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Emerging Gene and Cell-Based Therapies and Their Prospects for the Treatment of Animal Diseases

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ABSTRACT

Animal diseases caused by pathogens such as viruses, bacteria and nematodes account for major losses in livestock production and can have dramatic socioeconomic consequences in agriculture-dependant countries. Moreover, in recent years, the unpredictable worldwide outbreaks of pandemic infections such as mad-cow disease and bird flu, and the risks that these diseases pose for the human population, have raised public awareness about the importance of developing strategies for their treatment. The rapid development that cell and molecular genetics has experienced in the past 25 years has provided scientists with powerful new weapons which, unlike the more traditional medical approaches, can directly target the underlying molecular causes of disease. In this review, we will outline the most recent advances in cell and gene therapy applied to the treatment of animal diseases, with a particular focus on livestock animals. Relevant examples have been used to illustrate these novel treatment options and current restrictions, and future prospects for their application to combat animal diseases will be discussed.

Keywords: animal diseases, DNA vaccines, gene therapy, somatic cell nuclear transfer, transgenic animals

Abbreviations: BHV-1, bovine herpesvirus 1; **BSE**, bovine spongiform encephalopathy; **BTV**, bluetongue virus; **BVDV**, bovine viral diarrhoea virus; **CRA**, conditionally replicative adenoviruses; **ES cells**, embryonic stem cells; **FDA**, Food and Drug Administration; **FE**, facial eczema; **FMDV**, foot and mouth disease virus; **HAC**, human artificial chromosome; **HAT**, human African trypanosomiasis; **HR**, homologous recombination; **IHNV**, infectious haematopoietic necrosis virus; **MAC**, mammalian artificial chromosome; **MHC**, major histocompability complex; **PrP**, prion protein; **QTL**, quantitative trait loci; **RNAi**, RNA interference; **SCC**, somatic cell counts; **shRNA**, small hairpin RNA; **SLE**, systemic lupus erythematosus; **SCNT**, somatic nuclear cell transfer; **SIT**, sterile insect technique; **SMT**, sperm-mediated transgenesis; **SPAG**, sporozoite surface antigen; **VHSV**, viral haemorrhagic septicaemia virus; **VSV**, vesicular stomatitis virus; **VWD**, Von Willebrand disease; **ZFN**, zinc finger nuclease

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INTRODUCTION

Since the early domestication of animals, men and livestock have developed a mutual dependence; the human became not only the collector of the products domesticated animals provided, but also the guardian of their wellbeing in exchange for a better and sustainable food supply. Thus, even today, any disease severely affecting livestock will negatively impact on society as a whole.

Infectious and parasitic diseases which adversely affect livestock have a major impact both upon production, animal welfare and human health (see **Table 1**). While a drop in quantity might be just an economic burden, in particular for food animals, any compromise in the quality of the food produced poses a significant health risk, which is reflected in the general premise that only healthy animals produce safe food. Furthermore, at least some of the animal diseases as bovine spongiform encephalopathies (BSE) (Will *et al.* 1996) or avian influenza (Koopmans *et al*. 2004) also have the potential to transfer to humans.

The costs of existing endemic diseases are estimated to account for 17% of the turnover of the livestock industries in the developed world, and up to 50% in developing countries (Whitelaw and Sang 2005). The resulting economic

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Invited Review

Table 1 Summary of livestock relevant animal diseases which have been described and used as examples to illustrate emerging treatment options.

Disease	Animal	Cause	Current Treatments Being Investigated	Impact[a]
Acute bovine gastroenteritis	Cattle	Bovine rotaviruses	DNA vaccines	US$95 million/year losses in the USA[b].
Avian influenza	Domestic poultry	Influenza A virus	DNA vaccines	Avian influenza's last outbreak in 2004 is still threatening domestic poultry in Asia, Europe, the Near East, and Africa. Mortality rate can reach 90-100%. Transmittable to humans with a mortality of 60%.
Bluetongue	All livestock	Bluetongue virus (BTV)	Pox-based vaccine	Vast economic effects in many parts of the world, due to the high morbidity and mortality rates of the virus, and its long asymptomatic viremia.
Bovine Spongiform Encephalopathy (BSE), scrapie	Cattle, sheep, goat, deer	Prion PrP^{SC}	Knockout/reduced expression of PrP gene by different strategies: • Gene targeting + SCNT • Introduction of SNPs • RNAi + SCNT	167000 cows dead from the disease in the UK. Britain's US$7.5 billion beef industry crippled in 1996. 4000 cows sacrificed in the USA.
Bovine viral diarrhoea (BVD)	Cattle	BVD virus	DNA vaccines	NZ$37 million annual loss to dairy farmers in New Zealand.
Facial eczema	All livestock	Fungus toxin (sporidesmin)	Zinc prophylaxis Breeding of genetically resistant animals	Losses between NZ$63 and $126 million annually in New Zealand from 1983 to 1988[c].
Flystrike	Sheep	Blowfly (*Lucilia cuprina*)	Mulesing Sterile insect technique (SIT) Breeding of "easy-caring sheep"	An estimated 3 million sheep/year die in Australia without mulesing.
Foot and mouth disease (FMD)	Cloven-hoofed livestock	FMD virus	DNA vaccination Vaccine-producing transgenic plants	The most contagious disease of mammals. Endemic in parts of Asia, Africa, the Middle East and South America. Periodic outbreaks in FMD-free countries cause billions US$ in losses (e.g. UK in 2001).
Mastitis	Cattle	Bacterial infection in the mammary gland	Antibiotics Transgenics for increased resistance (lysostaphin, lactoferrin)	2 billion annual US$ losses in USA.
Nagana	All livestock	Tsetse-transmitted protozoan *Trypanosoma*	Vaccines Genetic modification of *Trypanosoma* SIT	Tsetse infests 10 million square kilometres and affects 37 countries, mostly in Africa.
Theileriosis	Cattle	Tick-transmitted protozoan *Theileria annulata*	Live attenuated vaccine	Important constraints to the improvement of the livestock industry in large parts of the Old World.
Toxoplasmosis	All livestock	Tick-transmitted protozoan *Toxoplasma gondii*	Antiparasitic drugs Vaccine	Cause of abortion in sheep, goats and pigs. High prevalence in many African countries, like Ethiopia (up to 22% cattle affected).

