the pig has become well established in many areas of research and training. For instance, in the past 20 years the pig has replaced the dog as a model for surgical training and has also gained FDA approval for the testing of surgical implantation devices intended for human use (Swindle et al., 2012; Schook et al., 2015a). Minipig models, which are much smaller in size compared to the domestic farm breeds, offer lower operating costs compared to other large animal models and also reduce the concern of ethical acceptance given the already widespread use of pigs in agriculture (Bollen and Ellegaard, 1997; Swindle et al., 2012).

Pigs offer many exciting applications, including stem cell research, tissue engineering and xenotransplantation. Although incredible advances in transgenic pigs harboring various engineered alterations designed to minimize graft versus host rejection (Lai et al., 2002; Phelps et al., 2003, 2009; Klose et al., 2005; Takahagi et al., 2005; Hauschild et al., 2011; Petersen et al., 2011; Jeong et al., 2013), much work remains to be accomplished since multiple genes need to be manipulated given the various types of tissue rejection reactions (Takahagi et al., 2005; Whyte and Prather, 2011; Jeong et al., 2013). Porcine induced pluripotent stem cells (iPSCs) have been produced (Esteban et al., 2009) and chimeric pigs were generated using iPSC (West et al., 2010, 2011). This is highly relevant since study of porcine iPSCs have eventual human applications (Esteban et al., 2009), such as cell-based therapies. However, the mechanisms of cellular reprogramming, directed cell differentiation and species-specific cell culture requirements necessitate further investigation (Ezashi et al., 2012). The

International Society for Stem Cell Research has indicated in their guidelines for translational use that validation must occur in both small and large animal models (Aigner et al., 2010). Tissue repair is another potential application of engineered pig models. Cartilage tissue grafts have been created using chondrocytes isolated from infant minipigs (Deponti et al., 2014), and mandibular condyle grafts have been generated from Yucatan minipig adipose-derived mesenchymal stem cells (Abukawa et al., 2003). There has also been successful regeneration of bone defects using engineered bone graft tissues in minipig models (Gröger et al., 2003). If custom donor transgenic minipig strains can be created, this could open the doors to other engineered tissue replacements for human uses. For example, the use of blastocyst complementation and pluripotent stem cells has been applied to direct the development of otherwise missing organs in pigs (Matsunari et al., 2013). This has increased the hope that it may one day be possible to create non-immunogenic donor organs in pigs using human iPSCs (Matsunari et al., 2013; Feng et al., 2015). Finally, similarities in the porcine and human immune system have sparked interest in vaccine development and efficacy testing in pigs (Meurens et al., 2012).

The completion of the porcine genome project in 2012 has further facilitated the use of pigs in research. Data from this project has enabled the comparative analysis of genetic sequences and development of the necessary tools to create and validate targeted genetic alterations in the porcine genome (Gun and Kues, 2014; Schook et al., 2015a). In addition, the development of RNASeq technology has facilitated transcriptome analysis, which further improves our ability to identify important targets related to certain phenotypic traits (Ropka-Molik et al., 2014). Other recent achievements in the pig include the use of inducible or conditional systems to control transgene expression (Kues et al., 2006; Klymiuk et al., 2012), and tissue-specific expression of the Cre recombinase (Li et al., 2009; Luo et al., 2014). These advances will ensure the continued development of various pig strains for research, similar to what has already been accomplished in mice.

Summary

It is clear that the use of the pig as a biomedical model is increasingly gaining approval due to physiopathological similarities with humans. However, some obstacles remain to be overcome in order to realize the full potential of the porcine species in developing new diagnostic and therapeutic approaches. Despite the sequencing of the porcine genome, full annotation has yet to be completed. This is essential to facilitate interrogation of the pig genome and investigation of less characterized genes. Efforts to develop a complete porcine proteome map as well as epigenome map are currently underway (Meurens et al., 2012; Schook et al., 2015a). These databases are necessary to understand disease pathogenesis (Meurens et al., 2012; Schook et al., 2015a). Moreover, the availability of both inbred and outbred breeds of minipigs extends the utility of these species as a viable large animal model. Continuing refinements and adaptation of technologies for genome editing, cell/tissue-specific gene targeting strategies, stem cells and somatic cell cloning will further facilitate the creation of specialized pig strains for biomedical research.

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REVIEW
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Engineered Swine Models of Cancer

Adrienne L. Watson[1]*, *Daniel F. Carlson*[1], *David A. Largaespada*[1, 2, 3, 4], *Perry B. Hackett*[1, 3, 5] *and Scott C. Fahrenkrug*[1]

[1] Recombinetics, St. Paul, MN, USA, [2] Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA, [3] Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA, [4] Pediatrics, University of Minnesota, Minneapolis, MN, USA, [5] Center for Genome Engineering, University of Minnesota, Minneapolis, MN, USA

Over the past decade, the technology to engineer genetically modified swine has seen many advancements, and because their physiology is remarkably similar to that of humans, swine models of cancer may be extremely valuable for preclinical safety studies as well as toxicity testing of pharmaceuticals prior to the start of human clinical trials. Hence, the benefits of using swine as a large animal model in cancer research and the potential applications and future opportunities of utilizing pigs in cancer modeling are immense. In this review, we discuss how pigs have been and can be used as a biomedical models for cancer research, with an emphasis on current technologies. We have focused on applications of precision genetics that can provide models that mimic human cancer predisposition syndromes. In particular, we describe the advantages of targeted gene-editing using custom endonucleases, specifically TALENs and CRISPRs, and transposon systems, to make novel pig models of cancer with broad preclinical applications.

Keywords: swine models, preclinical cancer models, genetically engineered swine, cancer genetics, genome engineering

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***Correspondence:**
Adrienne L. Watson
adrienne@recombinetics.com

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INTRODUCTION

Cancer is the second leading cause of death in the United States. The National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) data, estimated that in 2012 there were more than 13.7 million people living with cancer in the U.S. (Siegel et al., 2012). Trends suggest that in 2015 there will be over 1.6 million new cancer diagnoses in the U.S. and a staggering 589,000 deaths due to cancer (SEER). Throughout history there have been dramatic improvements in the methods by which we detect, diagnose, and treat cancer, yet the 5-year survival for all types of cancer remains at a dismal 66.5% (N.C.I. Surveillance Research Program; Mukherjee, 2011; Siegel et al., 2012). While the overall trends in cancer mortality in the U.S. have been reduced over the past decades, there are still several types of cancer for which the prognosis is very poor and for which few improvements have been made (**Figure 1**). Indeed, it has been suggested that the apparent increase in 5-year survival rates is due to earlier diagnoses, rather than improvements in treatment, for many types of cancer. The lifetime risk of developing cancer in the U.S. is over 40%, which emphasizes the need to better understand this deadly disease and improve outcomes for patients diagnosed with cancer (Siegel et al., 2012).

Cancer is a genetic disease in which cells acquire or inherit mutations, leading to uncontrolled growth of cells in the blood or solid organs (Hanahan and Weinberg, 2011). Animal models, and specifically mouse models, have played a major role in our understanding of the genetic basis of cancer and the role of specific genes and gene mutations in the development and progression of cancer. Mice led the way for the identification of new therapies to treat cancer owing to advances in constructing specific mutants in the late 20th century. Hundreds of mouse models of cancer have

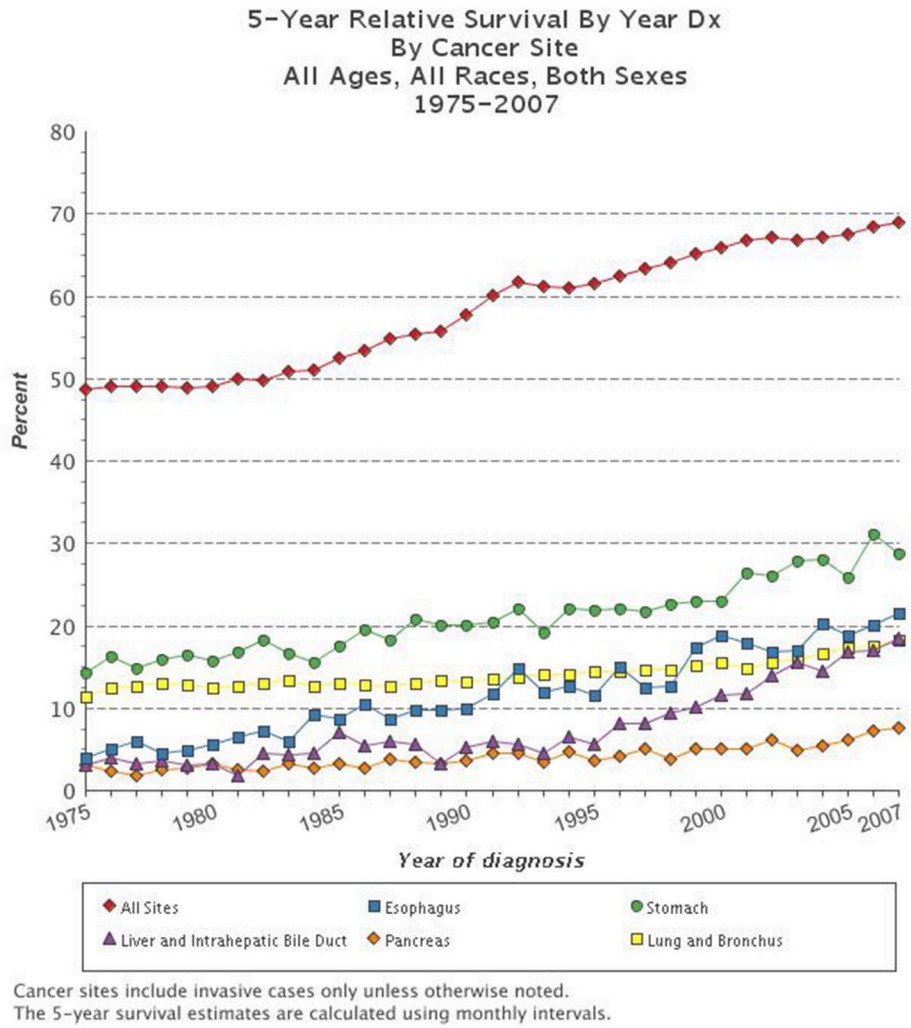

FIGURE 1 | Five-year survival rate based on cancer type. The combined 5-year survival rates of all types of cancer have improved over the past decades, yet survival for the most deadly types of cancer including esophageal, stomach, liver and intrahepatic bile duct, pancreatic, and lung and bronchial remain very dismal (www.cancer.gov; N.C.I. Surveillance Research Program).

been made and studied. However, gaining a complete understanding of cancer, which turns out to be an astonishing number of variant diseases, and translating this knowledge to better treatments and ultimately a cure has been elusive. Clearly, there are limitations to using rodents to model human diseases including large differences in their size, anatomy, physiology, drug metabolism, chromosome structures, and their genetics. Most cancer studies done in mice involve inbred lines of mice in which every locus is homozygous—a condition that inhibits translation of murine studies to humans (Hunter, 2012). To augment studies in the mice, new animal models of cancer are needed. Swine may turn out to be the best alternative models due to their size, physiological, genetic, and biochemical similarities to humans (Prather et al., 2008; Schook et al., 2008; Ganderup et al., 2012; Swindle et al., 2012; Flisikowska et al., 2013; Helke and Swindle, 2013). High-throughput genome sequencing and a collection of precision-genetic tools combined with tools for bioinformatics analysis, and profiling of gene expression/proteomics can be applied in swine. The ability to modify mammalian genomes through transgenesis and targeted nucleases, united with the development of advanced reproductive technologies including cloning, allows researchers to create complex and unique models of cancer in swine that are more applicable to human disease.

THE LIMITATIONS OF RODENT MODELS OF CANCER

Due to the vast differences between rodents and humans, the ability to model the complex diseases such as cancer is quite limited (Cheng et al., 2014). Humans are 3,000 times larger than mice, live 30–50 times longer, and therefore undergo about 10^5 more cell divisions in a lifetime (Rangarajan and Weinberg,

2003). Without genetic modification, mice develop cancers of mainly mesenchymal origin, such as sarcomas and lymphomas, whereas humans have a bias toward the development of epithelial cancers with age (Rangarajan and Weinberg, 2003). The small size and short lifespan of mice means that loss of certain tumor suppressor genes is insufficient to result in development of cancer in a highly penetrant manner, particularly when such mutations are heterozygous. Accordingly, investigators have used the Cre-Lox system to homozygously inactivate tumor suppressors in a tissue or cell type-specific manner. While this is often sufficient to drive tumor formation, such a situation does not mimic the disease course in patients in which rare loss of heterozygosity (LOH), a genetic condition in which one copy of a gene (or genetic locus, portion of chromosome, etc.) is lost or deleted due to a mutation or chromosomal event, occurs in a field of heterozygous cells. LOH is a common phenomenon in cancer, resulting in homozygous loss of tumor suppressor genes in a subset of cells in the body, often leading to the development of a tumor or the progression of an existing tumor. Because mouse chromosomes are telocentric, LOH often occurs in mouse models by loss of the entire chromosome carrying the wild type tumor suppressor gene allele in cells heterozygous for a tumor suppressor gene mutation (Luongo and Dove, 1996). However, in human tumors LOH usually occurs via sub-chromosomal deletions covering the wild type tumor suppressor gene locus (Thiagalingam et al., 2001; Petursdottir et al., 2004).

On a cellular level, murine cells have a lower threshold for genetic and/or epigenetic changes that lead to transformation in culture, which demonstrates fundamental differences in the mechanistic properties of cancer development between mice and humans (Holliday, 1996). Arguably, the most profound difference between rodent models and humans is the essentially 100% homozygosity of every locus in inbred mouse lines, which may represent only a single individual in the entire population, making extrapolation back to entire human populations challenging (Kaiser, 2015). Mouse cells are immortalized much more readily than are human cells (Rangarajan and Weinberg, 2003). It has also been suggested that mouse cells respond to oncogenic Ras expression in a different manner than human cells; RAS oncogenes require RAL signaling in human cells, whereas the requirement for this signaling pathway is much reduced in RAS oncogene transformation of mouse cells (Hamad et al., 2002). Laboratory mouse strains have very long telomeres, and readily re-express TERT, in contrast to human cells (Holliday, 1996; Kim Sh et al., 2002). Moreover, mice do not develop the same kinds of genetic instability that human cells do during tumorigenesis, perhaps due to their shorter lifespan that could restrict the number of sequential mutations that accumulate in human tumors (Kim Sh et al., 2002).

Many organ systems vary so greatly between rodents and humans that certain types of cancer cannot be accurately modeled. For example, when one copy of the tumor suppressor gene adenomatous polyposis coli (*APC*) is inherited in humans, LOH leads to polyps in the large intestine that progress to invasive carcinoma. In contrast, mice that are heterozygous for *Apc* develop polyps in the small intestine that rarely show disease progression (Karim and Huso, 2013). Such differences in cancer development are due to inherent biological differences between man and rodent and are not limited to the intestinal polyps, but are seen in many mouse models of cancer (**Table 1**). There are fundamental differences in how tumorigenesis occurs in rodents and humans. This is well illustrated by variations in tumor spectrum when certain tumor suppressor genes, known to cause specific cancers in humans, are knocked out in mice (**Table 1**). Specifically, the five deadliest cancers in the U.S. (**Figure 1**) either cannot be modeled in rodents, or have ineffective models for identification of treatments that translate to the clinic.

The size limitation in rodents makes the development of novel imaging modalities and surgical techniques nearly impossible, yet these are key techniques needed to diagnose and treat a wide variety of tumor types in patients. Moreover, the rate of metabolism is much, much higher in mice compared to humans (Rangarajan and Weinberg, 2003). These differences mean that the pathways by which tumor progression occurs can vary dramatically when comparing mouse models to human cancer. As a consequence, the tumors that develop in a mouse model may respond differently to therapy. For the genetic and physiological reasons, including vast differences in drug metabolism and xenobiotic receptors, rodents also poorly model toxicity, sensitivity, and efficacy when used in preclinical drug studies (Swanson et al., 2004). The ability to establish toxicity and drug sensitivity pre-clinically in animal models is immensely

TABLE 1 | Comparison of knockout mouse models to human patients.

Gene	KO mouse	Patients	References
APC	Small intestine polyps which do not typically progress	Large intestine polyps that progress to invasive carcinoma	Groden and Burt, 2012
BRCA1	No cancer development	80% risk of breast cancer, 55% risk of ovarian cancer	Evers and Jonkers, 2006
BRCA2	No cancer development	80% risk of breast cancer, 25% risk of ovarian cancer	Evers and Jonkers, 2006
NF1	Leukemia, pheochromocytoma	Plexiform neurofibromas, malignant peripheral nerve sheath tumors, optic nerve glioma, astrocytoma, leukemia	Gutmann and Giovannini, 2002
NF2	Bone tumors, lymphoma, lung adenocarcinoma, hepatocellular carcinoma	Schwannomas, meningiomas, ependymomas	Gutmann and Giovannini, 2002
RB	Pituitary tumors	Retinoblasoma, osteosarcomas, prostate, breast cancer	Taneja et al., 2011
TP53	Osteosarcoma, soft tissue sarcoma, lymphoma	Breast cancer, brain tumors, osteosarcoma, soft tissue sarcoma	Taneja et al., 2011

important because less than 8% of cancer drugs translate successfully in Phase I clinical trials from animal models (Mak et al., 2014). While mice have provided numerous insights into the biology of cancer, their historical limitations emphasize the need to develop new models for cancer research, such as swine.

ADVANTAGES OF USING SWINE CANCER MODELS

The anatomical, physiological, and genetic similarities between swine and humans are striking, suggesting that disease modeling in this large animal may better represent the development and progression of cancer seen in human patients (Swindle et al., 2012). Swine have been widely used in many areas of biomedical research due to such a high resemblance in organ systems. For these reasons pigs are commonly used in cardiovascular research where models of atherosclerosis, thrombosis, and myocardial infarction have been used to understand these health conditions in patients and to develop therapeutic and medical device interventions (Dixon and Spinale, 2009; Vilahur et al., 2011). The similarity in size and anatomy of the swine cardiovascular system allows design and testing of stents and tissue engineering of blood vessels (Bedoya et al., 2006; Gyongyosi et al., 2006). Further, comprehensive studies of the skin, urinary, integumentary, and digestive systems demonstrate extensive similarities to humans (Swindle et al., 2012). This history suggests that swine may be extremely useful as models of human cancer.

Perhaps of greater importance is the degree of genetic variation in pigs, including those used for disease models. Numerous genetically distinct lines of pigs exist and are available for model development, with various levels of diversity and inbreeding. Cultivation and characterization of these lines provides the opportunity to address both basic science and preclinical research needs. Lines that are low in variation provide a predictable platform for the development of therapeutics and toxicology research. But, like patients, many swine herds are highly outbred, with tremendous genetic and phenotypic heterogeneity that is more reflective of the patient population. This heterogeneity has two major consequences. First, genes that act as "drivers" or are otherwise critical to cellular transformation are more likely to be evident in pigs because they must act in the presence of other genetic variations. Also, therapeutic interventions that show efficacy will have to operate in many genetic environments in pigs, likely more accurately predicting safety and efficacy in patients.

The swine genome (*sus scrofa*) has been completely sequenced and, as expected, it shares considerable homology to the human genome (Schook et al., 2005; Groenen et al., 2012). Extensive conservation between pigs and people at the protein and primary sequence level, and extensive chromosomal synteny provide opportunities to address the initiation and progression of cancers, including frequently observed indels, inversions, and translocations, an outcome prohibitive in rodents, where synteny is more fragmented (Schook et al., 2005; Groenen et al., 2012). For instance, one study identified conservation between human and pigs of 112 loci, wherein a human amino acid that is implicated in a human disease is the same in swine (Groenen et al., 2012). Further, gene expression profiling and proteomics have been rapidly advancing in swine (Garbe et al., 2010). With the full genomic sequence in swine, the advancement of bioinformatics tools, the ability to modify somatic swine cells with transgenesis and targeted nucleases, and the development of techniques such as somatic cell nuclear transfer (SCNT), we are now able to create genetically engineered swine models of human disease (Prather et al., 2008). The homology between human and swine genes will be a guide for engineering exact human disease alleles into the swine genome. In the past 4 years, a platform for genetically engineering the swine genome using targeted nucleases and homology-dependent repair (HDR) has been developed (Carlson et al., 2012a; Tan et al., 2013). As described below, this technology allows the replication of precise amino acid changes and/or truncating mutations in oncogenes and tumor suppressor genes known to drive initiation, progression, or metastasis in human cancer. The use of targeted nucleases to engineer swine genocopies, or exact mutant alleles that cause cancer in human patients, represent a more accurate animal model than removing entire exons using standard knockout strategies or overexpressing oncogenes using transgenesis, which have been the mainstays in murine models for decades. Further, recent precision genetic technologies can support the development of single-gene modifications and complex, multiple-gene changes as well as chromosomal translocations in a single generation in swine.

In addition to recent advances in making precise genetic modifications to large animal genomes, there has been significant progress in technologies for testing consequences of genetic changes. Imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) can be easily applied to large animals such as pigs, whereas application of analogous clinical protocols is difficult in rodents (Sieren et al., 2014). By applying these imaging modalities to swine models of cancer we can improve detection techniques, better monitor progression, and more accurately measure response to therapy. The size of the pig allows for radiation-directed therapies to be tested and optimized. Surgical resection is the first line of defense and often the standard of care for many cancers. The size of the pig allows for refinement of surgical techniques and studies of local tumor recurrence, both of which are difficult or impossible to perform in rodents. Tumor natural history is an area that is difficult to study in rodents due to their short lifespan, about $1/30^{th}$ that of humans (Rangarajan and Weinberg, 2003). Swine can live up to 10 years thereby enabling researchers to carefully follow the development of tumors, tumor progression, invasion, and metastasis in the absence of intervention over time. Additionally, the identification of biomarkers may be feasible in these animals due to the facile nature of accessing blood and tissue samples, the abundance of sample material and the ability to perform longitudinal blood sampling over long periods of time. Understanding tumor heterogeneity may be well suited for a large animal, as samples could be collected from many different tumors over time and followed for variations in

somatic mutations, gene expression, or differential responses to treatment.

One of the main drawbacks of rodent models of cancer has been their inability to identify safe and effective drugs to treat cancer. Mouse models of cancer have been poor predictors of drug safety, toxicity, and efficacy (Gould et al., 2015). Further, routes of administration in mice are largely limited to intravenous (i.v.), intraperitoneal (i.p.) or oral gavage. Pigs have been widely used in preclinical drug toxicology, and are a standard large animal model for preclinical toxicology prior to human studies (Ganderup et al., 2012). The size and ease in handling pigs allows for drugs to be administered in the same way that patients are given them, including orally, intravenous (i.v.), intraperitoneal (i.p.), inhalation, dermal absorption, subcutaneous, intramuscular, and transmucosal. Longitudinal blood sampling can be performed to assess drug exposure and metabolism over long periods of time, and the amount of blood samples that can be taken from swine, in a short period of time, enhances the ability of pharmacologists to get precise kinetic data following drug exposure. There is significant homology in xenobiotic receptors in swine and human that regulate drug metabolism and pharmacokinetic properties (Myers et al., 2001). The cytochrome P450 (CYP) superfamily of proteins play a critical role in the processing and metabolism of drugs, and again, many studies have shown parallels in the structure and function of these molecules in pigs and humans (Myers et al., 2001). Importantly for pediatric cancer drug studies, juvenile pigs have been shown to have human-similar pharmacokinetic responses to certain drugs that cannot be modeled in other animals (Roth et al., 2013). The use of pigs in preclinical drug testing may identify safer and more effective therapies as well as establish dosing and routes of administration for new drugs prior to human clinical trials. Furthermore, a facile porcine genome engineering platform enables future humanization of drug metabolism in swine models.

APPLICATIONS OF PRECISION GENETICS TO MODEL CANCER IN SWINE

There have been three main types of disease models in swine that have been applied toward cancer modeling—spontaneous, induced, and genetically modified. Spontaneous models of pig cancers are rare, because like humans, pigs develop cancer with age, and as an agricultural animal produced mostly for food, most pigs do not survive to the age where cancer would be commonly seen. Sinclair miniature white swine have been a valuable model of malignant melanoma, which was identified as occurring in these animals in 1967 and has since been selected for by breeding (Oxenhandler et al., 1979). Another study identified 92 cases of leukemia in 3.7 million pigs tested, and 58% of those cases were in pigs under 6 months of age (Anderson and Jarrett, 1968). A range of rarer cancers have been described in older pigs (Brown and Johnson, 1970). However, owing to the economic necessities of keeping costs low in animals of agricultural importance, pigs harboring diseases due to rare mutations are euthanized without further study. Induced models of cancer in swine have been developed and are providing valuable insights into triggers of tumorigenesis found in some agricultural environments. In one study, researchers exposed pigs to N-nitrosodiethylanime to induce hepatocellular carcinoma (HCC) that resembles human HCC (Li et al., 2006). Another induced model took advantage of a naturally occurring severe combined immunodeficiency (SCID)-like Yorkshire pig line and transplanted human melanoma and pancreatic carcinoma cells, demonstrating the usefulness of these animals as human tumor xenografts (Basel et al., 2012).

Genetic engineering and gene editing technologies are being developed for cancer modeling in pigs (**Figure 2**, **Table 2**). A transgenic pig carrying the *MMTV-v-Ha-ras* oncogene was developed by microinjection of DNA into embryos, although no tumors developed in these animals (Yamakawa et al., 1999). A basal cell carcinoma model was created by making a transgenic pig in which *Gli2* was expressed under control of a keratinocyte-specific promoter (McCalla-Martin et al., 2010). Standard gene-targeting methods developed in mice have been applied to pigs and led to the development of a familial adenomatous polyposis (FAP) model in which gene-targeting was used to introduce premature termination codons (PTCs) in *APC* by electroporation of linearized vector DNA into mesenchymal stem cells and subsequent SCNT to produce animals (Flisikowska et al., 2012). Similarly, gene-targeting was used to introduce a dominant-negative missense mutation, R167H, in the swine tumor suppressor gene *TP53* using a recombinant adeno-associated virus (rAAV) in fetal fibroblasts, which were cloned to produce animals that when bred to homozygosity for the R167H mutation developed lymphomas, osteogenic tumors, and renal tumors at varying rates (Sieren et al., 2014). These animals represent a great model for humans with germline mutant *TP53* mutations, seen in Li-Fraumeni patients, as they develop hematopoietic malignancies, bone tumors, and adrenal gland tumors in the kidney as well. A breast cancer model was attempted in the pig using rAAV-mediated *BRCA1* knockout, but no phenotype was observed due to death of the animals by unknown causes before they reached 3 weeks of age (Luo et al., 2011).

