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Original article

The minipig as a platform for new technologies in toxicology

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ABSTRACT

The potential of the minipig as a platform for future developments in genomics, high density biology, transgenic technology, in vitro toxicology and related emerging technologies was reviewed. Commercial interests in the pig as an agricultural production species have driven scientific progress in these areas. There is no equivalent economic driver for progress in the dog or the monkey. As a result the available knowledge-bases are much greater for pigs (than for dogs or monkeys) in many areas (physiology, disease, genetics, immunology etc). Fundamental genomic knowledge and phenotypic characterization in regard to the pig is well in advance of the dog or the monkey and basic knowledge of the pig is therefore likely to stay ahead of the other two species. While the emerging technologies are essentially "species neutral" and can in principle be applied to all species, for all the technologies that we examined, basic knowledge and technical capabilities are greater for the pig than the dog or monkey. In concrete terms, in application to safety testing we have seen that: (i) The Göttingen minipig is well positioned for the performance of toxicogenomics studies, (ii) The close sequence homology between pigs and humans suggest that minipigs will be useful for the testing of biotechnology products (and possibly for *in silico* toxicology) and (iii) the minipig is the only non-rodent toxicology model where transgenic animals can be readily generated, and reproductive technologies are well developed in the pig. These properties should also make the minipig an interesting model for the testing of biotechnology products. These factors all support the idea that the minipig is well placed to meet the challenges of the emerging technologies and the toxicology of the future; it also seems likely that the minipig can be an advantageous model for the testing of biotechnology products.

1. Introduction

1.1. Introduction

In this article we review the potential of the minipig as a platform for future developments in genomics, high density biology, transgenic technology, *in vitro* toxicology and related emerging technologies, as a contribution to the *RETHINK* project (Forster, Bode, Ellegaard & van der Laan 2010a-this issue, 2010b-this issue). The impact of some of these technologies on toxicology and safety evaluation of new medicines and chemicals is already evident. We expect that in the coming years, these new technologies will be the drivers for significant developments and changes in the way that we perform safety studies, and will provide new tools for safety assessment. It is therefore essential, if the minipig is to play a role in the safety assessment of the future, that it is well adapted to these new technologies.

The specific technologies that were identified for evaluation by the authors included biosensors, genomics and toxicogenomics, genetic manipulation, stem cell technology, manipulation of the immune system and *in vitro* toxicology. For each of these topics in this article we have reviewed the current state-of-the-art with regard to pigs and minipigs, and the potential for future developments and applications in this animal model.

1.2. Application of the 3Rs

In what way does this part of the *RETHINK* project also contribute to the application of the 3Rs? The evaluation of the minipig as a platform for emerging technologies in toxicology is a relevant discussion for the application of the 3Rs for several reasons.

The opinions expressed in this paper are those of the authors and not necessarily those of the organizations they represent.

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- The focus of the *RETHINK* project is to evaluate the impact of greater use of the minipig in toxicity testing, and we do not therefore expect to directly address issues of Replacement in this article.
- Technological developments can have a positive impact on Refinement; an example could be the application of minimally or non-invasive measuring systems reducing or eliminating the need for surgery or other interventions. Non-invasive measurements of this kind can be achieved through the use of transgenic animals and imaging systems. Furthermore, such approaches may permit the use of fewer animals in a study (e.g. using time course analysis from single animals rather than using a set of animals) and may therefore bring a benefit in terms of Reduction.
- More sophisticated analysis systems such as genomic tools or multiplex analyses that provide considerably greater and more detailed outputs of information from single animals in comparison to current experimental approaches can be expected to result in a corresponding positive impact in terms of Reduction.
- In most cases the emerging technologies discussed in this article are neutral in terms of species, and can be equally applied to the minipig or to dogs, monkeys or other non-rodent animals used in toxicity testing. Nevertheless, it was repeatedly noted that the current knowledge base for most of the technologies is significantly greater for the pig than the dog or primate. This is true for genomics, reproductive biology, immunology and genetic manipulation. In terms of future animal use, therefore, there is a potential benefit in building on the existing well developed porcine knowledge base and a potential negative impact of using the dog or primate, since extensive experimentation would be required to reach the same general state of advancement.

1.3. Origin, history and genetics of the Göttingen minipig

A review of the origins and genetic management of the Göttingen minipig is presented in an accompanying article (Simianer & Köhn, 2010-this issue). The review provides valuable background information both for the present article, and gives a unique insight into the history of this animal model. Some important and relevant conclusions emerge from the accompanying article, including the following:

- The Göttingen minipig is an almost unique resource, since the entire population history is extremely well documented back to the early development of the population in the 1960s.
- The genetic management of the entire breeding population of the Göttingen minipig is assured by the Institute of Animal Breeding and Genetics of the Georg August University of Göttingen, with objectives to maintain the genetic integrity and uniformity of the population, to balance adverse effects of inbreeding and to pursue desired breeding objectives.
- The proportional dwarfism of the Göttingen minipig is probably mediated by modulation of the actions of growth hormone and/or insulin-like growth factor 1 and is not associated with any genetic defects or disturbances of development.
- The light skin colour of the Göttingen minipig is due to a "dominant white" genetic status; the molecular basis of this is not entirely understood (Marklund, Kijas, Rodriguez-Martinez, Rönnstrand, Funa, Moller et al., 1998). The Göttingen minipig is not an albino and melanin is present in the skin, and the retina of Gottingen minipigs is pigmented.

