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Transgenic animals resistant to infectious diseases

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Summary
The list of transgenic animals developed to test ways of producing livestock resistant to infectious disease continues to grow. Although the basic techniques for generating transgenic animals have not changed very much in the ten years since they were last reviewed for the World Organisation for Animal Health, one recent fundamental technological advance stands to revolutionise genome engineering. The advent of technically simple and efficient site-specific gene targeting has profound implications for genetically modifying livestock species.

Keywords
Infectious disease – Transgene – Transgenic livestock.

Introduction

Strictly speaking, a transgenic (TG) animal is one that contains one or more 'foreign' genes introduced by methods distinct from conventional genetic crossing or hybridisation. A '*trans*gene' is a gene that has come *across* into the animal's genome from a different species or been produced by synthetic means, and it is implicit that this required the use of molecular biological genetic modification techniques. It is now possible to insert a transgene precisely wherever it is intended in the recipient host's genome. The transgene is usually inserted into the germ-line of the animal so that it is perpetuated in subsequent generations. By this definition, all transgenic animals and their descendants are genetically modified organisms. These same techniques can now be applied more subtly to make precise changes to the animal's own genes by altering specific single nucleotides or inserting/deleting short regions to disrupt, modify or repair individual genes. This is referred to as 'genome editing'. Because no foreign DNA is introduced, the animal is not formally transgenic. A similar argument can be made for gene knockout approaches. It is a matter of semantic dispute whether such animals are considered to be genetically modified or not.

The World Organisation for Animal Health (OIE) has reviewed the progress in transgenic livestock research on several occasions (1, 2, 3). This paper will concentrate on the current state of the art for genome modification techniques that are now being applied to generate transgenic livestock resistant to infectious disease.

Transgenic technologies

The way in which a new gene is inserted into the germ-line of a target species varies depending on the species concerned. The popularity of the different techniques waxes and wanes: new techniques are used frequently when first introduced, but they tend to fall out of favour when simpler and more efficient techniques are developed. Direct pronuclear microinjection of modified DNA into newly fertilised oocytes (4), once the dominant methodology, is now relatively uncommon. Current methods modify cells grown in the laboratory and then manipulate them to regenerate embryos that can be implanted into surrogate mothers. This allows the genome of the modified cells and expression of the transgene to be characterised beforehand. All well-established DNA delivery techniques may be used to introduce the DNA into these cells, e.g. electroporation, transfection (5) or transduction using viral vectors such as lentivectors (6).

For some species, embryonic stems (ES) or primordial germ cells (PGCs) can be maintained *in vitro*, genetically modified and then injected into a normal developing blastocyst/embryo. These cells populate the embryo and produce mosaic offspring. Some of these cells become

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germ cells, so a proportion of the offspring from these animals will be hemizygous transgenic. ES and PGCs are derived from early embryos and are difficult to obtain from species other than humans and mice. This obstacle may be overcome in future if methods developed to produce the closely analogous 'induced pluripotent stem' (iPS) cells can be applied to animal species (7). The current method of choice for generating transgenic livestock (and animal cloning procedures) is somatic cell nuclear transfer (SCNT) (8). Fetal fibroblast cells are readily cultured, modified and characterised in the laboratory and then used to donate their nuclei. Introducing a donor nucleus into an enucleated ovum and triggering fusion with the ooplast (by chemical or electrical means) reprogrammes the nucleus to produce a viable blastocyst that can be implanted in a surrogate mother animal. This is still a complex, technically challenging and inefficient process.

Lentiviral vectors have grown in popularity due to the ease of use and their high-efficiency gene delivery (9). They can be used to modify cells in culture or used directly by infection into pre-implantation embryos, PGCs or, in the case of chickens, early embryonic discs (10). They have the attractive property of apparently preferentially targeting cells destined to be germ cells and integrating their transgene DNA into transcriptionally active regions of the chromosome. Consequently, they produce transgenic animals with relatively high efficiency that express the transgene stably over multiple generations. However, they have the disadvantages of integrating randomly and having limited packaging capacity. The efficiency of transgenesis is highly dependent on the titre of the vector (6), which decreases significantly when its total length exceeds 9.5–12 kilobases (kb) (11).

