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PERSPECTIVE

The new pig on the block: modelling cancer in pigs

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Abstract The molecular mechanisms underlying many human cancers are now reasonably well understood. The challenge now is to bridge the gap between laboratory and clinical oncology, so these accomplishments can be translated into practical benefits for human patients. While genetically modified mice have played a prominent role in basic research, they are less suitable for many preclinical studies. Other animals can provide important complementary resources to aid the development, validation and application of new medicines and procedures. Powerful methods of genetic engineering have now been extended to physiologically more relevant species, particularly the pig, opening the prospect of more representative, genetically defined, cancer models at human scale. We briefly review the field and outline our program to generate gene-targeted pigs carrying mutations in tumour suppressor genes and proto-oncogenes that replicate key lesions responsible for a variety of human cancers. We also highlight some important issues for the future development and usefulness of porcine cancer models.

Keywords Disease model · Cancer · *APC* · p53 · Swine · Gene targeting

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Need for cancer models

Around the world many people are living longer with increased expectations of health and wellbeing. The broad demographic shift towards aging populations, combined with other health trends such as increased obesity, presents an enormous challenge to healthcare systems. Recent decades have seen significant improvements in the treatment of important age-related causes of death such as cardiovascular, cerebrovascular and respiratory diseases. The decline in cancer mortality has in contrast been modest and largely restricted to cancers that can reliably be diagnosed at early stage. This is a major concern because cancer incidence is predicted to rise significantly as more people enter later life (http://www.cancerresearchuk.org). It has been estimated that more than 40 % of people alive today will be diagnosed with some form of cancer during their lifetime (Howlader et al. 2011).

Years of research and advances in cancer genetics and medicinal chemistry have provided a wealth of knowledge that could revolutionise cancer detection and treatment. Arguably this also applies to many other fields of biomedicine, as reflected by increasing use of the term 'translational research' in the literature.

The safe and effective transition of new biomedical procedures from laboratory to clinic often requires testing in animals. Humane and ethical treatment of animals is an important priority for all biological research, particularly when disease conditions are

being modelled, and strenuous efforts should be made to reduce the number of animals used. However, no in vitro system currently available can adequately model human whole body physiology or a functional immune system, and none are in immediate prospect. Preclinical studies using animals are thus unavoidable for the foreseeable future. It therefore makes ethical and practical sense to increase the predictive value of animal trials as much as possible, to ensure they provide high quality information directly relevant to human disease. This is best achieved using well-characterised, physiologically relevant animal models.

Mice, and to a lesser extent rats, are the most common and often the only animal species used for preclinical studies before trials proceed in human subjects. However rodents differ considerably from humans in size, general physiology, anatomy and lifespan, which can reduce their usefulness. For example, small size effectively precludes their use for the development of most human surgical procedures. It is also technologically difficult to scale down necessary equipment, e.g. for endoscopic imaging technologies. Modelling radiation or photodynamic tumour therapy in rodents is confounded by uncertainties regarding appropriate scaling of energy dose and tumour size (Adam et al. 2007).

Recent decades have also seen genetically modified mice come to dominate research into cancer and many other human diseases. This is not because of their particular predictive value, but rather low cost, convenience and the technical ease of engineering interesting mutations. As mammals, mice and humans clearly share many fundamental similarities, but there are differences in cancer biology. Murine cells are more easily transformed in vitro than human cells (Hooper 1998; Rangarajan et al. 2004), and a different set of genetic events is required for tumorigenesis (Kendall et al. 2005). Dissimilar protein interactions, physiology and anatomy can thus lead to different disease phenotypes from similar genetic lesions. For example, mutations in the human adenomatous polyposis coli(*APC)* gene initiate polyposis predominantly in the colon and rectum that progresses to metastatic cancer, but *Apc* mutations in mice cause non-invasive, non-metastatic neoplasia in the small intestine (Boivin et al. 2003). Data from a wide variety of areas is revealing the limitations and shortcomings of mouse models, for example in drug metabolism (Martignoni et al. 2006), cystic fibrosis (Guilbault et al. 2007) and breast cancer (Vargo-Gogola and Rosen 2007). A recent systematic study of inflammatory diseases highlighted the lack of correlation between mouse data and human conditions (Seok et al. 2013) and provoked critical comments in the popular press.