2. Pig structural genomics

In this article questions are addressed such as: what is the current state-of-the-art with regard to pig gene mapping, sequencing, bioinformatics and functional analyses? Can genomic data derived from domestic/farm pigs be extrapolated to minipigs used as toxicology models? What future developments can be foreseen in this area? How does this compare with the dog, monkey and other toxicology models?

Because of the important economic role of the pigmeat industry both in Europe and around the world, an enormous amount of basic and applied animal breeding, genetics and genomics research has been conducted in the pig. This has led to in-depth characterization of many phenotypic traits, with particular emphasis on those traits of importance to sustainable production (growth, feed efficiency, meat quality, fertility, disease-related traits etc.). Understanding of the genetic basis of these traits, and the genetic prediction of phenotype is of major economic importance for the pigmeat industry and this has provided a great incentive to develop new tools to study the pig genome.

The genome of the pig comprises 18 autosomes, and 2 sex chromosomes (X and Y). The genome is similar in size to that of humans and is estimated at 2.7Gb (Gigabases). Through the EU-supported Pig Gene Mapping Project (PiGMaP, PiGMaP2 and GENETPIG, all funded under EC Framework III programmes) and through research activities in the U.S, the basic tools for studies in pig genomics have been developed. These tools comprise several thousand microsatellite markers and carefully built linkage maps (www.thearkdb.org/arkdb/; www.marc.usda.gov/genome/swine/swine.html); well characterized radiation hybrid panels (www.toulouse.inra.fr/lgc/pig/RH/IMpRH.htm); and comparative maps (http://www.toulouse.inra.fr/lgc/pig/compare/compare.htm). The comparative maps show that there is extensive conserved homology with the human genome. The blocks of pig–human synteny are on average larger (Frönicke, Chowdhary, Scherthan & Gustavsson, 1996) than both the dog–human and the mouse–human synteny blocks (Breen, Thomas, Binns, Carter & Langford, 1999; Nilsson, Helou, Walentinsson, Szpirer, Nerman & Ståhl, 2001), indicating that fewer chromosomal rearrangements have occurred since divergence of the pig and human lineages.

These genetic tools have been used to identify and infer the functions of individual genes (e.g. Fujii, Otsu, Zorzato, DE Leon, Khanna, O'Brian et al., 1998; Meijerink, Fries, Voegeli, Masabanda, Wigger, Stricker et al., 1997; Van Laere, Nguyen, Braunschweig, Nezer, Collette, Moreau et al., 2003; Jørgensen, Cirera, Anderson, Archibald, Raudsepp, Chowdhary et al., 2003) and to identify quantitative trait loci (QTL) of importance to sustainable breeding (see, for example, the pig QTL database at: www.animalgenome.org/QTLdb/pig.html). Numerous QTL studies have been performed in pigs, with many concordant results, indicating that there are good possibilities for correlating phenotypes and genotypes. This will provide new functional knowledge about gene function and gene interaction.

Whole genome sequences are an important resource in biomedical research and today genome sequences are available for many species including humans, cattle and mice. Efforts are also underway to sequence the porcine genome. This work is coordinated through the International Swine Genome Sequencing Consortium (http://piggenome.org/) and progress on this project can be inspected at the website (www.sanger.ac.uk/Projects/S_scrofa/). As of Spring 2008, ten chromosomes have been assembled and it is anticipated that the remaining chromosomes will be assembled soon.

In addition to genomic sequencing, efforts have also been made to generate sequence information on cDNA. The Sino–Danish Pig Genome Sequencing Consortium has, for instance, generated approximately 1 million porcine EST's (Expressed Sequence Tags) (Gorodkin, Cirera, Gilchrist, Paniz, Jørgensen, Scheiby-Knudsen et al., 2007). Parallel to the generation of more sequence information, oligo arrays have been developed for commercialisation by Affymetrix and Qiagen, permitting gene expression studies as described below.

Genotyping arrays with Single Nucleotide Polymorphisms (SNPs) have also been established. The first porcine SNP chip has been developed within the context of the EU SABRE project. This microarray contains 7.5 K informative SNPs on an Illumina chip (the porcine SNP chip is not commercially available). Within the coming 6–12 months it

is expected that a 50 K SNP chip will be available. This tool will enable a full characterization of the minipig genome and provide genotyping at the "individual animal" level.

New developments in sequencing technologies are transforming this field and opening up possibilities for even more rapid progress in genomics. New low-cost high-throughput techniques based on pyrosequencing chemistry (commercialised by Solexa and 454 Life Sciences) make it possible to perform resequencing at a very high scale. This should increase our ability to make a direct connection between information on phenotype and information about the genes underlying the phenotype in question. In future gene mapping projects the outcome will not only be identification of candidate regions, as is often the case now, but rather identification of the actual genes and molecular mechanisms underlying the traits in question.

2.1. Applicability of pig genomics mapping and tools to the minipig

The various tools and resources established for research in genomics outlined in the previous section are applicable to all pig breeds including minipigs. This point has been amply demonstrated by studies in pig resource populations produced by crossing divergent breeds (e.g. Andersson, Haley, Ellegren, Knott, Johansson, Andersson et al., 1994; SanCristobal, Chevalet, Haley, Joosten, Rattink, Harlizius et al., 2006).

A predominant feature of the pig as a biomedical model is that because of its economic and agricultural importance, a wealth of functional genomics information has been established in this species. This provides a rich background of existing data for interpretation of new results generated in genomics studies using domestic pig breeds or minipigs.