[a] The data on this column, unless stated otherwise, have originated from the World Organisation for Animal Health webpage (OIE); [b] House 1978; [c] Morris *et al*. 2004

and social impacts, in particular for countries strongly reliant on primary industry sectors, are enormous and highlight the need to develop strategies to combat these diseases.

Globalisation with its complex international trading networks, most notably the increased mobility of the world's population, has promoted the outbreaks of epidemic diseases which have reached previously unknown levels. Infections such as BSE in the UK and the rapid spread of an avian influenza outbreak in 2003, both with the proven ability to cause similarly severe diseases in humans (Koopmans *et al*. 2004; Areechokchai *et al*. 2006), are just two of the most outstanding examples in recent years which illustrate the scale and potential threat originating from animal diseases. Similarly, the highly contagious Foot and Mouth Disease Virus (FMDV), traditionally endemic as during its most recent discovery in the U.K. (Cressey 2007), dramatically demonstrated its potential to trigger an epidemic during an outbreak in the UK in spring 2001 (Crispin *et al*. 2002). The attempt by the British authorities to halt the disease entailed the slaughter, incineration and burial of around seven million sheep and cattle. The unpredictability of these outbreaks, often associated with an immediate risk for human health, and the enormous scale of the necessary measures to control them, are stark reminders that research into animal health is of utmost importance.

Veterinary medicine (as well as human medicine) was initially limited to treatments with drugs that were not very effective and could only alleviate the symptoms of some of the most common illnesses. This all changed at the end of the 18th and beginning of 19th century with two major breakthroughs in the fight against three of the smallest but most dangerous killers – viruses, bacteria, and fungi. Vaccination against viruses and the application of antimicrobials to counter bacteria and fungi provided highly effective treatments and went on to revolutionise medicine. Although extensively used to treat human diseases, their use in livestock animals poses both economic and biological problems. Mass vaccination is an expensive measure, especially in developing countries, and access to vaccines is not widespread. Excessive and/or inappropriate use of antibiotics, on the other hand, is suspected to contribute to the development of resistant microorganisms, can contaminate animal derived foods with residues of antibiotics and thus poses a risk to public health and food safety.

Today, the increased understanding of the underlying molecular basis of diseases has provided scientists with powerful new weapons with the ability to target the molecular cause of the disease. The emergence of biotechnology has allowed the introduction of genetic modifications to alter or improve existing genetic traits, foremost by adding exogenous DNA sequences. Numerous methods for germ line transmitted genetic changes have been described. They include pronuclear microinjection, cell transduction using retroviral vectors, or sperm mediated DNA transfer (Wall *et al*. 2005). Non-heritable genetic or cellular alterations in somatic cells have been achieved by DNA vaccination and immunization with antigens providing increased tolerance or resistance towards certain diseases.

In addition to this so-called gene therapy approach, a new therapeutic avenue based on cells opened up with the

discovery of embryonic stem cells (ES cells). ES cells have the unique ability for self-renewal and the plasticity to differentiate into any cell type. Thus, they have the potential to replace defective cells and can essentially repair a diseased tissue by repopulating affected tissues with healthy cells. The newest addition to this toolbox is adult stem cells which have similar properties but are more restricted in their ability to differentiate into other cell types.

The current lack of bona fide ES cells from livestock species has resulted in the development of somatic cell nuclear transfer (SCNT). The first demonstration of SCNT in sheep (Wilmut *et al*. 1997) overturned the previous concept that the restricted developmental plasticity of somatic cells would not allow the cloning of mammals by nuclear transfer. Since then successful SCNT has been demonstrated in the major livestock species including cattle, goats, pigs and deer (for a complete chronological list, see (Berg *et al*. 2007)). Today, both stem cells and SCNT, in particular in combination with genetic modifications are opening exciting prospects for treatment and protection against animal diseases.

In this review, we will present a general outline of the most recent advances in gene and cell therapy and their current applications for animal health. The main emphasis is on the treatment of diseases affecting livestock animals in accordance with their considerable impact on the global economy and human health. Relevant data from companion animals which illustrate novel therapeutic approaches will also be presented.

ENGINEERING ANIMAL GENOMES FOR DISEASE RESISTANCE

Animal diseases are typically caused by the pathogen's interference with often well characterized proteins or signal transduction pathways. This usually results in the alteration or elimination of cellular activities leading to the disease phenotype. Progress in livestock genomics has resulted in the elucidation of those mutations that correlate with either increased susceptibility or resistance towards certain diseases. This knowledge has been exploited not only for breeding programs but also for the generation of disease resistant transgenic animals.