The ability to activate or inactivate genes in a temporal or spatially-specific manner has been a critical aspect of many murine models of cancer, and this technology is now being developed in the pig by use of the Cre-Lox system (Flisikowska et al., 2013; Schook et al., 2016). There have been three swine models that use the Cre-Lox system for inducible cancer gene expression (Leuchs et al., 2012; Li et al., 2015; Schook et al., 2015). A pig was engineered to harbor a latent $TP53^{R167H}$ mutation by the insertion of a transcriptional termination signal between two LoxP sites upstream of the gene, allowing expression of $TP53^{R167H}$ only in the presence of Cre recombinase. Cre can be given in a time- or tissue-specific manner to induce recombination and results in the expression of the dominant negative *TP53* allele, and in the absence of Cre Recombinase, the latent form of the gene is a knockout; however, at this time, the effects of this allele *in vivo* have not been reported (Leuchs et al., 2012). A second inducible model was generated using

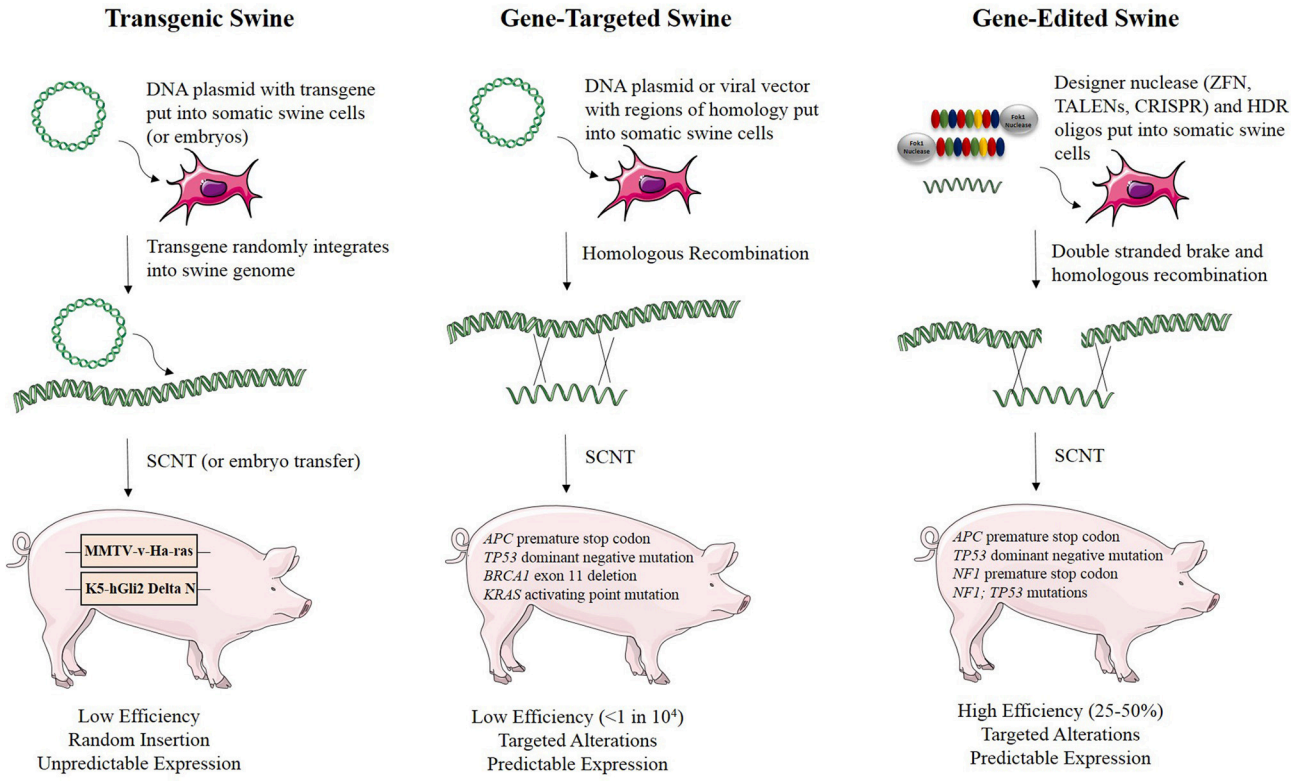

FIGURE 2 | Methods for developing genetically engineered swine models of cancer. There are three methods for developing genetically engineered swine. Transgenic swine are created by randomly integrating exogenous DNA into the swine genome, resulting in random insertion and unpredictable expression. Gene-targeted swine are created using large, homologous DNA sequences to integrate exogenous DNA into the swine genome at targeted loci, but this method has low efficiency, often requiring the introduction of some exogenous markers such as drug resistance genes. Gene-edited swine are made using designer nucleases, such as TALENs or CRISPRs, and use a double stranded break at a specific locus to induce efficient homologous recombination to make a targeted alteration in the genome (Yamakawa et al., 1999; McCalla-Martin et al., 2010; Luo et al., 2011; Flisikowska et al., 2012; Leuchs et al., 2012; Sieren et al., 2014; Li et al., 2015).

TABLE 2 | Genetically engineered swine models of cancer.

Gene	Mechanism	Method	Phenotype	References
RAS	MMTV-v-Ha-ras transgene	Transgenesis	No tumor development	Yamakawa et al., 1999
GLI2	K5-hGli2 Delta N transgene	Transgenesis	Basal cell carcinoma-like lesions	McCalla-Martin et al., 2010
APC	Premature Stop codon at 1311	Gene-targeting by linearized vector DNA	Low- and high-grade dysplastic adenomas in large intestine	Flisikowska et al., 2012
APC	Premature Stop codon at 1061	Gene-targeting by linearized vector DNA	No tumor development at 1 year of age	Flisikowska et al., 2012
TP53	R167H dominant negative allele	Gene-targeting by rAAV	Lymphoma and osteogenic tumors	Sieren et al., 2014
TP53	R167H dominant negative allele with floxed termination signal	Gene-targeting by vector DNA	TBD	Leuchs et al., 2012
BRCA1	Loss of exon 11	Gene-targeting by rAAV	Pigs died by 18 days	Luo et al., 2011
KRAS	Floxed G12D activating allele	Gene-targeting by promoter trap gene targeting vector	TBD	Li et al., 2015
KRAS; TP53	Floxed, bicistronic $KRAS^{G12D}$ cDNA and $TP53^{R167H}$ cDNA	Transgenesis	Mesenchymal tumor formation upon AdCre injection	Schook et al., 2015

gene-targeting to express a Cre-activated $KRAS^{G12D}$ mutation, although the effect of Cre-induced activation of this allele has yet to be tested in these pigs (Li et al., 2015). Most recently, a transgenic "oncopig" was developed in which a Cre- inducible transgene expressing $KRAS^{G12D}$ and $TP53^{R167H}$ was engineered, in hopes to model the many types of human cancers that have

KRAS and *TP53* mutations (Schook et al., 2015). Indeed, upon transgene activation, porcine cells were transformed in culture, formed tumors in immunodeficient mice, and led to tumors of mesenchymal origin when activated by AdCre injection directly into these animals (Schook et al., 2015). In addition to using the Cre-Lox system for conditional gene expression, as described in the models above, this system can also be applied to conditional deletions of short coding sequences and used as a strategy for inducing chromosomal rearrangements (Schook et al., 2016).

USING SITE-SPECIFIC NUCLEASES FOR GENE-EDITING TO MODEL CANCER IN PIGS

The use of designer nucleases is the latest technological platform being used to modify the germline of model species. This technology includes zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs). All of these methods to engineer precise changes into genomes have been used to make genetically engineered mouse models. In short, site-specific nucleases are designed to bind to user-defined regions of the DNA. ZFNs utilize a zinc finger domain which generally contains 3–6 zinc finger repeats recognizing 9–18 base pairs of DNA (Pabo et al., 2001). TALENs utilize a DNA binding domain contains repeated amino acid sequences, each which harbors a Repeat Variable Diresidue (RVD) (Boch et al., 2009). The RVD sequence gives specific nucleotide recognition, allowing TALENs to bind in a sequence specific manner (Boch et al., 2009). Typically, both ZFNs and TALENs utilize a cleavage domain with a bacterial type IIS restriction endonuclease, FokI, which requires dimerization in order for DNA cleavage to occur, creating even a higher level of specificity for site-specific nucleases (Pabo et al., 2001; Boch et al., 2009). The CRISPR/Cas9 system, derived from the prokaryotic immune system, consists of guide RNA (gRNA) sequences that guide Cas9, an RNA-guided DNA endonuclease, which then cleaves the DNA at these recognition sequences (Jinek et al., 2012; Mali et al., 2013). All three site-specific nuclease systems result in a double stranded break in the DNA, which can be repaired by non-homologous end joining (NHEJ) or homologous recombination (HR) when a repair template is provided. NHEJ often results in small insertions and deletions which can be used to disrupt the function of a gene, where HR allows users to engineer defined genetic changes at specific sites within genome (Carlson et al., 2012a,b; Tan et al., 2012, 2013). These genome engineering systems can be applied to primary cells, which, upon modification, can be used for SCNT to generate animals with germline genetic changes (Carlson et al., 2012a; Tan et al., 2012, 2013). Alternatively, these custom nuclease systems can be applied directly to embryos for *in vivo* modification (Bedell et al., 2012; Tan et al., 2012; Lillico et al., 2013).

The use of custom nucleases to enhance the efficiency of HR in swine has been dramatic from less than 10^4 using standard HR to rates as high as 25–75% using a recently developed TALEN/homology dependent repair (HDR) platform (Carlson et al., 2012a; Tan et al., 2013). Our group has used CRISPRs and TALENs to engineer several pig models of human disease, including models of infertility and atherosclerosis (Carlson et al., 2012a; Tan et al., 2013). Despite these advantages, a disadvantage to using the various site-specific nucleases, is the potential of undesirable collateral mutations that can accompany those that are desired (Kim et al., 2013; Lin et al., 2014; Mussolino et al., 2014; Frock et al., 2015; Hendel et al., 2015). Because the RNA-guided site-specific platforms (e.g., CRISPR-based) may allow U-G base-pairing, their fidelity may be lower than the protein-based platforms (e.g., TALENs). Consequently, although we have used most of the site-specific nuclease platforms, for fidelity and efficiency, we find TALEN-induced cleavages are the best balance for reliable gene-editing (Carlson et al., 2012a; Tan et al., 2013). TALEN technology allows replication of exact cancer mutations found in patients in pigs. Indeed, we have used TALENs to construct a swine models of the cancer predisposition disease, familial adenomatous polyposis by engineering a premature termination codon in *APC* (Tan et al., 2013).

TALEN-based genome editing for making swine genocopies of human cancer mutations is demonstrated for colorectal cancer in **Figure 3**. More generally, for a given type of cancer or known cancer driver gene, the method begins with the identification of common mutations within a gene of interest (**Figures 3A,B**; Cerami et al., 2012; Gao et al., 2013). Second, the location of the human mutation must be identified in the swine gene using bioinformatic approaches in which the amino acid sequence of the human and swine genes are aligned and the mutated human amino acid in humans is identified in swine (**Figure 3C**; Flicek et al., 2014). Third, custom nucleases such as TALENs are designed to induce a DSB at the appropriate site and HDR-oligonucleotides are designed to introduce the desired mutation (**Figures 3D,E**; Cermak et al., 2011; Doyle et al., 2012). We can employ a strategy in which the HDR-oligo design includes not only the mutation of interest, but a novel restriction length polymorphism (RFLP) that allows facile screening of a large number of cells for the desired mutation, although introduction of a single point mutation in the absence of an RFLP allele can be engineered as well. Fourth, TALENs and HDR-oligos are transfected into primary swine fibroblasts, where they will cut the DNA and induce HDR to introduce the desired mutation (**Figures 3F–H**). Transfected cells can be easily screened by RFLP and sequenced to confirm that the intended mutation is present (Carlson et al., 2012a; Tan et al., 2013). In the last step, these cells are used for SCNT to produce gene-edited swine that have the precise human cancer-causing mutation. It should be noted that this technology has also be applied to gene-editing in pig embryos, in addition to somatic cells, avoiding the need for the somatic cell nuclear transfer step (Lillico et al., 2013; Whitworth et al., 2014; Wei et al., 2015).

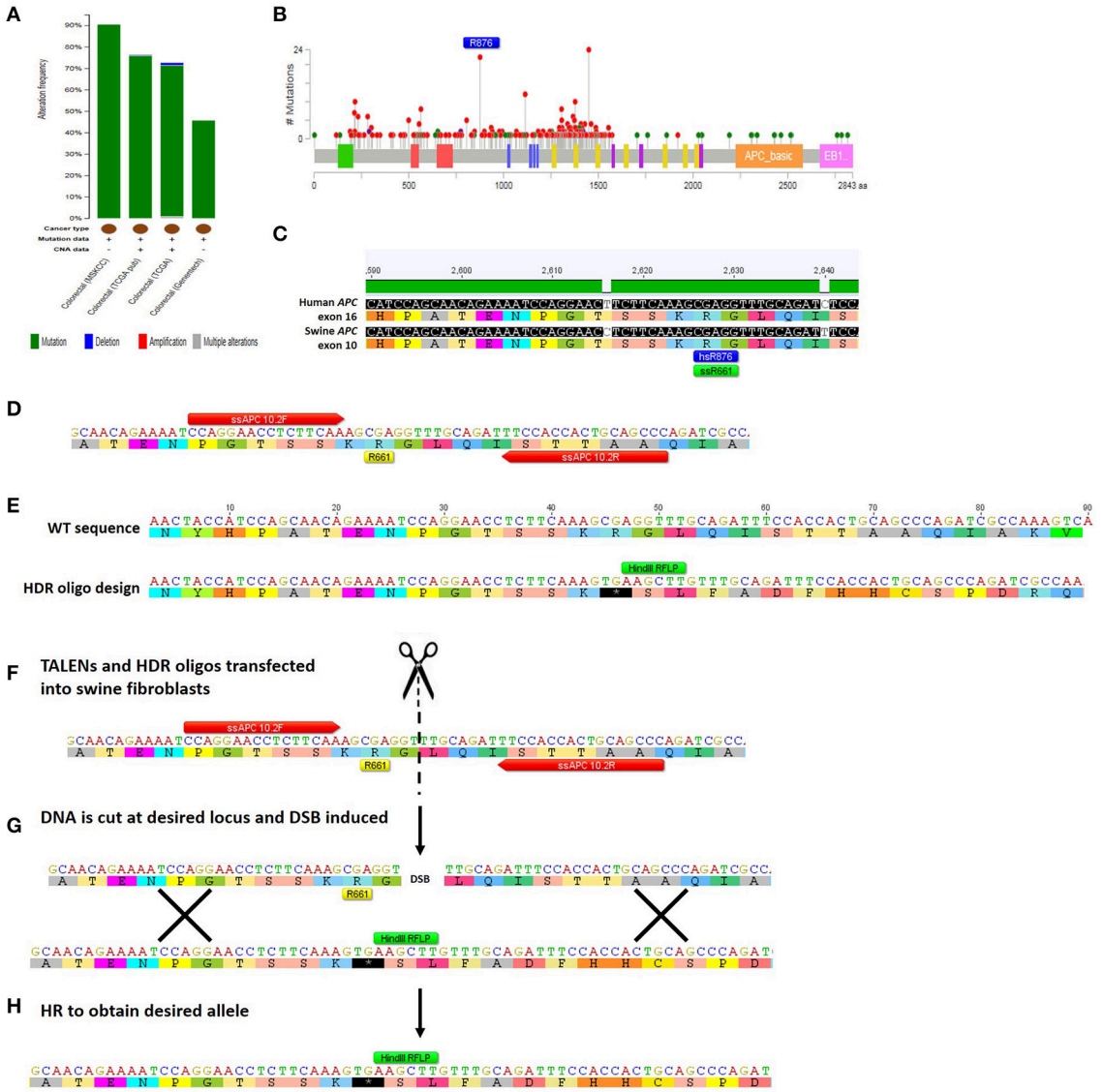

FIGURE 3 | Method for TALEN-mediated gene-editing to make a swine colorectal cancer model. (A) The tumor suppressor gene *APC* is commonly mutated in colorectal cancer at a rate of 46–91% depending on which study is assessed (Cerami et al., 2012; Gao et al., 2013). The majority of the mutations are mutations resulting in truncation of APC protein (Cerami et al., 2012; Gao et al., 2013). **(B)** This figure shows the rate of specific mutations across the *APC* gene, with truncating mutations shown in red and missense mutations shown in green (Cerami et al., 2012; Gao et al., 2013). One of the most common mutations in colorectal cancer is an R876X truncation mutation that was seen in 22/727 mutations analyzed (Cerami et al., 2012; Gao et al., 2013). **(C)** Human and swine *APC* genes were aligned and human exon 16 where R876 is found (shown in blue) aligns with swine amino acid 661 in exon 10 of *APC* (shown in green) (Flicek et al., 2014). **(D)** The locus surrounding swine *APC* R661 was inputted into software that identified TALENs that would bind and cleave the DNA near this site (TALEN binding sites shown in red; Cermak et al., 2011; Doyle et al., 2012). **(E)** A 90mer HDR-oligo was designed with homology upstream and downstream of targeted mutation including the TALEN binding sites. A C →T base pair change was introduced to create a premature termination codon. Additionally, four nucleotides were added which would cause a frameshift and a novel RFLP (*Hin*dIII) site (shown in green). **(F)** TALENs and HDR-oligos are then transfected into primary swine fibroblasts. **(G)** TALENs cut at the specified location resulting in a double-stranded break (DSB) and the HDR-oligo acts as a template for homologous recombination (HR). **(H)** HR results in the desired allele with the novel premature STOP colon, RFLP site, and frameshift incorporated into the genome of the swine fibroblasts (Carlson et al., 2012b; Tan et al., 2013).

UNIQUE OPPORTUNITIES FOR GENE-EDITED PIG CANCER MODELS

The ability to engineer specific human mutations into the swine genome is critical for accurately modeling cancer in the pig. While gene-targeting with either rAAV or other vectors has the ability to generate animals with swine genocopies of human disease alleles, these methods generally require the introduction of exogenous DNA into the swine genome in the form of antibiotic resistance genes such as puromycin- or neomycin-resistance since standard gene targeting via HR in swine is very inefficient with rates less than 1 in 10^4. In

contrast, the method of gene editing by employing TALENs and HDR-oligos of ∼90 nucleotides is highly efficient, with homologous recombination occurring at an average efficiency of about 45% across several TALEN pairs tested (Tan et al., 2013). This allows facile isolation of cellular clones that are heterozygous or homozygous for the engineered allele of interest.

We identified several types of cancer-causing mutations that can be engineered into swine using TALEN-mediated gene editing (**Figure 2**). Premature STOP codons can be introduced, resulting in truncated gene products, a common phenomenon for tumor suppressor genes in cancer (**Figure 3**). Tumor suppressor genes have also been shown to contain point mutations that result in a dominant negative protein product, as is the case for the human *TP53* mutation R175H, which functions as a dominant negative in swine (R167H) and can be introduced efficiently using TALEN-mediated HDR (Leuchs et al., 2012; Sieren et al., 2014). Similarly, oncogene activation can be modeled by introducing point mutations such as $KRAS^{G12V}$ using TALEN-mediated HDR. TALEN-mediated gene-editing and HDR repair can be used to create large deletions encompassing one or more genes, which are also seen in several types of cancer, such as micro-deletions of *NF1* seen in Neurofibromatosis Type 1 patients (Pasmant et al., 2010).

In addition to making point mutations using custom nuclease-mediated HDR, TALENs can be utilized to increase the efficiency of HDR with much larger constructs such as HDR templates with homology arms of 750 bp or larger that include LoxP sites flanking large exons. By inducing a double-stranded break, HDR occurs at a much higher efficiency than when plasmids or viral vectors are put into cells alone (Shin et al., 2014). Using TALENs to induce large HDR events has many applications in developing swine models of cancer. Many oncogenes and tumor suppressor genes are somatically mutated and therefore they must be expressed or disrupted in specific tissue types, at specific times. Using custom nuclease-mediated HDR, one could introduce LoxP flanked exons of tumor suppressor genes, or introduce latent oncogenes that would need to be activated by Cre-recombinase to support tissue/temporal regulation. This method allows for the introduction of transgenes at specific loci in the genome thereby avoiding unwanted insertional mutagenesis effects. In order to introduce multiple genetic changes that occur in cancer, TALEN-mediated, site-specific mutagenesis can be used to introduce simultaneous targeted disruption of a tumor suppressor gene along with one or more transgenes for oncogene expression.

The ability to edit multiple genes at one time is necessary due to multiple genetic alterations in each cancer cell. The efficiency at which the TALEN-mediated gene-editing and small HDR-oligo platform works, allows this technology to be applied toward multiplex gene-editing to model more complex genotypes of human tumors. Epidemiological data and mathematical models in colorectal cancer has suggested that it takes about five to seven rate limiting "steps" for transformation to occur (Renan, 1993). More recently, whole exome sequencing analysis in colorectal and breast cancer has shown that tumors have an average of 90 mutant genes, with 11 of these mutations being "cancer-causing" (Sjoblom et al., 2006). There are many types of cancer for which mutations in two or more genes are clearly demonstrated (**Figure 4**; Cerami et al., 2012; Gao et al., 2013). Recapitulating this phenomenon would be ideal when engineering animal models of cancer. Developing these multi-hit models is virtually impossible by standard gene-targeting techniques due to such low efficiencies, and would therefore require either serial cloning or animal breeding to obtain multiple alleles in one animal. Both of these alternatives are expensive and time-consuming in large animals such as swine. In contrast, the efficient custom nuclease-stimulated HDR allows the engineering of multiple cancer genes in a single generation.

Multiplex gene editing can also be applied in making models of cancer with associated co-morbidities. Treating patients with cancer becomes quite complex when the patient is suffering from other diseases as well, and this phenomenon of co-morbidity is quite common (**Table 3**; Sogaard et al., 2013). A study of 15,962 patients showed various types of cancers that are more highly associated with comorbidities (Ogle et al., 2000). The overall frequency of any single comorbidity occurring in this population of cancer patients was 68.7% and 32.6% of patients had two or more comorbidities (Ogle et al., 2000). Some of the effects of these comorbidities on cancer patients and their subsequent survival and cancer treatment are shown in **Table 3** (Sogaard et al., 2013). Several studies have demonstrated that patients with comorbidities are less likely to complete chemotherapy, more likely to suffer complications from treatment and/or surgery, and the 5-year mortality hazard ratio for cancer patients with comorbidities ranges from 1.1 up to 5.8 (Sogaard et al., 2013). A multiplex gene editing approach can be taken to model complex disease associations with cancer to understand the impact on survival and treatment approaches. For example, we have used our gene-editing technology to develop swine models of hypercholesterolemia, heart failure, and hypertension and have the ability to generate models of various cancer types in conjunction with these common comorbidities (Carlson et al., 2012a; Tan et al., 2013). Using multiplex gene-editing, it is possible to engineer swine models of cancer in the background of other comorbidity diseases in a single generation.

Genomic rearrangements, and specifically, chromosomal translocations are a common occurrence in cancer (**Table 4**) (Nambiar et al., 2008). The ability to model cancer-causing translocations has been limited to the expression of gene-fusion transgenes in mice and has yet to be demonstrated in swine. Chromosomal translocations can be induced by double-stranded DNA breaks and TALEN technology allows engineering of exact human translocations at endogenous loci in the swine genome resulting in expression from the native promoter (**Figure 5**). Indeed, cancer translocations have been previously engineered using TALENs and ZFNs in human cells (Piganeau et al., 2013) and could also be applied in porcine cells. Applying TALEN-mediated site-specific mutagenesis to swine opens up a broad field of new research into the mechanism of oncogene activation via genomic rearrangements, the pathogenicity of chromosomal translocations in various cancer types, and investigations into therapies targeting novel gene-fusions and mechanisms of resistance in translocation-driven cancer types.

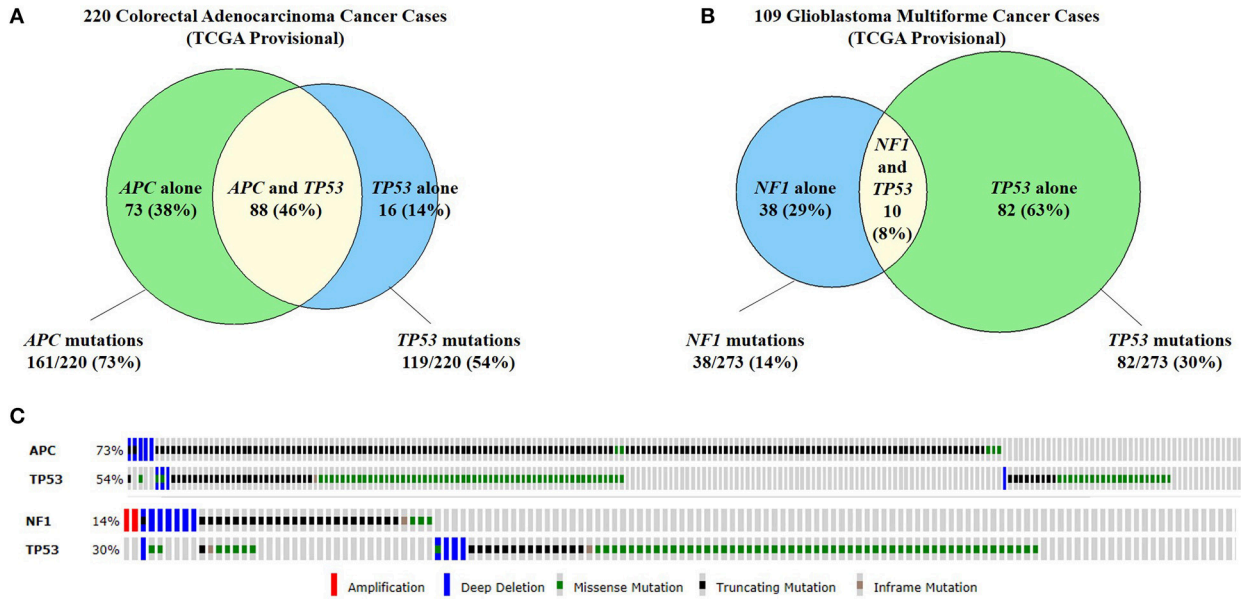

FIGURE 4 | Commonly co-mutated genes in colorectal adenocarcinoma and glioblastoma multiforme. (A) A study of 220 colorectal adenocarcinoma cases found that 73% of cases had *APC* mutations and 54% of cases had *TP53* mutations. Forty-six percent of cases had both *APC* and *TP53* mutations. **(B)** Similarly, in 109 cases of glioblastoma multiforme (GBM), 14% of cases has *NF1* mutations and 30% has *TP53* mutations. 8% of cases had both *NF1* and *TP53* mutations. **(C)** The types of mutations seen in *APC* and *TP53* in colorectal cancer (top) and GBM (bottom). Because all of these genes are tumor suppressor genes, the majority of mutations are truncating mutations or missense mutations (Cerami et al., 2012; Gao et al., 2013).

PIGS AS MODELS FOR CANCER XENOGRAFTS

An additional application of TALEN-based multiplex gene editing is the ability to simultaneously knock out and add in genes involved in immune system development to facilitate even a broader range of cancer research applications. We and others have developed a severe combined immunodeficiency (SCID) swine model by knocking out genes necessary for both B-cell and T-cell development (**Figure 6**) (Shultz et al., 2007; Suzuki et al., 2012; Watanabe et al., 2013; Huang et al., 2014; Ito et al., 2014). These animals will allow xenograft experiments to proceed in which one could engraft cells or tissues from human tumors into the pig and monitor these xenografts for growth and development properties, as well as for efficacy studies with novel therapies. These animals can also be engrafted with human immune cells by either blastocyst complementation or transplantation, making a "humanized pig." This animal would serve several purposes including to investigate the role of the immune system in response to chemo- and radio-therapies for the treatment of cancer and the role of the human immune system in cancer development and progression if combined with a tumor xenograft (Zitvogel et al., 2011). These animals may also have a major impact on immunological research and treatments including the evaluation of: (1) immune-modulatory drugs (Pardoll, 2012; Ileana et al., 2013), (2) cell-based therapies (Fischbach et al., 2013), (3) adoptive T-cell transfer (June, 2007), (4) autologous immune enhancement therapy (Rosenberg, 1984), (5) genetically engineered T-cells (Restifo et al., 2012), and (6) studies of inflammation and infectious disease in the context of cancer (Cibelli et al., 2013).

An alternative approach to using swine as cancer xenograft models is by a method called *in utero* cell transplantation (Fisher et al., 2013). This method relies on the ability of a fetus to become tolerized to foreign antigens by exposing an immunologically immature fetus to xenogenic cells (Fisher et al., 2013). This allows the recipient fetus to recognize human cells as "self," when the foreign cells are injected prior to population of the pig thymus by CD3+ lymphocytes (Sinkora et al., 2000). This approach has allowed stable and long-term engraftment of both allogenic and xenogenic cells into immunocompetent host animals (Flake et al., 1986; Zanjani et al., 1992a,b, 1994). Using this method, one could perform *in utero* injections of a xenograft cell line of interest, tolerizing the host pig, and allowing for engraftment of human cancer cells in the pig post-natally. The xenogenic cells could be established human cancer cell lines, or human cells engineered with specific mutations and/or transgenes to determine their contribution to tumor formation, tumor growth, or drug responsiveness.

APPLYING TRANSPOSON SYSTEMS IN PIGS TO STUDY CANCER DEVELOPMENT, PROGRESSION, IMMUNE RESPONSE, AND DRUG RESISTANCE

Transposable elements have been widely used in in forward genetic screens to identify genes involved in cancer, as well as

TABLE 3 | Cancer comorbidities (Ogle et al., 2000).