Several characteristics of the pig combine to facilitate identification of the genetic basis of phenotypic traits. The phenotypic diversity within pig breeds, together with breeding history and population structure, offer unique advantages for the molecular dissection of multifactorial traits. In only a few thousand years, selective breeding has produced pig breeds that thrive in diverse environments and climatic conditions, convert energy to muscle mass efficiently and rapidly, and tolerate specific pathogens. Since the effective population size is smaller than that of humans the genetic complexity of the traits will in most cases be reduced. Furthermore, since population-wide linkage disequilibrium (LD) in livestock extends over tens of centimorgans (rather than subcentimorgan regions as typically observed in humans), locating genetic determinants is considerably easier in the pig compared to man. In many respects, breeds of pigs are similar to human ethnic groups with diverse geographic origins, but with more exaggerated phenotypic diversity.

Finally, there can be little doubt that the understanding of the factors that make porcine breeds differ with respect to reproductive efficiency, bone structure, growth rates, fat deposition, and resistance to specific pathogens will be important to understanding basic biological processes important to human health. The general advancement of genomics together with the favourable characteristics of the species, suggest that progress could be rapid.

2.2. Relevance of pig genomic data for man and human biomedical research

Although less close to man in evolutionary terms than non-human primates, the pig is generally considered a good model in biomedical research because of its anatomical and physiological similarity to humans with respect to a variety of organs and functions. A recent phylogenetic study based on rare genomic changes has clustered primates and rodents together (Murphy, Pringle, Crider, Springer and Miller, 2007). However, while it has taken many short (evolutionary) branchings to reach the present day rodents, the branches leading to both humans and pigs are much shorter (Springer, Murphy, Eizirik & O'Brien, 2003). It is important to keep in mind that the differences between DNA sequences of two species are not solely a function of the elapsed time since their divergence. It has recently been shown that the evolutionary distance in sequence space between the porcine and human genome sequences is smaller than the distance between mouse and human (Grønlund, Hobolt, Hornshøj, Bendixen, Fredholm & Schierup, 2005; Wernersson, Schierup, Gorodkin, Panitz, Jun, Stærfeldt et al., 2005). These studies are based on comparison of human, mouse and pig full length cDNA alignments comprising more than 700,000 nucleotides and approximately 0.7× coverage from the pig genome respectively.

The Göttingen minipig is predisposed to obesity, unlike production pigs which have been strongly selected for low fat content and leanness. Thus, backcrossing and intercrossing minipigs with production pigs will provide ideal families for identification of genes involved in obesity, since it can be predicted that the traits and genes involved in obesity will segregate. Genes underlying other traits that differ between the two breeds (e.g. size, growth, age of sexual maturity) will also segregate in the crossbred animals. This is therefore an interesting model with great potential to generate knowledge about the molecular basis of range of different traits.

3. Toxicogenomics

Toxicogenomics is the study of gene expression modulation after exposure to a test item with the objective of gaining a deeper mechanistic understanding of toxic actions, and developing predictive tools to rank, select and evaluate new drugs and chemicals. Genome-based technologies such as DNA microarrays allow the simultaneous analysis of the expression of many thousands of genes in a single experiment. Whole genome microarrays are available for several species, including humans and laboratory animal species routinely used in safety evaluation such as the rat, mouse, primate and also the pig. The study of thousands of parameters in a single sample can give new insights into the assessment of the effects which a chemical or drug can cause, whether beneficial (pharmacology or efficacy) or harmful (toxic). Drug induced changes in the regulation of gene expression can occur within few hours after drug exposure. This opens the way for the deployment of short term toxicogenomics studies in different ways and toxicogenomics approaches can provide:

- A basis for compound classification: compounds with the same pharmacological or toxic mechanism of action generate similar changes in gene expression profiles,
- A better understanding of toxicological mechanisms through the study of the function of the genes that are modulated by a drug treatment, as well as the signalling pathways or ontologies in which proteins encoded by these modulated genes are classified.
- The discovery of new pharmacological/efficacy/toxicity biomarkers: consistently regulated genes across several compound time/dose combinations may be good candidates as markers for drug effects,
- The generation of toxicity signature databases, by correlating the early gene expression profiles induced by reference compounds with traditional toxicology endpoints such as histopathology or clinical pathology findings. These toxicity signature databases can be composed of several tens of genes and are built by taking out the genes that are regulated in common by several drugs that produce the same toxicological phenotype.
- The early prediction of new compound toxicity by transcriptional profile comparisons with existing (in-house or commercially available) toxicity signature databases.
- The discovery of new pharmacological actions of existing drugs, permitting the repositioning of these drugs for new therapeutic indications.

3.1. Gene expression and toxicogenomics studies in the pig

Toxicogenomics studies need up-to date genome annotation databases in order to mine the huge amount of data generated by

microarray technologies and to derive mechanistic information. The integration of structural (i.e., genetic) and functional data is also very useful in order to understand the individual variability in the gene expression profiles generated by a treatment. The context for gene expression studies in the pig is very favourable since much annotation data is available from mapping exercises together with functional information on genes (as described above). This is demonstrated by the results of a rapid bibliographic comparison using PubMed:

<"expressed sequence tags" and rat> yielded 485 results

<"expressed sequence tags" and porcine> yielded 144 results

<"expressed sequence tags" and canine> yielded 38 results

<"expressed sequence tags" and "non human primate"> yielded 14 results

Compared with the dog and the monkey, non-rodent laboratory species typically used in toxicology and safety evaluation, a significantly greater volume of genomic work is being performed in the pig.