Transmissible spongiform encephalopathies

Some of the best characterized diseases in livestock linked to a single gene are transmissible spongiform encephalopathies, which are characterized by neuronal degeneration. They are induced by an aberrantly folded isoform of the cellular prion protein (PrP^{C}), so called PrP^{Sc}. Prominent examples include scrapie in goat and sheep, BSE in cattle (also referred to as "mad cow disease"), chronic wasting disease in deer and elk, and Creutzfeldt-Jakob disease in humans. The outbreak of BSE in the U.K. in the 1980s and 1990s was linked to the emergence of a novel Creutzfeldt-Jakob like disease in humans, which demonstrated the risk of laterally-transmitted prion diseases. An initial study in mice showed that the knockdown of the PrP gene is sufficient to prevent the onset of scrapie following exposure to pathogenic PrP^{Sc} (Bueler *et al.* 1993). Surprisingly, the reduction of PrP expression also resulted in enhanced resistance to scrapie suggesting that close to normal levels of PrP are required for susceptibility to scrapie. One obvious concern for the knockout or reduction of PrP is interfering with its anti-oxidative and anti-apoptotic cellular role (for review, see Sakudo *et al*. 2006). Indeed, several studies have reported phenotypic changes in PrP knockout mice, including sleep cycle disruption and ataxia (Tobler *et al.* 1997; Li *et al.* 2000). While the effect of knockout or modifications of the PrP gene in production animals remains to be fully investigated, the reduction of the PrP protein level is currently considered the most promising genetic approach to produce disease-resistant animals.

The first successful interference with the PrP gene in livestock was demonstrated in sheep (Denning *et al*. 2001). One allele of the PrP gene was functionally disrupted (PrP+/-) in cultured cells that were subsequently used for SCNT. Most likely related to SCNT and not PrP+/-, the only viable $PrP^{+/-}$ lamb died after only 12 days which prevented any developmental or behavioural studies or the further dissemination of the disrupted PrP allele by conventional breeding. In a newer study, the feasibility of generating live heterozygous $PrP^{+/-}$ goats was reported (Yu *et al*. 2006). Using essentially the same cell-mediated transgenesis approach, five viable goats were produced. Up to the age of three months, no abnormal development or behaviour was observed in these animals. It will be interesting to see whether homozygous $PrP^{-/-}$ goats, to be produced by conventional breeding using the heterozygous $PrP^{+/-}$ animals, will display scrapie resistance and/or show any abnormalities due to the lack of the prion protein. Another SCNT based approach for obtaining homozygous PrP knockout cows was described by Kuroiwa (Kuroiwa *et al.* 2004). By performing multiple rounds of gene targeting and SCNT using rejuvenated fibroblasts from day 45 embryos, Kuroiwa *et al*. succeeded in knocking out both alleles of the PrP gene. In a subsequent study $PrP^{-/-}$ cattle derived from such homozygous knockout cells were extensively characterised but no abnormalities were observed, which suggests that PrP is dispensable for normal development in cattle (Richt *et al*. 2006). It was further shown that brain tissue homogenates derived from these animals are resistant to prion propagation. *In vivo* studies for prion propagation are under way and will answer the question whether the $PrP^{-/-}$ animals are indeed resistant to BSE.

An alternative approach for conferring resistance towards scrapie in sheep is the introduction of single nucleotide polymorphisms (SNPs) shown to be associated with scrapie resistance. Selective breeding programs have identified three SNPs (A136V, R154H, and Q171R/H) which determine resistance or susceptibility to scrapie (Baylis and Goldmann 2004). The genotype ARR for the codons 136, 154, and 171 has been correlated with resistance towards scrapie. In addition to breeding those animals with the ARR genotype, its targeted introduction by genetic engineering could provide a faster and more permanent way towards resistant animals. However, recent discoveries suggest that the selected sheep might be susceptible to different strains of the disease, with atypical forms of scrapie been reported in 'resistant' sheep in several European countries (Baylis *et al*. 2004). In spite of this, similar experiments in mice are clearly encouraging. Perrier *et al* showed that mice engineered to express only PrP Q167R were resistant to scrapie (Perrier *et al.* 2002). In addition, PrP Q167R acted as a dominant negative isoform when co-expressed with wt PrP in mice, dramatically slowing PrP^{Sc} accumulation in these Mice (Perrier *et al.* 2002). If these results were replicated in livestock, the random integration of dominant negative PrP isoforms could provide a simpler way for obtaining resistance in livestock avoiding the need to directly target the PrP gene.

RNA interference (RNAi) has proved very successful for gene specifc knockdown in mammals in recent years. Therefore, it comes as no surprise that the expression of PrP has also been targeted at the level of its transcript. Short hairpin RNAs (shRNAs) were designed against the caprine PrP transcript and used for generating stably transfected goat fibroblasts (Golding *et al*. 2006). Due to the low number of healthy pregnancies following SCNT, one fetus was surgically removed and analyzed for its PrP expression. PrP levels in the transgenic fetus were reduced by more than 90% when compared with non-transgenic control fetuses. In summary, even though RNAi has yet to be demonstrated in live livestock animals, harnessing the cellular RNAi pathway may provide another method for reducing expression of specific proteins.

Mastitis

Mastitis is a disease caused by bacterial infections of the mammary gland in dairy animals, predominantly cattle. Numerous bacteria have been found in the udders of mastitis-infected cows such as coagulase-negative staphylococci, *Streptococcus uberis*, *Staphylococcus aureus* (*S. aureus*), *Corynebacterium bovis*, *Staphylococcus agalactiae* and *E. coli*. Infections have not only been associated with diminished milk quality, a loss in milk production, and extended calving intervals, but have also been shown to severely affect the animal's wellbeing, often leading to the culling of severely infected animals. The cost for the loss in milk production and treating mastitis-infected animals has been estimated at about 2 billion $ in the United States (Sordillo and Streicher 2002). Traditional approaches to combat mastitis have involved the use of antibiotics which are prone to leak into milk, making their use controversial. Recently, transgenic livestock with increased resistance towards mastitis have been generated. Interestingly, the endopetidase lysostaphin, which naturally occurs in *Staphylococcus simulans*, has been shown to be effective in killing *S. aureus*, the microorganism responsible for up to 30% of mastitis cases. Building upon an earlier finding that transgenic mice carrying the lysostaphin gene are resistant towards *S. aureus* infections (Kerr *et al*. 2001), transgenic cows with copies of the lysostaphin gene were produced by SCNT (Wall *et al*. 2005). In these animals, lysostaphin was secreted in milk and shown to provide effective protection against *S. aureus* infections when compared with non-transgenic control animals.