Expenditure on pharmaceutical research and development has increased significantly in recent decades, but the number of new pharmaceutical products approved each year has not risen accordingly (Kaitin 2010), a worrying reduction in productivity and a failure to meet human clinical needs. Analysis of a large database of pharmaceutical research and development projects pursued since 1990 reveals that a major proportion fail in phase II and III clinical trials (Pammolli et al. 2011). It has been argued that this is in large part due to companies seeking broadly efficacious 'blockbuster' drugs for more difficult clinical problems while facing ever stricter regulatory environments (Mittra and Tait 2012). One proposed solution is a more tailored approach where drugs are carefully targeted to those patient subpopulations most likely to benefit. Key to this is the identification of biomarkers to inform the design and structure of smaller, more refined clinical trials. Preclinical trials are clearly a vital part of this process and likely to become more so.

Preclinical studies would clearly be improved by reducing the overreliance on mice. Rats are one alternative. They have long been used as a laboratory species, but have until recently resisted gene targeted modification. Technological advances in embryonic stem cells, induced pluripotent stem cells and genome editing have now removed this block (reviewed by Huang et al. 2011). Genetic modification campaigns in rats are underway and will no doubt yield a substantial panel of mutant strains. Nevertheless, rats share with mice similar drawbacks of size, diet and physiology. How well rats model human cancers also remains to be seen. It is notable that p53 mutant mice, rats and humans exhibit quite a different spectrum of tumours (Jacks et al. 1994; van Boxtel et al. 2011; Goh et al. 2011).

No single species is likely to provide the best model for all human disease, each has advantages and disadvantages. There are good arguments for investigating a variety of animal models. Interspecific studies provide useful insight into the genetic bases of disease

and disease predisposition. Comparative analysis of gene expression data can identify evolutionarily conserved networks of expression and gene regulatory regions, and unravel the complex interactions between genetic, environmental and lifestyle factors that influence disease pathology. Canine oncology is already providing a useful complementary perspective. Genetic modification technology is little used in dogs, but large numbers of companion dogs receive veterinary attention for sporadic cancers, and many owners participate in clinical trials in the interest of their pets. Particular breeds are known to be susceptible to cancers, and canine cancer is increasing for similar reasons as in humans, with better care extending life and revealing age-related diseases. This has allowed veterinary oncologists to develop substantial repositories, biobanks, of canine cancer samples, and findings show direct relevance to human treatments (Paoloni and Khanna 2008).

Pigs have not so far played a major role in experimental oncology, but are ever more important for a wide range of preclinical research. Pigs share many similarities with humans in body size, anatomy, and their physiological and pathophysiological responses. They are already widely used to study newly designed equipment and instruments at human scale and to develop procedures such as endoscopic and laparoscopic surgery (Swindle 2007). Pigs are also relatively long lived, enabling longitudinal studies in individual animals under conditions that mimic the human patient. This will allow important clinical parameters to be followed, such as tumour progression and remission, response, toxicity or failure of drug therapy, and the acquisition of drug resistance by cancer cells.

Many practical requirements necessary for the use of pigs as models of human cancer and cancer predisposition are in place. Conditions for raising pigs in a pathogen-free environment have been established. Key enabling techniques originally developed in other livestock, such as transgenesis and gene targeting using somatic cell nuclear transfer (Schnieke et al. 1997; McCreath et al. 2000), were rapidly extended to pigs (Dai et al. 2002). Nuclear transfer with its attendant difficulties remains the main way of generating genetically modified large animals, but this is changing with the use of genome editing directly in fertilised oocytes using synthetic highly specific endonucleases such as zinc finger nucleases (Flisikowska et al. 2011) and transcription activator-like effector nucleases (TALENs) (Carlson et al. 2012). RNA-guided endonucleases provide yet another new tool (see review by Mussolino and Cathomen 2013), further increasing the range of methods available.