Comorbidity	Common cancers with comorbidity	Frequency (%)	Consequences
Cardiovascular Disease (CVD)	Urinary bladder	31.2	• Chemotherapy and radiation can worsen CVD
	Stomach	30.3	• Chemotherapy has cardiotoxic effects
	Lung	30.1	• Concerns with bleeding and thrombocytopenia in patients with stents or prosthetic valves may complicate treatment
	All sites	28.9	
Diabetes	Liver	12.7	• Diabetes significantly increases mortality in cancer patients
	Eye	10.1	• Steroids given with chemotherapy can elevate glucose levels
	Mesothelioma	8.5	
	All sites	6.1	
Hypertension	Colon	46.8	• Hypertension results in an overall higher risk of cancer death
	Eye	44.5	• Hypertension puts patients at risk during surgery and radiation therapy for hypertensive crisis
	Stomach	43.5	
	All sites	41.2	• Chemotherapy and some targeted therapies can increase hypertension
Respiratory Disease	Lung	37.1	• Increased odds of complications with cancer
	Mesothelioma	31.8	• Patients less likely to undergo surgery
	Esophagus	25.1	
	All sites	25.1	
Cerebrovascular disease	Stomach	10.0	• Development of cerebrovascular disease may be provoked by cancer treatment
	Lung	9.1	
	Esophagus	8.5	• Endothelium toxicity and abnormalities of coagulation factors with chemotherapy can induce stroke
	All sites	7.9	
Any single comorbidity	All cancers	68.7	
Any two comorbidities	All cancers	32.6	

TABLE 4 | Common cancer-associated chromosomal translocations (Nambiar et al., 2008).

Cancer type	Translocation		Human location	Pig location
Burkitt's lymphoma	*c-myc*	*IGH@*	$t_{(8;14)(q24;q32)}$	$t_{(4;1)}$
Follicular thyroid cancer	*PAX8*	*PPARg1*	$t_{(2;3)(q13;p25)}$	$t_{(2;1)}$
Acute myeloblastic leukemia	*ETO*	*AML1*	$t_{(8;21)(q22;q22)}$	$t_{(4;13)}$
Chronic myelogenous leukemia/acute lymphoblastic leukemia	*ABL1*	*BCR*	$t_{(9;22)(q34;q11)}$	$t_{(1;14)}$
Ewing's sarcoma	*FLI1*	*EWS*	$t_{(11;22)(q24;q11.2-12)}$	$t_{(9;14)}$

in reverse genetic studies to produce transgenic animals and determine the contribution a gene or set of genes makes in the development of cancer (Tschida et al., 2014). Transgenesis via transposon systems have produced transgenic mice, rats, fish, frogs, and more recently, pigs (Clark et al., 2007; Carlson et al., 2011; Garrels et al., 2011; Jakobsen et al., 2011). Using cytoplasmic or pronuclear injection, transposon systems can efficiently deliver genes of interest into the porcine genome, allowing for the development of transposon-mediated transgenic porcine cancer models (Carlson et al., 2011; Garrels et al., 2011).

Another application of transposon systems in swine is to utilize the ability of transposon mutagenesis systems to screen for genes involved in cancer. Historically, these studies have been done in mice in which one chromosome contains a concatemer of transposons, and upon expression of transposase, these transposons randomly integrate throughout the genome (Moriarity and Largaespada, 2015). By random chance, certain cells will have the right combination of oncogenes activated and/or tumor suppressor genes inactivated to cause a tumor to form (Moriarity and Largaespada, 2015). These studies depend on the development of many, many tumors, which are then sequenced to determine genes that were activated or inactivated by transposon mutagenesis (Moriarity and Largaespada, 2015). Bioinformatics analysis can predict which genes are potential drivers in the development of cancer, because they will undergo mutagenesis at a rate higher than would be expected by random chance (Moriarity and Largaespada, 2015).

The pig offers unique opportunities for applying transposon mutagenesis screens. For example, due to the large size of the pig, one could engineer a swine model harboring transposon concatemers and expressing a transposase, and look for tumors by imaging using MRI, CT, PET, or ultrasound analysis. By using sophisticated imaging techniques, coupled with the large size of the pig and availability of tissue samples, one could

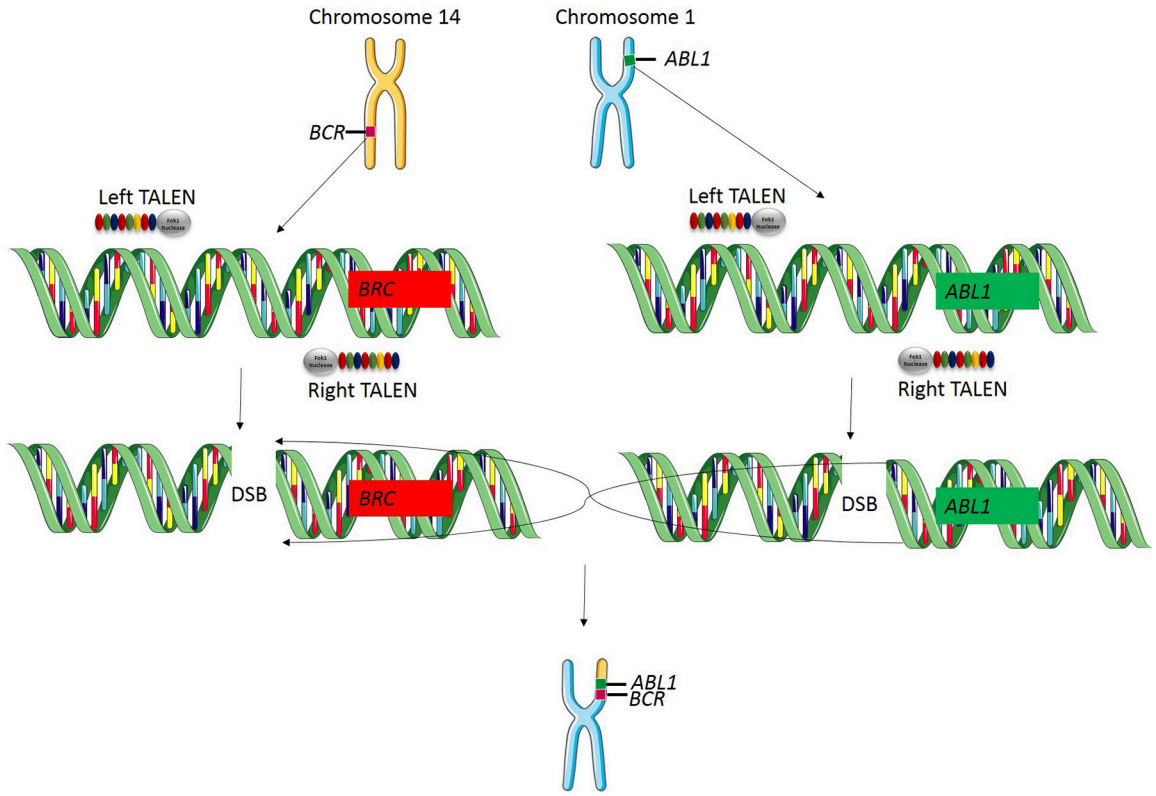

FIGURE 5 | TALEN-induced translocations. TALEN technology can be applied for inducing targeted translocations in the swine genome. In this example, a translocation event is known to cause the *BCR-ABL1* fusion gene resulting in chronic myelogenous leukemia. A TALEN pair is designed to cut at the known breakpoint in the *BCR* gene and a second TALEN is designed to cut at the known breakpoint in *ABL1* gene. When these two double stranded breaks occur simultaneously, chromosomal repair will induce a targeted translocation and result in the expression of the *BCR-ABL1* fusion gene at the endogenous loci in the swine genome.

biopsy tumors, and look at transposon insertion sites over time to determine tumor evolution genes involved in development, progression, and metastasis (DeNicola et al., 2015). Further, pigs that develop tumors could be treated with drugs, and their tumors monitored over time to look for tumor regression followed by development of resistance. Tumors that show initial regression to a certain drug could be sequenced to identify genes involved in drug sensitivity. Similarly, tumors that develop resistance to a drug or therapy could be sequenced to identify genes involved in the development of drug resistance. Lastly, new and innovative applications of transposon mutagenesis screens can be applied to swine models of cancer. For example, as described previously, the Sinclair miniature white pig develops a spontaneous form of malignant melanoma (Oxenhandler et al., 1979). Interestingly, these pigs show a nearly 100% spontaneous regression of cutaneous melanomas (Oxenhandler et al., 1982). This spontaneous development and regression of melanoma model is a perfect opportunity to utilize a transposon mutagenesis system to identify genes involved in the suppression of regression. Lastly, methods for transposon-mediated transgene delivery to somatic cells have been developed in the mouse and could be applied in the pig as well (Wiesner et al., 2009).

PIG MODELS OF CANCER: UNANSWERED QUESTIONS AND LOOKING FORWARD

Cancer remains the second leading cause of death in the U.S. There is a chronic need to understand the etiology and biology of this collection of diseases as well as identify new treatments. The anatomical, physiological, and genetic variations between mice and humans limit the prospects of meeting the needs of patients by modeling cancer in rodents. However, for any novel animal model to be useful in cancer research, it must be adopted and fully tested in many laboratories under many circumstances. Even though pigs may turn out to be better models to investigate cancer and potential therapeutics, the considerable expense associated with large animals over their extended lifetimes coupled with the perceived need to run experiments under various experimental and controlled conditions may impede the rate of their widespread adoption into mainstream science. That will be determined by the scientific community plus funding and private entities that support cancer research and therapeutic development. As a large animal with striking similarities in anatomical structure, physiological function, and genetic makeup to humans, we expect the pig will become an improved model animal to advance decades of cancer research studies conducted

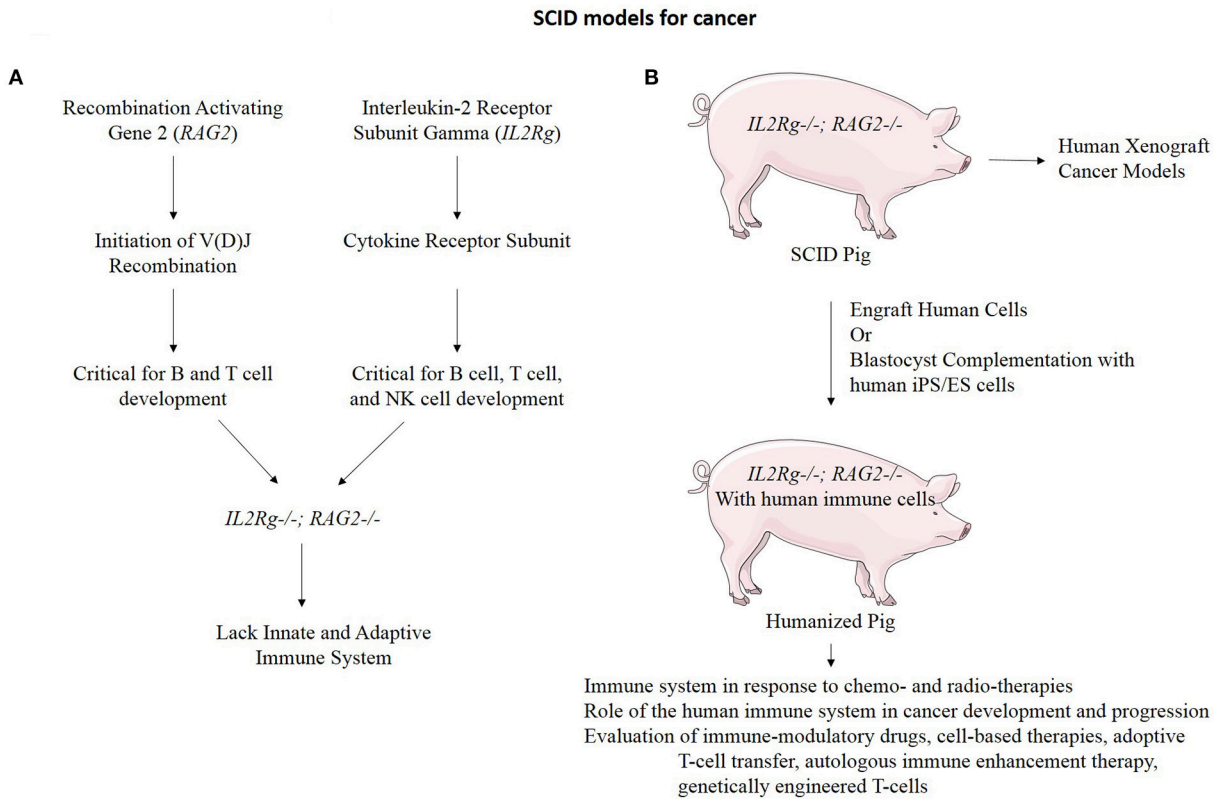

FIGURE 6 | Immunodeficient swine for cancer research. (A) *RAG2* and *IL2Rg* function in the development of B cells, T cells, and Natural Killer (NK) cells. When both of these genes are mutated, the resulting organism lacks both the innate and adaptive immune system function (Shultz et al., 2007). **(B)** An *IL2Rg/RAG2* knockout pig to model SCID has many practical applications for cancer research.

in rodents. Custom endonucleases, such as TALENs, coupled with cloning, enable the engineering of swine genocopies of human cancers mutations, providing a myriad of unique and exciting opportunities in cancer research that may ultimately better model cancer seen in human patients and lead to novel biological insights into the mechanism of cancer and more effective treatments for patients.

AUTHOR CONTRIBUTIONS

AW provided intellectual content and drafted the review article, figures and tables. DC, DL, PH, and SF contributed significant ideas and intellectual content including conception and design and provided critical review and editing.

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Emerging Technologies to Create Inducible and Genetically Defined Porcine Cancer Models

Lawrence B. Schook[1,2], Laurie Rund[2], Karine R. Begnini[3], Mariana H. Remião[3], Fabiana K. Seixas[3] and Tiago Collares[3]**

[1] University of Illinois Cancer Center, University of Illinois at Chicago, Chicago, IL, USA, [2] Department of Animal Sciences, University of Illinois at Urbana–Champaign, Champaign, IL, USA, [3] Postgraduate Program in Biotechnology, Biotechnology Unit, Technology Development Center, Federal University of Pelotas, Pelotas, Brazil

There is an emerging need for new animal models that address unmet translational cancer research requirements. Transgenic porcine models provide an exceptional opportunity due to their genetic, anatomic, and physiological similarities with humans. Due to recent advances in the sequencing of domestic animal genomes and the development of new organism cloning technologies, it is now very feasible to utilize pigs as a malleable species, with similar anatomic and physiological features with humans, in which to develop cancer models. In this review, we discuss genetic modification technologies successfully used to produce porcine biomedical models, in particular the Cre-*loxP* System as well as major advances and perspectives the CRISPR/Cas9 System. Recent advancements in porcine tumor modeling and genome editing will bring porcine models to the forefront of translational cancer research.

Keywords: oncopigs, cancer, biotechnology, transgenesis, genome editing

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****Correspondence:***
Lawrence B. Schook
schook@illinois.edu;
Tiago Collares
tiago.collares@pq.cnpq.br

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INTRODUCTION

Animal models have played a central role over the centuries in scientific investigations of human disease and treatment strategies. Genetic strategies for the development of cancer models using human mutations in targeted oncogenic pathways demonstrated that porcine fibroblasts could be transformed *in vitro* and could be tumorigenic with four to six gene alterations (Adam et al., 2007). The authors used retroviral vectors carrying pairs of human and murine oncogenic cDNAs (hTERT and $p53^{DD}$, cyclin D1 and $CDK4^{R24C}$, and c-Myc^{T58A}, and H-Ras^{G12V}) to transform porcine fibroblasts. These altered cells showed a transformed phenotype in culture and formed tumors following autologous transfer. These induced changes demonstrated that the pig/tumorigenic pathway recapitulated those observed in human much more closely than murine cells (Adam et al., 2007). Although this approach was limited because the animals needed to be immuno-suppressed for tumors to grow *in vivo*, this work was the first to demonstrate that genetically defined tumors could be induced in a large animal (Schook et al., 2015a).

Recent innovations in reproductive, cloning and transgene technologies have enhanced efficacy and efficiency or producing targeted porcine genome modifications. With the successful cloning of animals by somatic cell nuclear transfer (SCNT), it is now possible to produce genetically modified pigs from genetically engineered somatic donor cells using a wide variety of techniques from random genomic insertion of plasmid DNA (Hyun et al., 2003; Watanabe et al., 2005), to genomic integration of transduced retroviral or lentiviral vectors (Lai et al., 2002; Park et al., 2002), and to modern genome editing with molecular methods using endonucleases such as transposases,

recombinases, and programmable nucleases (Zhou et al., 2015). Genetic modification technologies successfully used to produce porcine biomedical models, in particular the Cre-*loxP* System as well as major advances and perspectives the CRISPR/Cas9 System will be presented in this mini review.

Cre-*loxP* SYSTEM

The ability to activate latent genes in defined tissues and at defined times is a key factor to enable the development of inducible temporally and spatially regulated cancer models. The activation of an oncogenic mutation(s) in a chosen tissue could mimic the spontaneous somatic events that initiate many human cancers and enable replication of diverse cancer types using the same mutant gene(s) (Flisikowska et al., 2013). Those conditional gene expressions are now well established in mice using site-specific recombinase (SSR) systems that allow the precise recombination between genomic sites, resulting in deletion or inversion of the intervening sequences (Frese and Tuveson, 2007; Oh-McGinnis et al., 2010). The use of SSR technology in genome manipulation has been demonstrated to effectively resolve complex transgene insertions to single copy, remove unwanted DNA, and precisely insert DNA into known genomic target sites (Wang et al., 2011). Site-specific recombination occurs at a specific sequence or recognition site and involves cleavage and reunion leading to integration, deletion or inversion of a DNA fragment without the gain or loss of nucleotides (Wang et al., 2011). Because of the efficiency of the SSR systems, it can be applied to conditional deletions of relatively short coding sequences or regulatory elements but also to more extensive chromosomal rearrangement strategies (Oh-McGinnis et al., 2010).

Cre-recombinase system is one of the best-studied and most commonly used SSR in mammalian cell cultures. Since its first use for mammalian genome editing in 1988 (Sauer and Henderson, 1988) many adaptations have expanded the utility of the Cre system from flies to mammalian cells beyond mouse to include porcine and humans cell lines (Lanza et al., 2012). Cre-recombinase is derived from the bacteriophage P1 and recognizes a distinct sequence-specific motif termed as recombination target sites (*loxP*) catalyzing efficient conservative DNA rearrangements (Wirth et al., 2007). The *loxP* site is a 34 bp palindromic sequence with an 8-bp asymmetric spacer region (Feng et al., 1999; Siegel et al., 2001; Araki et al., 2002; Sauer, 2002; Schnutgen et al., 2003; Garcia-Otin and Guillou, 2006) and acts upon the neighboring DNA sequences. The Cre-*loxP* system is a bidirectional tyrosine recombinase that enables the recombinase-mediated genetic cross-over between two identical *loxP* recognition sites promoting intermolecular or intramolecular recombination. Intermolecular recombination is a translocation between two DNA fragments with corresponding *loxP* sites, while the intramolecular recombination involves removal of genetic material between two *loxP* sites, with the last one been the preferred function of Cre-recombinase (Feng et al., 1999). Because of the identical nature of the recognition sites, the recombination reaction is fully reversible, although intramolecular recombination (excision) is highly favored over intermolecular reactions (integration) (Wang et al., 2011).

One of the most powerful and widely used applications of the Cre/*loxP* system is in conditional gene expression (Gu et al., 1994). This strategy allows for tissue and time-specific gene expression when recombination is triggered by Cre-recombinase, and is even more important in cancer models where oncogenic activation in a chosen tissue could mimic the spontaneous somatic events that initiate many human cancers (Schook et al., 2015a). Endogenous engineered mice are usually conditional alleles constructed by the insertion of a transcriptional and translational LoxStopLox 'stop' cassette between the promoter and first coding exon of the oncogenic allele. Providing the expression of an active Cre-recombinase, the stop cassette is excised and the mutant oncogene is subsequently expressed (de Alboran et al., 2001; Jackson et al., 2001). In pigs, this conditional gene expression strategy has been used to promote oncogenic expression in three cancer models (Leuchs et al., 2012; Li et al., 2015; Schook et al., 2015b). Leuchs et al. (2012) have generated gene-targeted pigs with a conditionally activated oncogenic mutant form of p53, which in latent form is a gene knockout. The construction used a porcine BAC vector with CAGGS promoter-mCherry cassette (in reverse orientation) as a fluorescent counter-selectable marker; a short arm of homology corresponding to a region of TP53 intron 1 from a point of exon 2 to a *PmlI* restriction enzyme site of exon 2; a floxed transcriptional termination cassette (LSL); and a region extending from the *PmlI* site in intron 1 to a point of exon 11 that includes a G to A substitution in exon 5 changing arginine to histidine in codon 167 (R167H) (Leuchs et al., 2012). In this same model, viable gene-targeted pigs carrying a latent $Kras^{G12D}$ mutant allele that could be activated by Cre-recombinase was constructed (Li et al., 2015). The KRAS-neo vector comprised: a short homology arm in *KRAS* intron 1; a transcriptional stop cassette comprising: a *loxP* site; adenoviral splice acceptor; promoterless neomycin phosphotransferase resistance gene (*neo*); three poly-adenylation signals derived from SV40, bovine growth hormone and cytomegalovirus; and a second *loxP* site inserted into a *ClaI* site in KRAS intron 1; and a region of porcine KRAS extending from the *ClaI* site in intron 1 to a *SacI* site in intron 2, which also included an engineered G to A point mutation within exon 2 that results in a glycine to aspartic acid substitution at codon 12 (G12D) (Li et al., 2015). Both *KRAS* and *TP53* transgenic pigs cells were transduced with 5 μM of Cre protein produced *in vitro* with the vector pTriEx-HTNC (Addgene plasmid 13763; Leuchs et al., 2012; Li et al., 2015).

Transgenic oncopigs (**Figure 1**) have also been engineered to contain oncogenic $Kras^{G12D}$ and dominant-negative $p53^{R167H}$ downstream of a LoxP-polyA(STOP)-LoxP sequence (LSL) and CAG promoter (Schook et al., 2015b). Site-directed mutagenesis was then used to introduce the oncogenic G12D mutation into the porcine *KRAS* cDNA and the R167H mutation was chosen for *TP53* as its human equivalent (R175H) is commonly found in human cancers as well as the cancer predisposition Li-Fraumeni Syndrome. These two cDNAs were then introduced into a Cre-inducible vector, followed by the aforementioned LSL sequence,

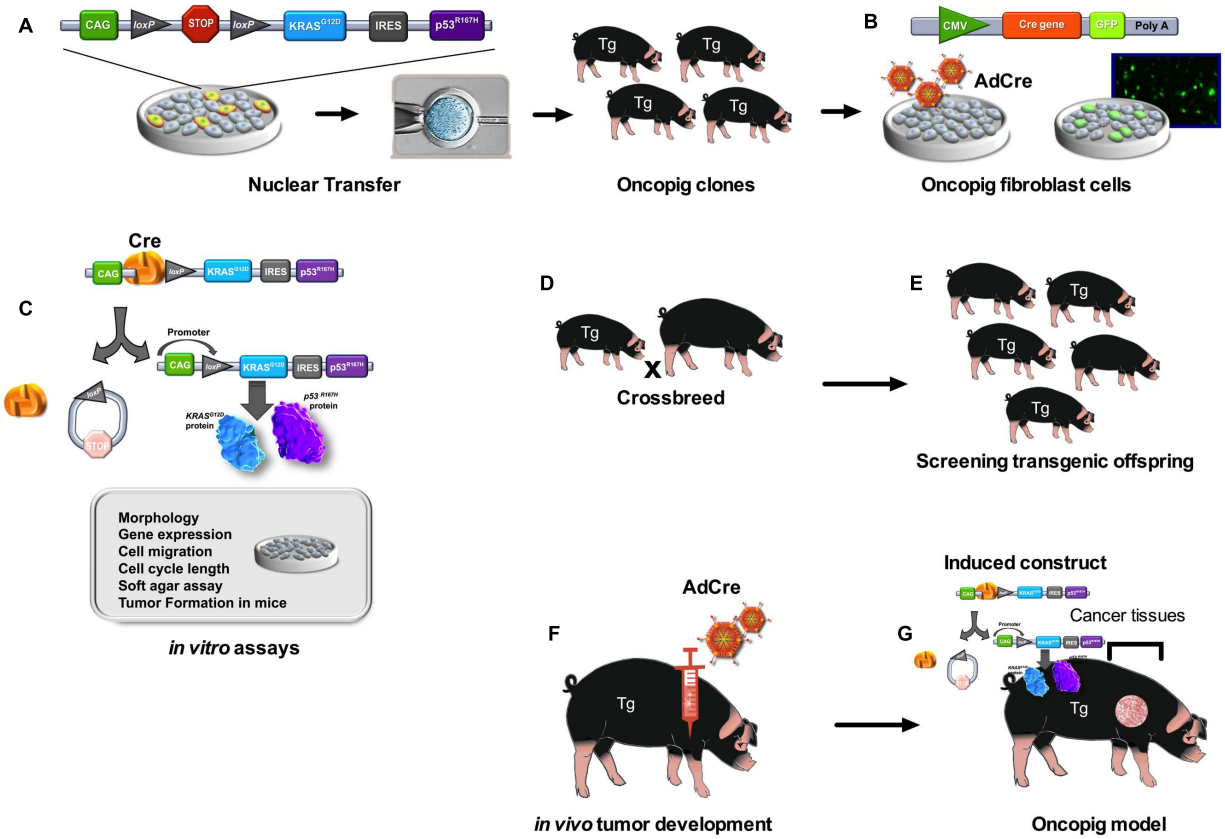

FIGURE 1 | Development of the Oncopig using the Cre-*loxP* inducible system. Pigs were cloned from fibroblast cells with this transgene construct **(A)**. Fibroblast cell lines were established from oncopig offspring. *In vitro* work utilized fibroblast cell lines isolated from transgenic oncopigs which were then infected with adenovirus encoding Cre recombinase (AdCre) and the marker green fluorescent protein (GFP) **(B)**. AdCre induced removal of the STOP codon allowing for expression of both transgenes. This expression altered the phenotype of the cells as was demonstrated in numerous *in vitro* assays **(C)**. Oncopig clones (founders) were crossbreed with York females **(D)**. The transgenic offspring (Tg) **(E)** were injected with AdCre at various sites including intramuscular, subcutaneous and intratesticular sites **(F)**. Tumors were induced at each site of injection in transgenic oncopigs **(G)**.

KRAS^{G12D}, an IRES sequence to allow for bicistronic expression, *TP53*^{R167H} and a poly A sequence. This design allows for co-expression of both *KRAS*^{G12D} and *TP53*^{R167H} in ostensibly any cells of the pig by transient expression of AdCre (Ad5CMVCre-eGFP, AdGFP, Gene Transfer Vector Core; Schook et al., 2015b). These pig models have resulted in tumorigenic profiles *in vitro* (Leuchs et al., 2012; Li et al., 2015) and *in vivo* (Schook et al., 2015b) and the results obtained with these three cancer pig models are shown in **Table 1**.

CRISPR/Cas9 SYSTEM

The discovering of molecules that recognize specific sequences of DNA was one of the most important advances in gene editing technology allowing site specific genetic modifications to be made. These DNA binding proteins include the zinc fingers and transcriptional activator-like effector (TALE; Wood et al., 2011; Gaj et al., 2013). When they are fused to nucleases, they generate a double-strand break (DSB) in the DNA at the desired genomic loci, triggering the endogenous DNA repair machinery (Gaj et al., 2013; Zhu et al., 2014); if fused to transcription factors or inhibitor molecules, they can bind to promoter regions of target genes, modulating gene expression (Gilbert et al., 2014; Kearns et al., 2014). However, there is a disadvantage of utilizing these proteins that interact with DNA: production of these proteins involves a complicated and more expensive assembly process (Pan et al., 2014).

An easier, cheaper, and yet highly efficient tool for directed genome edition appeared to be more worthwhile and profitable than proteins: the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR associated proteins) system. This system is simpler then zinc fingers and TALEs because the CRISPR/Cas system uses the RNA-DNA interaction for genome loci recognition, which is more specific than protein-DNA (Gasiunas and Siksnys, 2013; Pan et al., 2014).

CRISPR/Cas system has been recently discovered as an adaptive immune system of some bacteria and archaea and protects them against invading viruses and plasmids (Barrangou et al., 2007). The transcription of the repeat-spacer elements from CRISPR locus generates a precursor non-coding CRISPR RNA (pre-crRNA) that later will be cleaved to have short CRISPR

TABLE 1 | Porcine models for cancer developed by different strategies.