Sperm-mediated gene transfer exploits the property common to many animal species whereby their sperm can bind, take up and possibly integrate foreign DNA into their genome. Binding and uptake can be enhanced by transfection methods and by using 'linkers' (monoclonal antibodies that enhance binding of the exogenous DNA to the sperm surface) (12). Sperm containing exogenous DNA may carry this as an integrated or episomal form into the ovum during normal fertilisation. Alternatively, intra-cytoplasmic injection of DNA-treated sperm heads can lead to efficient production of transgenic progeny (13). Although the concept of sperm-mediated transgene delivery has been around for over 30 years, the controversy surrounding its original exposition, inconsistent success rates and the sense that it is too good to be true have apparently discouraged its wider adoption. Recently the use of cultured spermatogonal stem cells (SSCs) for transgenesis has been demonstrated in mice. Sterile donor animals repopulated with modified SSCs produce transgenic sperm with 100% transgene transmission (14). If SSCs from other species can be manipulated in the same way, this will be a major advance in animal transgenesis.

Site-specific gene targeting

The majority of transgenic approaches applied to livestock species integrate the transgene into random locations in the recipient genome. This has major disadvantages: each integration site must be characterised to determine the local effects on transgene expression and potential consequences for nearby genes; expression of the transgene is variable and difficult to control; the efficiency of producing transgenic animals is often very low; usually it is not practical to eliminate, modify or repair defective endogenous genes.

Efficiently targeting genetic modification to specific genome locations was previously only practical in ES cells (and therefore limited to mice and humans), which have more prominent homologous recombination (HR) repair activity. Systems now exist that work well in ordinary somatic cells enabling efficient production of TG animals by SCNT. Indeed, these methods are becoming so advanced, it is now feasible to directly inject DNA into the cytoplasm of a zygote (technically much simpler than pronuclear injection or SCNT) and achieve efficient site-specific integration of the transgene (15, 16).

The systems exploit the combined effect of exogenous site-specific nucleases and endogenous HR DNA repair mechanisms. The principle is very simple: a site-specific nuclease is introduced into suitable cells (e.g. PGCs, fibroblasts intended for SCNT or a zygote). The nuclease cleaves the genomic DNA at a specific site and this 'damage' activates the cells' DNA repair mechanisms. Double-stranded DNA breaks are recognised and repaired by one of two processes, namely, non-homologous end-joining (NHEJ) and HR. NHEJ is the process whereby broken ends of DNA are stuck back together again and it is usually the dominant process in somatic cells. It is an error-prone process that produces insertions, deletions and point mutations at the site of cleavage. This has some useful but limited applications. However, if the site-specific nuclease is introduced in conjunction with a linear piece of double-stranded DNA homologous to the sequences surrounding the cleavage site, this exogenous DNA can be inserted by HR. Modified nucleases that produce single-strand rather than double-strand breaks ('nickases') result in HR-mediated repair in preference to NHEJ (17) or can be used in pairs to reduce off-target effects (18). This is beneficial, as it enables efficient gene targeting by HR in somatic/non-ES cells. The homologous piece of DNA can be designed to introduce specific point mutations to alter or repair the endogenous gene, or to delete a defined section of the genome or insert a transgene into a specific location. If the nuclease targets both copies of the gene in the diploid cells, a homozygous transgene can potentially be achieved in a single operation (15).

Initially, this technology made use of mega-nucleases such as I-Sce-1 and the Cre/Lox and Flp/FRT systems, which have large target sequences and consequently cleave at very rare sites in the chromosome. This permitted site-specific modification of genomic DNA, but seriously limited the locations that could be specifically altered. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) represented a major breakthrough, making it possible to couple nuclease domains together with assembled arrays of modular DNA binding domains to generate nucleases with customisable large target sequence specificity (reviewed in [19]). The production of ZFNs is technically more challenging and relatively expensive compared to TALENs, which quickly dominated this burgeoning technology. However, TALENs have themselves been superseded by the conceptual simplicity and ease of use of the CRISPR/Cas9 system.