Porcine cancer models

As in humans, spontaneous cancers are rare in pigs. Lymphosarcomas have been described in young animals (Anderson and Jarrett 1968; Stevenson and DeWitt 1973) and a range of cancers in older animals (Brown and Johnson 1970; Fisher and Olander 1978). Most pigs are of course raised for food and do not reach sufficient age for tumours to occur naturally.

In the absence of 'real' pig tumours, a variety of strategies have been adopted to aid development of tumour therapies at human scale. Mimic structures have been generated by injection of agarose, cellulose and glycerol into the liver to enable study of ultrasound thermal ablation (N'Djin et al. 2007). Liquid plastic has been injected into pig kidney to mimic exophytic kidney tumours for the development of laparoscopic nephrectomy (Hidalgo et al. 2005). Porcine tumours can also be induced artificially by introduction of chemical carcinogens (e.g. Li et al. 2006). While such procedures may be suitable for a limited set of applications, they are labour intensive, can raise ethical concerns and clearly do not provide fully representative models of human cancers.

Two pig tumour models based on natural inherited mutations are available for biomedical research, both predispose to melanoma. MeLiM (melanoblastoma-bearing Libechov) minipigs develop cutaneous melanomas of varying severity, including highly invasive and metastatic lesions (Borovansky et al. 2003). Most lesions develop in the first three months and share many histopathological and clinical features with human melanoma. Unlike in humans, these melanomas eventually spontaneously regress (Vincent-Naulleau et al. 2004). Sinclair miniature pigs also develop a variety of skin lesions in early life that again spontaneously regress (Greene et al. 1997). Sinclair pigs have been used to study molecular aspects of tumour regression (Pathak et al. 2000). Unfortunately the causative genetic lesions are undefined, making it difficult to draw parallels with human melanoma.

Adam et al. (2007) reported a porcine cancer model based on autologous transplantation of primary porcine cells transduced with retroviral vectors carrying

oncogenic cDNAs. Importantly this revealed that the changes necessary to convert porcine cells to a tumorigenic state resemble those required by humans more than those in mouse cells, suggesting that a similar process is involved (see also Kendall et al. 2005). However it falls somewhat short as a representation of human cancer. The use of viral cDNA constructs does not reliably reflect the expression and regulation of endogenous genes. Tumours arising from grafted cells also differ in important respects from autochthonous tumours. They typically lack complex tumour architecture, have poorly developed vascular and lymphatic system and are often composed of a single dominant cell clone as a consequence of selective pressures in culture. Tumours arising from grafted cell lines also tend to be poor predictors of clinical efficacy, for example anti-cancer drugs found to be effective on such grafts can be ineffective on real tumours (Zhou et al. 2009).

Our view is that livestock genetic engineering holds the real key to producing representative pig cancer models 'to order'. Cancer has not so far been a major focus of pig biotechnology, and we are aware of only three primary papers from other groups. Pigs carrying the *v-Ha-ras* oncogene directed by the mouse mammary tumour virus long terminal repeat promoter were generated by microinjection, but no phenotype was observed (Yamakawa et al. 1999). Constitutive expression of the Gli2 transcriptional activator in keratinocytes resulted in basal cell carcinoma-like lesions in young pigs, but these were euthanized due to bacterial infection before fuller investigation could be carried out (McCalla-Martin et al. 2010). The first gene-targeted pigs for cancer were generated by adeno-associated virus mediated gene inactivation of *BRCA1* (breast cancer associated gene 1) in fibroblasts (Luo et al. 2011). Animals were produced by nuclear transfer but none survived beyond 18 days, so their usefulness remains to be demonstrated.