Author	Gene	Technique	Inducible strategy	Survival	Location	Phenotype	Tumor progression
Yamakawa et al., 1999	*v-Ha-ras*	Pronuclear microinjection	No	–	–	No	No
McCalla-Martin et al., 2010	Gli2	DNA vector + SCNT	No	Euthanized due to bacterial infection	–	Not informed	Not informed
Luo et al., 2011	BRCA-1 (KO)	Adenovirus + SNCT and HMC	No	18 days	Breast	Not informed	Not informed
Flisikowska et al., 2013	*APC*	DNA vector + SCNT	No	At least 1 year	Intestinal polyps	Yes	Yes
Leuchs et al., 2012	*TP53*	DNA vector + SNCT	Cre-recombination	Not informed	–	Yes	Not yet
Sieren et al., 2014	*TP53*	rAAV + SCNT	No	Not informed	Several locations	Yes	Yes
Li et al., 2015	*Kras*	DNA vector + SNCT	Cre-recombination	Yes	–	Not informed	Not informed
Schook et al., 2015b	*TP53* + *Kras*	DNA vector + SNCT	Cre-recombination	Yes	Several locations	Yes	Yes

SCNT, Somatic Cell Nuclear Transfer; HMC, Handmade Cloning.

RNAs (crRNA) (Garneau et al., 2010; Jinek et al., 2012). The crRNA will be homologous to the DNA or RNA from foreign sequences, and when the invasion occurs, the crRNA will be directed just by Watson-Crick base pairing (Jinek et al., 2012; Wade, 2015). There are different types of CRISPR systems in different organisms (I–III), and the one that have been most developed as a new tool for genome editing, the CRISPR/Cas9 system, is the type II CRISPR originating from *Streptococcus pyogenes* SF370 (Jinek et al., 2012; Qi et al., 2013). The type II is different from types I and III, that crRNA hybridize with another RNA molecule, the *trans*-activating crRNA (tracrRNAs), to direct Cas9 protein to specific DNA sequences (Jinek et al., 2012; Mali et al., 2013; Doudna and Charpentier, 2014). For genome editing, the researchers created a single chimeric guide RNA's (sgRNA), which is a fusion of a precursor crRNA and a transactivating crRNA (tracrRNA) (Jinek et al., 2012; Pan et al., 2014). Beyond the polymerization, the genome sequence from invader has a complementary genome sequence containing a tri-nucleotide protospacer adjacent motif (PAM) that will be required for initial binding of Cas9 protein (Guilinger et al., 2014). Cas9 protein has an endonuclease activity that cleaves on both strands a few nucleotides away from the PAM generating DSB, preventing the invader genome translation (Jinek et al., 2012). This has been used to generate knockin and knockout transgenic animals, as the DSB activates the endogenous DNA repair machinery by non-homologous joining (Ma et al., 2014; Flemr and Buhler, 2015; Yang, 2015; Zhu et al., 2015).

However, the study of CRISPR/Cas9 identified a new application for Cas9: without its nuclease activity, Cas9 protein, attached to a molecule that modulates gene expression, could bind to the promoter region of some gene of interest, changing the genic expression pattern (Qi et al., 2013). The catalytically dead Cas9 (dCas9), lacking endonuclease activity, contains two mutations in the nuclease domains (D10A and H840A) (Choudhary et al., 2015). Since dCas9 was reported, new studies have been described using it for genome regulation creating different segments to use this tool: CRISPRi, for gene interference, and CRISPRa, for activation of gene translation. When these strategies uses an effector domain attached to dCas9, it can be called CRISPRe. For gene interference (CRISPRi), dCas9 recognizes sgRNA attached to the promoter region of target gene, impairing transcription (Qi et al., 2013). However, this strategy is not efficient for gene repression in eukaryotic cells, so dCas9 can be fused to a transcription repression domain to enhance gene knockdown (Gilbert et al., 2013). The most described strategy for CRISPRi is dCas9 fused to a KRAB (Krüppel- associated box domain of Kox1), a repressive chromatin modifier domain, which have been demonstrating increased gene expression repression in relation to dCas9 alone (Gilbert et al., 2013, 2014). Some authors mention that CRISPRi can be an alternative strategy to RNAi for repressing gene expression in mammalian cells (Gilbert et al., 2013).

Another approach for using dCas9 is fused to transcriptional activator domains, which can be called CRISPRa (Gilbert et al., 2014) or CRISPR-on system (Cheng et al., 2013a), to induce expression of target genes. To achieve that, dCas9 fused to the transcriptional activator is guided by the sgRNA complementary

to the promoter region of the gene. The well-characterized tetramer of herpes simplex virus protein, VP16 (VP64) is one of the most reported transcription activator attached to dCas9 and it has been shown to induce gene expression in eukaryotic cells, including human cells (Gilbert et al., 2013, 2014; Maeder et al., 2013; Perez-Pinera et al., 2013; Kearns et al., 2014). Some studies also report that target genes can be simultaneity artificially activated by just adding complementary sgRNAs of promoters of each one of the interest genes (Cheng et al., 2013b; Maeder et al., 2013). This strategy has been tested in human and mouse transformed cells, as well as in ES cells, in one-cell embryo (Cheng et al., 2013b).

The use of CRISPR/Cas9 strategy to build an animal for model of cancer disease is a recently developed approach. For lung adenocarcinoma, Maddalo et al. (2014) describe a methodology of *in vivo* chromosomal rearrangement using CRISPR/Cas9 delivered by virus infection. Rearranging chromosomes by fusing EML4 and ALK genes generated a new murine model for lung adenocarcinoma. An *in vivo* somatic cancer mutation in adult animals was described by Xue et al. (2014), which they developed a different strategy using a hydrodynamic delivery of plasmids with CRISPR components that occasioned to efficient hepatocyte transfection to edit oncogenes and suppressor-tumor genes.

Most frequently, rodents are used to test new strategies for genome editing with CRISPR/Cas9 system to develop cancer and other biomedical models of human disease. However, a new strategy for enrichment of cells with chromosomal deletions made by CRISPR/Cas9 to generate cancer genotype was developed in porcine embryonic fibroblasts (He et al., 2015). For employment in xenotransplants, CRISPR/Cas9 technology has already been applied to inactivate porcine endogenous retroviruses in porcine kidney epithelial cell line (Yang et al., 2015).

Not only modifications in genome sequence can induce cancer phenotype, epigenetic modifications can also be a target to develop animal models for cancer. Falahi et al. (2015) supposes that dCas9 can contribute for epigenome engineering to develop animals for cancer study. Effector domains attached to dCas9 could generate epigenetic mutations known to evolve to different cancer types. Also using dCas9, attached or not to KRAB domain, initial studies in human cells HEK293 and HEK293T, showed repression of TP53 (Lawhorn et al., 2014).

The recent advances generated by CRISPR/Cas9 system in genome editing are extremely important for development of new strategies to generate animal models of cancer. The simplicity, low cost, and low off-target effects put this strategy as one alternative not only for ZFN and TALEN, but also for RNAi technology and Cre-*loxP* systems.

PERSPECTIVES

To unite Cre-*loxP* and CRISPR/Cas9 system has been a promising approach to develop animal models for cancer. Cre-*loxP* affords to conditional gene expression, while CRISPR/Cas9 can be used for target gene insertion and also for gene expression regulation. Some promising works already showed how these technologies can be used together. Using Cre-*loxP* system for induced expression, Sánchez-Rivera et al. (2014) used a system with CRISPR/Cas9 and Cre recombinase to evaluate new candidates for cancer genome, developing adenocarcinoma by editing tumor-suppressor genes sequences in mice models. A different association of both techniques is a study that a mouse model had Cas9 expressed by Cre dependence, and when expressed in conjunction with sgRNAs for Kras, p53, and LKB1 genes, it generated a change of function of those proteins, taking to macroscopic tumors of adenocarcinoma pathology (Platt et al., 2014). Probably, the next step is to standardize those techniques and employ them for a next-generation models for human cancer (Sanchez-Rivera and Jacks, 2015), and pigs fits for those purpose.

AUTHOR CONTRIBUTIONS

TC: acquisition of data, data analysis/interpretation, drafting of the manuscript and figure; FS: acquisition of data, data analysis/interpretation, drafting of the manuscript; KB: acquisition of data, data analysis/interpretation, drafting of the manuscript and table; MR: acquisition of data, data analysis/interpretation, drafting of the manuscript; LR: critical revision of the manuscript, drafting of the manuscript and figure; LS: critical revision of the manuscript. All authors approved the manuscript.

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A porcine model system of BRCA1 driven breast cancer

*Howard Donninger[1], Katharine Hobbing[2], M. L. Schmidt[3], Eric Walters[4], Laurie Rund[5], Larry Schook[5] and Geoffrey J. Clark[2]**

[1] Department of Medicine, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA, [2] Department of Pharmacology and Toxicology, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA, [3] Department of Biochemistry, University of Louisville, Louisville, KY, USA, [4] Division of Animal Sciences, National Swine Resource and Research Center, University of Missouri, Columbia, MO, USA, [5] Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA

BRCA1 is a breast and ovarian tumor suppressor. Hereditary mutations in BRCA1 result in a predisposition to breast cancer, and BRCA1 expression is down-regulated in ∼30% of sporadic cases. The function of BRCA1 remains poorly understood, but it appears to play an important role in DNA repair and the maintenance of genetic stability. Mouse models of BRCA1 deficiency have been developed in an attempt to understand the role of the gene *in vivo*. However, the subtle nature of BRCA1 function and the well-known discrepancies between human and murine breast cancer biology and genetics may limit the utility of mouse systems in defining the function of BRCA1 in cancer and validating the development of novel therapeutics for breast cancer. In contrast to mice, pig biological systems, and cancer genetics appear to more closely resemble their human counterparts. To determine if BRCA1 inactivation in pig cells promotes their transformation and may serve as a model for the human disease, we developed an immortalized porcine breast cell line and stably inactivated BRCA1 using miRNA. The cell line developed characteristics of breast cancer stem cells and exhibited a transformed phenotype. These results validate the concept of using pigs as a model to study BRCA1 defects in breast cancer and establish the first porcine breast tumor cell line.

Keywords: breast cancer, BRCA1, miRNA, SV40 LT, transformation

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****Correspondence:***
Geoffrey J. Clark, Department of Pharmacology and Toxicology, James Graham Brown Cancer Center, University of Louisville, Room 417, CTR Building, 505 S. Hancock St., Louisville, KY 40202, USA
gjclar01@louisville.edu

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Introduction

Breast cancer is a leading cause of death in women and is one of the most common cancers in the world today. Up to 40,000 women are expected to die of breast cancer annually in the US alone (Siegel et al., 2011). The underlying causes of breast cancer development remain very much under investigation, but we now know that the BRCA1 tumor suppressor gene plays an important role in many breast cancers. Women who carry a BRCA1 germ line mutation have a cumulative lifetime risk of 50–85% of developing breast cancer (King et al., 2003). Although somatic BRCA1 mutations are rare in sporadic breast cancer, BRCA1 expression is down-regulated in ∼30% of sporadic cases by allele loss or epigenetic mechanisms (Welcsh and King, 2001; Yang et al., 2001).

The function of BRCA1 remains poorly understood. It has a ubiquitin ligase activity and can control the stability/activity of proteins such as Claspin (Sato et al., 2012) and estrogen receptor alpha (Savage and Harkin, 2015). It is also a key player in modulating DNA repair (Zhang and Powell), replication fork stability (Pathania et al., 2011), senescence (Tu et al., 2013), oxidative stress (Marks, 2013), genomic stability (Savage and Harkin, 2015), and checkpoint induced cell

cycle arrest (Huen et al., 2010). The complex role of BRCA1 in cellular homeostasis has made elucidating its key functions in cancer difficult.

Mouse models of BRCA1 deficiency have been developed in an attempt to understand the role of the gene *in vivo* (Ma et al., 2010). Although BRCA1 knockout provokes embryonic lethality in mice, conditional knockout of BRCA1 in breast tissue leads to tumor development after a long latency. The latency period can be strongly reduced by introducing defects in the p53 tumor suppressor to the animal system. These animal models have allowed the validation of therapies designed against BRCA1 defective tumors. However, even therapeutic approaches that were effective resulted in the emergence of resistant tumors (Ma et al., 2010). Further studies to examine approaches to overcome the resistance are limited by the short lifespan of the mice. Moreover, the subtle nature of BRCA1 function and the well-known discrepancies between human and murine breast biology (Dine and Deng, 2013) and cancer genetics (Kendall et al., 2005) may limit the utility of mouse systems in defining the function of BRCA1 in human cancer.

In contrast to mice, pigs exhibit very similar cancer genetics to humans (Adam et al., 2007). Moreover, their physiology and biochemistry is similar (Swindle et al., 2012) and their lifespan extends for decades. Consequently, a porcine model for breast cancer could prove a powerful tool for validating breast cancer therapies, preventative strategies and the clinical response to the emergence of drug resistance.

In order to validate the use of porcine systems in breast cancer research, we generated an immortalized porcine breast cell line using the SV40 LT oncoprotein (Chen and Hahn, 2003). We then used BRCA1 miRNA to generate a stable matched pair of cell lines that are positive or negative for BRCA1 expression. Characterization of the cells showed that BRCA1 knockdown induced enhanced growth and induced a transformed phenotype on the cells. Moreover, the transformed cells expressed markers characteristic of cancer stem cells. These results establish the first porcine breast cancer cell line and validate the concept of using porcine systems as a model to study BRCA1 defects in breast cancer.

Materials and Methods

Porcine Cell Lines and Transfections

Primary porcine breast epithelia cells were isolated as described in Prather et al. (1999) using a protocol approved by the IACUC of the University of Missouri-Columbia, Columbia, Missouri. They were transfected with pbabe puro SV40LT (Addgene #13970) using Lipofectamine 2000 (Invitrogen, Carlsbad CA) according to the manufacturer's instructions. Cells were selected in puromycin (Sigma, St Louis, MO) at 1 μg/ml. miRNA sequences corresponding to two different regions of porcine BRCA1 were designed using the Block-iT™ RNAi Designer (Invitrogen). Two single-stranded DNA oligonucleotides were designed for each sequence, one encoding the target pre-miRNA (top strand) and the other, its compliment (bottom). Each oligonucleotide also contained five nucleotides (TGCTG) derived from the endogenous miR-155 at the 5′ end and 19 nucleotides derived from miR-155 to form a terminal loop. The sequences of the two different oligo sets are as follows: #1 Top: 5′-TGCTGATTGTTTGCAAACTGCAATCCGTTTTGGCCAC TGACTGACGGATTGCATTGCAAACAAT-3′, #1 Bottom: 5′-C CTGATTGTTTGCAATGCAATCCGTCAGTCAGTGGCCAA AACGGATTGCAGTTTGCAAACAATC-3′; #2 Top: TGCTG TATTAAAGCACCATGAGGGTCGTTTTGGCCACTGACTG ACGACCCTCAGTGCTTTAATA-3′; #2 Bottom: 5′-CCTGTAT TAAAGCACTGAGGGTCGTCAGTCAGTGGCCAAAACGA CCCTCATGGTGCTTTAATAC-3′.

The corresponding single-stranded oligos were annealed to generate a double-stranded oligo which was then cloned into the pcDNA™ 6.2-GW/EmGFP-miR vector (Invitrogen). Generation of the double-stranded oligos and cloning into the expression vector were performed using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen) as described by the manufacturer. Stable transfectants were generated by transfecting the transformed pig mammary epithelial cells with 2 μg of the two different miRNA expression vectors, as well as a negative control consisting of a miRNA to LacZ, using Lipofectamine 2000 according to the manufacturer's instructions and selecting with Blasticidin (4 μg/ml).

qRT-PCR

qRT-PCR was performed on total RNA isolated from the cells with Trizol using an iCycler Real-Time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) with the Quantitect SYBR Green RT-PCR Kit (Qiagen, Inc., Valencia, CA) as per the manufacturer's instructions. The fold change for each gene was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with GAPDH as the reference gene. The primers used were BRCA1 For: 5′-GTCCAAAGCGAGCAAGAGAA -3′, BRCA1 Rev: 5′- ACAGAAGCCCCACAGAGGA -3′; GAPDH For: 5′- CGATGCTGGTGCTGAGTATG- 3′, GAPDH Rev: 5′-GAAGGGGCAGAGATGATGAC- 3′.

Western Blots

Total cell lysates were prepared by lysing the cells in modified RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% NP-40) supplemented with 100 μg/ml leupeptin, 100 μg/ml aprotinin and 1 mM sodium orthovanadate. BRCA1 and ALDH1 antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA., Actin antibodies were from Sigma (St. Louis MO) and EpCAM antibodies were from AbCam. HRP conjugated Trueblot secondary antibodies were purchased from eBioscience (eBioscience Inc. San Diego, CA) and western blots were developed using a Pierce ECL detection system (Thermo Scientific, Rockford IL).

Growth Curves

2×10^4 cells/well were plated in six-well plates in normal growth medium and incubated for 6 days. Cell number was determined each day by counting the number of viable cells. Experiments were performed twice in duplicate.

Matrigel

Fifty micro liters of Matrigel (BD Biosciences, San Jose, CA) was plated in a 96 well plate and allowed to set. Cells were trypsinized,

washed in growth medium and plated at 5000 cells per well in 100 μl of growth medium. Hundred micro liters of medium +4% Matrigel was added and the medium changed every 4 days.

Soft Agar

Six well plates were prepared with 2 ml bottom agar (16 ml 1.8% molten Difco Bacto agar cooled to 42°C and mixed with 1.6 ml serum, 1.6 ml 10X PBS and mixed with 30.3 ml DMEM) and allowed to set. Cells were trypsinized, washed, and 3×10^4 cells suspended in 1.5 ml growth medium. Three milli liters of liquid bottom agar was added to the cell dilution and 1.5 ml aliquoted into each well to set.

Anoikis

Twelve well plates were treated with polyHEMA (Sigma) and allowed to dry overnight. 1×10^6 cells were plated in each well and the cell viability measured after 48 h by trypan blue exclusion.

Results

Generation of an Immortalized Porcine Epithelial Cell Line

Primary pig breast epithelial cells were isolated as described previously (Prather et al., 1999) and transfected with an SV40 LT expression vector. Transfected cells were isolated by selection in puromycin and surviving colonies pooled. As the cells were passaged, the SV40 LT transfected cells lost the senescent morphology apparent in the parental cells (**Figure 1**). They were then serially passaged to determine if they had been immortalized. Transfected cells have been passaged more than 26 times without apparent loss of viability. In contrast, parental cultures lose proliferative capacity by passage 8.

Identification of an Effective Porcine BRCA1 miRNA

The Block-iT™ RNAi Designer tool from Invitrogen was used to identify potentially effective miRNA sequences against porcine BRCA1. Two were generated and cloned into the vector pcDNA GW 6.2 EmGFPmiRNA. The vectors were then transiently transfected into the immortalized breast epithelial cells and assayed for the degree of knockdown by RT-PCR. Only one of the miRNAs proved effective (**Figure 2A**). This miRNA and the empty vector were stably transfected into the immortalized pig breast cells to generate a matched pair +/− for BRCA1. Western analysis confirmed that the miRNA transfected cells had almost completely lost BRCA1 protein expression (**Figure 2B**).

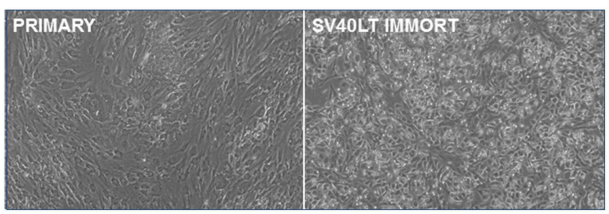

FIGURE 1 | Immortalization of pig mammary epithelial cells. Primary pig breast epithelial cells were stably transfected with an SV40 LT expression construct and selected in puromycin. Surviving cells were serially passaged to confirm immortalization.

Suppression of BRCA1 Enhances Porcine Epithelial Cell Growth

As the cells were passaged, the BRCA1 suppressed cells progressively adopted a noticeably different morphology than the vector control cell line (**Figure 3A**). To characterize the effect of the BRCA1 suppression on the cell cycle, we measured the relative growth of the matched pair of cell lines transfected with vector or miBRCA1. Cells were plated and counted every day for 1 week. The BRCA1 suppressed cells exhibited an enhanced growth rate (**Figure 3B**).

Suppression of BRCA1 Alters Differentiation

Non-transformed human breast epithelial cell lines can be induced to differentiate into acini with hollow lumens when plated in 3D in matrigel. This differentiation is thought to mimic the process that occurs during the development of breast ducts. The process is disrupted by suppression of BRCA1 (Furuta et al., 2005). To examine the loss of BRCA1 in porcine cells on this process, we plated the BRCA1+/− matched cell lines in matrigel for 10 days. After 10 days, the immortalized cells transfected with vector alone formed acini, reminiscent of human immortalized breast cells. The BRCA1 knockdown cells mostly grew as disordered masses (**Figure 4**).

Suppression of BRCA1 Promotes Transformation

The BRCA1 knockdown appeared to have induced enhanced growth and reduced differentiation (**Figures 3**, **4**). In order

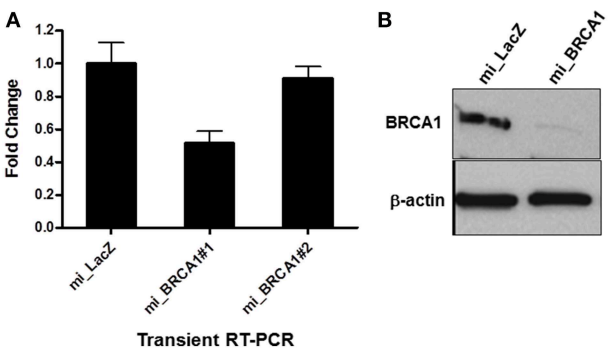

FIGURE 2 | miRNA-mediated BRCA1 knockdown in the immortalized pig mammary epithelial cells. (A) The immortalized pig breast epithelial cells were transiently transfected with expression constructs for two BRCA1 miRNAs and a LacZ control. Forty-eight hours later, BRCA1 mRNA levels were determined by qRT-PCR analysis. **(B)** The immortalized pig mammary epithelial cells were transfected with BRCA1 miRNA#1 or the miLacZ control and selected with blasticidin to obtain cells that were stably knocked down for BRCA1. Western blot analysis confirmed efficient knockdown. β-actin served as control for equal protein loading. Error bars show standard error, $p < 0.05$ for miRNA#1, mRNA #2 was not significant.

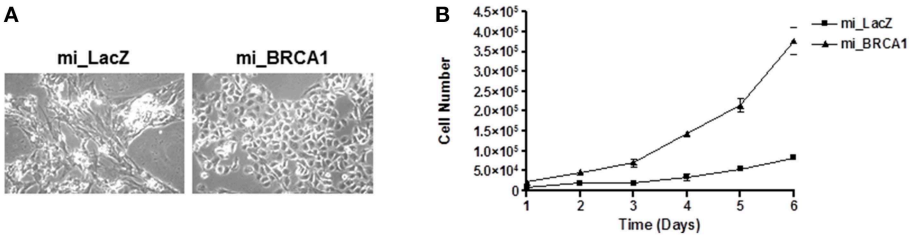

FIGURE 3 | Loss of BRCA1 enhances pig mammary epithelial cell growth. (A) Serially passaging the pig mammary epithelial cells stably knocked down for BRCA1 resulted in an altered morphology compared to those cells stably expressing the LacZ miRNA. **(B)** 2×10^4 cells/well were plated in 6-well plates and cell growth was determined by counting the number of cells at the indicated times. Error bars show standard error, $p < 0.05$.

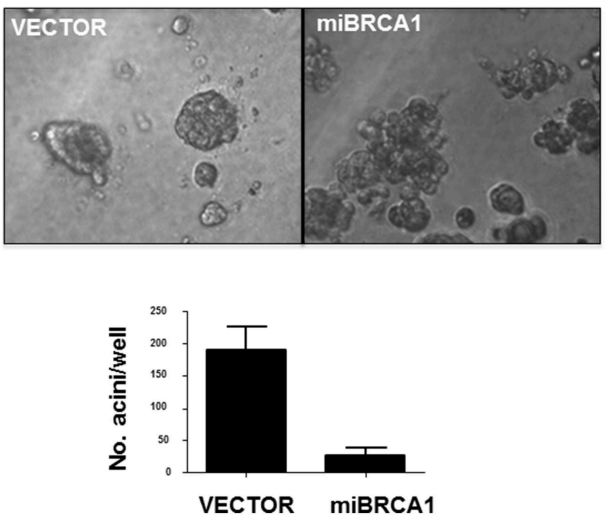

FIGURE 4 | Loss of BRCA1 inhibits acini formation. The −/+ BRCA1 pig mammary epithelial cells were plated in matrigel and allowed to grow for 10 days. Control cells formed acinus-like structures after 3D growth whereas the cells stably expressing BRCA1 miRNA grew as disordered masses. Error bars show standard error, $p < 0.05$.

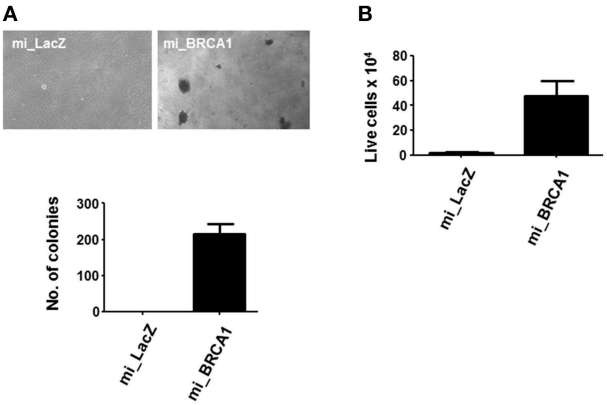

FIGURE 5 | Loss of BRCA1 enhances the transformed phenotype of pig mammary epithelial cells. (A) The pig breast epithelial cells stably expressing BRCA1 miRNA were plated in soft agar and scored for growth 14 days later. Representative photomicrographs are shown in the top panel and data from three independent experiments quantitated in the bar graph in the lower panel. **(B)** 1×10^6 cells/well were plated in polyHEMA-coated 12-well plates and cell viability assessed 48 h later by trypan blue staining. Error bars show standard error, $p < 0.05$.

to determine if it was sufficient to induce the tumorigenic phenotype, we plated the cells in soft agar and counted colony formation after 14 days. Anchorage-independent growth is one of the hallmarks of cell transformation and is considered the most accurate and stringent *in vitro* assay for detecting malignant transformation of cells (Colburn et al., 1978). **Figure 5A** shows that the BRCA1 positive cells failed to form colonies in agar. In contrast, the BRCA1 knockdown cells formed numerous, large colonies, indicative of highly transformed cells.

Suspension of normal cells results in the induction of apoptosis, a process called anoikis. Transformed cells typically resist anoikis, and this may contribute to their ability to proliferate when suspended in soft agar (Guadamillas et al., 2011). Examination of the ability of the cells to survive suspension showed that the BRCA1 knockdown cells were resistant (**Figure 5B**).

BRCA1 Knockdown Promotes a CSC Phenotype

In primary breast cells, knockdown of BRCA1 blocks the differentiation of stem/progenitor cells and enhances their proliferation (Furuta et al., 2005; Ma et al., 2010). Moreover, the ability to grow in soft agar is typically associated with the cancer stem cell (CSC) population of a transformed culture (Colburn et al., 1978). To determine if the knockdown of BRCA1 had promoted the development of CSC phenotype, we performed Western analysis for the expression of the CSC markers EpCAM (Dawood et al., 2014) as well as ALDH1 (Moreb, 2008). We found that in the BRCA1 knockdown cells, the EpCAM CSC marker was massively upregulated, and ALDH1 was upregulated three-fold (**Figure 6**). Actin served as a loading control. In these experiments, we had included miRNA against a second tumor suppressor, RASSF1A (Donninger et al., 2007), as an additional negative control. Whereas, the RASSF1A miRNA had no obvious effect on EpCAM, it did upregulate ALDH1, although less than the miBRCA1. Thus, RASSF1A may also be involved, to some extent, in CSC regulation.