CRISPR/CAS9 nuclease

Clustered regularly interspaced short palindromic repeats (CRISPRs) are repetitive DNA elements found in the genomes of most bacterial species. They are components of a system providing 'adaptive immunity' against bacteriophage infection and plasmid invasion. At least three distinct classes of CRISPR systems exist which differ in their detail, but all operate on the same fundamental principle. In a two-stage process they are first acquired from invading foreign DNA and then used to specifically target that DNA and interfere with phage or plasmid replication.

Naturally, CRISPRs are acquired by the action of Cas proteins recognising and cleaving foreign DNA and incorporating fragments of it into 'CRISPR loci' in the bacterial genome. Each fragment (referred to as a 'spacer') is inserted bounded by a palindromic repeat, hence their name. In the interference stage, these loci are transcribed and the resulting RNAs processed by other Cas proteins to generate 'CRISPR RNAs' (crRNAs). crRNAs form complexes with other Cas proteins that have nuclease activity. The sequence of the crRNA determines the specificity of the nuclease. The result is a ribonucleoprotein complex that can interrogate foreign DNA by unwinding it and specifically cleaving it where it contains a sequence complementary to the crRNA. Because the CRISPR loci has gained a new CRISPR in the acquisition process, the bacterium is now permanently protected against invaders with that particular sequence. This in itself is a fascinating system that exemplifies a form of Lamarkian evolution in bacteria, but it is its application to eukaryotic genome engineering that has brought it to prominence. The most fully developed system so far is CRISPR/Cas9, based on the one found in *Streptococcus pyogenes*.

Exploiting the CRISPR system for genome engineering purposes is an elegantly simple process (for a comprehensive review see [17]). The first component is a source of the protein with the interrogator/nuclease function (in this case, purified Cas9 protein, synthetic messenger RNA (mRNA) or a plasmid that expresses the Cas9 gene, depending on which is most appropriate for the application). The second component is the crRNA (or 'guide RNA' in the CRISPR/Cas9 system). This matches the sequence of the DNA target plus a region that interacts with Cas9. The combination of guide RNA and Cas9 produces a complex with an almost entirely customisable large target sequence specificity. The third component is the linear piece of DNA with homology to the region spanning the target site and the genetic modification that the user wishes to introduce. This will be the substrate for the HR system to repair the DNA damage (the site cut by the nuclease) and thus incorporate the modification.

The pace at which this technology is developing means that this review will be out of date within months. It is reasonable to expect that integrated viral delivery systems for the three components and reconstituted synthetic *in vitro* systems for delivering guide RNAs, nucleases and transgene DNA will be in general use in the near future. This has truly opened the door to genome editing and transgenic manipulation of a wide range of species. It is now feasible to envisage precise and sophisticated approaches to alter host species genetics to control a range of infectious diseases.

Strategies for interfering with replication and pathogenesis of infectious diseases

Muller (3) reviewed a variety of different approaches to genetically suppress infectious diseases in livestock. These will be reprised here under three conceptual categories with up-to-date examples of where these have been applied to livestock (Table I).

Molecular spanners

The term 'molecular spanner' comes from the idiom 'to throw a spanner in the works' (an unsophisticated disruption of the normal functioning of a machine by jamming it with something). Mechanisms of natural genetic resistance may involve multiple genes and require subtle knowledge of the pathogen/host interaction in order to be exploited transgenically. However, only basic knowledge about how the pathogen operates is needed for researchers to design molecules to interfere with it, e.g. molecules that can bind to essential components and stop them working. These include: molecular decoys, short interfering RNAs (small interfering RNAs [siRNAs], short-hairpin RNAs [shRNAs] and micro RNAs [miRNAs]), transdominant