Our group has set out to model human cancers and cancer predispositions in pigs as accurately as possible. Similar genetic alterations in a small set of genes are thought to be responsible for several tumour types; see for example the catalogue of somatic mutations in human cancer compiled by the Wellcome Trust Sanger Institute (Futreal et al. 2004). It should thus be possible to replicate a variety of cancers by combining and activating defined oncogenic mutations in chosen tissues. We are in the process of generating animals with a set of defined genetic lesions known to play important roles in human cancers.

One of our first priorities was cancer of the colon and rectum because it is very common, the affected tissue is well characterised and the molecular basis of disease initiation understood. Colorectal cancer causes an estimated 600,000 deaths or more per year worldwide (Ferlay et al. 2010) and is increasing in countries with traditionally low risk, mainly due to adoption of westernised diets. The tumour suppressor gene *APC* plays a vital role in maintaining intestinal homeostasis. Disruption of *APC* function is key to initiating the sporadic form of the disease (Powell et al. 1992) and an inherited predisposition, familial adenomatous polyposis (FAP) (Kinzler et al. 1991; Groden et al. 1991). FAP varies widely in severity, but patients typically develop hundreds of adenomatous polyps in the colorectum between puberty and twenty years old, which almost inevitably progress to cancer in mid life (Croner et al. 2005). Although disease onset in FAP is sooner than the more common sporadic form, the origin and progression of colorectal cancer are thought to be the same.

We recently reported the first viable gene-targeted 'oncopigs'. These carry truncating mutations in the *APC* gene at sites orthologous to the most frequent FAP germline mutations (Flisikowska et al. 2012). Examination of a one year old founder animal carrying the mutation APC^{1311} (orthologous to a severe FAP mutation, APC^{1309}) revealed polyps in the colon and rectum that closely resembled human adenomas by a variety of criteria (Flisikowska et al. 2012). Results accord with the location and early onset of human FAP, and contrast with *Apc* mutations in mice (Boivin et al. 2003). We are currently analysing F1 generation animals to monitor the pattern and time course of polyposis and progression to cancer. Figure 1 shows rectal endoscopy of wild-type and APC^{1311} sibling pigs at 7 months old. Evidence so far indicates that porcine FAP mirrors the development of the human disease.

We have also generated gene-targeted pigs with a conditionally activatable oncogenic mutant form of p53, which in latent form is a gene knockout (Leuchs et al. 2012). These are vital components in our program because p53 plays such an important role in human cancers. Evidence so far indicates that the porcine $TP53^{R167H}$ mutation, orthologous to human $TP53^{R175H}$ and mouse $Trp53^{R172H}$, confers similar changes as in

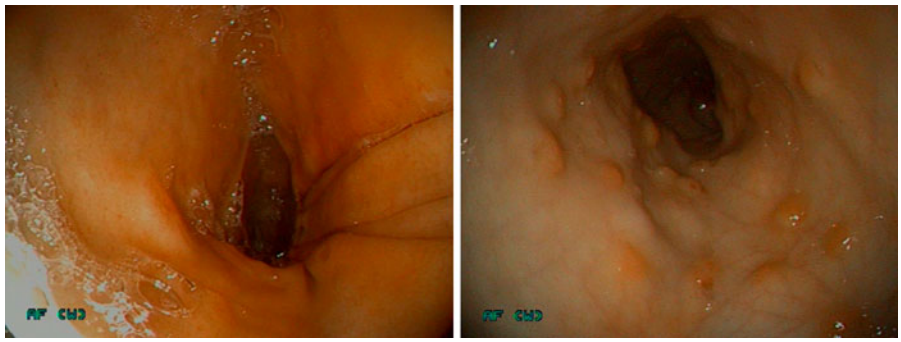

Fig. 1 Endoscopic imaging of the rectums of two sibling F1 generation male pigs at 7 months old. The animal on the *left* is wild type, the animal on the *right* carries the APC^{1311} mutation in heterozygous form

humans and mice. Mutant porcine p53-R167H protein accumulates in affected cells, indicating failure of normal p53 degradation (Midgley and Lane 1997). The mutation also confers resistance to the chemotherapeutic drug doxorubicin. Pigs carrying the latent $TP53^{R167H}$ allele in heterozygous form are viable and healthy, but are continually monitored for signs of disease. Given the variations in p53 tumour phenotypes across species, we are keen to see how closely the mutant pigs resemble human Li–Fraumeni syndrome.