The number of available porcine ESTs has greatly expanded in the last five years, and more than one million porcine ESTs have now been deposited in the NCBI GenBank (Gorodkin et al, 2007). Recent progress in the porcine sequencing project, as well as porcine genome annotation, has improved the identification of differentially expressed genes in experiments using Serial Analysis Gene Expression (SAGE), Differential Display or Subtractive Hybridization approaches (i.e., non-microarray approaches). Academic groups have used these technologies in order to understand the molecular mechanisms involved in pig livestock improvement, such as muscle growth and development (Janzen, Kuhlers, Jungst & Louis, 2000) or the response to infection (Wang, Hawken, Larson, Zhang, Alexander & Rutherford, 2001).

Several academic and/or industrial laboratories have also developed custom porcine microarrays using these ESTs. Transcriptomics studies have been performed, principally aimed at livestock improvement, and dealing with muscle development (see Kim, Chang, Hong, Jung, Kwon, Cho et al., 2005), reproduction (Whitworth, Agca, Kim, Patel, Springer, Bivens et al., 2005) and infection (Moser, Reverter, Kerr, Beh & Lehnert, 2004).

Porcine microarray chips are available commercially (Affymetrix GeneChip® Porcine Genome Array, interrogating approximatively 23,256 transcripts from 20,201 *S. scrofa* genes). Operon Biotechnologies has developed a set of 13,297 annotated "ready to spot" oligonucleotides specific for *S. scrofa*. These DNA fragments can be used to manufacture DNA chips for pig transcriptomics studies, which can be used for the generation of reliable toxicogenomics and/or pharmacology data in minipigs.

Nevertheless, it appears that very little gene expression work has been done in pigs in the context of toxicology and safety assessment. The principal animal models used for toxicogenomics studies and predictive toxicology are rats, reflecting the predominant position of the rat in regulatory toxicology. Several companies (including Iconix and Gene Logic) have developed toxicity signature databases by correlating the early gene expression profiles induced by reference compounds in key target tissues of the rat (liver, kidney and heart) with traditional toxicology endpoints such as histopathology or clinical pathology findings.

Unpublished and confidential studies performed by pharmaceutical companies and CRO's will also constitute a significant proportion of the work in this area. Here also we anticipate that the rat and the non-human primate will be the principal models used in toxicogenomics studies.

The potential advantages of the pig in the area of toxicogenomics remain unexploited at the present time. As for other animal models, we may expect that the application of toxicogenomic approaches to the pig and to the minipig will lead to valuable insights and the identification of useful efficacy and/or toxicity biomarkers.

3.2. Porcine genomics and toxicogenomics: technical gaps

No significant technical gaps were identified in terms of genomics tools that could potentiate the role of the pig (or minipig) in toxicogenomic investigations. The principal technical issue impeding the wider application of genomics and related technologies to the minipig is the deficit in the bioinformatics infrastructures for storage, analysis and use of the porcine gene expression data (both data already available and that which will be generated in the future). This is not an issue specific to porcine genomics, and the comment is equally applicable to the development of genomics studies in other animal species.

Data mining in transcriptomics studies relies on existing annotation as well as expression databases. Predictive toxicology studies particularly need the comparison of transcription profiles with drug signature databases. Up to now, no such database for the pig and/or minipig has been generated and this leaves a very important gap in the development of genomics tools for predictive toxicity and/or pharmacology studies. The value of the minipig as a model in toxicogenomics would be greatly enhanced by availability of toxicity databases and validated toxicity signatures (on the model of the Gene Logic and Iconix databases for the rat, described above). Initiatives in this area, through collaborative exercises, or through private or public funding, could have a significant catalysing role in the use of the minipig as a model for toxicogenomic studies, and hence permit the toxicology community to benefit from the strong pre-existing genomics knowledge base for the pig.

Initiatives are needed to open up data generated in livestock context for exploitation in other areas of research such as safety assessment. Genomics studies in livestock context provide new information concerning gene functions that may also provide insights in the field of safety assessment. The generation of focused databases should be encouraged.

3.3. Genomics and toxicogenomics: conclusions

Much research in the animal breeding, genetics and genomics of the pig has been undertaken, driven largely by the economic and agricultural importance of the pig. As a consequence the genomics knowledge base for the pig is strong, and the tools and databases required to facilitate pig genomics studies are well developed. These tools and the genomic data generated for the domestic pig are equally relevant and applicable to different pig breeds including minipigs. The context for gene expression and toxicogenomics studies in the pig is therefore very favourable. We anticipate that this general conclusion is also true for the application of other –omics technologies such as proteomics and metabolonomics. Nevertheless, it appears that very little gene expression work has been done in pigs in the context of toxicology and safety assessment and the potential advantages of the pig in the area of toxicogenomics remain unexploited at the present time. Initiatives to make available toxicity databases and validated toxicity signatures could have a significant catalysing role in the use of the minipig as a model for toxicogenomic studies.

The pig genome shows extensive homology with the human genome and evidence from sequence studies suggest that the evolutionary distance in sequence space between the porcine and human genome sequences is smaller than the distance between mouse and human (and by implication, probably also the rat and the human).

4. Genetic manipulation in the pig

4.1. Introduction

The ability to add or remove genetic material from the germline of mammals has been possible since the late 1970s. Such genetically

modified animals are termed transgenic. Among mammalian species, in addition to transgenic mice, gene transfer technology has enabled the generation of transgenic rats, sheep, goats, cattle and pigs. There are two reports of transgenic monkeys (Chan, Chong, Martinovich, Simerly & Schatten, 2001; Sasaki, Suemizu, Shimada, Hanazawa, Oiwa, Kamioka et al., 2009) and there are no reports of transgenic dogs. Transgenic minipigs have been generated (Uchida, Shimatsu, Onoe, Matsuyama, Niki, Ikeda et al., 2001) and arguments presented for their use in biomedical research (Vodicka, Smetana, Dyorankova, Emerick, Xu, Ourednik et al., 2005).