Table I
Examples of livestock animals with disease-resistance transgenes

Target species	Disease or pathogen	Gene transfer method	Random or site-specific integration	Insertion locus	Inhibitory strategy	Gene target	References
Cattle	Bovine TB	SCNT	SS (TALEN)	M-S locus	IIG	Mouse SP110	(20)
Cattle	BSE	SCNT	SS (HR KO)	PrP	Intrinsic (KO)	PrP	(21)
Cattle	*Staphylococcus aureus*	SCNT	SS (ZFN)	CSN2 (β- casein)	IIG	Lysostaphin	(22)
Cattle*	Trypanosomosis	Hydrodynamic transfection	Random (somatic)	N/A	IIG	Baboon APOL1	(23)
Cattle	*S. aureus*	SCNT	Random	N/A	IIG	Lysostaphin	(24)
Chickens	Influenza A	Lentivector	Random	Ch2:21,481,758	RNA Decoy	Flu 5'3'NCR	(25)
Goats	Mastitis	PNI	SS (RecA)	β-lg	IIG	Lysozyme	(26)
Goats	Mastitis	SCNT	Random	N/A	IIG	β-defensin	(27)
Goats	BSE	SCNT	Random	N/A	Intrinsic (RNAi)	PrP shRNA	(28)
Goats	FMDV	SCNT	Random (SB transposon)	Unknown	shRNA	3D	(29)
Pigs	ASFV	ICI	SS (TALEN/ZFN)	Rel-A	IIG or Intrinsic?	Rel-A	(30)
Pigs	Influenza A and CSFV	SCNT	Random	Unknown	IIG	Porcine Mx1	(31)
Pigs*	PRV	ZMI	Random	Unknown	Receptor decoy	Nectin-1	(32, 33)
Pigs	Influenza A	PNI	Random	Unknown	IIG	Murine Mx1	(34)
Pigs	*Escherichia coli*	SCNT	Random	Unknown	shRNA	FUT-1	(35)
Pigs	CSFV	SCNT	Random	Unknown	shRNA	NS3 NS5	(36)
Pigs	PRRSV	SCNT	Random	Unknown	shRNA	PRRSV	(37)
Sheep	BVDV	SCNT	Random	Unknown	shRNA	BVDV	(38)
Sheep	FMDV	SCNT	Random	Unknown	shRNA	1D/VP1	(39)

* as yet, research has been carried out using mouse models only
ASFV: African swine fever virus
BSE : bovine spongiform encephalopathy
BVDV: bovine viral diarrhoea virus
CSFV: classical swine fever virus
FMDV: foot and mouth disease virus
HR: homologous recombination
ICI: intracytoplasmic nuclear injection
IIG: innate immunity gene
KO: knockout
PNI: pronuclear injection
PrP: prion-related protein
PRRSV: porcine reproductive and respiratory syndrome virus
PRV: pseudorabies virus
SB: Sleeping Beauty transposon
SCNT: somatic cell nuclear transfer
shRNA: short- hairpin RNA
SS: site-specific
TALEN: transcription activator-like effector nuclease
TB: tuberculosis
ZFN: zinc finger nuclease
ZMI: zygote microinjection

inhibitor proteins, and single-chain intracellular antibodies (intrabodies).

Inhibiting viruses using molecular decoys

Influenza virus

Transgenic chickens constitutively expressing influenza-specific RNA decoy molecules have been produced by microinjection of lentiviral vectors into the embryonic disc of fertilised eggs (25). The decoys mimic the structures located at the ends of the viral genome that are the binding sites for the viral polymerase and conserved in all strains of influenza A. Chickens expressing these decoys were challenged with highly pathogenic avian influenza virus (H5N1) to compare the infection dynamics between TG and non-TG (NTG) birds. Susceptibility to primary infection by direct inoculation or contact exposure to infected NTG birds was identical for the TG and NTG chickens. Mortality, tissue-distribution, pathogenesis, and virus shedding were also unchanged. However, transmission from the TG birds to secondary contact birds was absent, even when the secondary contacts were themselves non-transgenic. The hypothesis to explain this phenotype is that the TG birds shed virus that is defective in some way that restricts its transmission from bird to bird while still allowing virus to

spread within individual birds. Tests of this hypothesis have yet to be reported.

Pseudorabies virus

Transgenic mice expressing a secreted form of the membrane receptor Nectin-1 involved in pseudorabies virus (PRV) entry and cell-to-cell spread were resistant to infection by PRV (32, 33). However, the transgenic mice showed ocular developmental defects consistent with the transgene interfering with the normal intracellular adhesion function of Nectin-1. This problem was overcome by reducing the Nectin-1 component to just a single domain. However, it highlights one of the problems posed by using molecular decoys corresponding to cellular components.