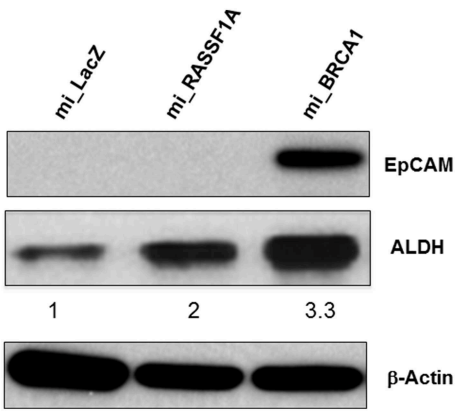

FIGURE 6 | BRCA1 knockdown in pig mammary epithelial cells alters CSC marker expression. Equal amounts of protein lysates from control and BRCA1 knockdown cells were fractionated on SDS polyacrylamide gels and western blotted with an anti-EpCAM antibody and an ALDH1 antibody. β-actin was used a protein loading control. Mi_lacZ and a non BRCA1 miRNA transfected cell line (mi_RASSF1A) served as negative control cell lines.

Discussion

Women who carry a BRCA1 germ line mutation have a cumulative lifetime risk of 50–85% of developing breast cancer (King et al., 2003). Although somatic BRCA1 mutations are rare in sporadic breast cancer, BRCA1 expression is down regulated in ∼30% of sporadic cases (Yang et al., 2001). Its mode of action appears complex, subtle and remains only partially understood. It has been shown to modulate DNA repair, DNA damage checkpoints, stability of Claspin and Estrogen receptor alpha, and to modulate cell adhesion and motility (Wang, 2012; Christou and Kyriacou, 2013). Its loss of function in human cells is thought to promote genetic instability, hence leading to the development of cancer. It has been shown to synergize with the p53 tumor suppressor in mouse models and human cell tissue culture experiments (Brodie and Deng, 2001; Hartman and Ford, 2003).

Although mouse model systems have proven to be powerful tools in the investigation of the nature of cancer *in vivo*, they suffer from a major drawback. Murine cancer genetics is much simpler than that of humans. Murine cells are much easier to transform than human cell systems. Whereas, human cells require at least five genetic lesions to convert from a normal cell to a tumor cell, mouse cells can be induced to transform by just two oncogenic lesions (Rangarajan et al., 2004; Kendall et al., 2005). Thus, mouse models may prove inaccurate when trying to model human cancer. In contrast, porcine cancer genetics is very similar to human cells. Pig cells require five or more oncogenic mutations to undergo transformation, much like humans (Adam et al., 2007). Thus, a pig cancer model is more likely to accurately reflect the human condition.

Nothing is known about the role of BRCA1 in porcine cells and whether its ablation phenocopies the human state. Here, we have attempted to address the issue by generating the first immortalized porcine breast cell line by introducing an SV40 LT expression plasmid into primary breast cells derived from a pig. SV40 LT can immortalize human cells impairing the function of both the p53 and the Rb tumor suppressors (Ahuja et al., 2005). In experimental human cell systems, SV40 LT transduction has been shown to promote a transcriptional fingerprint which is quite reminiscent of that observed in triple negative breast cancer primary tumors (Deeb et al., 2007), suggesting the lesion is a relevant model. We found that it is also effective in a porcine system. We then examined the effects of inactivating BRCA1 in the immortalized cells.

To knockdown BRCA1, we used a stable miRNA expression approach. Although we assayed two different miRNA sequences, only one was really effective as measured by qRT-PCR, and so this is the sequence we used in the experiments. Subsequent examination of BRCA1 protein levels by Western blot showed that this miRNA rendered the BRCA1 protein almost undetectable. The knockdown of BRCA1 in a background where SV40LT has impaired p53 and Rb function was sufficient to promote enhanced growth and a dramatic transformation of the cells, as measured by colony formation in soft agar. Thus, we have created the first porcine breast epithelial tumor cell line.

BRCA1 down-regulation has been implicated in the development of a cancer stem cell-like phenotype in breast cells (Liu et al., 2008). *In vitro*, it appears that it is the CSC population that provides the ability to form colonies in soft agar (Colburn et al., 1978). When we examined the cells we found that the inactivation of BRCA1 in the SV40 LT background induced the upregulation of the CSC markers EpCAM (Munz et al., 2009) and ALDH1 (Moreb, 2008). This suggests that breast cancer CSC in humans and pigs are regulated in a similar manner by BRCA1.

This work establishes the first porcine model system for studying BRCA1 and breast cancer. It validates the concept that porcine transgenic animal models may be valuable for the study of human breast cancer and the development of novel therapeutics for the treatment of breast cancer driven by BRCA1 defects. In particular, due to the human-like life span of pigs, a porcine model of BRCA1 driven breast cancer could allow the testing of long term preventative measures, as well as strategies to counter the persistence of minimal residual disease after treatment. Attempts have been previously made to develop such an animal (Luo et al., 2011). Unfortunately, no animal's survived BRCA1 knockout long enough to determine any biological effects on breast cancer. These experiments suggest that a future porcine BRCA1 system would need to involve a tissue specific knockout, as has been the case in transgenic mouse systems.

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Myeloid Leukemias and Virally Induced Lymphomas in Miniature Inbred Swine: Development of a Large Animal Tumor Model

Raimon Duran-Struuck[1], Abraham J. Matar[2] and Christene A. Huang[3]*

[1] Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA, USA, [2] University of Central Florida College of Medicine, Orlando, FL, USA, [3] Center for Transplantation Sciences, Massachusetts General Hospital, Boston, MA, USA

The lack of a large animal transplantable tumor model has limited the study of novel therapeutic strategies for the treatment of liquid cancers. Swine as a species provide a natural option based on their similarities with humans and their already extensive use in biomedical research. Specifically, the Massachusetts General Hospital miniature swine herd retains unique genetic characteristics that facilitate the study of hematopoietic cell and solid organ transplantation. Spontaneously arising liquid cancers in these swine, specifically myeloid leukemias and B cell lymphomas, closely resemble human malignancies. The ability to establish aggressive tumor cell lines *in vitro* from these naturally occurring malignancies makes a transplantable tumor model a close reality. Here, we discuss our experience with myeloid and lymphoid tumors in major histocompatibility characterized miniature swine and future approaches regarding the development of a large animal transplantable tumor model.

Keywords: swine, CML, PTLD, model, cancer, transplantation immunology

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****Correspondence:***
Raimon Duran-Struuck
rdura@upenn.edu

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INTRODUCTION

Malignancies of the hemolymphatic system in swine were first reported as early as 1865(Bostock and Owen, 1973) but generally, there are limited reports describing neoplasias in swine. This is partly due to the fact that most swine die at a relatively young age, either slaughtered for food or used in biomedical research. As of yet, there is no large animal tumor model available that can be reliably induced and consistently reproduced. The vast majority of documented cases of leukemias and lymphomas in veterinary oncology are in the domestic population (Schiffman and Breen, 2015). Liquid neoplasias have been reported in farm animals; however, these are uncommon due to the lack of desire for a clinical workup and preference for euthanasia to minimize animal suffering. Here, we will discuss how swine provide an attractive large animal model for the study of cancer biology and its treatment. Specifically, the Massachusetts General Hospital (MGH) miniature swine herd retains unique genetic characteristics that facilitate the study of hematopoietic cell (HCT) and solid organ transplantation (SOT; Hanekamp et al., 2011). A significant incidence of spontaneous chronic myeloid leukemias and herpesvirus associated B cell lymphomas have been reported in the MGH miniature swine herd, making it a viable option for the development of a large animal tumor model (Hanekamp et al., 2011).

SWINE AS A LARGE AMINAL RESEARCH MODEL

The use of large animal models in biomedical research remains controversial. Ethical justifications and selection of a less expensive model must continuously be addressed to private, government, and academic reviewers. However, the use of large animal models is important as murine studies often fail to translate clinically (Hunter and Williams, 2002; Adelman et al., 2006). Among the available large animals in biomedical research, primate models have the obvious advantage of physical and physiological similarity to humans, but there are several barriers to their use including strict regulation standards, expense, negative societal impression, difficulty in breeding and handling, prolonged time to sexual maturity and potential for infectious disease. Canine models are a more practical option and have been widely used (Cain and Champlin, 1989; Ladiges et al., 1990; Storb et al., 2001; Zaucha et al., 2001). However, no canine tumor model exists yet, and compared to swine, their use is less favored because of their status as a common companion animal. Alternatively, swine are an ideal experimental model for several reasons including ease of breeding and handling, short gestation periods, large litters, short time to sexual maturity, and an anatomy and physiology that closely resembles that of humans (Laber et al., 2002; Swindle et al., 2012). However, as with any animal model, there are limitations to the swine model. The ability to consistently reproduce findings in outbred species, though clinically relevant, remains the biggest challenge in terms of developing a tumor model.

Although murine studies have historically been critical in the study of cancer biology and immunological diseases, attempts at extrapolation to large animals or clinical studies have often been unsuccessful, especially with respect to studies of transplantation (Bortin, 1970; van Bekkum, 1984; Storb, 2003). This can be appreciated in studies of immunological tolerance, in which numerous approaches to allograft tolerance have been developed in mice, but very few have proven successful in clinical studies (Storb, 2003). Over 30 years ago, NIH researcher Dr. David Sachs initiated a selective breeding program of miniature swine to develop and maintain a large animal model for studies of transplantation biology (Sachs et al., 1976). Through years of selective breeding, Sachs et al. (1976) were able to "fix" the major histocompatibility (MHC) genes of the miniature swine herd, while retaining variability to minor antigens (miHAs; Pennington et al., 1981a,b; Mezrich et al., 2003). The homozygosity of MHC genes has made the MGH miniature swine a valuable model in that different clinical transplant scenarios can be mimicked (full MHC match, complete MHC mismatch, haploidentical match, etc). One line of swine was selectively inbred, which will refer to as the "SLA^{dd}" line (swine leukocyte antigen - dd), aiming to achieve complete syngeneity (Hanekamp et al., 2011), as has been done in mice. Currently, the SLA^{dd} line has reached a coefficient of inbreeding of >94%. Despite not yet being 100% genetically identical, skin and organ allografts transplanted between animals within this line are consistently accepted without any immunosuppression (Mezrich et al., 2003). Extrapolating from these data, spontaneous tumors arising in this line can be harvested expanded *in vitro* and cryopreserved for *in vivo* transfer studies, providing the foundation for a transplantable swine tumor model. Here, we discuss our experience with myeloid and lymphoid tumors within the MGH miniature swine and future goals of a large animal tumor model.

CHRONIC MYELOGENOUS LEUKEMIA IN SWINE

Recently, we reported a significant incidence of spontaneous myeloid leukemias in the inbred SLA^{dd} line of the MGH miniature swine herd and demonstrated that swine chronic myelogenous leukemias (CML) closely resembled human CML (Duran-Struuck et al., 2010). In our study, two swine CML cell lines were karyotyped to assess the presence of a specific translocation or mutation, similarly to the t(9;22) translocation, or philadelphia chromosome (Ph+), which is well documented in the majority of human CMLs (Oettel et al., 1994; Marks et al., 2010; Chereda and Melo, 2015). In both cases, a shortened chromosome arm was identified (Duran-Struuck et al., 2010), reminiscent of the classical Ph+ in humans. PCR was performed using BCR–ABL gene-specific primers to determine whether the genetic change was identical (or similar) to the Ph+ chromosome in human CML (Chereda and Melo, 2015). Sequences surrounding the known chromosomal breakpoint of the BCR and ABL genes in the human K562 CML cell line were compared to the available porcine or bovine sequences to identify conserved regions for primer design. Two bands of 300 and 500 nucleotides were detected in the pig while a single distinct band of 450 nucleotides was present in the K562 sample. Due to differences in chromosome numbers between humans and swine (23 pairs for humans and 19 for swine), we could not directly translate the t(9;22) translocation observed in humans CMLs. Future genetic studies of these swine tumors may provide a platform for novel therapeutic approaches for human tumors sharing similar genetic defects.

AN INDUCIBLE SWINE CML MODEL

Central to the development of many murine tumor models has been the establishment of *in vitro* oncologic cell lines. Similarly, characterization of tumor cell lines derived from inbred miniature swine and adaptation for *in vivo* growth is a possibility. Several CML cell lines from the SLA^{dd} inbred line were previously isolated from affected animals (Cho et al., 2007; Duran-Struuck et al., 2010), and aggressive subclones were selected out by serial passages *in vitro*. *In vivo* growth of a CML cell line originating in animal 14736 was assessed after direct inoculation into naïve swine conditioned with gamma irradiation (ranging from 100 to 500 cGy). Subcutaneous (SQ) injection of the 14736 CML cell line into an animal conditioned with 300 cGy total body irradiation (TBI) resulted in SQ tumor growth, but not systemic growth (Cho et al., 2007). Systemic tumorigenesis (with mostly lung involvement) required at least 500 cGy of TBI. Though non-myeloablative, 500 cGy of TBI proved to be significantly

immunosuppressive and animals often died of infections and not due to the induced neoplasm. If animals received less irradiation (<500 cGy), tumor cells did not grow *in vivo* (Duran-Struuck, unpublished data). Injection of CML cell lines directly into the bone marrow (intra-BM) of SLA^{dd} swine conditioned with low levels of TBI (100–200 cGy) also did not lead to systemic leukemic growth. BM biopsies from one animal that had been infused with intra-BM CML were cultured *in vitro*. The resulting cell line was phenotypically and morphologically similar to the injected CML but could not be differentiated from a potential *de novo* CML. Difficulties in achieving CML disease in this model may be explained by the presence of minor antigen incompatibilities (miHAs), which may exist between host and tumor despite being MHC matched. Inbreeding can induce the loss (or gain) of expression of an immunogenic protein (secondary to a mutation) to which the animal may have not been made tolerant during thymic T cell education. Thus, host "rejection" of infused tumor cells can occur despite being MHC matched in the context of insufficient immunosuppression. This is supported by SQ tumor growth in the animal conditioned with high amounts of irradiation (500 cGy) while animals that received lower amounts of irradiation (100–300 cGy) did not exhibit any tumor growth. Two other explanations for failed tumor growth can be attributed to the loss of growth characteristics (growth factors, adhesion molecules, etc.) of the *in vitro* passaged CML tumor cells and the requirement of a longer time to develop *in vivo* than what was designed in the IACUC protocol.

To assess whether the *in vitro* culture process affected tumor cell growth capacity *in vivo*, cell lines were passaged *in vivo* in mice. Tumors have historically been expanded across xenogeneic barriers in immunodeficient mice (NOD/SCID) and have been successful in selecting for aggressive tumor subclones (Waller et al., 1993; Adam et al., 2007; Schook et al., 2015). Though not ideal to expand tumor cell lines in animals different from the original host species, this approach ensures that tumor cells retain their *in vivo* growth capacity. 14736 CML tumor cells did not grow in NOD/SCID mice (Cho et al., 2007), but did lead to CML disease in NSG (NOD/SCID gamma −/−) mice (Schenk et al., manuscript in preparation), albeit requiring over 4 months. These results suggest that the innate immune system of NOD/SCID mice may have been sufficient to "reject" the tumor cells, as NSG mice lack macrophages and NK cells. Thus, spontaneous swine CML lines can be successfully expanded *in vivo,* and transfer studies into swine are forthcoming.

PORCINE LYMPHOTROPIC HERPESVIRUS (PLHV) INDUCED B CELL LYMPHOMAS

A major area of study in MGH miniature swine for the past 30 years has been the use of HCT to induce mixed hematopoietic chimerism without GVHD, both for solid organ tolerance and treatment strategies of hemolymphatic neoplasias. Currently, a major complication of both HCT and SOT is the development of post-transplant lymphoproliferative disease (PTLD). PTLD is observed in immunosuppressed transplant patients, but similar lymphoproliferative processes can present in those naturally immunosuppressed, such as AIDS patients (Bollard et al., 2004; Abu-Elmagd et al., 2009). Under the cover of immunosuppression and depressed CD8+ T cell immunity, the B cell population aggressively expands as a result of primary infection or reactivation of a herpes virus, most commonly Epstein Barr virus (EBV; Heslop et al., 1996; Lucas et al., 1996). Unfortunately, the variability of the human patient population, both clinically and pathologically, complicates the ability to study this disease (DiNardo and Tsai, 2010). Murine models of PTLD involving immunodeficient mice injected with human PTLD lines and mice infected with murine gamma herpesvirus are unreliable and do not accurately model human disease (Schiffman and Breen, 2015).

In contrast, swine have been shown to be an excellent model for the study of PTLD. Immunosuppressed swine undergoing HCT or SOT develop B-cell expansions with a clinical presentation that closely resembles human PTLD (Huang et al., 2001; Matar et al., 2015). Similarly to human PTLD's association with EBV, swine PTLD is associated with primary infection or reactivation of a gamma herpesvirus, porcine lymphotropic herpesvirus-1 (PLHV-1; Doucette et al., 2007). In a model of haploidentical HCT, immunosuppressive regimens consisting of T-cell depletion using CD3-immunotoxin, 1000 cGy of thymic irradiation, and a 30–60 days course of cyclosporine A consistently (40–50%) resulted in the development of B cell lymphomas post-transplant (Cho et al., 2004; Cina et al., 2006; Matar et al., 2015). When thymic irradiation was eliminated as part of the conditioning regimen, only 1/23 animals developed PTLD. However, in the absence of thymic irradiation, T cell depletion was poor, resulting in inconsistent stem cell engraftment. 100 cGy of TBI was added to the conditioning regimen in an attempt to decrease the incidence of PTLD while allowing for stem cell engraftment. Subsequently, only 15% developed PTLD, while the majority of animals successfully engrafted (Matar et al., 2015). Matar et al. (2015) recently explored the effect of thymic and TBI on the incidence of PTLD in this model and concluded that thymic irradiation was a risk factor for PTLD development via its depleting effect on the absolute number of T cells. Further, the use of LDH as a serum marker for swine PTLD was validated (**Figure 1**). As in humans, B cell expansion in the context of swine PTLD is mirrored by increases in LDH (Boothpur and Brennan, 2008), even before clinical signs of PTLD such as lymphadenopathy (**Figure 2**). This was also shown to be diagnostically valuable in swine CMLs (Duran-Struuck et al., 2010) which mirrored the LDH increases observed in human CMLs and reinforcing the swine tumor model.

In clinical practice, the standard treatment approach for PTLD is the reduction of immunosuppression and sometimes followed by a second treatment modality if necessary, such as rituximab (anti-CD20 mAb) or chemotherapy (DiNardo and Tsai, 2010). Removing immunosuppression in the transplant setting is often complicated by organ rejection or graft-versus-host disease in the setting of allogeneic bone marrow transplantation (BMT). Chemotherapy remains an effective, yet toxic treatment option, and rituximab though effective, does not control PTLD in all

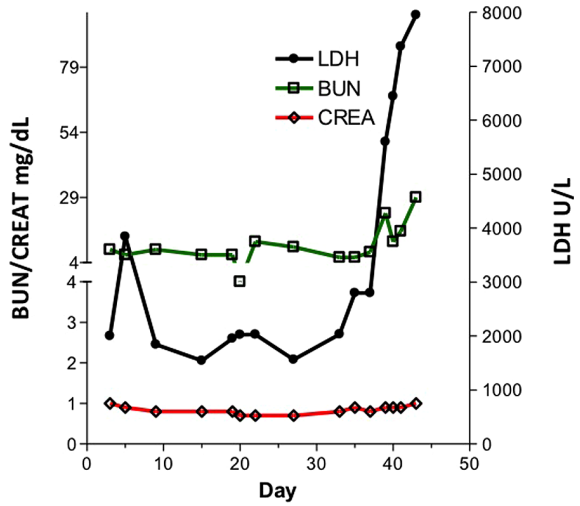

FIGURE 1 | LDH, BUN and Creatinine in a pig developing post-transplant lymphoproliferative disease (PTLD). Kidney values remained relatively constant while LDH levels rose acutely.

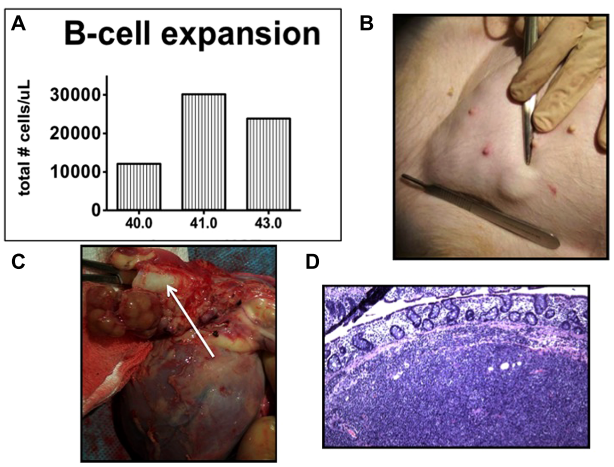

FIGURE 2 | Post-transplant lymphoproliferative disease in miniature swine. (A) Total B cell burden in an animal developing PTLD. B cells tripled in number within 24 h. **(B)** Lymphadenopathy (inguinal area). **(C)** Perihilar lymphadenopathy (arrow shows trachea). Cranial aspect of the animal is to the left of the image. **(D)** H & E section demonstrating cellular infiltrate in a lymph node (20×).

cases. In the context of swine PTLD following BMT, reduction or discontinuation of immunosuppression only sometimes leads to PTLD resolution, but GVHD is a common consequence (Duran-Struuck et al., manuscript in preparation). In the study cited above, of 11 animals that developed PTLD, only two cases resolved after discontinuation of cyclosporine, and those two animals subsequently developed GVHD. This naturally induced model of PTLD following BMT can be used to study novel treatment approaches such as new antivirals or the use of *in vitro* primed host CD8+ T cells as a cellular therapy for primary or refractory PTLD.

SWINE PTLD TUMOR LINES

Although this naturally induced model offers a clinically relevant opportunity which to study PTLD, it is limited by the inconsistency in PTLD incidence (40–50%) and the logistics and cost involved in a BMT or SOT. Alternatively, swine PTLD tumors have been successfully harvested from various involved organs, including lymph nodes and spleen, and expanded *in vitro* with the intention of establishing an immortal cell line that can reliably induce PTLD when introduced into a naïve animal from the same inbred line. To test the ability of these cell lines to grow *in vivo*, as a preliminary experiment, a PTLD B cell lymphoma line was established from animal 13271 and infused intravenously into unconditioned NSG (NOD/SCID IL-2r gamma−/−) mice (Schenk et al., manuscript in preparation). In general, an average of 10×10^6 PTLD cells was infused per mouse. At this dose, we observed successful "engraftment" of tumor cells with 100% lethality within 57–70 days. Subsequently, the same PTLD cell line was tested in two MHC matched swine. Animals were preconditioned with a non-myeloablative protocol that has previously been permissive for the induction of PTLD. Animals received 100 cGy of TBI on day −2, T cell depletion with a recombinant CD3-immunotoxin twice daily from day −4 to day −1, and were maintained under cyclosporine coverage for 60 days. In total, each animal received three doses of approximately 300×10^6 tumor cells/kg over a period of 1 week, totaling 900×10^6 tumor cells/animal. Tumor cells were infused intravenously (IV) and/or intraosseously (IO), with the intention of overwhelming the animals' tumor clearing capacity and allowing for successful "engraftment" of tumor cells. Unfortunately, none of the animals developed PTLD. B cell counts normalized soon after infusion and an increase in B cells was only observed during the peri-infusion period as determined by flow cytometry analysis. Thus, PTLD tumor cells selected *in vitro* for their growth ability and which had successfully engrafted in NSG mice did not cause overt PTLD in immunocompromised miniature swine. A limitation of this non-myeloablative approach is the potential for radiation resistant T cells to "reject" the tumors via minor antigen incompatibility (Nadazdin et al., 2011; Zhang et al., 2012) or TLR ligation (Yu et al., 2012). Thus, conditioning regimens that have a stronger depleting effect on T cells, such as the use of thymic irradiation which has been shown to be very conducive to the development of PTLD, may be required to better induce tumor growth.

From an immunologic standpoint, it is crucial to understand the method of "graft" (tumor) loss in this model. There are several possibilities for the lack of tumor cell engraftment including an active rejection of tumor cells by residual host defenses, evasion of host immune responses by "hiding" in an immune privileged site (such as the bone marrow), simply being ignored by host defenses in the circulation, or alternatively, tumor cells may have died due to lack of fitness, without an immunological attack. In our studies, mixed lymphocyte reactions (MLR) and cell mediated cytolysis (CML) assays did not suggest a cellular sensitization against tumor antigen(s). This implies the mechanism of graft loss was possibly non-immunologic, either via clearance from the circulation or lack of fitness in the swine environment.

Due to the fact that host and tumor cells are fully MHC matched, it is difficult to distinguish tumor cells after infusion, as they could be residing in the marrow or lymph nodes without surviving in the peripheral blood. As a method to distinguish and monitor tumor cells *in vivo*, a green fluorescent protein (GFP) gene was transduced into the PTLD tumor cell line using a lentivirus vector. GFP(+) tumor cells were then sorted and expanded. Interestingly, GFP+ tumor cells grew faster *in vitro* compared to GFP(−) tumor cells. When NSG mice were challenged with GFP(+) tumor cells, we observed a faster onset of disease as well as more extensive organ involvement, suggesting a more aggressive tumor. One MHC matched swine was infused with GFP(+) PTLD under the same conditioning regimen as used previously. The cells were monitored via flow cytometry and were undetectable after 48 h, suggesting they were either cleared from the circulation or sequestered. Again, no sensitization was observed by MLR or CML assays indicating a non-immunologic mechanism of graft loss (Schenk et al., manuscript in preparation).

CONCLUSION AND FUTURE DIRECTIONS

Spontaneously arising hemolymphatic tumors in the MGH miniature swine herd, specifically myeloid leukemias and B cell lymphomas, closely resemble human malignancies, making the MGH swine an valuable model for the development of a clinically applicable large animal tumor model based on their unique genetic characteristics. Future approaches focusing on reproducibility will include several strategies, including; (i) optimizing transplant protocols to induce tumor cell engraftment, (ii) *ex-vivo* transduction of porcine hematopoietic stem cells with known oncogenes (Adam et al., 2007), and (iii) the introduction of oncogenes via retroviral vectors (Adam et al., 2007). Alternatively, backcrossing the MHC characterized mini-swine with the first naturally occurring severe combined immunodeficient (SCID) pig line. This SCID pig has already been shown to accept human tumor xenografts, and thus can enhance the engraftment of allogeneic tumor transfer studies (Waide et al., 2015). In summary, the importance of a consistently reproducible large animal tumor model cannot be understated, as it will facilitate the study of these lethal malignancies and test reliably novel therapeutic strategies for clinical applications.

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REVIEW
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The MeLiM Minipig: An Original Spontaneous Model to Explore Cutaneous Melanoma Genetic Basis

Emmanuelle Bourneuf[1,2]*

[1] LREG, CEA, Université Paris-Saclay, Jouy-en-Josas, France, [2] GABI, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France

Melanoma is the deadliest skin cancer and is a major public health concern with a growing incidence worldwide. As for other complex diseases, animal models are needed in order to better understand the mechanisms leading to pathology, identify potential biomarkers to be used in the clinics, and eventually molecular targets for therapeutic solutions. Cutaneous melanoma, arising from skin melanocytes, is mainly caused by environmental factors such as UV radiation; however a significant genetic component participates in the etiology of the disease. The pig is a recognized model for spontaneous development of melanoma with features similar to the human ones, followed by a complete regression and a vitiligo-like depigmentation. Three different pig models (MeLiM, Sinclair, and MMS-Troll) have been maintained through the last decades, and different genetic studies have evidenced a complex inheritance of the disease. As in humans, pigmentation seems to play a prominent role, notably through MC1R and MITF signaling. Conversely, cell cycle genes as *CDKN2A* and *CDK4* have been excluded as predisposing for melanoma in MeLiM. So far, only sparse studies have focused on somatic changes occurring during oncogenesis, and have revealed major cytological changes and a potential dysfunction of the telomere maintenance system. Finally, the spontaneous tumor progression and regression occurring in these models could shed light on the interplay between endogenous retroviruses, melanomagenesis, and adaptive immune response.