Lysozyme, as lysostaphin, acts as an endopeptidase that causes the lysis of bacteria by breaking down their cell wall. Lysozyme is present in human milk at levels up to 3000 times higher than in livestock. This made it an attractive target protein since its additional expression in livestock animals was expected to result in milk with increased antimicrobial properties. In a pilot study, copies of the gene for human lysozyme were introduced into goat fibroblasts. Following the selection of transfected cells and SCNT, five transgenic goats were obtained (Maga *et al*. 2006b). Though not a direct measure for mastitis infections, somatic cell counts (SCC) are typically used as an indicator whether animals are healthy or infected. Interestingly, SCCs were significantly lower for the transgenic goats secreting lysozyme at about 2/3 of the level found in human milk compared with a control herd of non-transgenic animals. Furthermore, milk from the transgenic animals was shown both *in vitro* and *in vivo* to inhibit the growth of mastitis-causing strains of *E. coli* and *S. aureus* (Maga *et al*. 2006a).

Another antibacterial compound expected to confer enhanced mastitis resistance is lactoferrin. In addition to its antibacterial properties, lactoferrin has antifungal, anti-endotoxin, and antiviral activities and is also implicated in iron absorption. Considering its multiple functions it is not surprising that human lactoferrin was one of the first proteins to be expressed in the milk of transgenic cows (van Berkel *et al*. 2002). To test its bactericidal effect, human lactoferrin purified from the milk of these transgenic cows was injected into mice infected with either *S. aureus* or *Klebsiella pneumoniae*. The injection resulted in a significant reduction in the number of bacteria compared to animals injected with BSA which indicates the antibacterial activity of the recombinant human lactoferrin. The emphasis of this study was on the production of a novel milk for human consumption that provides an added health benefit, therefore no data for enhanced mastitis resistance were reported for the transgenic animals. Extrapolating the above mentioned results from infected mice, one would, however, expect the additional expression of lactoferrin to also result in an enhanced protection against mammary gland infections.

Mastitis is caused by various bacterial infections, therefore dramatically enhanced protection from, or susceptibility to, the disease is unlikely to be linked to a single genetic locus. Not surprisingly, therefore, it was shown that the heritability of mastitis incidence in dairy cattle is low (Morris 2006). However, thanks to a comprehensive field recording system for all veterinary treated cases of mastitis in Norway, quantitative trait loci (QTL) associated with mastitis incidences have been pinpointed to chromosome six (Klungland *et al*. 2001). Furthermore, it has also been shown that alleles of the major histocompatibility (MHC) complex DRB beta locus are correlated with lower somatic cell scores in milk, which suggests that the MHC plays a role in protection against mastitis (Sharif *et al*. 1999). Further analysis and confirmation of any of the above mentioned genetic loci are necessary, but the introduction of SNPs linked to QTLs with a lower incidence of mastitis may be envisioned enabling the production of transgenic animals with a lower susceptibility for mastitis.

Facial eczema

Facial eczema (FE) is a costly disease in New Zealand affecting all traditionally farmed animals. It is caused by sporidesmin A, the major toxin of the fungus *Pithomyces chartarum*, and results in the occurrence of skin lesions and liver damage due to the development of free oxygen radicals following exposure to strong sunlight. The disease is moderately heritable and candidate genes and chromosomal locations correlated to FE have been located in sheep (for a review see Morris *et al*. 2004). In cattle, markers on three chromosomal regions have been found (C.A. Morris, pers. comm.). The ongoing fine-mapping of these loci is expected to assist both further breeding programs and the future generation of transgenic animals with enhanced protection towards FE.

Viral Infections

Livestock animals are very susceptible to viral infections causing millions of dollars in damage due to reduced productivity, culling of animals or decreased fecundity. Additionally, viral animal infections also pose a risk for lateral viral transfer to the consumer. The main approach to protect from viral infections, vaccination, will be discussed in detail in the next section. An alternative strategy based on improving the host defence mechanism has previously been proposed and is outlined below in one example. Viral infections are known to trigger the synthesis of alpha/beta interferons (IFN-alpha/beta) which, besides acting on multiple other signal transduction pathways, induce the expression of Mx proteins. Mx proteins from various species are involved in the natural antiviral defence and become up-regulated following the infection with RNA viruses such as vesicular stomatitis virus (VSV), herpes virus, influenza (Jung and Chae 2006), rabies or rotavirus (Muller-Doblies *et al*. 2002). Although we currently lack a clear understanding of the role of Mx proteins during viral infections, the expression of Mx proteins in cell culture has been shown to confer enhanced protection against VSV (Baise *et al*. 2004) and rabies (Leroy *et al*. 2006). If it is possible to transfer these findings to livestock, the introduction of additional copies of Mx genes, either from the same or different species, could be a means to generate animals with enhanced resistance to RNA viruses. Unfortunately, one published attempt to introduce the mouse Mx1 protein, known to be sufficient to confer resistance to influenza viruses, did not result in the detection of the transgene in transgenic pigs (Muller *et al*. 1992). However, the recent progress in expressing transgenes in livestock enables a more controlled production of Mx proteins in transgenic livestock, which may turn this approach into an efficient strategy to protect livestock from viral infections in the near future.