Inhibition of pathogens using RNA interference

The conceptual simplicity of RNAi-based inhibitory transgenes makes this a very popular approach for directly (or indirectly) targeting virus (or host) gene expression, respectively. Direct RNA interference is unlikely to be effective against non-viral pathogens as they present few, if any, RNA targets within the host cell. A considerable number of viral targets have been investigated in cell culture and animal model systems. However, comparatively few have been tested in livestock species. Off-target and secondary effects (such as innate immune activation) must be considered as potential problems affecting the viability of the transgenic animal. Easy escape by the virus mutating the target site (40) requires careful selection and combined use of multiple targets.

Inhibiting foot and mouth disease virus using RNAi

Proof of principle experiments in cell culture and mouse models confirmed the effectiveness of foot and mouth disease virus- (FMDV-)specific shRNAs and miRNAs (41, 42). Theoretically, any accessible site within the viral genome is a potential target for RNA interference. Selecting the most highly conserved and unstructured regions (e.g. 2B and 3D regions) broadens the range of target strains and may reduce the options for virus escape mutants (43). Targeting the αv-integrin gene (a receptor for FMDV) is an example of indirect targeting, whereby the target is a host gene and therefore much less likely to mutate and lose the target site. miRNAs targeting the integrin αv receptor in TG PK15 cells showed ~ 2–3 log reduction in virus titre over 24–48 h and TG suckling mice showed 30% survival in contrast to 5% survival in NTG mice (44). Knockdown rather than knockout of the receptor was chosen because of possible effects on host viability.

Currently, there are several encouraging examples of TG livestock carrying FMDV-suppressing inhibitory RNAs. Goats produced using pronuclear injection of the Sleeping Beauty transposon carry an shRNA expression cassette targeting the 3D polymerase region of FMDV. Virus challenge in live animals has not been reported yet, but fibroblast cell lines from these animals showed modest (reportedly statistically significant) reductions in FMDV reporter assay expression (10–55%) (29).

Pigs produced by SCNT carry multiple copies of an shRNA transgene targeting a conserved location, namely, the 1D(VP1) region of FMDV. *In vivo* challenge experiments showed delayed and substantially reduced lesion scores and >90% reduction in virus replication. The TG animals had normal levels of interferon- (IFN-)stimulated gene (ISG) expression and showed no evidence of shRNA-induced IFN expression (39).

Inhibiting porcine reproductive and respiratory syndrome virus with RNAi

Pigs produced by SCNT carry shRNA transgenes targeting a highly conserved (1b/polymerase) region of the genome of the porcine reproductive and respiratory syndrome (PRRS) virus. Although the TG pigs were not fully protected against PRRS challenge, they showed delayed mortality (the pigs died 3–5 days after NTG controls) and reduced virus shedding (37).

Inhibiting classical swine fever virus using RNAi

Two lines of transgenic piglets produced by SCNT carry shRNA transgenes against the NS3 and NS5 genes of classical swine fever virus (CSFV). Fibroblasts prepared from the tails of the TG pigs showed reduced virus gene expression when infected. However, it is unclear whether cells from negative control pigs expressed an irrelevant shRNA or not. This is important, as the authors reported that the shRNAs triggered adverse reactions in the cells. Elevated levels of IFN and ISG expression were noted, as well as aberrant miRNA-processing enzyme levels that resulted in early lethality in the TG pigs (36).

Inhibiting bovine viral diarrhoea virus using RNAi

Lambs produced by SCNT carrying shRNA cassettes targeting bovine viral diarrhoea virus (BVDV) died shortly after birth, but their kidney cells showed reduced BVDV replication in cell culture (38). The reason for the death of the lambs was not investigated, but it was speculated that it may have been due to placental abnormalities that have been reported for SCNT previously (45).