Keywords: porcine model, predisposition genes, cutaneous melanoma, QTL, endogenous retrovirus

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***Correspondence:**
Emmanuelle Bourneuf
emmanuelle.bourneuf@cea.fr

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STATE OF THE ART OF MELANOMA GENETICS

Melanoma is a tumor arising from melanocytes, highly differentiated cells notably found in the skin of Vertebrates. Melanocytes are derived from the neural crest of the embryo, from which melanoblasts migrate to colonize the skin of the entire body, the eye, as well as other structures. Later, melanoblasts differentiate into functional melanocytes, which main role is pigment production (Cichorek et al., 2013). Melanins (black eumelanin and red pheomelanin) are synthesized mainly to prevent DNA damage due to UV radiation (Miyamura et al., 2007). However, sun exposure, as well as other environmental or intrinsic factors, can lead to malignant transformation of melanocytes, thus turning into melanoma. Several clinical types of melanoma prevail: cutaneous melanoma, uveal melanoma developing in the eye, acral melanoma observed

on palms of hands, soles of feet and nails, and mucosal melanoma, located in the nose, mouth, vagina, urinary tract, and rectum for example. Other less frequent subtypes are described, and all these different forms of melanoma have different causalities, molecular mechanisms and outcomes (Schadendorf et al., 2015). This review will focus only on cutaneous melanoma, which also displays heterogeneous histological features. For example, the two main subtypes are Nodular Melanoma [or Vertical Growth Phase melanoma (VGP)], and Superficial Spreading Melanoma [or Radial Growth Phase Melanoma (RGP)]. Other melanocytic lesions can be observed (nevi, lentigo, atypical melanocytic proliferations. . .) but most remain benign and do not lead to malignant transformation.

The incidence of cutaneous malignant melanoma has been increasing for decades, reaching in 2011 a rate of around 30 per 100,000 per year in United States whites, Swedish and Norwegian populations, a rate of 19.8 per 100,000 in United Kingdom, and up to 51 per 100,000 in Australian and New Zealand populations (Whiteman et al., 2016). Mortality associated to melanoma has also been increasing in all six populations, although at a slower pace. The highest incidence is observed in fair-skinned populations; however dark-skinned individuals also present cutaneous melanoma, usually diagnosed at a later stage with a greater Breslow thickness, and associated with a poorer outcome (Hore et al., 2010). Also, non-caucasian populations have a higher incidence of acral lentiginous melanoma, thus independent of sun exposition (Stubblefield and Kelly, 2014). The only efficient treatment for cutaneous melanoma is *in situ* resection of the primary tumor, when no cells have spread away in the body. Once a metastatic process has started, most of classical therapies used in oncology do not improve survival (Schadendorf et al., 2015). Nevertheless, recent developments in immunotherapy have shown exciting results, with the use of antibodies targeting immune checkpoints such as CTLA-4 and PD-1 (Boutros et al., 2016). Melanoma is among the most immunogenic cancers, with an important mutational load, probably explaining a high rate of partial tumor regression. Nevertheless, to circumvent this potential antitumor response, melanoma cells profit from immune checkpoints such as CTLA-4 and PD-1 pathways. These surface molecules, expressed notably by T lymphocytes, are negative regulators of immune function, and prevent an over-activation of immune response in physiological conditions. Thus, immune checkpoints inhibition stimulates the host immune response, via diverse mechanisms: CTLA-4 pathway blockade allows for proliferation and activation of more T-cells, while PD-1 blockade restores of the antitumor activity of T cells. These treatments have shown unprecedented responses and antitumor activity in advanced melanoma patients, and extensive clinical trials are currently underway (Boutros et al., 2016).

Cutaneous melanoma is mainly due to exposure to UV radiation, and strong prevention campaigns around a limited use of sunbeds and extensive protection with sunscreens has proven efficient in the last years (Lo and Fisher, 2014). However, genetic susceptibility has been shown to be another factor promoting melanoma development (Aoude et al., 2015b), in around 10% of patients (Florell et al., 2005). Thus, sporadic melanomas can be distinguished from familial melanoma cases, for which genetic studies can be considered. Other factors of importance are for example a high number of nevi on the body, or the patient's skin phototype, with an increased risk of melanoma if carrying >50 moles or in fair-skinned people (MacKie et al., 2009).

In human, early linkage analyses performed in large families have led to the identification of two high-risk genes, namely *CDKN2A* and *CDK4*, both cell cycle regulators. *CDKN2A* is the most important gene described so far, and around 20% of familial cases are due to deleterious mutations in this gene (Hussussian et al., 1994; Kamb et al., 1994; Potrony et al., 2015 for review). Loss-of-function mutations thus discard the inhibitory role of the two *CDKN2A* products, namely $p16^{INK4A}$ and $p14^{ARF}$ on cell cycle progression, leading to enhanced proliferation. The *CDK4* R24C activating mutation is also highly penetrant, although it concerns only a few families worldwide (Zuo et al., 1996; Soufir et al., 1998): this non-synonymous substitution changes an amino acid essential for binding of CDK4 to $p16^{INK4A}$, leading to an increased proliferation. Early studies have also designated *MC1R* as a low-risk gene (Valverde et al., 1996; Palmer et al., 2000). *MC1R* codes for a G-protein-coupled receptor, which physiological ligand is α-MSH. A binding to MC1R leads to an increase in cAMP production, and therefore an activation of subsequent cascades in the cell. In melanocytes, *MC1R* expression controls the balance between pheomelanin and eumelanin production and thus regulates the pigmentation of the individual (Dessinioti et al., 2011). For example, a series of polymorphisms, named RHC variants for Red Hair Color variants, are carried by individuals with light skin, blue eyes and red hair, poor tanning ability and sensitivity to sunburn (Flanagan et al., 2000). These patients also display a higher risk of melanoma, initially related only to the role of *MC1R* in pigmentation. Further studies have shown a much more complex picture of *MC1R* effects in the melanocyte: in addition to the UV-protective effect of melanin, *MC1R* can influence melanoma beyond pigmentation, through the positive effect of cAMP on repair of UV-induced damage (Hauser et al., 2006; Kadekaro et al., 2010). In two recent studies focusing on somatic mutations in melanoma, the authors reported a more important mutational burden in tumors of patients carriers of 1 or 2 RHC alleles compared to non-RHC patients (Robles-Espinoza et al., 2016; Johansson et al., 2017). Also, this higher mutation rate is observed for all mutation classes, and not only the UV damage-associated C>T transitions, indicating other potential mutational processes linked to *MC1R* germline variation.

Since 2011, the *MITF* gene (Microphtalmia-Associated Transcription Factor) is considered as a medium-risk gene, since two studies involving linkage analysis followed by sequencing, showed the functional impact of the E318K rare mutation on melanoma and renal cell carcinoma risks (Bertolotto et al., 2011; Yokoyama et al., 2011). The mutation impairs the SUMOylation of MITF proteins, leading to a differential transcriptional activity of the target genes. More recently, Bonet et al. (2017) demonstrated that human melanocytes carrying the *MITF* E318K mutation could no longer undergo $BRAF^{V600E}$-induced senescence, thus promoting melanoma development.

Horn et al. (2013), mutations in *TERT* promoter were shown to modify melanoma risk in familial melanoma. In addition, TERT promoter is frequently mutated in sporadic melanomas as well as other tumor types (Aoude et al., 2015b).

With the advent of high-throughput technologies such as SNP genotyping and sequencing, numerous Genome-Wide Association Studies have been performed in large case-control designs, for melanoma occurrence *per se*, as well as connected phenotypes such as number of nevi and pigmentation (reviewed in Law et al., 2012). These approaches have pointed at several genes which role in melanoma development remains to be elucidated. However, a number of genes associated with melanoma are involved in pigmentation (*ASIP, OCA2, TYR, TYRP1,* and *MTAP*), or in cell cycle regulation (*CCND1*, *CDKAL1*). Other function classes include immune response (*HLA*, *IRF4*) and metabolism (*FTO*, *VDR*). Most of these results have been confirmed in a recent international meta-analysis (Law et al., 2015).

Finally, successful candidate genes approaches led to the identification of other high risk genes more recently, like for example *BAP1* for which germinal mutations are found in a wide spectrum of neoplasms (Wiesner et al., 2011; Carbone et al., 2013). Since the discovery of predisposing variants in *TERT* promoter, research on melanoma genetics has focused on germline mutations in genes coding for components of the telomere maintenance complex. Mutations in *POT1* (Robles-Espinoza et al., 2014; Shi et al., 2014), and in *ACD* and *TERF2IP* (Aoude et al., 2015a) have been associated with melanoma increased risk in around 1% of predisposed families (Potrony et al., 2015). As a consequence, a renewed interest has grown for telomere maintenance in tumors as a potential therapeutic target, despite previous pitfalls (Zanetti, 2017).

Overall, different approaches allowed the identification of low to high risk genes, and of common variants showing a very limited effect on disease risk when taken alone. One of the major difficulties in GWAS of complex traits is genetic heterogeneity of the cases, which requires the use of very large cohorts and careful clinical classification of the patients. A possible way to bypass this critical issue is the use of animal models to better understand the complex genetic and molecular mechanisms leading to melanomagenesis.

ANIMAL MODELS OF MELANOMA

The most frequently used model for melanoma is the mouse. Spontaneous melanoma is very rare in mouse, but the model is notably used as a support for patient-derived xenografts (PDXs) in immunocompromised animals. PDX mice properly model the human disease and can guide personalized therapy decisions (Hartsough and Aplin, 2016). Another essential application of mouse as a model is the relatively easy manipulation of its genome. Therefore, a large set of GEM (genetically engineered mice) models were developed and used for a fine dissection of molecular pathways governing melanocyte transformation and melanoma progression. For example, Mann et al. (2015) used transposon mutagenesis in a $BRAF^{V600E}$ mouse model to determine a set of genes cooperating with the BRAF mutation to drive melanoma progression. Those fine studies performed in GEM mice can give crucial information about pivotal pathways and potential therapeutic targets for human melanoma.

A major limitation to the use of mouse as a model is that melanoma incidence is often very low and appears at late onset (van der Weyden et al., 2015). In addition, murine melanocytes reside in the dermis, and not at the basal layer of the epidermis. This latter aspect should be taken into account since a large part of melanoma biology (and cancer in general) is modulated by surrounding stromal cells and immediate environment. To circumvent this anatomical issue, another interesting mouse model has been developed, by overexpressing the HGF/SF (hepatocyte growth factor/scatter factor) under the metallothionein promoter (Takayama et al., 1997). In the HGF/SF mouse model, melanocytes colonize not only the dermis, but also the epidermis. In addition, HGF/SF mice harbor different histological types of lesions, including sporadic melanomas arising following UV exposition. Nevertheless, despite a very convenient handling, and the undeniable advantage of genetic engineering to modulate single genes function, mouse models may not recapitulate all the features of a complex disease. The lack of translatability to human of mouse results obtained on inflammatory diseases illustrates this aspect (Seok et al., 2013). Naturally occurring models may better reflect complexity and might be closer to reality (**Table 1**).

Among non-rodent models for cutaneous melanoma, zebrafish is a very attractive model to decipher precise mechanisms (Michailidou et al., 2009), perform *in vivo* imaging (Heilmann et al., 2015) and drug screening (Xie et al., 2015). However, as in rodents, melanoma development is induced by genetic manipulation of oncogenes or ENU-induced mutagenesis, which is a major limitation to study genetic predisposition. Naturally occurring models include some horse breeds, for which aging is associated with graying of the coat color, mucosal melanomas and depigmentation. Melanoma development has been attributed to a mutation in the intron 6 of *STX17* (Rosengren Pielberg et al., 2008), leading to an activation of the ERK pathway (Jiang et al., 2014). Dogs show a quite high incidence of melanoma, and different breeds can serve as models for multiple melanoma subtypes. For example, cutaneous melanoma is found frequently in Beauce shepherds, while poodles are more prone to developing oral tumors (Gillard et al., 2014). Moreover, the structure of dog breeds has been shown as a very efficient tool for trait mapping, even for complex diseases (Rimbault and Ostrander, 2012). These extraordinary features also make the dog an exciting model for melanoma predisposition.

SWINE MODELS OF MELANOMA

Pigs and large animals in general are recognized as compelling models for human diseases. This wonderful potential has had a limited impact because of the poor availability of genomic

TABLE 1 | Examples of animal models of melanoma, and some of their advantages/disadvantages to explore melanomagenesis in human.

Species	Advantages	Disadvantages
Mouse	Genetic manipulation possible Different genetic backgrounds available Easy breeding and handling Vast genetic and genomic resources Many examples of molecular pathways dissection	Late onset Low incidence No spontaneous melanoma, genetic modifications needed Melanocytes in dermis
Pig	Cutaneous melanoma Early onset of multiple tumors No environmental effect Same inheritance as humans Common histological and clinical features with human melanoma, including metastatic invasion Spontaneous and complete regression Melanocytes on the basal layer of the epidermis	Major susceptibility genes identified in human are not predisposing in pigs Early onset and UV-independent, thus not reflecting a large part of human cases occurring in the elderly, on sun-damaged skin Cell biology tools are limited (antibodies for example)
Dog	Several possible clinical types (mucosal, cutaneous, acral, uveal) Veterinary records Anti-cancer treatments and clinical trials Shared environment with human Somatic mutations similar to human ones Breed genetic structure should facilitate association analysis	Often benign (except melanomas from the oral cavity) Cell biology tools are limited (antibodies for example) Genetic basis remains poorly described
Horse	Presence of nevi and melanomas Dermal melanomas can eventually metastasize The genetic basis of melanoma development in gray horses is partly known Activation of ERK pathway, as seen in human	Correspond to rare melanomas in human Late metastatic evolution in gray horses

resources for domestic species. However, recent technological breakthroughs now circumvent these pitfalls. For example, swine models of mutations found in human can now be produced to decipher the mutation effect *in vivo*. Thus, the very recent development of "oncopigs" is of major importance (Schook et al., 2015), but remains complementary to spontaneous models to reflect and model natural complexity.

One of the major advantages of swine models is the tightly controlled breeding process, so that the genetic determinism can be studied independently from potentially interacting environmental factors. Thus, a spontaneous porcine model of melanoma, bred totally indoors, can help deciphering melanoma genetics, without any influence of the UV-dependent mechanisms. Also, pig breeds harbor a limited genetic heterogeneity, mimicking to some extent a complex susceptibility background, but still allowing genetic studies with reasonable number of samples.

The pig has been used for skin physiological studies for decades, given its properties comparable to human skin (Vodicka et al., 2005). One of the most interesting point using pig as a model for a cutaneous melanoma is the location of the melanocytes, sitting on the basal layer of the epidermis, as in humans, and contrary to rodents where they are found in the dermis. Thus, swine skin is expected to better reflect the microenvironment of the healthy and transformed cells. This fundamental aspect is illustrated for example by a recent study showing that melanoma vertical invasion is governed by contact with keratinocytes in human (Golan et al., 2015). Another advantage of the swine model is the early onset of melanoma and high incidence in some specific breeds. As a consequence, clinical observations and sampling can be performed in the first weeks of the animals and do not require producing a large number of animals and waiting for tumor appearance. Yet, one should keep in mind that early onset melanoma is only rarely observed in human, and generally originates from a giant congenital nevus (Kinsler et al., 2017). Other pediatric melanomas appear rarely before puberty, and share features with adult melanomas developing on an intermittently sun-exposed skin (Lu et al., 2015).

Pigs bearing cutaneous melanoma have been described as early as in the 30s (Nordby, 1930). Commercial breeds also show a low incidence of melanoma. For example, a few cases were described in the progeny of a cross between a Duroc male and a Slovak White sow (Levkut et al., 1995), in the Hampshire breed (Empringham and Wilkins, 1979), or in slaughterhouses, without any mention on the breed (Bundza and Feltmate, 1990; Vidal et al., 2015). Frequent cases have been documented in Duroc swine, and in Duroc X Iberian cross (Thirloway et al., 1977; Hordinsky et al., 1985; Mishima et al., 1989; Perez et al., 2002). Interestingly, there is no mention in the literature of any melanoma lesions in Asian breeds.

In addition to these animals, three models have been selectively bred for cutaneous melanoma studies (**Table 2**). Their origins go back to two different breeds, Hormel and Hanford (Köhn, 2011). The first line is the Munich miniature swine (MMS) Troll, maintained and studied at the Institute of Veterinary Pathology, University of Munich (Germany). The animals are derived from Hanford and Columbian miniature swine, and have been selectively bred since 1986 (Müller et al., 2001). The Sinclair pig originates from the Hormel swine, and was first described in Millikan et al. (1974), when authors mentioned a melanoma incidence of 21% in the herd back in

TABLE 2 | Pathological and genetic features of the three main swine breeds bearing cutaneous malignant melanomas.

		MeLiM	Sinclair	MMS-Troll
Breed features	Origin	Hormel	Hormel	Hanford
	Coat color	Red or black		
Melanoma traits	Age of onset	At birth or in the first weeks		
	Clinics	Single or multiple lesions, clinical ulceration, local or distant metastasis		
	Histology	SSM and NM subtypes, presence of flat benign lesions, Clark's level I-V, histological ulceration, skin invasion to dermis or subcutaneous adipose tissue, heavily pigmented melanoma cells and presence of pigment-laden macrophages		
	Regression	Spontaneous and total		
	Depigmentation	Partial or total, affecting hair, skin, and eye		
	Mode of inheritance	2–3 loci, or complex autosomal dominant with incomplete penetrance	One major unmapped locus + SLA "B haplotype"	For nevi: one major gene and polygenic background For melanoma: two to three recessive genes No influence of SLA
	Reference	Geffrotin et al., 2004; Hruban et al., 2004; Vincent-Naulleau et al., 2004; Du et al., 2007	Millikan et al., 1974; Hook et al., 1979; Tissot et al., 1987; Green et al., 1992; Misfeldt and Grimm, 1994; Gomez-Raya et al., 2007; Ho et al., 2010	Müller et al., 2001; Dieckhoff et al., 2007

the 1960s. Since then, Sinclair melanoma was studied in different laboratories and is maintained now in the Sinclair Research Center in the United States http://www.sinclairresearch.com/. Finally, the Melanoblastoma-bearing Libechov Minipig (MeLiM) model was originally maintained in the Libechov Institute in Czech Republic, and further distributed to a French unit belonging to INRA and CEA (Geffrotin et al., 2000). Since then, only a few animals from the Czech herd have been imported to France, but animals still remain comparable. Horak et al. (1999) described the appearance of tumors in these animals: pigs from Hormel origin were crossed with Göttingen minipigs, with a white with black spots coat color, and later with four additional breeds (Canadian Landrace, Cornwall, Large White, and Vietnamese). The objective was to increase genetic variation in a herd designed for blood group variability studies, while maintaining a miniature phenotype. Cutaneous melanoma appeared in the herd in the 80s and was further selected for, so that the melanoma incidence reached more than 50% after some years. The fact that at least two of the three models (Sinclair and MeLiM) come from the Hormel swine farm would indicate shared genetic variants, potentially including the melanoma predisposing variants. Studying both models in parallel would therefore reinforce findings.

In the three breeds, as well as sporadic cases above mentioned, melanoma exclusively occurs on colored animals (solid red or black coats). This observation is explained by the absence of melanocytes in the skin of white pigs, due to a complex mutation of the *KIT* gene (Johansson Moller et al., 1996). This gene codes for a tyrosine kinase receptor expressed at the cell surface and regulating several intracellular processes. KIT is notably present at the surface of melanoblasts and regulates the migration of cells in the embryo. Deleterious mutations in *KIT* thus impair melanoblasts migration, leading to a white coat color. Therefore, melanoma predisposing variants could not have an effect on a white animal that does not possess the cell of origin of the tumor.

In all animals, tumors appear early in life, and even before birth. In Sinclair, Beattie et al. (1988) have shown the presence of melanocytic hyperplasia as early as the 11th week of gestation. Tumors are exclusively cutaneous and no uveal or mucosal melanoma has been reported so far in pigs. There is no predilection for cutaneous tumor location on the body, reflecting the absence of environmental influence. In human, predictably, cutaneous melanomas are more frequently observed in sun-exposed areas of the body, although non-CSD (chronically sun-damaged) melanomas are not negligible (Shain and Bastian, 2016). Also, no sex difference has been observed in transmission of the disease in the three swine models.

The overall clinical presentation also shares features between breeds. Indeed, animals can carry multiple lesions, some being flat and benign, others showing obvious malignancy signs and eventually leading to metastasis (Millikan et al., 1974; Hook et al., 1979; Horak et al., 1999). Vincent-Naulleau et al. (2004), realized a more extensive clinical and histological description of the melanocytic lesions found in the MeLiM pig, along with a comparison with human classification. Three types of lesions are observed, from benign to highly invasive. The benign flat melanocytic lesions show no metastatic invasion, and are histologically similar to atypical melanocytic proliferations for the vast majority of them. A second histological subtype consists of raised and pigmented lesion but without malignant evolution (no ulceration, slow growth, no metastasis). Finally, heavily pigmented tumors with a rapid growth correspond to invasive melanoma. Some are exophytic and often exhibit ulceration, and eventually lymph nodes and visceral metastasis. Histologically, they correspond to SSM or NM, with a larger proportion of NM compared to human.

Importantly, the different models share the fascinating phenomenon of tumor regression. The regression process has been described in details, both clinically an histologically in MeLiM (Vincent-Naulleau et al., 2004). This corresponds to a spontaneous and complete disappearance of tumors and metastasis, without any treatment. Regression commonly occurs after a few weeks of age, between 2 and 4 months after birth. While the immune system intervention is indisputable, transcriptomic analyses performed in a MeLiM time-course experiment has shown a potential cell cycle arrest of the melanoma cells, occurring before the infiltration of the tumor by lymphocytes (Rambow et al., 2008a,b). The possible involvement of immune checkpoints in swine tumor regression has not been established yet.

Along with this fascinating process, a partial or total depigmentation of skin, hair and iris occurs, starts around the regressing lesions, and eventually propagates to the whole body for the totally depigmented individuals. Some pigs remain "spotted," while others become totally white with blue iris. Misfeldt and Grimm also mention depigmentation in black Sinclair (Misfeldt and Grimm, 1994) and hypothesize that it could reflect an immune response toward melanocytes and melanoma. The nodal and visceral metastases also regress, giving way to fibrotic tissue. Overall, only a 4% mortality rate is observed, likely due to metastatic complications appearing before the regression onset (Vincent-Naulleau et al., 2004).

MELANOMA INHERITANCE IN PIGS

Several studies have been conducted in the three models, with different approaches, and leading to different conclusions. In the MMS Troll, a first model is proposed for the inheritance of flat benign lesions (defined as nevi in this breed), i.e., the influence of one major gene on a polygenic background. For melanoma tumors however, two to three recessive genes may be involved (Müller et al., 2001). In Sinclair, several publications (Tissot et al., 1987; Blangero et al., 1996) describe a model with a major unmapped gene and a specific SLA (Swine Leukocyte Antigen) haplotype, noted as the "B haplotype," or a modifying gene co-segregating with SLA. Later, Ho et al. (2010) have shown that the B haplotype actually corresponds to the SLA1 0201 and 0701 alleles. In MeLiM, a three-genes mode of inheritance was proposed by Hruban et al. (2004). More recently, different approaches were used to decipher melanoma occurrence, considering it as a complex trait, as detailed below for the MeLiM swine.

GENETIC SUSCEPTIBILITY IN THE MeLiM MODEL

Most of the genetic studies performed on the MeLiM model so far rely on an experimental backcross design. Briefly, four affected MeLiM were crossed to five healthy Duroc pigs, to produce an F1 generation. As described before, the Duroc breed presents a very small incidence of melanoma, and animals used were checked for the absence of cutaneous tumors. However, one cannot rule out the possibility that the Duroc pigs used previously harbored small tumors that underwent regression, or that without exhibiting lesions, they still carry predisposing variants that can be transmitted to the progeny. Sick F1 individuals were further backcrossed to Duroc animals to create a first backcross generation of 331 individuals (Geffrotin et al., 2004; Du et al., 2007). This three-generation pedigree was therefore used to test linkage and association of various genes with melanoma development.

Two genome-wide quantitative trait loci (QTL) analysis were performed after microsatellite genotyping of the MeLiM X Duroc cross (Geffrotin et al., 2004; Du et al., 2007). A first observation is that the inheritance of melanoma in this model is complex, likely autosomal dominant with an incomplete penetrance. Despite what is postulated in other models, linkage studies have shown that a 2 to 3-genes model is probably too simple to explain all the extent of the disease. However, the small incidence of melanoma in Duroc herds may add complexity to the results. The first linkage study, using only one MeLiM founder and its backcross progeny ($n = 123$), discovered 4 QTLs, on SSC1, SSC2, SSC7, and SSC8 (Geffrotin et al., 2004). The second QTL analysis used the extended backcross pedigree and focused also on more specific traits, such as clinical ulceration and invasion, or presence of metastasis for example. This linkage study led to the identification of various QTLs, some of which being detected for different phenotypes and thus potentially corresponding to genes with a pleiotropic effect (Du et al., 2007). All these studies were based on microsatellite genotyping. The availability of high-density SNP chips in pig should allow a more accurate detection of genomic regions associated to melanoma development and sub-phenotypes. An integration of genome-wide association results obtained in different melanoma-prone breeds would also be extremely powerful to detect major genes involved in melanoma predisposition in pigs.

On the other hand, a few candidate gene studies have targeted major actors of melanoma susceptibility in human. First, Le Chalony et al. (2003) used newly identified microsatellite markers in and around the *CDKN2A* locus to test the gene for involvement in melanoma susceptibility in the MeLiM pig. Association, linkage and haplotype analyses all excluded *CDKN2A* as predisposing in the progeny of one sick founder. However, the complex QTLs identified by the linkage analysis suggest the presence of one or several susceptibility gene(s) in the vicinity of the microsatellites tested. In humans, among cases linked to the HSA9p21 region (which encloses *CDKN2A*), half are not due to mutations in *CDKN2A*, thus suggesting the presence of another gene in the vicinity. A fine-mapping strategy could be worth setting up on the MeLiM model to try to evidence this other gene. In the first linkage study, Geffrotin et al. (2004) also excluded *CDK4* and *BRAF* as high risk genes, since microsatellites located near the loci did not segregate with melanoma. No linkage signal was observed in the second study for either gene (Du et al., 2007). The second QTL analysis highlighted a QTL on the swine chromosome 13, directly above *MITF* locus. However, subsequent studies showed no association between variants in the gene and melanoma phenotypes (Bourneuf et al., 2011).

Also, the authors showed that the locus was not amplified in tumor samples, contrary to what is seen in humans. Yet, *MITF* expression seems tightly regulated during the course of the disease, showing as in humans, the probable central role of *MITF* in pig melanocyte biology.

A microsatellite located close to *MC1R* was linked to melanoma, even when the analysis was corrected for coat color, proving that pigmentation variation was not the only effect (Du et al., 2007). Two alleles were evidenced in the MeLiM swine: the allele *MC1R* E^{D1} in black animals and the E^{P} allele in red animals. These alleles correspond to the Asian black and the European black spotting alleles, respectively. Despite its name, a wide variety of colors exist for pigs homozygous for the latter allele (Fang et al., 2009). The *MC1R* E^{D1} allele, associated with melanoma in MeLiM pigs, carries a Leu102Pro polymorphism. This mutation is equivalent to the *sombre* mutation in mice, coding for a constitutive MC1R receptor (Robbins et al., 1993). Such a constitutive receptor induces a constant production of cAMP and steady activation of the subsequent signaling cascade. This would explain why a dark color is favorable to melanoma, which is opposite to what is seen in humans, where light phototypes have an increased risk. Interestingly, Tibetan pigs also carry the *MC1R* allele E^{D1} (Liu et al., 2016), but no skin lesion has been described in this breed to our knowledge. This confirms that *MC1R* is not sufficient to promote melanoma development but contributes to an increased penetrance.

In the first linkage study, a significant association was observed between melanoma development and a region on SSC8. The study evidenced that the Duroc alleles were promoting melanoma, when introduced on a MeLiM background. The *KIT* gene is located in this area, and is of utmost importance in pigment cell function as mentioned earlier. Also, *KIT* is mutated in different cancer types, including certain subtypes of melanoma. An association of a SNP in the exon 19 was shown, once again with the Duroc allele promoting melanoma. The exact mechanism explaining this association remains to be elucidated. An association between SLA microsatellites and melanoma was also observed. However, this result has not been confirmed by adding new individuals to the analysis. New methods for accurate genotyping of SLA (PCR-SSP) could be used to determine the specific haplotypes segregating in the MeLiM population and how they could influence melanoma development.

TUMOR GENETICS IN THE MeLiM MODEL

So far, comparative genomic hybridization (CGH) was the only large-scale experiment performed to decipher somatic changes occurring during tumorigenesis in the MeLiM pig (Apiou et al., 2004). In this work, Apiou et al. (2004) laser-microdissected tumor cells, amplified DNA by DOP-PCR and compared it to genomic DNA extracted from pig lymphocytes. Some genomic gains were shared by NM and SSM subtypes of melanoma. However, only nodular melanoma showed a loss of material, located at 13q31-49. This loss was confirmed by I-FISH, which also indicated polyploidy of the tumors. This result is consistent with an hyperploidy that has been observed in cell lines from Sinclair melanoma. However, only a few gains and no loss were detected in both models, but experiments were performed on tissues in MeLiM, and cell lines for Sinclair.