The great potential for the application of genetic engineering to generate disease resistant livestock animals has been validated through the characterisation of a growing number of concepts. However, efficient introgression of beneficial disease resistant traits into livestock animals on

the large scale of modern agriculture still represents a major challenge for its successful implementation. In addition, as discussed in more detail later, the low acceptance of genetically modified animals by the general public is a major factor that currently limits the research effort in this field and any application beyond the confines of a research project.

COMBINING GENE TECHNOLOGY AND VACCINATION: REVOLUTION OF A SUCCESSFUL STRATEGY

An alternative to the production of resistant animals using transgenesis/gene therapy is to target the disease-causing pathogens. The classical approach is to prime the animal's immune system by vaccination against a specific pathogen. Subsequent exposure to the pathogen will trigger an immune response targeted against the invading pathogen. Traditionally, complete pathogenic organisms, modified to ensure safety, were used as vaccines. The first animal vaccines contained either dead or attenuated infectious particles which can be obtained following heat or chemical treatments or the selection of spontaneous mutations. The modified agents maintain their ability to produce infection, but have a reduced or absent ability to induce the disease (Rogan and Babiuk 2005). However, the overall safety profile of these vaccines is far from ideal, and some of them show a tendency for mutational reversions and secondary infections. Apart from their inherent risks, traditional vaccines also have a high cost of production, which is only justifiable for the supply of large markets, like in the case of avian coccidiosis (>$300 million market), toxoplasmosis, cattle anaplasmosis and lungworm (Dalton and Mulcahy 2001). Thus, the current focus is to make vaccines more affordable and safer.

Recombinant vaccines

Recent advances in molecular biology have resulted in the sequencing and identification of genes associated with the virulence and replication of many pathogenic organisms which provide new targets for intervention. For example, transcriptional profiling and bioinformatic analysis have enabled the identification of candidate antigens for a cross protective vaccine in *Dichelobacter nodosus*, the principal causative agent of footrot in ruminants (Myers *et al*. 2007). The sequencing of the genomes of a growing number of pathogenic organisms has also facilitated the mutation or deletion of specific sequences from pathogens resulting in much more stable and safer attenuated vaccines than the ones previously available. The use of non-pathogenic organisms expressing heterologous antigens derived from the pathogen – also known as recombinant live vectored vaccines – greatly reduced the risks of reversion and secondary infections. These organisms, which can be viruses, bacteria and even parasites, deliver the heterologous antigens to the host, which produces only a mild infection and induces immune responses to the pathogenic organism (Staats *et al*. 1994).

The first and most commonly used recombinant live viral vector has been the vaccinia virus, with a number of pox-based vaccines presently on the market, including the vaccine against several capsid and core proteins of the Bluetongue virus (BTV), one of the most serious diseases transmitted by blood-sucking arthropods. Bovine adenoviruses are able to elicit effective immune responses and can accommodate large inserts of foreign DNA, which makes them one of the most utilised components in this vaccination strategy (Babiuk and Tikoo 2000).

A novel and original strategy for developing vaccines against livestock parasites/pathogens involves the use of a specific component derived from the parasite which under normal circumstances would not be capable of eliciting an immune response. This strategy, described in one example below, results in an immune reaction from the host which destroys the parasite, ideally before it causes any negative effect to the animal. In the case of blood-sucking parasites, this can be achieved using components of the parasites' gut wall. In their natural context, these components are not exposed to the host's immune system and antibody conferred immunity does not occur. The vaccination ensures that, when the parasite bites the host to feed, it will ingest antibodies which will destroy its gut wall and, consequently, kill the parasite. This technique has been used to develop a genetically engineered *E. coli*-expressed vaccine against the cattle tick *Boophilus microplus*, economically the most damaging bovine ectoparasite in Australia (Willadsen *et al*. 1995). Similar vaccines that have been developed against livestock parasites include avian coccidiosis, sheep toxoplasmosis, cattle anaplasmosis and lungworm (Dalton and Mulcahy 2001).

Another early attempt to heterologously express antigens in *E. coli* was described by van Die *et al*. (1988) who replaced part of the hypervariable regions of the bacteria's main fimbria protein (P fimbrillin) with an epitope from FMDV, one of the most contagious diseases in cloven-hoofed mammals. The resulting recombinant bacteria produced fully functional fimbriae which were recognized and bound by FMDV-specific antibodies *in vitro*; however, whether this vaccination strategy can provide significant protection against FMDV infections still awaits experimental *in vivo* validation. Though *E. coli* is the most ubiquitous recombinant Gram-negative bacteria, it does not elicit a significant immune response due to its natural occurrence in the colorectal tract in most mammals, which makes it less ideal for the development of efficient vaccines. Other bacteria tested for the cellular response to heterologously expressed antigens are *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* (Rogan and Babiuk 2005).

Nucleic acid vaccines

Along with progress in the fields of gene therapy and the transformation of mammalian cells, vaccination technology has seen a major breakthrough in recent years with the development of DNA vaccines. A DNA vaccine can be defined as a DNA fragment encoding an antigenic protein which is delivered into the cells of the host animal to direct the transcription and translation of the exogenous DNA (**Fig. 1**). Since the host's immune system has not been exposed to the protein during early development, the protein is not recognised as self but as foreign, in spite of being produced by its own cells. This simple and elegant strategy mimics a viral infection in the sense that an exogenous DNA penetrates the host cells and uses the cellular machinery to drive its expression. Therefore, DNA vaccines effectively transform the vaccinated host into a mammalian bioreactor for the production of its own vaccine (Rogan and Babiuk 2005).