Inhibiting *Escherichia coli* by RNAi

Although direct targeting of bacteria by RNAi is unlikely to occur, indirect targeting of receptors required for colonisation has been demonstrated (35) using shRNA to target the alpha(1,2)-fucosyltransferase (FUT1) gene. This is a candidate gene for the expression of the receptor

required by enterotoxigenic *Escherichia coli* F18 (a cause of post-weaning diarrhoea and oedema disease in piglets). This work went as far as generating TG embryos by SCNT, but nothing further has been reported yet

Inhibiting transmissible spongiform encephalopathies by RNAi

shRNAs targeting bovine prion-related protein (PrP) mRNA achieved 90% reduction of PrP expression in 81 day-old TG goat fetuses (28).

Intrinsic resistance

The modification or removal of a host factor essential for the propagation or pathogenesis of a pathogen should render the transgenic animal intrinsically resistant. Simple ablation of the host factor is only possible where the gene is not essential to the host. More subtle modification of the host factor to prevent the host/pathogen interaction while retaining the normal function of the protein has wider application. Thus, detailed knowledge about critical host/pathogen protein or ligand interactions obtained through basic research can potentially be exploited to engineer animals in which such interactions no longer occur.

Receptor knockouts

Pig strains that naturally lack the receptor for K88-Ag-positive *E. coli* are intrinsically resistant to colonisation by that strain (46). Similarly, the FUT-1 gene is a candidate gene in pigs for producing the receptor for enterotoxigenic *E. coli* F18 (47).

Influenza viruses show preferences for α2-3 and α2-6 linked terminal sialic acid receptors (48). Gene knockout of the specific sialyl transferases would be expected to alter the susceptibility of livestock species such as pigs and chickens to the prevailing strains that infect them. However, predictions based on this facile view of influenza receptor tropism may not be so straightforward (49).

Perhaps the anticipated undesirable effects of knocking out receptors have discouraged the adoption of such approaches. To date, no transgenic livestock species with receptor knockouts have been reported. Gene editing, whereby the receptor is modified rather than knocked out entirely, is now a realistic possibility, but nothing has been published on this yet. However, recent RNAi approaches to knock down the αv-integrin FMDV receptor and the FUT-1 receptor show that this is an area of renewed interest (35, 44).

Intrinsic resistance to transmissible spongiform encephalopathies

In transmissible spongiform encephalopathies (TSEs) such as bovine spongiform encephalopathy (BSE), a mis-folded form of the PrP protein is considered to be the infectious agent that propagates itself by inducing the mis-folding of the protein produced by the endogenous PrP gene. Hence, the PrP protein is essential for susceptibility and propagation of TSEs. The function of PrP is not fully understood, but it is clear that the gene is not essential (50).

Cattle lacking prion protein have been produced using a sequential gene knockout approach to eliminate both alleles in successive rounds of transfection of bovine fetal fibroblasts, screening and SCNT to regenerate embryos for the next round of transfection (21). Despite not using ES cells or gene-targeting nucleases, they were able to achieve an adequate efficiency of homologous recombination. The cattle reached over 20 months of age and were phenotypically normal. Brain tissue homogenates did not support *in vitro* prion amplification and the cattle are likely to be completely resistant to BSE infection. The demand for PrPc-deficient cattle as livestock does not warrant large-scale expansion of such animals; however, the authors believed that they would be a useful model for prion research and as a source of PrP-free bovine products.

Augmenting innate and acquired immunity

Influenza

Mx proteins (reviewed in [51]) are IFN-inducible dynamin-like GTPases that disrupt virus replication by binding to viral nucleoprotein complexes. Mx1 was first identified because of its dominant, overriding importance in controlling influenza infection in mice and its activity against a wide range of influenza strains when transferred into cells of other species. Homologues have been found in many vertebrate species. Not all work against influenza virus (52), some have antiviral activity against a wider range of viruses whereas others are apparently not antiviral at all.

Transgenic pigs carrying constitutive and IFN-inducible versions of the mouse Mx1 gene were produced (34). Unfortunately, constitutive expression of the gene appeared to be detrimental, as the efficiency of transgenesis was very low and all TG offspring had gene rearrangements. TG pigs carrying the IFN-inducible transgene expressed TG mRNA but no protein was detectable.