Although the vast majority of transgenic work has been performed in the mouse, much of the transgenic technology established in this species is applicable to large animal species such as the pig, dog and monkey. However, differences in the reproductive processes in each species and the extent of understanding of their reproductive physiology, results in significant differences in the ability to generate transgenic models in these different species.

4.2. Gene transfer methods in the pig

A variety of transgene delivery methods have been established for the generation of genetically modified animals (Clark & Whitelaw, 2003). All were initially developed in mice and subsequently applied to larger animal species.

The original method of transgene delivery involves the direct (micro)-injection of DNA into the pronuclei of fertilised eggs. This method has been applied to pigs for the last two decades. Although this method is less efficient in the pig than in the mouse, pronuclear injection still enables the robust generation of transgenic pigs (Brem & Springmann, 1989; Clark & Whitelaw, 2003). Recently a more efficient transgene delivery route has been established, involving the use of replication-defective lentivirus vectors (Whitelaw, 2004). This method is at least 10-fold more efficient than pronuclear injection when assessed in terms of the number of transgenic founder (F_0) animals, and has been used to generate transgenic pig models (Hofmann, Kessler, Ewerling, Weppert, Vogg, Ludwig et al., 2003; Whitelaw, Radcliffe, Ritchie, Carlisle, Ellard, Pena et al., 2004). The increased efficiency can be expected to result in an overall reduction in the number of animals needed to generate a transgenic pig model.

In the mouse, gene transfer can be achieved using cultured embryonic stem (ES) cells which can be genetically modified prior to introduction into mouse embryos. This approach permits targeted genetic modifications by homologous recombination. Unfortunately equivalent ES cell lines do not currently exist for pigs or dogs (Clark & Whitelaw, 2003) and ES cell technology cannot be applied to these species.

Recent work by groups in Japan and the US has pioneered the way to reprogram somatic cells, such as fibroblasts, to become pluripotent. This involves as yet unknown signalling cascades through the simple transfection of four genes — *myc, sox2, klf4 and Oct4*. In this way somatic cells are reprogrammed to become ES-cell like in nature and action, designated induced Pluripotent Stem cells (iPS). This has been demonstrated for mouse fibroblasts and more recently for human cells (Takahashi, Tanabe, Ohnuki, Nariat, Ischisaka, Tomoda et al., 2007), thus opening up the possibility that the 4-gene approach may be applicable to a range of animal species, which is clearly a stimulating proposition. Concurrently, primate ES cells have been generated using a nuclear transfer technique (Byrne, Pedersen, Clepper, Nelson, Sanger et al., 2007).

In the absence of ES approaches for pigs and dogs, gene targeting in these non-rodent species can be achieved using nuclear transfer (cloning) technology (Wilmut, Schnieke, McWhir, Kind & Campbell, 1996), since the donor cell can be genetically modified prior to transfer. Several transgenic pig models have been produced in this way (Vajta, Zhang & Machaty, 2007). This method allows for gene deletion (and thus removal of gene activity) and is currently being utilised in the development of animals for xenotransplantation studies (Bucher, Morel & Bucher, 2005).

Building on the initial and highly debated claims of sperm-mediated gene transfer (Wall, 1999), intracytoplasmic sperm injection using transgenic sperm cells has emerged as an alternative transgene delivery route and has been recently demonstrated in pigs (Yong, Hao, Lai, Li, Murphy, Rieke et al., 2006). The emergence of spermatogonial stem cell culture (Kanatsu-Shinohara, Ikawa, Takehashi, Ogonuki, Miki, Inoue et al., 2006) may further contribute to this approach by facilitating the ability to introduce transgenes into the donor cells.

Looking to the future, it is anticipated that the recent trend to develop more precise and efficient transgene delivery methods will continue to have a positive impact in terms of the 3R's. Given the state of knowledge of reproductive processes and technical capabilities in embryology manipulation it is likely that transgenesis in the pig will remain more advanced than transgenesis in the monkey and dog.

4.3. Transgenic animals and reproductive technologies in toxicology

In the 25 years since the first production of a transgenic mouse by Gordon and Ruddle in 1981, transgenic approaches have already begun to play a significant role in regulatory toxicology. One established area of use of transgenic mice is in short-term tests for carcinogenic properties. After a large scale collaborative study to evaluate the use of four carcinogenesis-prone transgenic mouse strains in the testing of new medicines, these model are now accepted by regulatory authorities as part of the "weight-of-evidence" evaluation of carcinogenic properties (Jacobson-Kram, Sistare & Jacobs, 2004). Transgenic mice also increasingly find a role in routine toxicology testing of biotechnology products, in those cases where the molecular targets of biologicals are only present in humans and are not present in laboratory animals. In such cases, genetic modification may offer a route to develop an appropriate model for toxicology testing, where otherwise no appropriate model exists (Ma & Lu, 2007). Another potentially valuable area of application is the use of transgenic strains with modified metabolic capabilities, including hepatic cytochrome P450 reductase null mice (Henderson & Wolf, 2003). Numerous further applications may be proposed: transgenic reporter animals that enable sensitive and rapid read-out of toxic insults could contribute to early toxicity screens (Maggi, Ottobrini, Biserni, Lucignani & Ciana, 2004), and reporter systems enabling non-invasive and/or real time read-out are being developed.