FUTURE DIRECTIONS

To go further in the comparison of the swine model with human melanoma, a more precise exploration of the tumor genome would be needed. A first question to address concerns the mutational burden in these tumors. Genome modifications should be limited since (i) regression systematically occurs, and thus may indicate that tumors do not escape immune surveillance using favorable mutations in specific genes (ii) the environmental UV-signature that is classically encountered in the genome of human cutaneous melanomas should be absent. Acral lentiginous and mucosal melanomas do not carry a genomic UV signature, but rather somatic signatures observed in other cancer types of unknown etiology, and are more subject to structural variants (Hayward et al., 2017). More specifically, the existence of recurrent mutations in oncogenes/tumor suppressor genes frequently found in human tumors should be investigated. In particular, a survey of coding mutations leading to neo-antigens would be a priority, since MeLiM pigs exhibit an efficient antitumor activity, notably through the humoral response. Preliminary studies in the model have shown the presence of anti-melanoma antibodies and the identification of targeted peptides is underway (Blanc et al., 2016). The TCGA network (The Cancer Genome Atlas Network) has now established a classification of cutaneous tumors according to their mutational status for genes BRAF, NRAS and NF1, all known as major actors of tumor initiation for melanoma. Tumors that do not fall into these categories are defined as "triple wild-type" and are more prone to focal amplifications or structural rearrangements (Cancer Genome Atlas Network, 2015). It is thus crucial to determine to which class the MeLiM tumors (and other swine melanomas) could be assigned. Also, the identification of somatic mutations in MeLiM that are similar to recurrent variation in human melanoma could pave the way to pharmacogenomics studies.

The recent CRISPR/Cas9 revolution has completely modified the landscape of genetic modification in many species, including pig. It is now feasible to introduce and target variation in the swine genome and produce quite efficiently modified animals, as shown in pilot studies (Lai et al., 2016). In the frame of the melanoma study, such a tool could have two attractive applications. First, genome modification could help validating a potential causal mutation for melanoma development. Second, CRISPR technology could help investigating the effect of a known mutation in human in a predisposed background and regressing model.

As mentioned previously, telomere function is currently a very active area of research in oncology. *TERT* (telomere reverse transcriptase) and some other components of the telomere maintenance complex have been identified as risk genes for several cancer types, including melanoma. In addition, *TERT*

TABLE 3 | Summary of findings on candidate gene studies in swine melanoma models.

Candidate gene	Model and approach	Results	Reference
Cell cycle			
CDKN2A	MeLiM; linkage, association and haplotype analysis	No linkage/association of the genes with melanoma occurrence	Le Chalony et al., 2003
CDK4	MeLiM, linkage analysis	No linkage with melanoma occurrence	Geffrotin et al., 2004; Du et al., 2007
Pigmentation			
MC1R	MeLiM; association analysis and sequencing	Association of *MC1R*2* allele with melanoma occurrence	Du et al., 2007
MITF	MeLiM; linkage and association analysis, gene expression, somatic status	No linkage/association with melanoma occurrence; changes in gene expression compatible with human melanoma data; no genomic amplification in tumors	Bourneuf et al., 2011
KIT	MeLiM; association analysis	Association with melanoma occurrence	Fernández-Rodríguez et al., 2014
Proliferation			
BRAF	MeLiM; association analysis	No association with melanoma occurrence	Geffrotin et al., 2004
Telomere biology			
TERT	Sinclair; cytogenetics and functional assays	Karyotypic abnormalities, no telomerase activity in tumors	Pathak et al., 2000
Endogenous retroviruses	MMS Troll; cell biology	Increased expression of transcripts in melanoma and metastasis; release of viral particles	Dieckhoff et al., 2007

promoter is very frequently mutated in a wide range of tumors, enhancing *TERT* transcription, and thus favoring the reactivation of the telomerase system (Fredriksson et al., 2014). Among the 115 samples analyzed by the TCGA consortium to establish the genomic classification of cutaneous melanoma, 65% were found with an activating mutation in *TERT* promoter, and 7% with a focal amplification of TERT locus (Cancer Genome Atlas Network, 2015). In the Sinclair swine model, Pathak et al. (2000) evidenced a lack of telomerase activity and an increased number of abnormal karyotypic figures in cell lines derived from tumors. In MeLiM, no study has been conducted concerning telomere length and telomerase activity, however, Apiou et al. (2004) evidenced hyperploïdy in tumors. The absence of reactivation of the telomerase complex during oncogenesis in those pigs could be a factor participating to spontaneous regression. Also, one of the rare tumor types undergoing a spontaneous and complete regression in human, the neuroblastoma 4S, is associated with a range of factors, including an absence of telomerase activity (Brodeur and Bagatell, 2014). In conclusion, given that melanomas from the MeLiM model all regress spontaneously only a few months after the progression starts, it would be interesting to investigate the telomere length, and expression and functionality of the different components of the maintenance complex. A defect in the process could very well be a key to the regression phenomenon.

Finally, more advantage of the MeLiM model should be taken for better understanding the pathway alterations and molecular changes occurring between normal melanocytes and transformed melanoma cells. In addition, such a work could help evidencing new biomarkers to be tested in different species. Indeed, Egidy et al. (2008) explored the gene expression differences between a melanocyte cell line (PigMel) and a primary culture of melanoma lung metastasis from MeliM. This study pointed at *GNB2L1*, coding for RACK1, as a potentially interesting gene in melanoma characterization. Later, this scaffold protein with multiple functions has been described as a good marker for differentiating melanocytoma from melanoma in dog (Campagne et al., 2013) and horse (Campagne et al., 2012). More recently, Campagne et al. (2017) showed that RACK1 could cooperate with $NRAS^{Q61K}$ mutation to accelerate melanoma onset and metastasis formation in transgenic mice. This example illustrates the usefulness of such a model to describe the role of pivotal molecules in specific pathologies.

STUDY OF ENDOGENOUS RETROVIRUSES IN SWINE MELANOMA

Recent reports have focused on human endogenous retroviruses (particularly of subtype HERV-K) and tumor biology. Endogenous retroviruses represent around 8% of the human genome, and correspond to an ancient integration of viral DNA in the germline genome. Several studies have shown a re-activation of HERV-K in various solid tumors, including melanoma (reviewed in Downey et al., 2015). HERV-K transcripts and proteins have been observed in melanoma cell lines and tissue (Buscher et al., 2006), but are absent from normal human melanocytes (Serafino et al., 2009). Current studies aim at better describing the regulation of expression of the different ERV transcripts, and their interaction with melanomagenesis. Notably, a correlation between HERV-K expression and the MEK-ERK and p16INK4A/CDK4 pathways was found (Li et al., 2010), and MITF-M was found to regulate the transcription of HERV-K LTR sequences (Katoh et al., 2011).

More recently, Lemaitre et al. (2017) showed that HERV-K (HML-2) could even induce the ERK1/2 pathway *in vitro*, through upregulation of several transcription actors. The global hypomethylation of the genome observed in many tumors is likely to explain the reactivation of HERV transcription, usually silenced by CpG methylation of the LTRs (Stengel et al., 2010). Among the possible oncogenic mechanisms, HERV-K could participate to cell transformation by insertional mutagenesis, through its rec and np9 oncogenic proteins, or even by modulating the immune response (Gonzalez-Cao et al., 2016). However, even if HERVs are released as particles (which is rarely the case), they remain non-infectious (Denner, 2016). Nevertheless, many studies have identified a modulation of adaptive immune response mediated by ERV reactivation (Downey et al., 2015). In recent reports, the HERV-K env protein has been used as chimeric antigen receptor (CAR) expressed by T cells, therefore capable of inducing a promising anti-tumor response in murine models of melanoma (Krishnamurthy et al., 2015) or breast cancer (Zhou et al., 2015).

The existence of porcine endogenous retroviruses (PERVs) in the pig genome is one of the main barriers to pig-to-human xenotransplantation (Groenen et al., 2012). Indeed, even if most of PERVs are considered as defective, a risk of re-activation remains. For example, a higher transmission of PERV has been observed in mice xenografted with PERV-producing cells, in particular under an immunosuppressant treatment. Also, this PERV infection was correlated with a decrease of T cells proportion, especially CD4+ subset (Kim et al., 2016). In addition, SLC52A1 and SLC52A2 molecules are described as receptors for PERV-A particles on human cells *in vitro* (Colon-Moran et al., 2017), providing a demonstration of a possible infection of human cells by PERVs. In order to circumvent this infectivity, Yang et al. (2015) recently described the creation of a model of pigs where 62 PERVs have been knocked-out by the CRISPR-Cas9 technology. This work illustrates a renewed interest for heterologous transplantation, enabled by new methodologies. The existence and expression of PERVs were investigated in MMS swine (Dieckhoff et al., 2007). The authors found an enhanced expression of PERV transcripts in melanoma and metastasis compared to normal skin, as well as a release of viral particles in metastasis-derived cell cultures. Whether transcriptional activation of endogenous retroviruses in porcine melanoma is an initiator or a consequence of malignant transformation remains to be determined. Overall, swine models recapitulating tumor progression, an efficient immune response and a genome containing many active endogenous retroviruses could thus be of great help to decipher such intricate mechanisms.

CONCLUSION

Swine models of melanoma exhibit several common features with human disease. Clinical and histopathological studies have shown a range of lesions that are comparable to different subtypes in human. Also, tumor heterogeneity has been observed at cellular and molecular levels. Genetically, pig melanoma is a complex trait with incomplete penetrance, and although high-risk genes remain to be discovered, *MC1R* has been involved beyond pigmentary phenotypes (**Table 3**). Efforts have to be pursued in order to fine-map already evidenced QTLs.

Of course, differences exist between the two species. One of the main discrepancies is the early age of onset of melanoma in pigs. While in human, most of tumors are caused by UV radiations and appear late in life, pigs are not exposed to sunlight and most tumors have a prenatal origin. The swine model can therefore help deciphering molecular mechanisms leading to melanocyte transformation independently of UV radiation. A survey of swine tumor genome variation may reveal recurrent mutations worth investigating in. Similarly, sampling and sequencing several lesions and metastasis from a same individual may illustrate precisely the heterogeneity and clonal origin of melanoma cells. Also, spontaneously developing and regressing porcine tumors could represent a valuable tool to study complex interactions between endogenous retroviruses, oncogenesis and adaptive immune response.

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The author confirms being the sole contributor of this review and approved it for publication.

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Establishing the pig as a large animal model for vaccine development against human cancer

Nana H. Overgaard[1], Thomas M. Frøsig[1], Simon Welner[1], Michael Rasmussen[2], Mette Ilsøe[1], Maria R. Sørensen[1], Mads H. Andersen[3], Søren Buus[2] and Gregers Jungersen[1]*

[1] Department of Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark, [2] Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, [3] Center for Cancer Immune Therapy, Department of Hematology, Copenhagen University Hospital, Herlev, Denmark

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Tiago Collares, Federal University of Pelotas, Brazil

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Christina Stuelten, National Cancer Institute, USA
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***Correspondence:**
Thomas M. Frøsig, Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, Building B, 1870 Frederiksberg C, Denmark
thomfr@vet.dtu.dk

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Immunotherapy has increased overall survival of metastatic cancer patients, and cancer antigens are promising vaccine targets. To fulfill the promise, appropriate tailoring of the vaccine formulations to mount *in vivo* cytotoxic T cell (CTL) responses toward co-delivered cancer antigens is essential. Previous development of therapeutic cancer vaccines has largely been based on studies in mice, and the majority of these candidate vaccines failed to induce therapeutic responses in the subsequent human clinical trials. Given that antigen dose and vaccine volume in pigs are translatable to humans and the porcine immunome is closer related to the human counterpart, we here introduce pigs as a supplementary large animal model for human cancer vaccine development. IDO and RhoC, both important in human cancer development and progression, were used as vaccine targets and 12 pigs were immunized with overlapping 20mer peptides spanning the entire porcine IDO and RhoC sequences formulated in CTL-inducing adjuvants: CAF09, CASAC, Montanide ISA 51 VG, or PBS. Taking advantage of recombinant swine MHC class I molecules (SLAs), the peptide-SLA complex stability was measured for 198 IDO- or RhoC-derived 9-11mer peptides predicted to bind to SLA-1*04:01, −1*07:02, −2*04:01, −2*05:02, and/or −3*04:01. This identified 89 stable ($t_{½} \geq 0.5$ h) peptide-SLA complexes. By IFN-γ release in PBMC cultures we monitored the vaccine-induced peptide-specific CTL responses, and found responses to both IDO- and RhoC-derived peptides across all groups with no adjuvant being superior. These findings support the further use of pigs as a large animal model for vaccine development against human cancer.

Keywords: immune therapy, cancer vaccines, cytotoxic T cells, animal model, peptide-MHC stability, adjuvants, immunologic

Introduction

Therapeutic anti-cancer vaccines are expected to be important in the future immunotherapeutic treatment of cancer, either alone or in combination with, e.g., administration of drugs targeting the checkpoint inhibitors cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) (Hodi et al., 2010; Brahmer et al., 2012; Topalian et al., 2012; Hamid et al., 2013; Wolchok et al., 2013). Previously, vaccines have mainly been comprised of full proteins; however especially in terms of safety, peptide-based vaccines are preferable, as reviewed in Purcell et al. (2007). Many cancer-associated targets have been described (Cheever et al., 2009; Andersen et al., 2012), and peptide vaccinations have previously generated significant immune responses, although these only rarely correlated with the clinical outcome (Dalgleish and Whelan, 2006; Becker et al., 2012; Inderberg-Suso et al., 2012). One limitation to peptide vaccination is that peptides in general generate weaker responses *in vivo* as compared to full protein, and the immunogenicity of peptides alone is not sufficient to generate a strong immune response; therefore adjuvant systems are included to enhance such response in order to increase the likelihood of a clinical effect. Numerous adjuvant systems have proved the ability to strongly stimulate the unspecific immune system in a cancer setting (Butterfield, 2015) hence supporting the need for studies identifying optimal Th_1-inducing adjuvants in combination with cancer antigens.

Various mice models are currently the golden standard for early pre-clinical studies, even though important differences in terms of immunology and physiology between mice and humans exist. It is now well established that "mice lie" and recent studies have shown the pig immunome to be much more similar to the human counterpart (Dawson et al., 2013; Seok et al., 2013); pigs must therefore be considered a highly relevant supplementary model when studying human immune activation. Furthermore, the difference in body size and metabolism makes studies on the dose effect of adjuvants and peptides impossible to extrapolate from mice studies to human vaccine formulation. Relating specifically to cancer, six genetic defects are required for converting both normal porcine and human cells to their cancerous counterparts (Hahn et al., 1999; Adam et al., 2007), while only two mutations are required to convert a mouse cell (Rangarajan et al., 2004). Over the last decade the toolbox of swine immunological reagents has expanded considerably, which contributes further to the usefulness of pigs as a relevant model for human diseases (Meurens et al., 2012). In this study we introduce outbred pigs as a large animal model for human cancer vaccine development.

The immune response to cancer is complex and responses can either be in favor or disfavor of cancer development and progression. Major players in the anti-cancer immune response are the $CD8^+$ cytotoxic T lymphocytes (CTLs), which specifically recognize peptides derived from cancer-specific or over-expressed proteins when presented by the Major Histocompatibility Complex (MHC) class I molecules on the surface of the transformed cells or cross-presented by dendritic cells. Mounting strong and effective CTL responses against such peptide-MHC complexes is thus a goal of vaccine development against cancer. Previous work has shown indoleamine 2,3-dioxygenase (IDO) and Ras homolog gene family member C (RhoC) to be promising antigen targets for inclusion in vaccines against multiple cancer forms (Wenandy et al., 2008; Sørensen et al., 2009). To investigate whether it is possible to mount immune responses toward the above mentioned cancer antigens, we immunized 12 healthy outbred pigs holding the swine leukocyte antigen (SLA)-1*04:01, SLA-3*04:01, SLA-1*07:02, and/or SLA-2*05:02 MHC class I alleles with 20mer overlapping peptides spanning the entire sequence of IDO and RhoC. The pigs were divided in four adjuvant groups receiving the 20mer peptide library formulated in either poly(I:C) decorated dimethyldioctadecylammonium (DDA)/monomycoloyl glycerol (MMG) cationic liposomes referred to as the cationic adjuvant formulation (CAF)09 (Korsholm et al., 2014), a porcine/human modification of the combined adjuvant for synergistic activation of cellular immunity (CASAC) containing CpG, monophosphoryl lipid A (MPL), IFN-γ, CD40 ligand (CD40L), and CD40L enhancer in an oil/water formulation (Wells et al., 2008), Montanide ISA 51 VG water/oil (Iversen et al., 2014) (hereafter referred to as ISA 51 VG) or phosphate buffered saline (PBS). These adjuvants were chosen based on their previous ability to mount CTL responses in mouse and/or man. CAF09 has shown promising results in a mouse tumor model, where it generated responses to multiple antigens in parallel (Korsholm et al., 2014) and has previously been used several times in pigs (data not published). CASAC has shown very promising results in mice, where it generated high numbers of antigen-specific $CD8^+$ T cells (Wells et al., 2008). Among numerous human cancer studies, ISA 51 VG has together with a short IDO-derived peptide been shown to induce clinical responses in human metastatic lung cancer patients (Iversen et al., 2014) and has been used in various other clinical trials for cancer treatment (Tsuji et al., 2013; Lennerz et al., 2014).

In order to compare the vaccine-induced antigen-specific $CD8^+$ CTL response toward IDO and RhoC between the adjuvant groups, we predicted 198 9-11mer ligands by use of the NetMHCcons prediction server (Karosiene et al., 2012) in combination with the Position Scanning Combinatorial Peptide Library (PSCPL) method (as exemplified in Pedersen et al., 2011). Out of these, a total of 89 stable peptide-MHC complexes were subsequently identified using *in vitro* stability measurements. Pigs were blood sampled at various time points before and after immunizations and the IFN-γ responses following ∼70 h of peripheral blood mononuclear cell (PBMC)-peptide co-culture suggested generation of CTL responses to cancer antigens following peptide immunization. All adjuvants were capable of generating some CTL responses although none of the adjuvants was found to be superior. Taken together this first vaccine

Abbreviations: CAF09, Cationic Adjuvant Formulation 09; CASAC, Combined Adjuvant for Synergistic Activation of Cellular Immunity; CTL, Cytotoxic T cell; IDO, Indoleamine 2,3-dioxygenase; ISA 51 VG, Montanide ISA 51 VG; MHC, Major Histocompatibility Complex; PBS, Phosphate Buffered Saline; PBMC, Peripheral Blood Mononuclear Cell; RhoC, Ras homolog gene family member C; SEB, Staphylococcal Enterotoxin B; SLA, Swine Leukocyte Antigen; SPA, Scintillation Proximity Assay.

trial supports the use of pigs as a large animal model for human anti-cancer vaccine development. Stronger and more consistent responses are, however, warranted indicating the relevance of further studies on adjuvant and peptide dose, number of immunizations and more detailed characterization of the immunological response profile.

Materials and Methods

Animals

Outbred Danish Landrace/Yorkshire/Duroc pigs were obtained from a Danish production farm (Askelygaard, Roskilde, Denmark). Upon arrival to the National Veterinary Institute, the pigs were housed in groups of six animals using straw as bedding material with water freely available and food supplied once a day. No additional environmental enrichment was provided. All procedures of animal handling and experimentation were internally and externally approved by the institutional committee and the Danish Animal Experiments' Inspectorate, respectively.

Blood Sampling

Blood samples from pigs were collected at day −35, −9, −2, 12, 33, 40, and day 54. The PBMCs were isolated using Lymphoprep gradient separation in SepMate tubes (both from Stemcell Technologies, Grenoble, France) from blood samples obtained at day −2, 12, 33, 40, and 54 and used directly in the IFN-γ release assay.

SLA-typing of Candidate Pigs

Five weeks old, non-sex matched pigs were blood sampled and SLA-typed prior to purchase. Sanger sequencing based SLA-typing: Genomic DNA was extracted from blood samples obtained at day -35 using DNeasy® Blood & Tissue Kit (Qiagen, Cat. No. 69504) according to the manufacturer's instructions. PCR with sequence specific primers and subsequent sequencing of the positive amplicons (by Eurofins, Ebersberg, Germany) allowed for detection of the alleles SLA-1*04:01, SLA-1*07:02, SLA-2*04:01, SLA-2*05:02, and SLA-3*04:01 as previously described (Pedersen et al., 2014b).

NGS-based SLA-typing and Expression Analysis: To confirm the presence of the SLA class I genes found by the previously described SLA-typing, we used next generation sequencing of PCR amplicons spanning exon 2 and 3 of SLA class I genes, which also allowed for expression analysis of the transcripts. RNA from blood samples obtained at day -9 was purified using PAXgene Blood RNA Kit (PreAnalytiX, Cat. No. 762174) according to the manufacturer's instructions. After enzymatic digestion of genomic DNA, the RNA was transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Cat.No.205311). The cDNA was used as a template in a PCR with barcoded primers designed in conserved areas of the exon 2 and 3 of all known SLA class I genes. After sequencing on the MiSeq™ 250PE platform (The National High-throughput DNA Sequencing Centre, University of Copenhagen, Denmark) the sequences were de-multiplexed, pair mate joined, quality checked and sorted into clusters showing the expression levels of each allele (Ilsøe et al., manuscript in preparation). This was followed by alignment against a library containing all previously described SLA class I alleles to determine the allele identity.

20mer Overlapping Peptide Library for Immunization

Fifty-nine 20mer peptides with 10mer overlap covering the entire IDO and RhoC amino acid (aa) sequence were purchased from Genscript (New Jersey, U.S) or Pepscan Presto BV (Lelystad, the Netherlands). Due to dissolving problems, peptide $IDO_{330-350}$ was omitted and only 58 peptides were included in the immunization protocol (**Table 2**). Peptides were dissolved to a concentration of 5 mM in milliQ water, N-methyl-2-pyrrolidone or 3% ammonia water in accordance with the supplier's recommendations, for further details see Supplementary Table 1.

Immunizations

Based on their SLA profile 12 animals (12 weeks old) were divided in four groups each containing three pigs, hence maximizing the MHC class I allelic coverage in each group. The animals were primed at day 0 and boosted at day 19 with the full 20mer overlapping peptide library in combination with either an adjuvant system or PBS. Each pig received 50 μg for priming and 25 μg for boosting of each peptide with the exception of certain peptides (Supplementary Table 1). The CAF09 adjuvant (Korsholm et al., 2014) was a generous gift from Dennis Christensen at the State Serum Institute, Copenhagen, Denmark and vaccine doses for this group of animals were formulated by gentle mixing of 1 ml peptide library diluted in 10 mM Tris buffer with 1 ml CAF09. A porcine/human modification of CASAC (Wells et al., 2008) was prepared with the MegaCD40L® [1 μg recombinant human CD154 and 2 μg CD154 enhancer (Enzo Life Sciences, NY, U.S.)], 500 μg CpG ODN2007 (ODN 2007 Class B CpG oligonucleotide—bovine/porcine TLR9 ligand, InvivoGen, CA, U.S.) and 1 μg recombinant porcine IFN-γ (R&D Systems, UK) all formulated in PBS and mixed with peptides in a total volume of 1 ml followed by gentle mixing with 1 ml of Sigma adjuvant (Sigma Adjuvant System, Sigma Aldrich, Missouri, U.S.). Montanide ISA 51 VG (Seppic, Puteaux, France) vaccines were prepared by thorough mixing of 1 ml peptide library formulated in PBS and 1 ml adjuvant through an i-connector according to manufacturer's instructions. Finally, a vaccine formulated in PBS was produced with suspension of the peptide library in PBS only. All vaccines were formulated in a total volume of 2 ml and administered subcutaneously into the flank except for ISA 51 which was administered intramuscularly. Priming and boosting were both administered into the left side of the animals. Pigs were monitored for 5 days following priming and boosting, and all animals remained healthy following both injections.

9-11mer peptide Library for Immune Monitoring

Porcine IDO and RhoC aa sequences were obtained from the Uniprot database (http://www.uniprot.org/uniprot/F6K2E8 and http://www.uniprot.org/uniprot/F2Z5K4). Using PSCPL (Stryhn et al., 1996) and the NetMHCcons1.1 server (Karosiene et al., 2012) we identified 198 9-11mer peptides predicted to bind

to at least one of the five in-house SLAs (SLA-1*04:01, SLA-1*07:02, SLA-2*04:01, SLA-2*05:02, and SLA-3*04:01) with a rank score $\leq$ 2 % by at least one of the prediction methods. These were synthesized via Fmoc-based chemistry and purchased from Pepscan Presto BV (Lelystad, the Netherlands). For further information see Supplementary Table 2. These peptides were referred to as IDO1-IDO136 and RhoC1-RhoC62.

Test of Peptide-MHC Complex Stability

The stability of the selected 198 peptides in complex with relevant SLA class I molecules and β_2m was determined using a scintillation proximity assay (SPA), as previously described (Harndahl et al., 2011). Briefly, biotinylated recombinant MHC class I heavy chains were attached to a streptavidin-coated scintillation microplate together with iodinated (^{125}I) β_2m and candidate peptide resulting in a scintillation signal, which was consecutively measured every 40 min by a scintillation plate counter. The duration of this signal is directly correlated to the stability of the peptide-MHC class I-β_2m complex under dissociating conditions and in the presence of excess unlabeled β_2m (Harndahl et al., 2011). Peptides with a half-life $\geq$ 0.5 h were selected as stable binders.

In vitro Peptide Stimulation of Porcine PBMCs

In a 96 well plate, 2×10^5 PBMCs/well were individually cultured with 5 μg/ml of each of the 80 peptides previously found to form a total of 89 stable complexes with SLA class I heavy chain and β_2m. Cells co-cultured with Staphylococcal Enterotoxin B (SEB) (1 μg/ml) and media alone (RPMI 1640 (Gibco, Life Technologies) supplemented with 10 % fetal calf serum) were used as positive and negative controls, respectively. Cells were cultured for 67.5–70.0 h and the supernatant was harvested, frozen at −20°C and subsequently analyzed for IFN-γ release by an ELISA method.

IFN-γ Release Assay

Quantification of IFN-γ in the supernatant from cells stimulated with 9-11mer peptides was carried out in a monoclonal ELISA as previously described (Riber et al., 2011) except that the plates were developed for 1–30 min with tetramethylbenzidine (Kem-En-Tec, Taastrup, Denmark) at RT. The absorbance at 450 nm was determined using a microplate reader (Thermo Scientific) and corrected for unspecific background by subtraction of the signal at 650 nm. The detection limit was established as 8.8 pg/ml and all measurements below this limit was set at 8.8 pg/ml for further calculations. A vaccine-induced response was defined as the increase from pre- to post-vaccination after subtraction of the background IFN-γ from media control cultures without added peptides. Samples from three pigs (numbers 2033, 2045, and 2107) were excluded from the analysis as negative control media cultures had high non-specific background with IFN-γ levels above 20 pg/ml. All positive control SEB cultures were above the 70 pg/ml cut-off.

Statistics

Due to the low number of animals, statistical analyses between different adjuvants are not meaningful and no statistical analyses to prove significant differences were attempted.

Results

Previous studies have confirmed the involvement of CD8$^+$ T cells in anti-cancer immune reactivity (Klebanoff et al., 2005; Sørensen et al., 2011b; Andersen, 2012; Joyce and Fearon, 2015; Rosenberg and Restifo, 2015) and anti-cancer vaccines are generally administered with the aim of enhancing this antigen- specific T-cell reactivity. To establish the pig as a large animal model for human cancer vaccine development, we constructed a monitoring platform for vaccine-induced T-cell reactivity. First, candidate pigs were blood sampled and SLA-typed in order to choose animals holding the relevant SLA class I molecules (**Figure 1**). Second, identification of proteins relevant for vaccination and prediction of candidate CD8$^+$ T cell epitopes from the full protein sequence were carried out. The *in vitro* stability of the candidate T cell epitopes in complex with relevant SLA class I molecule was then examined. Pigs were immunized with 20mer overlapping peptides and blood sampled at various time points pre- and post-immunization in order to monitor the T-cell reactivity *ex vivo* (**Figure 1**). To increase the knowledge obtained from this study, we stratified the pigs in four groups based on their SLA-profile and immunized each group with peptides in combination with an adjuvant system or PBS.