DNA vaccines have numerous advantages over the traditional inactivated or attenuated ones. They are not formulated with chemical adjuvants unlike most of the traditional vaccines; therefore they do not produce injection site reactions. Moreover, since they consist solely of nucleic acids encoding an antigenic protein they lack the ability to replicate. This prevents them from producing an infection or inducing disease, which renders them extremely safe. They also have the potential to provide protection from organisms like the Hepatitis B virus, which represented a major obstacle for the production of traditional vaccines because of the intrinsic difficulties in isolating and cultivating this pathogen.

The effectiveness of DNA vaccines depends mainly on the successful delivery of the DNA molecules to the recipient cells. There are several approaches to enhance DNA uptake, which are discussed below.

Transfection by electroporation was one of the first logical steps, as it had already been proven highly efficient when applied in cell culture. It has been successfully used in pigs to deliver a DNA vector encoding an antigen for

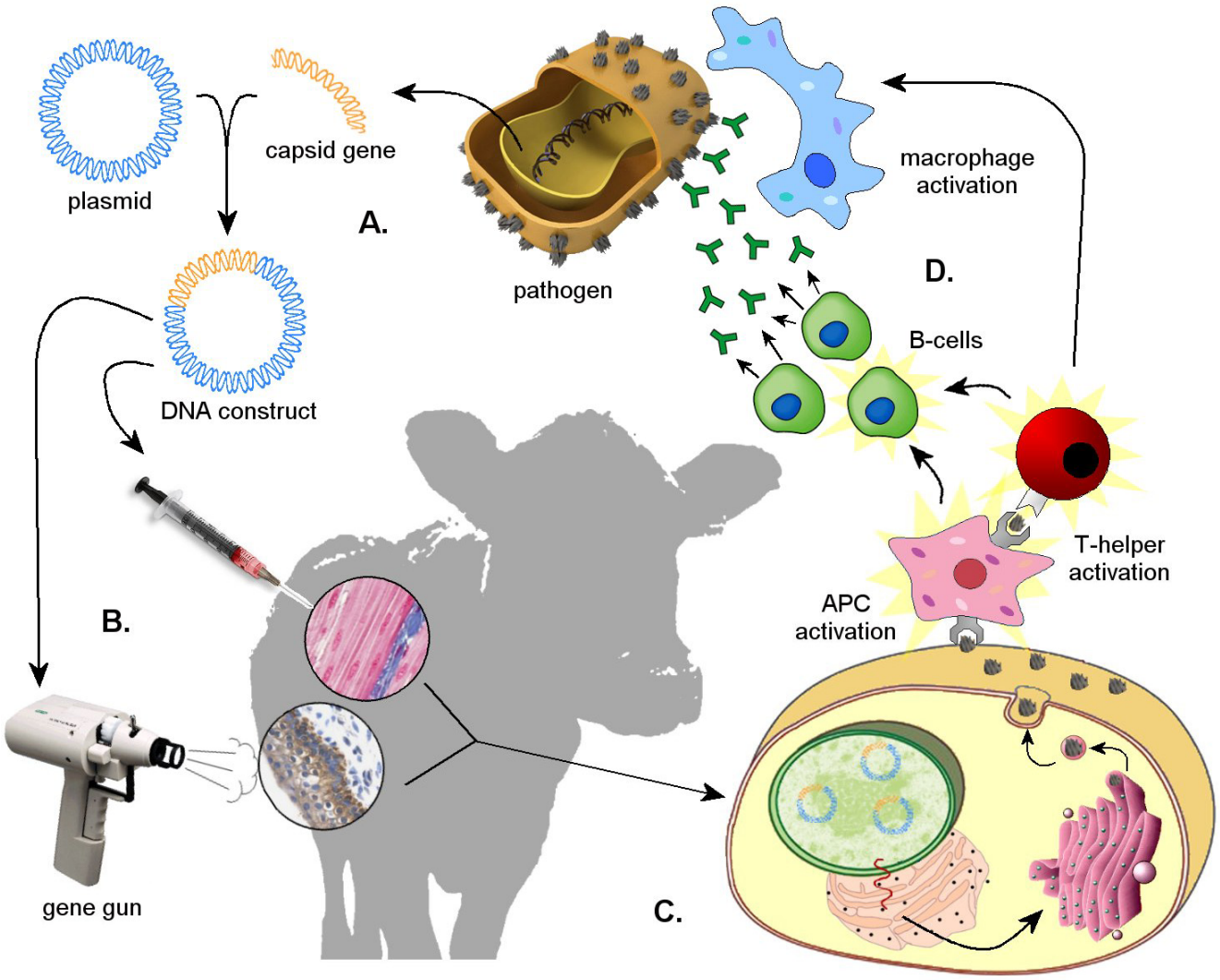

Fig. 1 General mechanism of a DNA vaccine. A. A pathogen's gene encoding one of its proteins is cloned into a plasmid. B. The complete DNA construct reaches the recipient's cells, either by direct intramuscular injection or bombardment of the skin using a gene-gun. C. The DNA construct enters the nucleus and is processed by the cell machinery. The resulting protein is transported from the Golgi via a vesicle which fuses with the cell membrane. D. Immune response: the recipient's immune cells detect the presence of the alien protein and activate the immune cascade, which culminates in the production of specific antibodies by B-cells and the activation of macrophages, all of which attack and destroy the pathogen.

bovine herpesvirus 1 (BHV-1) (Babiuk 2002). It is, however, an invasive method which requires the direct application of current to a live animal using an electrode.