At present these approaches have not been extended to include transgenic non-rodent approaches in toxicology, but in principle there is no reason why current transgenesis approaches could not also be applied to dogs and monkeys. In the case of the dog and the monkey, limitations are entirely technical and transgenic models are not generated in these species on a routine basis. Our knowledge of early reproductive processes in the dog is limited (Luvoni, Chigioni & Beccaglia, 2006), resulting in a lack of technical know-how for manipulation of the dog embryo. There is one report of a transgenic macaque monkey (Chan et al, 2001) and one report of a transgenic New World monkey, generated (Sasaki et al., 2009); both were generated using viral vector transgène delivery approaches. Although techniques for monkey species are not currently well-established in the laboratory, it is anticipated that methods to manipulate monkey embryos could be readily developed, building on our extensive knowledge of human embryonic development.

In contrast transgenic pigs have been generated for the last two decades and all the required procedures are now well established (Pursel, Pinkert, Miller, Bolt, Campbell et al., 1989; Golovan, Meidinger, Ajakaiye, Cotrill, Wiederkehr et al., 2001; Lavitrano, Bacci, Forni, Lazzereschi, Di Stefano et al., 2002; Whitelaw et al 2004; Kraft, Allen, Petters, Hao, Peng & Wong, 2005; Kues, Schwinzer, Wirth, Verhoeyen, Lemme et al., 2006; Deppenmeier, Bock, Mengel, Niemann, Kues et al., 2006; Hao, Yong, Murphy, Wax, Samuel et al., 2006). These approaches have also been applied to minipigs and transgenic minipigs have been

generated (Uchida et al, 2001). Current efforts focus on generating transgenic minipigs using nuclear transfer of transgenic donor cells (Gabor Vajta, personal communication). It seems very likely that techniques for transgenesis in the pig will remain more advanced than those for the monkey and dog in coming years. The moment may arrive in the future, where new technologies and new approaches in toxicology may in the future lead us to expect significant ethical and technical benefits through the development of transgenic non-rodent models (for example, through the generation of sensitive "reporter" models with non-invasive read-out of genomic responses). In these cases, our current knowledge base and experience indicates that the pig will be the species of choice.

4.4. Genetic manipulation in the pig: conclusions

In conclusion, transgenic germline manipulation is established and well developed in pigs and there are numerous examples of transgenic pigs, including transgenic minipigs. In contrast these approached are not well established in monkeys or dogs at the present time. A key tool in this area is the ES cell. Currently ES or ES-like cells only exist for mice and man. There are currently a number of experimental avenues being explored that lead to the generation of pluripotent cells and it is a realistic expectation that ES based approaches will be developed for other species beyond mice and man. Of the non-rodent species routinely used in regulatory toxicology testing (dog, minipig, monkey), the pig is the only species in which the generation of transgenic models is well established. This represents a real advantage for the testing of those biotechnology products which are highly species-restricted and for which no appropriate animal model exists, permitting the development of transgenic models for toxicology testing.

5. Cloning and reproductive technologies in the pig

5.1. Cloning in the pig

The term "cloning" refers to somatic cell nuclear transfer (SCNT) technology, which permits the generation of genetically identical "cloned" animals. The development of cloning technology (Wilmut et al, 1996) and the attendant publicity given to "Dolly" and other cloned animals has had an enormous impact on the public perception of research in this area, and led to widespread public debate of the ethical issues and finally the need for legislative processes (Suk, Bruce, Gertz, Warkup, Whitelaw, Braun et al., 2007). Cloning can be combined with genetic modification (transgenesis) but, without the use of genetically modified cells, represents replication of genetically identical animals. This can be viewed as analogous to normal reproductive processes during twinning (Fulka & Fulka, 2007). Unlike twinning, multiple cloned animals may be produced and cloning thus enables the generation of populations of genetically identical animals. This may also be achieved by inbreeding, but with attendant disadvantages resulting from consanguinity.

Numerous studies demonstrate the application of nuclear transfer cloning in pigs (Vajta et al, 2007, Pursel et al 1989; Kues et al 2006; Deppenmeier et al 2006) and recent efforts have resulted in the generation of transgenic minipigs using this technology (Kragh, Nielsen, Li, Du, Lin, Schmidt et al., 2009). The first report of cloning in dogs was surrounded by controversy but is now considered to be factual (Parker, Kruglyak & Ostrander, 2006; Luvoni et al, 2006). Cloned monkeys have been generated (Meng, Ely, Stouffer & Wolf, 1997) but the various difficulties encountered in producing non-human primate embryos by nuclear transfer indicate that cloning technology is still only poorly established for monkeys (Simerly, Navara, Hyun, Lee, Kang, Campuano et al., 2004). Nuclear transfer cloning cannot currently be considered to be routine in either dogs or monkeys. In contrast, cloning is well established in pigs and it is expected that this approach can easily be applied also to minipigs.

Cloning is potentially of interest in the development of animal models, since identical individuals can be generated without the accompanying disadvantages of consanguinity. This could potentially offer reduced animal to animal variability within groups of animals, and possibly provide opportunities for the experimental refinement and reduction in animal numbers.

5.2. Reproductive biotechnologies in the pig

Beyond cloning, several other reproductive biotechnologies impact on the possible use of animals in toxicology. Such technologies focus on the early mammalian embryo and include *in vitro* fertilisation (IVF) and *in vitro* maturation (IVM), embryo splitting and twinning.

The ability to perform IVF/IVM enables the use of surplus eggs (e.g. from a slaughterhouse). In contrast, for species where these technologies are not established, embryo manipulation requires *in vivo* fertilised embryos, probably after super-ovulation of the donor females. IVM/IVF capability is therefore associated with reduced animal numbers, reduced animal welfare concerns and reduced financial costs.