Transgenic Tibetan miniature pigs were produced that constitutively expressed an active isoform of their endogenous Mx1 gene (31). Hypothetically, constitutive expression might augment its activity against influenza and CSFV (both known to be susceptible to this isoform). The problem of gene rearrangement did not occur in this case, possibly because SCNT was used in preference to pronuclear injection and possibly because they used the porcine rather than murine Mx1 gene. The frequency of TG piglets was low (five piglets from >2,200 embryo transfers). Constitutive expression of full-length protein was observed,

and replication of influenza virus and CSFV was reduced by >90% in TG fibroblasts obtained from ear tissue. No animal challenge studies have been reported to date.

African swine fever resistance in pigs

It is hypothesised that natural resistance to African swine fever virus (ASFV) disease in warthogs is due to a single amino acid difference in the Rel-A gene (53). TALEN or ZFN-directed cleavage combined with intra-cytoplasmic injection of zygotes has been used successfully to generate pigs with Rel-A exchanged with the warthog gene. Pigs with truncated Rel-A (frame-shift mutation) have also been produced. The resulting genetic changes are so minor, that it is argued that both are examples of gene editing, not transgenesis. *In vivo* infection experiments are anticipated within the coming year (C.B. Whitelaw, personal communication; [30]). Warthogs are asymptomatic carriers of ASFV rather than truly resistant to infection and the mechanism for how warthog Rel-A protects against ASFV pathogenesis is not fully understood. These pigs will be important tools for investigating this.

Antibodies to transmissible gastroenteritis virus in milk

Passive immunity to infection is transferable from milk. The ability to target transgene expression to the mammary gland inspired the concept of TG animals that secrete pathogen-specific antibodies (Ab) in their milk. Proof of principle for transmissible gastroenteritis coronavirus (TGEV) was demonstrated by generating TG mice expressing a broadly neutralising mouse monoclonal antibody in early 1998 (54), but no transgenic pigs expressing TGEV-specific Ab have been reported. It is unclear whether TG expression of a single broad-specificity monoclonal Ab would offer sufficient benefit to newborn piglets compared to colostrum from a conventionally vaccinated sow.

Trypanosomosis in cattle

Innate resistance to trypanosome infection in primates is mediated through the action of the 'trypanosome lytic factors' (TLFs). The active component is the protein APOL-I (55). Human APOL-I protects us against *Trypanosoma brucei brucei* but not the major sub-species of the parasite *T. b. rhodesiense* and *T. b. gambiense* as they have evolved resistance. Baboon APOL-I is still active against all these strains. Proof of principle experiments (23, 56) using hydrodynamic transfection to introduce the transgene into somatic cells in the liver and blood vessels of mice showed that baboon APOL-I protects against *T. b. rhodesiense* and the cattle pathogen *Trypanosoma congolense*. Its effect is augmented by baboon haptoglobin-related protein (Hpr), which facilitates the uptake of TLF by the trypanosome. Work is in progress to generate cattle that express baboon APOL-I and Hpr using CRISPR/Cas9 and SCNT with the goal of producing animals that can be raised in large parts of Africa where this is normally precluded because of endemic trypanosome infection in wildlife (S. Kemp and J. Raper, personal communication). This has great potential to alleviate the burden of 'cattle-free' farming as well as eliminate a potential animal reservoir of *T. b. rhodesiense*.

Bovine tuberculosis

The combination of site-specific gene targeting and SCNT has been used to introduce the mouse SP110 gene into cattle to reduce susceptibility to bovine TB (20). SP110 (Ipr1) was identified as an innate host resistance factor active against *Mycobacterium bovis* in mice (57). Although its precise mechanism of action is not known, it promotes apoptosis in preference to necrosis in infected macrophages. The bovine orthologue appears to lack this activity. Heterozygous TG cattle carrying the mouse SP110 gene inserted into the M-S locus were generated by TALEN/SCNT methodology. Of 1,580 cultured embryos introduced, 13 calves reached 6 months of age. The TG cattle showed clearly increased resistance to *M. bovis*, with reduced bacterial multiplication, a shift from necrotic to apoptotic responses by infected macrophages, lower pathology and resistance to low-dose natural challenge. The transgene retained activity in the subsequent generation of animals.