A more intuitive method to deliver DNA vaccines is the direct injection of naked DNA into the recipient animal by intramuscular immunization, which was first used in the early 90s. This method gave acceptable results when performed with mice using DNA sequences encoding a highly conserved protein of influenza A (Ulmer *et al*. 1993). However, the application of this strategy to larger animals, including humans, revealed some intrinsic stumbling blocks: while much higher doses of DNA were required, much lower immune responses compared to the mouse model could be achieved. Degradation of free DNA before it reached the target cell seemed to be the main cause for this. This problem can be avoided with the use of carriers, like cationic lipids, which protect the DNA and direct it to its target cells, and helper molecules like polyethyleneimine (PEI), which induce endosome swelling and membrane destabilisation and increase the rate of lysosomal escape, promoting entry of DNA into the cytoplasm (Rosenecker *et al*. 2006).

The most efficient transformation method though is based upon the ballistic bombardment of tissues using a so called "gene gun". Gold particles are coated with DNA and explosively propelled through the skin and directly into the cytoplasm of target cells. This method can achieve an immunological response with 100-fold less DNA than required via traditional immunisation using a needle. Loehr *et al* (Loehr *et al*. 2000) proved, using the gene gun, that mucosal vaccination with one of the major glycoproteins of BHV-1 induced a stronger cellular immune response than intradermal immunisation in calves. These results suggest that vaccination via the mucosal route could constitute a good approach to treat mucosally-acquired diseases in cattle.

A particular class of production farm animals in which DNA vaccines have shown to be highly efficient are aquacultured fish, particularly salmonids. Although traditional vaccines have been shown to induce a certain level of protection against some of the most prevalent salmonid viruses, the cost of producing the vaccines based on inactivated viruses is higher than in other animals, due to the greater difficulty of working with cultured fish cells. Attenuated vaccines are economically more viable, but they have the risk of being able to occasionally inflict the disease. Moreover, the release of live vaccines into the water is often not compatible with veterinary and environmental control strategies. DNA vaccination has thus shown to be one of the most efficient protection strategies against fish diseases. The most successful vaccines to date have been the ones developed against fish rhabdoviruses, delivered by particle-mediated DNA transfer using the gene gun. A single intramuscular injection of microgram amounts of DNA induces rapid and long-lasting protection in farmed salmonids

against economically important viruses such as infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV). DNA vaccines against other types of fish pathogens, however, have so far had limited success (Lorenzen and la Patra 2005).

The use of translocatory proteins such as the Herpes simplex virus VP22, the antennapedia homeodomain from *Drosophila* and the HIV-1 tat protein transduction domain (Leifert and Whitton 2003) may provide a strategy to further improve the immunization of animals. Translocatory proteins have the unique ability to move between living cells and can act as antigen presenting proteins which makes them highly attractive for the development of vaccination strategies, especially for the induction of an effective CD8+ T cell response. Translocatory sequences would provide an obvious advantage to nucleic acid vaccinations to circumvent the need for targeting as many cells as possible during the initial transfection.

One possible drawback of DNA vaccines is the potential for an unintended permanent integration of the exogenous DNA into the host genome, jeopardising the efficiency of the vaccination strategy in subsequent generations. Ribonucleic acid vaccines (RNA vaccines) could provide an elegant solution to this problem. RNA-based vaccines meet most of the requirements for a good vaccine: they are even simpler than DNA vaccines, as the RNA represents the minimum of information required for the production of an antigenic protein. Furthermore, the injected RNA molecules can be directly translated into protein in the cytoplasm of the host cells. In contrast to DNA vaccines, no translocation to and transcription in the nucleus is required for RNA vaccines, which makes them independent of host transcription factors (Leitner *et al*. 2001). They are also safer, because RNA is a less stable molecule than DNA with a much shorter half-life and is unable to integrate into the genome of the transfected cell. However, a naked RNA vaccine would be prone to degradation by RNAses. To be effective in reasonable doses, RNA-vaccines must be protected by encapsulation that ensures their safe delivery to the recipient cell. Some of the delivery vehicles used in DNA vaccines, like liposomes and dendrimers, can also effectively protect and carry RNA (Pascolo 2004). Another strategy exploits the ability of viruses to safely deliver RNA molecules into cells. The combination of virus capsids with RNA can generate virus-like particles. In contrast to "real" viruses, the RNA in these particles contains incomplete or no genetic information for the viral capsid, thus making it impossible for the particle to replicate after the initial infection. The particles are produced in helper-cell lines that provide the capsid proteins. Because the particles do not encode capsid genes, they can only infect cells once. RNA vaccines against flaviviruses – human pathogens of world-wide medical importance – have been recently developed and evaluated in mice targeting the tick-borne encephalitis virus (Kofler *et al*. 2004). Other RNA vaccines currently under evaluation are the ones against *Mycobacterium tuberculosis* (Xue *et al*. 2004) and some types of tumours (Cheng *et al*. 2004).

Plant vaccines

In the last few years, an indirect and very host-friendly way of vaccination has emerged. This method eliminates the need for injections, electrodes or any other invasive methods; it is based on developing antigen-producing plants that can be fed to the animal together with their normal diet. Expression in seed – as in the case of cereals – also produces a vaccine immunogen in a form that remains stable at ambient temperatures, and can easily be stored long-term (Rogan and Babiuk 2005). Antigens produced in plants have the extra advantage of withstanding the passage through the stomach, since the plant cell wall confers protection against gastric secretions.