In the pig, despite considerable progress, IVM/IVF systems are still not optimal (Nagai, Funahashi, Yoshioka & Kikuchi, 2006). For the generation of transgenic pigs the lack of IVM/ICF capability can be offset by efficient transgene delivery systems, such as that offered by lentivirus vectors (Whitelaw et al, 2004; Sasaki et al, 2009). Currently for dogs the situation is even less favourable, principally due to our limited understanding of the necessary conditions for successful *in vitro* development of dog embryos (Luvoni et al, 2006). Building on the extensive human clinical use of IVF/IVM, procedures for these technologies in monkey are established (Yin, Duffy & Gosden, 2006).

The advantage of twinning is that genetically identical paired animals are generated. Embryo splitting and the generation of twins is not robust in monkeys (Schramm & Paprocki, 2004), and not at all practiced in the dog. In contrast, embryo splitting has been established for the pig for some time (Nagashima, Katoh, Shibata & Ogawa, 1988).

5.3. Cloning and reproductive biotechnologies: conclusions

In conclusion, nuclear transfer cloning and most other reproductive biotechnologies are well established in the pig, some approaches are developed for primates and very few have been applied to dogs. As a consequence a number of technical possibilities are available in the pig (and minipig) making this species an attractive research model. IVM/IVF systems are still not optimal in the pig, and there would be animal welfare benefits if progress could be made in this area. Overall the pig is well positioned for future developments in cloning and reproductive biology.

6. Biosensors and nanotechnologies

A range of "sensor" technologies could contribute in important ways to the way that toxicology is performed in the future. Developments in telemetry, remote sensors, biosensors, automated sampling and imaging have the potential to enrich the data obtained from animals used in regulatory toxicology studies. These approaches may replace some invasive techniques or techniques requiring sacrifice, and may allow *in vivo* monitoring and serial recording of physiological parameters and relevant toxicology biomarkers and/or biomarker signatures.

Biosensor readouts of this kind could permit investigators to obtain more extensive data from large animals both in terms of the range of endpoints/parameters that are measured, and in terms of the time points since continuous monitoring may be possible. Approaches of this kind may also permit reductions in the numbers of rodents (and satellite animals) that are required for the performance of studies. It is clear, therefore, that such approaches have clear implications for the application of the 3Rs in regulatory toxicology.

These technologies may be invasive; this covers approaches such as implanted devices, telemetry, cannulation, or analyses performed on using blood and urine samples, skin punches, biopsies etc. Other approaches are non-invasive, such as sensor patches, intracorporeal nanotechnology remote readout sensors and transgenic reporter genes (such as luciferase, green fluorescence proteins, urinary excreted reporters).

This is a fast moving area of research and it is not easy to accurately define what will become available or when these new technologies will result in useful applications. It was nevertheless the consensus opinion of the authors that these technologies can be expected to be neutral in terms of selection of animal models, and do not favour the use of any particular large animal species.

7. *In vitro* alternatives in toxicology testing

Given the swell of academic interest that has centred on *in vitro* alternatives in toxicology testing, and the generous funding that has been made available in this area, we can look forward to progress and new developments in the replacement of animal studies in coming years. *In vitro* tests have already met with some success in the replacement of animal studies that address a single well-defined endpoint (for example, skin irritation, skin corrosion, endotoxin detection, mutagenic activity etc.). For the time being, *in vitro* approaches have not been developed which can address issues of "general toxicity", where the target organ and possible harmful effects of a drug remain undefined. In the future, approaches may also be developed for this more complex problem, perhaps through the application of toxicogenomics.

Sources of tissues or cells (primary cells or permanent cell lines) are generally required for *in vitro* assay systems. Since the objective in regulatory toxicology is the prediction of hazard for humans, it will normally be most relevant to use human tissues and cells where they are available. There are cases where complex (genetic) manipulations have been performed on the biological test materials, or cell lines are used which have very specific phenotypes and characteristics, but apart from such cases, why not use human material if you can!

Some human tissues are hard to obtain, especially those tissues that are in high demand for transplantation, and there may be other circumstances where it is impractical to work with material of human origin. Where this is so, researchers should consider the close anatomical, physiological and biochemical similarities between pigs and humans. On account of these similarities, pigs may often be the most appropriate choice of species for the derivation of cells, and may provide the best prediction of human hazard.

A further practical advantage of the pig as a source of tissues and cells is that domestic pig materials may be readily available from the slaughterhouse, while it will be necessary to sacrifice animals in order to obtain tissues from non-food source animal species (e.g., monkey hepatocytes).

In conclusion, therefore, it was considered that, generally speaking, human tissues and cells will be the most appropriate biological material for in vitro tests intended to predict a hazard for humans. Where human material cannot be used, careful consideration should be given to the pig as a source of tissues and cells. Because of the close anatomical, physiological and biochemical similarities between pigs and humans, the use of pig materials may often provide the best prediction of a hazard for man. A further practical advantage is that porcine material can be readily obtained from the slaughterhouse, while non-food source species must be sacrificed to provide tissues.