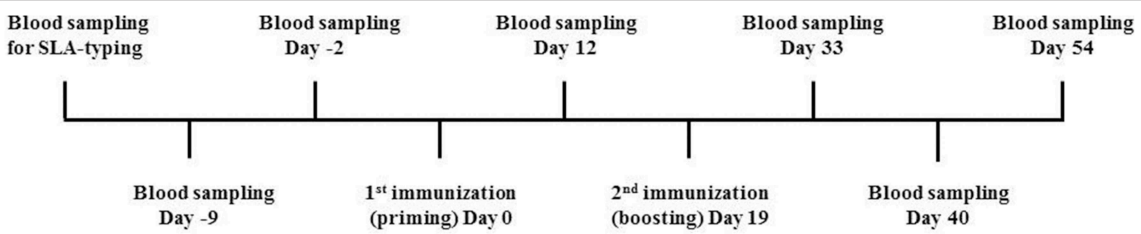

FIGURE 1 | Overview of the immunization strategy. Prior to initiation of the vaccine trial, candidate pigs were blood sampled and SLA-typed in order to select animals holding one or more of the following SLA alleles: SLA-1*04:01, SLA-1*07:02, SLA-2*04:01, SLA-2*05:02, and SLA-3*04:01. Pigs were then purchased and blood sampled at day −2 to determine the background level of IFN-γ. At day 0, pigs were primed with 58 20mer overlapping peptides in combination with either CAF09, CASAC, ISA 51VG or PBS. Blood samples were obtained at day 12, and all pigs were then boosted with another round of immunization at day 19. Blood samples were obtained three times following boost namely at day 33, 40, and 54.

SLA Class I Typing

We obtained blood from 24 animals and probed them for the presence of SLA-1*04:01, SLA-2*04:01, SLA-1*07:02, SLA-2*05:02, and SLA-3*04:01 by Sanger sequencing (**Table 1** and data not shown) corresponding to our in-house recombinant SLA library. Twelve pigs were positive for at least one of the desired alleles and further NGS-based SLA-typing, which also included expression analysis on RNA samples from these animals, confirmed part of these along with the identification of a few more expressed SLA molecules (**Table 1**). SLA-3*04:01 was found in nine of the pigs (75%) and seven pigs (58%) showed expression of SLA-1*04:01 (**Table 1**). SLA-1*07:02 was found in 50% of the animals, whereas SLA-2*05:02 and SLA-2*04:01 were found in only one (8%) and none of the pigs, respectively (**Table 1**).

Analysis of Peptide-MHC Class I Binding Stability

In order to determine peptides capable of forming stable ($t_{1/2} \geq 0.5$ h) complexes with the relevant SLA amongst the 198 peptides, the half-life of the peptide-MHC class I binding was determined for each of the 244 predicted peptide-SLA complexes using SPA analysis. For the widely distributed SLA-1*04:01, a total of 12 IDO and RhoC-derived peptides formed stable complexes with this SLA molecule (**Figure 2A**), and especially IDO122 showed very high binding stability ($t_{1/2} = 30.2$ h, **Figure 2B**). Also, 26 peptides formed stable complexes with the other well-distributed SLA-type amongst the pigs, namely SLA-1*07:02 (**Figure 2A**). Here, especially IDO21 ($t_{1/2} = 10.1$ h) and IDO88 ($t_{1/2} = 24.0$ h) were found to form highly stable complexes (**Figure 2B**). Strikingly, only five peptides were found to form stable complexes with SLA-3*04:01 in the SPA analysis (**Figure 2A**), and IDO105 was the only peptide being able to form a complex with a half-life longer than 1 h with this SLA molecule ($t_{1/2} = 14.7$ h) (**Figure 2B**). A total of 24 peptides were shown to form stable complexes with SLA-2*05:02 (**Figure 2A**) with IDO16 ($t_{1/2} = 12.5$ h) forming the most stable complex. IDO21 and IDO29 were both found to form stable complexes with SLA-2*04:01 exhibiting half-lives of 23.8 and 33.6 h, respectively (**Figure 2B**), and additional 20 peptides also formed stable complexes with SLA-2*04:01. To sum up, the SPA analysis revealed 89 stable peptide-SLA complexes (80 different peptides) from the 244 predicted high-affinity complexes (198 different peptides), and these were used in subsequent analyses of the $CD8^+$ T-cell reactivity.

TABLE 1 | SLA-profile of pigs included in the immunization trial.

Adjuvant	Pig	SLA-1*04:01	SLA-1*07:02	SLA-2*05:02	SLA-3*04:01
CAF09	2107				●
	2035	▲	●		■
	2038		■		
CASAC	2034	▲			■
	2037	▲	●		■
	2042		■		
ISA 51 VG	2046	▲	●		■
	2041	▲		▲	■
	2043	▲			■
PBS	2033				●
	2045	▲			■
	2040		●		

*SLA-typing of animals included in the immunization trial. Animals were blood sampled and tested for the presence of SLA-1*04:01, SLA-1*07:02, SLA-2*04:01, SLA-2*05:02, and SLA-3*04:01 at both the DNA and mRNA level. ●, determined by Sanger sequencing-based SLA-typing; ▲, determined by NGS-based SLA-typing and expression analysis; ■, determined by both methods. None of the animals were positive for SLA-2*04:01 hence this molecule was left out of the table. The Sanger sequencing-based SLA-typing was unsuccessful for SLA-1*04:01.*

IFN-γ Responses

To monitor the induction of specific T cell populations, co-cultures with peptide and PBMCs obtained at different time points prior to and after the immunizations were analyzed for IFN-γ release after ∼70 h. From the vaccine-induced responses, a biologically relevant IFN-γ response following peptide co-culture was defined as a 2-fold increase (stimulation index = 2) as compared to pre-immunization (day -2) and with a concentration of 25 pg/ml or more as depicted by the threshold lines (**Figure 2**). Three pigs were excluded from the analysis due to high non-specific background. For each animal, the peptide responses were divided into two groups based on the measured ability to form stable complexes with the SLA molecules found in the SPA analysis (**Table 1**, **Figure 2**). In general, we found responses in all pigs to both stable and non-stable binders (**Figure 3**, **Table 2**, Supplementary Table 2). Despite similar SLA profiles the pigs did not respond to the same peptides (**Table 2**). When comparing the total number of responses of all animals per adjuvant group, CASAC was shown to be slightly superior especially at day 12 after the first immunization; however in general the adjuvant systems performed similarly (**Figure 4A**). Comparison of the average IFN-γ production (total amount of IFN-γ divided by total number of responses) also revealed that the adjuvants induced a similar level of this cytokine (**Figure 4B**). Since the stability obtained in the SPA analysis has been used as a determinant for selecting the ligands most likely to be $CD8^+$ T cell epitopes, comparisons of the peptide-MHC class I half-lives and IFN-γ production (**Figure 4C**) as well as stimulation index (**Figure 4D**) were carried out. Surprisingly, no significant correlations could be drawn from this analysis. More data points are needed, especially for complexes with long half-lives, to fully determine this, but our findings apparently do not support the idea of stably binding peptides being more immunogenic as shown by Harndahl et al. (2012). It should be noted, though, that this study was performed with viral T cell epitopes. As shown in Supplementary Table 2 we find responses against the vast majority of stably binding peptides, however, for a significant part of these there was a mismatch between the predicted SLA restriction and the observed responses.

Discussion

To measure antigen-specific $CD8^+$ T cell responses, knowledge of the MHC class I alleles present in each individual is necessary. SLA-3*04:01 was found to be the most widely distributed of

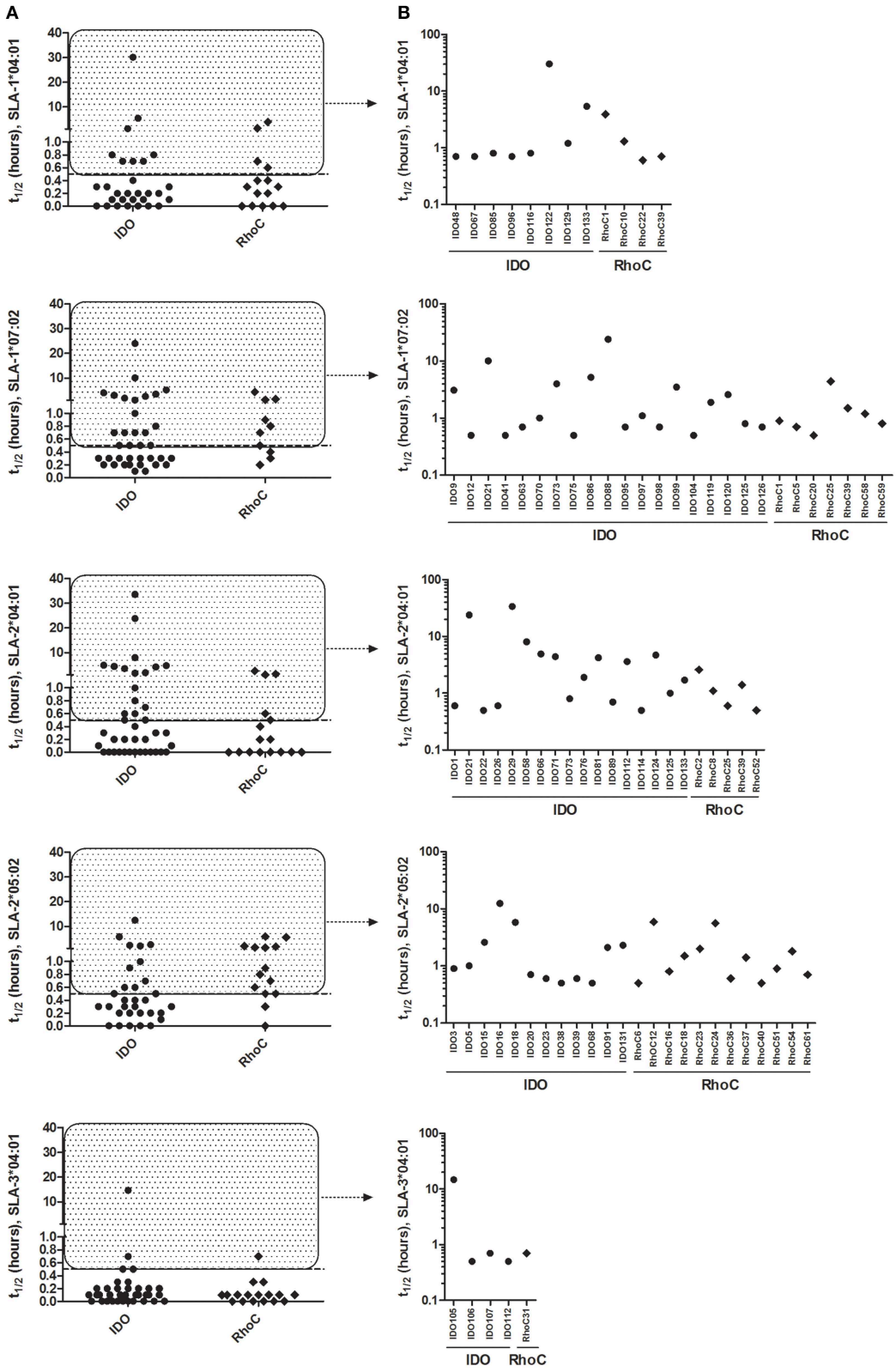

FIGURE 2 | Peptide-MHC class I binding stability as determined by SPA analysis. Stability of the predicted peptides in the 9-11mer peptide library with relevant SLA molecules measured by the Scintillation Proximity Assay. **(A)** SPA-determined half-life of peptides predicted as binders for each SLA molecule. **(B)** Individual peptides with SPA-determined half-life $\geq$ 0.5 h (stably binding) for each SLA molecule. Peptides derived from IDO and RhoC are shown in circles and diamonds, respectively.

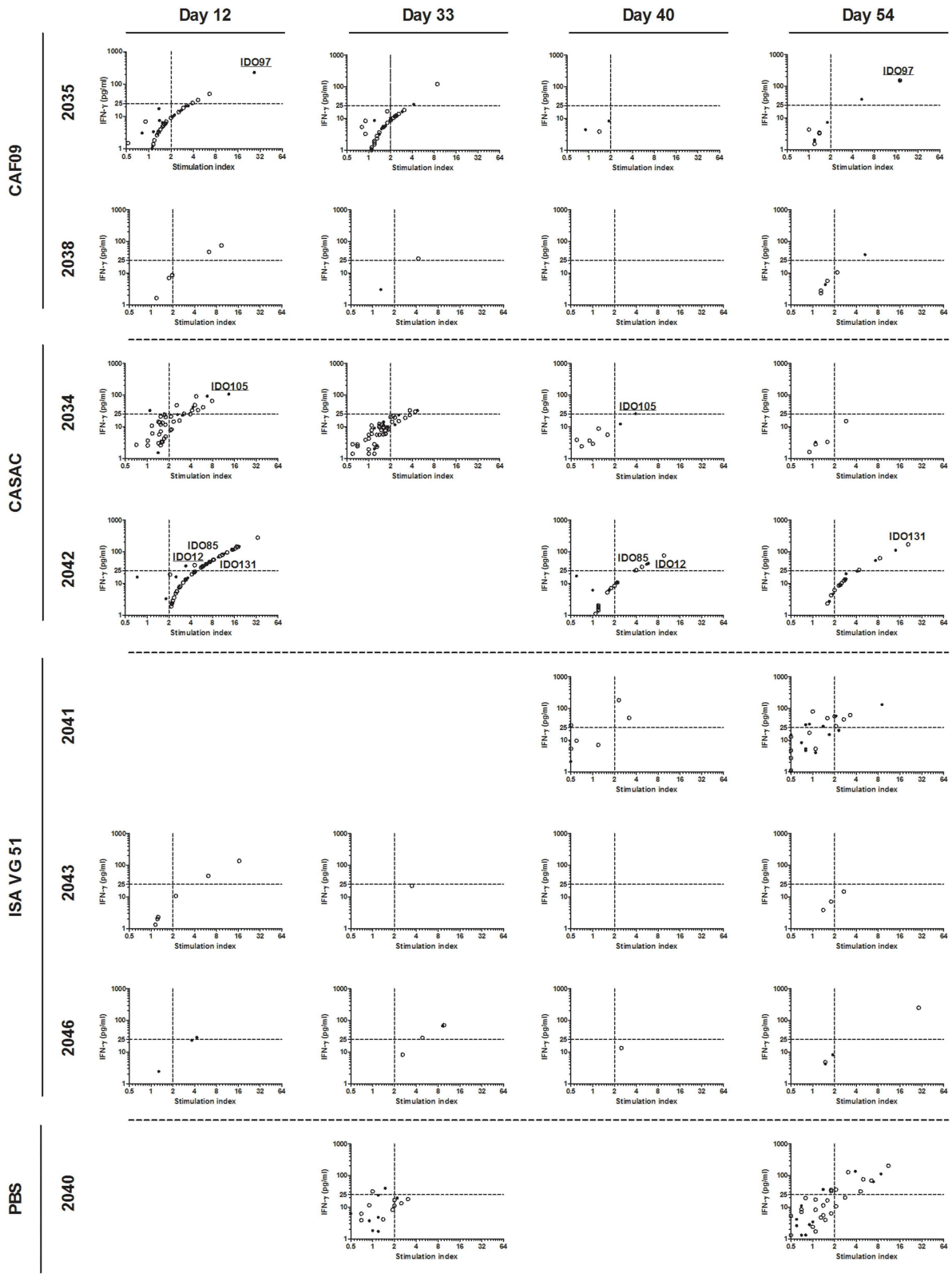

FIGURE 3 | IFN-γ responses to the stable binders in the 9-11mer peptide library. PBMCs purified from immunized pigs at day −2, 12, 33, 40, and 54 were stimulated with 80 peptides found to be stably binding to one or more of SLA-1*04:01, SLA-1*07:02, SLA-2*04:01, SLA-2*05:02, and SLA-3*04:01. A biological

(Continued)

FIGURE 3 | Continued
relevant vaccine-induced response was defined as a 2-fold increase as compared to day −2 (dashed line, x-axis) and a concentration of IFN-γ equaling at least 25 pg/ml (dashed line, y-axis). For each animal, peptides were divided into two groups: stable (filled circles) and non-stable (open circles) referring to the binding stability of a given peptide correlated with the SLA-profile of each pig. Responses occurring repeatedly in individual animals are highlighted by peptide name with stably binding peptides being underlined. Animals with no responses at any time point, and time points for included animals with data not fulfilling the quality parameters were left out of the analyses.

TABLE 2 | IFN-γ responses following co-culture with the 9-11mer peptide library.

SLA molecule	Pig	Adjuvant	Total number of responses				Stable binders			
			Day 12	Day 33	Day 40	Day 54	Day 12	Day 33	Day 40	Day 54
1*04:01 3*04:01	**2034**	CASAC	11	3	1	0	3(27.2%)	1(33.3%)	1(100.0%)	–
	2043	ISA 51 VG	2	0	0	0	0(0.0%)	–	–	–
1*07:02	**2038**	CAF09	2	1	0	1	0(0.0%)	0(0.0%)	–	1(100.0%)
	2042	CASAC	31	–	6	6	9(29.0%)	–	3(50.0%)	3(50.0%)
	2040	PBS	–	0	–	9	–	–	–	3(33.3%)
1*04:01 1*07:02 3*04:01	**2035**	CAF09	4	2	0	3	1(25%)	1(50.0%)	–	2(66.6%)
	2046	ISA 51 VG	1	3	0	0	1(100.0%)	1(33.3%)	–	–
1*04:01 2*05:02 3*04:01	**2041**	ISA 51 VG	–	–	2	6	–	–	0(0.0%)	2(33.3%)

Summarized responses from PBMCs co-cultured with the 9-11mer peptide library. Pigs are divided into four groups based on their SLA-profile. Adjuvant indicates immunization strategy, i.e., CAF09, CASAC, ISA 51 VG, or PBS injected animals. The total number of responses at day 12, 33, 40, and 54 are shown (column 4–7) as well as the number of responses generated from co-culture with a peptide shown to stably bind the given SLA molecule (column 8–11). The percentage of responses generated from these stable binders is shown in brackets for each time point. Animals with no responses at any time point are left out of the analysis. "–" indicates that the quality control thresholds of the assay were not fulfilled and therefore these data were left out of the analyses.

the five in-house MHC class I alleles; however the SLA-3*04:01 molecule has previously been shown to be unstable, most likely due to the presence of only one anchor position (Pedersen et al. manuscript in preparation), and NGS analysis revealed a significantly lower SLA-3*04:01 expression level compared to the expression of the SLA-1 and SLA-2 molecules (data not shown). The pigs were divided into the adjuvant groups based on their SLA-profile to stratify the study as much as possible and provide a similar possibility of measuring antigen-specific T-cell responses in all adjuvant groups.

To identify MHC class I ligands, we predicted binders (9-11mer peptides) from the full length sequences of IDO and RhoC to SLA-1*04:01, SLA-1*07:02, SLA-2*04:01, SLA-2*05:02, and SLA-3*04:01 by the combined use of NetMHCcons and PSCPL. We selected peptides in the upper 2 % rank by either of the methods. This ranking is a measure of the possibility that a random peptide would be a better binder to the relevant MHC complex. The resulting 136 and 62 predicted binders from IDO and RhoC, respectively, were purchased and investigated further (Supplementary Table 1). Some of the peptides ranked ≤2 % on more than one allele. The immunogenicity of a peptide has previously been linked to the affinity of its binding to MHC class I (Sette et al., 1994). However, it has recently been suggested that the stability of the peptide-MHC class I binding is a more accurate measure of the peptide's immunogenicity (Harndahl et al., 2012). Along with this note, the NetMHCstab server has recently been established (Jørgensen et al., 2014), but unfortunately this predictor does so far only work for a few human MHC class I molecules. Importantly, it has not previously been possible to measure the peptide-MHC class I stability in a high-throughput manner; however after development of the SPA analysis, an essentially label-free stability screening approach is now possible (Harndahl et al., 2011).

Vaccine efficacy can be determined in various ways, and a typical approach to evaluate Th1-inducing adjuvant systems is to measure the IFN-γ production. Importantly, the level of secreted IFN-γ has been used to evaluate the efficacy of the only FDA-approved therapeutic anti-cancer vaccine, PROVENGE® (sipuleucel-T), in a peptide-immunization study in mice (Saif et al., 2014). Several cell types have the ability to produce IFN-γ including Natural Killer T cells, Natural Killer cells, $CD4^+$ and $CD8^+$ T cells amongst others. Here, *in vitro* peptide co-culture of PBMCs purified pre- or post-immunization was done with *9-11mer* peptides. Therefore the IFN-γ production observed following this co-culture is expected to originate from *$CD8^+$ T cells* encountering antigen-presenting cells presenting peptides in the context of MHC class I. Since all animals were found to respond to both stable and non-stable peptides as determined by the SPA analysis, it could be indicative of peptides with lower half-life than 0.5 h also having immunogenic potential. Surprisingly, the animals generally did not respond consistently to the same peptides over time. This might however partly be due to blood sampling only showing a snapshot of what is circulating in the blood stream. Also, most pigs,

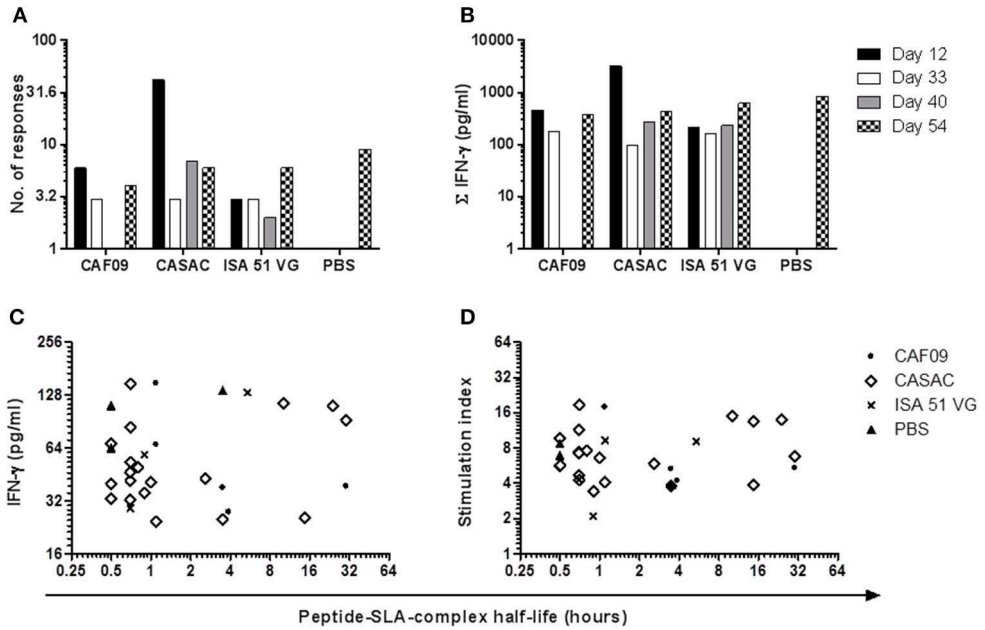

FIGURE 4 | Comparison of the adjuvant systems used in the immunization trial. The three Th1-inducing adjuvants CAF09, CASAC, and ISA 51 VG were compared on the basis of their ability to generate responses **(A)** and the total amount of peptide-specific IFN-γ produced from the culture **(B)** at the various time points following immunization. For peptides found to be stably binding to MHC class I in the SPA analysis, half-life of the peptide-MHC class I complex was correlated to the amount of IFN-γ **(C)** and the stimulation index **(D)**.

like humans, have three MHC class I gene loci constitutively expressed although duplication of the SLA-1 allele has been observed in a fraction of animals (Lunney et al., 2004). Therefore, most pigs express up to six different MHC class I molecules. We have selectively tested for the five SLAs in our in-house library; however the majority of the animals are expected to express additional SLA molecules and we most likely do not know their full SLA-profile. The additional SLA class I molecules might also bind certain peptides in the 9-11mer library, which could account for some of the responses to peptides not shown to bind stably to our in-house SLA molecules.

In the majority of the animals, especially pigs 2034, 2035, 2040, and 2042, there seemed to be a correlation between the stimulation index (as compared to day −2, pre-immunization) and the amount of peptide-specific IFN-γ produced. Unfortunately we had to exclude three pigs, two from the PBS and one from the CAF09 group, from these analyses due to high non-specific background. Recruitment of pigs from a background of higher sanitation status could provide a cleaner response window for future studies. The animal receiving peptides formulated in PBS only also showed responses to IDO and RhoC at day 54 post-immunization; however naturally occurring CTL responses to endogenous cancer antigens, among them IDO, have been frequently observed in healthy humans as well (Visseren et al., 1995; Jäger et al., 2001; Sørensen et al., 2011a; Frøsig et al., 2015). Further of notice, two of the previous peptide vaccine human trials with the most convincing clinical data used the granulocyte-macrophage colony stimulating factor as the only adjuvant (Inderberg-Suso et al., 2012; Walter et al., 2012). This cytokine functions as a growth factor and does not stimulate immunity directly as more common adjuvants do. Still, however, it seems the identification of an optimal adjuvant system will be highly beneficial.

Following immunization we found responses in most animals and for most time points; albeit the secreted amount of IFN-γ was fairly low, and the response magnitude can possibly be enhanced by further optimizing the immunization protocol. The generation of only a few specific T cells could, however, be enough to induce epitope spreading generating mutation antigen-specific T cells of higher avidity in a human cancer setting. The frequency of specific T cell populations measured in previous peptide vaccination studies in human cancer patients have also generally been low (Pollack et al., 2014; Køllgaard et al., 2015). This is probably due to the endogenous origin of the targets, but nonetheless, in some of these studies a clear clinical effect has been obtained. This was the situation in the IDO peptide immunization trial (Iversen et al., 2014) where 47 % of the treated patients developed a long-lasting partial response or stable disease, defined as 8.5 months compared to the expected 6–7 months of progression-free survival in this patient group. In addition, treated patients had significantly longer overall survival than a cohort of patients of similar shape not treated with the vaccine. Immunological activity was found *ex vivo* in this trial, but at quite low frequencies. In contrast, high-frequency immune responses are often found in cancer immunization studies using murine models (Wei et al., 2015) as exemplified in the study by Zhao et al. (2012),

where more than 1000 pg/ml IFN-γ was secreted for most peptides tested after two immunizations and only 48 h of peptide stimulation *ex vivo*. For comparison, the maximum level of secreted IFN-γ observed in our trial was 280 pg/ml IFN-γ (pig 2042, day 12, peptide IDO15, **Figure 3**) following 72 h of stimulation.

Here, we included three different Th1-inducing adjuvants, namely CAF09, CASAC, and ISA 51 VG and immunized three pigs with each along with a fourth group receiving peptides in PBS only. The PBS group was included as a control for the adjuvant efficacy. From this study, none of the adjuvants were found to be superior as they were all capable of generating a CTL response toward cancer antigens found to be important in human disease. Thus, despite very convincing data from mouse studies (Wells et al., 2008), this first porcine/human modification of CASAC was not superior in the pig model. The inconsistent response profile highlights, however, the importance of further optimization of peptide immunization protocols. In this study we set out to analyze the vaccine-induced immune reactivity on fresh material. Since the SPA analysis resulted in a peptide library of 80 stably binding peptides, a total of approximately 1000 stimulations and 2000 ELISA well analyses were carried out for each time point to measure the secreted IFN-γ. Due to this large screening setup, analyzing more cytokines in parallel was not feasible. To confirm the induction of antigen-specific $CD8^+$ T cells following an immunization protocol, MHC multimer staining for flow cytometry analysis is usually performed in humans and mice. Although we have developed porcine MHC multimers and staining protocols (Pedersen et al., 2014a), we are still in the process of developing a high-throughput MHC multimer screening system for porcine cells. We believe the pig model is highly appropriate to address questions relating to optimal adjuvant composition and formulation, peptide repertoire and dosing, as well as the route and number of administrations for endogenous peptide immunizations. Supplemented with the porcine immunological reagents, including recombinant SLA class I molecules and SLA multimers, we will further be able to characterize the immunological response profile following different immunization protocols and relate this to the immunological correlates of anti-cancer protection.

Author Contributions

NO and TF designed and performed the experiments, analyzed the data and wrote the article. GJ conceived the approach, designed the experiments, approved the analysis, provided financial support and co-wrote the article. SW, MR, MI, designed and performed experiments. MS, MR, and SW analyzed the data. SB and MA supervised the study.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fgene.2015.00286

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