Mastitis in dairy animals

The production of antimicrobial peptides or proteins in milk has the potential to control mastitis in diary animals. Five adult cattle that survived to adulthood (produced by SCNT from 650 embryos transferred to 330 recipient cattle) expressed lysostaphin in their milk (24). Infusion of *Staphylococcus aureus* into the udders resulted in only 14% infection rates compared with 71% in NTG cattle controls. The animal expressing the highest levels remained uninfected even after nine infusion attempts. The absence of an inflammatory response and a stable somatic cell count indicate that the TG cattle were resistant to *S. aureus* mastitis. The lysostaphin persisted in the milk through the cheese-making process which may have 'bioprotective' benefits for dairy food (58). Little has been heard of this development since 2009 but, recently, another group reported the construction of lysostaphin TG cattle with the gene inserted by ZFN/SCNT gene targeting into the beta-casein locus and achieved similar levels of expression in milk (22).

Similar strategies using human lysozyme (26, 59) and B-defensin (27) have been tested in goats. Both were active against a wider range of organisms including *S. aureus*, *E. coli* and *Streptococcus agalactiae in vitro*. B-defensin prevented infection by udder infusion of *S. aureus* and *E. coli*.

Concluding remarks

The examples provided in this review demonstrate that research into genetically modified (GM) disease-resistant

livestock has been progressing steadily but slowly. The technologies for precise genetic modification of animals have advanced substantially and several inventive GM strategies for suppressing diseases of livestock have been tested *in vivo*. However, it is notable that none of these have developed beyond the proof of principle stage and some have been stalled for years. The author has no doubt that the strong anti-GM sentiment prevalent in Europe and the 'political gamesmanship' that has hampered the introduction of Aquabounty GM salmon in the United States (60) has discouraged scientific investigation and inhibited the enthusiasm for GM in the boardrooms of livestock breeding companies. The public perception that GM food is intrinsically dangerous irrespective of the nature of the modification is obviously naive and wrong, but the efforts of scientists and educators to dispel this myth have been woefully ineffective (61). The tired cliché of 'Frankenfood' and the preoccupation with jellyfish-derived green fluorescent proteins (which no one seriously expects anyone to eat) continue to induce a collective groan in the GM-food scientific community.

The benefits of GM disease-resistant livestock are too great to remain suppressed in this way indefinitely. It is clear from the recent literature that China is investing substantially in this area and will become the dominant force driving the development of GM food. Hopefully, with this added impetus the field will gather momentum and escape the eddy it has been caught in over the past decade or more. It is regrettable that the Europeans dropped their paddle and may have to be towed to their senses.

Les animaux transgéniques résistants aux maladies infectieuses

L. Tiley

Résumé
La liste d'animaux transgéniques créés pour tester des moyens de produire des espèces d'élevage résistantes aux maladies infectieuses ne cesse de croître. Bien que les techniques de base pour créer ces animaux transgéniques n'aient pas beaucoup changé depuis la dernière synthèse publiée il y a dix ans sur le sujet par l'Organisation mondiale de la santé animale, une avancée technologique majeure mise au point récemment pourrait révolutionner le génie génétique. La capacité de modifier de manière spécifique des sites du génome ciblés au moyen d'une technique simple et efficace aura de profondes conséquences pour la modification génétique des espèces animales d'élevage.

Mots-clés
Animaux d'élevage transgéniques – Maladie infectieuse – Transgène.

Animales transgénicos resistentes a enfermedades infecciosas

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Resumen
La lista de animales transgénicos creados con la finalidad de ensayar formas de producción de ganado resistente a enfermedades infecciosas no deja de ir en aumento. Aunque las técnicas básicas para generar animales transgénicos no han cambiado mucho en los diez años transcurridos desde que la Organización

Mundial de Sanidad Animal las examinó por última vez, últimamente ha habido un avance tecnológico que está llamado a revolucionar la ingeniería genómica. El advenimiento de técnicas sencillas y eficaces para modificar sitios génicos específicos (*gene targeting*) influirá profundamente en las labores de modificación genética de especies ganaderas.

Palabras clave
Enfermedad infecciosa – Ganado transgénico – Transgén.

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