8. Conclusions and recommendations

8.1. Gaps and opportunities

This article has identified many potential opportunities for further research. Of these opportunities there are 3 that deserve particular mention

1. Breed minipigs for smoother temperament: Anybody who has worked with Göttingen minipigs will attest that they are not always easy to handle. The opportunity therefore exists to selectively breed minipigs for smoother temperament and greater tolerance of human contact. Much in the same way that reduced body size and "dominant white" skin colour of the Göttingen minipig were intended to adapt the minipig to laboratory use, breeding for temperament would bring further benefits in the laboratory environment. Better tolerance of human contact and greater amenability to handling would at the same time reduce the stress to animal handlers and scientific staff and also reduce the stress for the animals themselves. In consequence this would be a highly ethical development reducing anxiety during laboratory manipulation for both animal handling staff and for the minipigs and certainly contributing to enhancing the scientific quality of the work performed
2. Data base of drug-induced gene expression signatures in the minipig: Mini-pigs are very well positioned for use in toxicogenomic studies. Established experience in toxicogenomics studies would clearly favour the use of the minipig in routine regulatory toxicology studies, because of the additional interpretative data and mechanistic insight that it can bring. Data on the gene expression changes and signatures after treatment with reference toxicants would be very valuable in this respect, establishing the mini-pig as a toxicogenomics model and stimulating its further use. Predictive toxicology studies particularly need the comparison of transcription profiles with drug signature databases. Up to now, no such database for the pig and/or minipig has been generated and this leaves a very important gap in the development of genomics tools for predictive toxicity and/or pharmacology studies. The value of the minipig as a model in toxicogenomics would be greatly enhanced by availability of toxicity databases and validated toxicity signatures.
3. Progress in reproductive biology: further understanding of the reproductive biology of the pig/minipig focused on refinement of IVM/IVF techniques would bring animal welfare benefits. In particular it could permit the use of slaughterhouse-derived oocytes for embryo manipulation and genetic modification work, with a corresponding positive impact on the reduction of animal numbers used for experimental work

8.2. Application of 3Rs

This article describes a number of opportunities for 3R's benefits through technologies that can provide a greater volume of more relevant data from a smaller number of animals, or from the development of animal models that are more pertinent for the evaluation of safety.

The targeted breeding of minipigs for smoother temperament and better tolerance of human contact would be a highly ethical project. It would result in reduced stress and anxiety during laboratory manipulations for both animal handling staff and for the minipigs themselves and would certainly contribute to enhancing the scientific quality of the work performed.

8.3. Conclusions

In reviewing the potential of the minipig as a platform for future developments in genomics, high density biology, transgenic technology, *in vitro* toxicology and related emerging technologies, a number of features and advantages of this model emerge.

First, it is a repeated theme of this article that commercial interests in the pig as an agricultural production species have driven scientific progress in several research areas. There is no equivalent economic driver for progress in the dog or the monkey. As a result the available knowledge-bases are much greater for pigs (than for dogs or monkeys) in many areas (physiology, disease, genetics, immunology etc). Fundamental genomic knowledge and phenotypic characterization in regard to the pig is well in advance of the dog or the monkey

and basic knowledge of the pig is therefore likely to stay ahead of the other two species.

In the field of genomics a range of genomic tools is available including sequence data, SNP chips and whole genome arrays. These tools and the genomic data generated for the domestic pig are equally relevant and applicable to different pig breeds including minipigs. The context is propitious for studies in functional genomics and for the identification of genetic mechanisms associated with gene functions and genotypes. All the elements are in place for the exploitation of the pig in toxicogenomics. Nevertheless, it appears that very little gene expression work has been done in pigs in the context of toxicology and safety assessment and the potential advantages of the pig in the area of toxicogenomics remain unexploited at the present time. Initiatives to make available toxicity databases and validated toxicity signatures could have a significant catalysing role in the use of the minipig as a model for toxicogenomic studies.

The pig genome shows extensive homology with the human genome and evidence from sequence studies suggest that the evolutionary distance in sequence space between the porcine and human genome sequences is smaller than the distance between mouse and human (and by implication, probably also the rat and the human). These sequence similarities suggest that the molecular targets of the pig may conserve homology with human targets and that the minipig may find a role in the testing of biotechnology products. In the same way, the sequence similarity suggests that the minipig could play a leading role in approaches to in silico toxicology.

When we turn to genetic manipulation and reproductive technologies, transgenic germline manipulation is well established in pigs and there are numerous examples of transgenic pigs, including transgenic minipigs. In contrast these approached are not well established in monkeys or dogs at the present time. Of the non-rodent species routinely used in regulatory toxicology testing (dog, minipig, monkey), the pig is the only species in which the generation of transgenic models is well established. The ability to generate transgenic minipig models represents a real advantage for the testing of highly species-restricted biotechnology products. For such products, cases are often encountered where no relevant animal model exists, and the development of transgenic models is necessary in order to generate relevant animal models and permit safety testing.

Nuclear transfer cloning and most other reproductive biotechnologies are well established in the pig, some approaches are developed for primates and very few have been applied to dogs. Overall the pig is well positioned for future developments in cloning and reproductive biology.

In addition to the strong position of the minipig in genomics and in germline manipulation, it must also be remembered that the Göttingen minipig is a genetically managed model (unlike routinely used dog and monkey toxicology models), and that the basis of the small size of the Göttingen minipig does not involve defective genes.

While the emerging technologies are essentially “species neutral” and can in principle be applied to all species, for all the technologies that we examined, basic knowledge and technical capabilities are greater for the pig than the dog or monkey. In concrete terms, in application to safety testing we have seen that:

- The Göttingen minipig is well positioned for the performance of toxicogenomics studies.
- The sequence homology between pigs and humans suggest that minipigs will be useful for the testing of biotechnology products (and possibly for in silico toxicology).
- And finally the minipig is the only non-rodent toxicology model where transgenic animals can be readily generated, and reproductive technologies are well developed in the pig.

These factors all support the idea that the minipig is well placed to meet the challenges of the emerging technologies and the toxicology of the future.

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