The results outlined above are only the first steps. Fortunately the genetic bases of several serious human cancers are well enough understood to make them suitable targets for replication in pigs. More mutant cancer related genes are being generated and these will be brought into combination.

Issues for the future

As mentioned earlier in this article, appropriate biomarkers are key to the successful development of new diagnostic and therapeutic products. Genetically defined cancer-prone pigs provide a powerful resource for comparative 'omic analyses to identify new biomarkers that provide insight into disease mechanisms and drug testing based on genotype. Progress in this area will however depend on the parallel development of proteogenomic platforms in pig. High resolution proteome maps of many human tissues and body fluids have been generated, but porcine tissues are less well characterised and, until recently, the choice of tissues focussed on pig production rather than biomedicine (reviewed by Bendixen et al. 2010). A new porcine genome sequence assembly has now been published, providing a valuable new resource (Groenen et al. 2012). While the depth of coverage is reasonable, 15.3 × compared with 25 × for mouse and >12 × for human (Wellcome Trust Sanger Institute), more individuals of different pig breeds need to be included and a considerable amount of annotation has yet to be completed. For comparison, the current pig genome sequence is at version 10.2 (http://www.ensembl.org/Sus_scrofa/Info/Index), while the human and mouse genomes are at versions 37 and 38. Strengthening porcine functional proteomics and genomics is a priority if the emerging pig models of human disease are to be used to full advantage.

The technology available for livestock biotechnology is steadily improving, but one key tool is still missing: the ability to activate latent genes in defined tissues and at defined times. Activation of an oncogenic mutation(s) in a chosen tissue mimics the spontaneous somatic events that initiate many human cancers and enables replication of diverse cancer types using the same mutant gene. Conditional gene expression is well established in mice, based for example on Cre-mediated recombination (e.g. Hingorani et al. 2005; Frese and Tuveson 2007), but has not yet been extended to large animals. Mouse geneticists have available a large bank of engineered strains that express Cre recombinase under a wide variety of tissue specific and inducible promoters. Substantial efforts would be necessary to generate transgenic pigs that specifically express Cre in defined tissues, but these would provide valuable resources, not only for cancer modelling but many other diseases. Large animal biotechnologists are a scattered community and there is a strong case for international collaboration and coordination of efforts.

Cancer modelling in pigs is proceeding in parallel with other disease campaigns, many of which could provide useful synergy. For example, pig models are being developed for cardiovascular disease (Carlson et al. 2012) and diabetes (Renner et al. 2013). Co-morbidities are an important factor in designing safe and effective human treatments. Combination of disease models by cross breeding will offer unprecedented opportunities to study cancers and potential therapies in an ever more realistic manner.

An important question for the future usefulness of pigs as cancer models is how well they model the interaction between the immune system and cancer. The porcine immune system does differ from that in humans (reviewed by Scharek and Tedin 2007) but how this affects cancer biology has yet to be investigated. Immuno-therapeutics is the fastest growing sector in the pharmaceutical industry. A human scale immune competent animal model would be of considerable value for the development of innovative diagnostic and therapeutic approaches. Monoclonal antibody therapy is a relatively new and successful means of treating cancers (reviewed by Scott et al. 2012). Pigs could enable investigation of new means of monoclonal antibody production and delivery, new antibody–drug conjugates and their pharmacokinetics.

One issue that affects all new animal models is the need for validation. Simply providing a physiologically relevant animal model will not be sufficient for pharmaceutical and other medical companies to employ them in pre-clinical trials. Animal disease models will be useful only if fully validated by a body of accredited background data, enabling trials to be properly standardised. Gathering this data will require partnership between industry, academic and clinical research organisations and support from governments and medical charities.

Porcine oncology is a new and still very small field and there is much yet to be done, but the rewards for human health and wellbeing promise to be substantial.

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