To date, several transgenic plants have been generated for the production of animal disease specific immunogens. FMDV structural protein VP1, which carries critical epitopes responsible for the induction of protective neutralizing antibodies, has been successfully expressed in *Arabidopsis thaliana*, alfalfa and potato plants (Carrillo *et al*. 1998; Wigdorovitz *et al*. 1999; Dus Santos *et al*. 2005) and conferred protection against viral infection when administered orally in an experimental mouse model. Nevertheless, the concentration of the expressed protein in plants is relatively low, and achieving a higher concentration is essential to avoid the necessity of consuming large amounts in order to elicit an adequate immune response and protection. The fusion of a recombinant antigen-encoding gene with a screenable reporter gene proved to be highly effective in the identification of high expressing plant lines. Mice immunized with plants selected according to the expression levels of the β-glucuronidase (βGUS) reporter developed a strong and protective antibody response against virulent FMDV in experimental hosts (Dus Santos and Wigdorovitz 2005a).

Bovine rotaviruses are the principal cause of severe acute gastroenteritis in several mammalian species. The most susceptible individuals are newborn calves, which are difficult to immunize. However, colostral antibodies produced in rotavirus-vaccinated mothers can confer passive protection to the calf. The potential of a plant based rotavirus vaccination strategy has been demonstrated in mice. The rotavirus epitope eBRV4 has been effectively expressed as a βGUS fusion protein in alfalfa, and conferred good passive protection against the virus in adult mice (Wigdorovitz *et al*. 2004).

Bovine viral diarrhoea virus (BVDV) is a common disease around the world that infects cattle of all ages. This virus causes a complex of diseases in cattle which interfere with reproduction, affect the foetus and lead to mucosal disease. The BVDV genome encodes at least 10 structural proteins. One of them, E2, has been demonstrated to be an excellent immunogen. Using specific viral regulatory signals, a recombinant binary vector containing the E2 gene was transfected into alfalfa and produced plant lines with high E2 expression levels (Dus Santos and Wigdorovitz 2005b).

This and other plant produced glycoproteins under development have demonstrated the feasibility of this immunization concept. Further trials using livestock animals will be necessary to unravel its true potential in the control of major livestock diseases.

APPLICATION OF GENE TECHNOLOGY TO COUNTERACT INTERNAL PARASITES

Internal parasites represent a unique group of pathogens, comprised mainly of protozoans and helminths, which have undergone a specialised evolutionary process resulting in complete adjustment to their host. These pathogens usually show highly complex life cycles and are often carried by one or more vector organisms which themselves are not negatively affected by the pathogen before reaching their final hosts. The high specificity of these parasite-host relationships provides opportunities to intervene and break the cycle not only in the host, but at any of the intermediate steps, thereby preventing the pathogen from reaching the host.

Some of the most common transmitters of these kinds of pathogens are blood-sucking insects, such as flies, midges and mosquitoes, and some arachnids, such as mites. The use of insecticides, although effective, has resulted in the development of resistant populations and severe negative effects on the environment. Microinjection of transgene constructs into insect eggs enables the targeted expression of genes with the ability to interfere with the development of the pathogen, in particular when expressed in tissues where they can affect parasite viability (e.g. in salivary glands or hemolymph). The efficiency of these techniques was recently proven on the human malaria carrier, the mosquito Anopheles. A piggyBac-based vector was constructed for the expression of the SM1 peptide, which blocks the

development of the plasmodium in the midgut lumen of the mosquito, where the initial stages of plasmodium development take place. As a result, transgenic mosquitoes were on average 80% less susceptible to infection by *Plasmodium*, and even when infected, they transmitted the disease less frequently than wild type ones (Ito *et al*. 2002). A recently published follow-up study investigated the important issue of how the transgenic mosquitoes would perform in competition with wild type populations. In contrast to previous reports which had shown a distinct disadvantage for similarly modified mosquitoes, the transgenic mosquitoes expressing the SM1 peptide were able to outcompete their wild type counterparts as reflected by higher fecundity and lower mortality rates (Marrelli *et al*. 2007). These results are highly promising for developing vaccination strategies for insect-transmitted protozoan diseases affecting livestock.

The protozoan parasite *Theileria annulata* is responsible for the tropical theileriosis, a disease which affects cattle in Asia, North Africa and Southern Europe. It is transmitted by feeding ticks, which inoculate the infective sporozoite stage of the parasite. Sporozoites invade mononuclear leucocytes which subsequently become immortalised and eventually destroyed. This ability to immortalize mononuclear cells has made it possible to grow these parasites *in vitro*. It was found that the pathogenicity of the parasite was attenuated after continuous passages in culture; therefore, while they still can induce a strong immune reaction, their ability to induce disease is lost. These attenuated cell lines were first used to develop live attenuated vaccines in 1986 (Subramanian *et al*. 1986). More recent studies have proved a synergistic effect in vaccines containing a combination of the sporozoite surface antigen (SPAG) and the attenuated protozoan (Darghouth *et al*. 2006). This kind of attenuation in culture has also been reported to happen with other protozoan pathogens like *Babesia bovis* and *Plasmodium berghei*, and seems to be caused by genomic rearrangements, large chromosomal deletions and/or the selection of initially minor avirulent subpopulations (Carson *et al*. 1990; Janse *et al*. 1992).

The Tsetse fly is another well-known vector which transmits several pathogenic species of the flagellate protozoan *Trypanosoma*. This fly is infamous for the transmission of the Human African trypanosomiasis (HAT), commonly known as "sleeping sickness", which is caused by *T. brucei rhodesiense* and *T. b. gambiense*. Other species of the trypanosome are responsible for a livestock disease known as "nagana" (*T. vivax* and *T. congolense* in cattle and other ruminants, and *T. simiae* in domestic pigs). This animal disease has drastically restricted agricultural development and food resources in sub-Saharan Africa. Approximately 70% of the humid and semi-humid zones of sub-Sahara are devoid of cattle and farming systems, and only continuous efforts to control tsetse populations have allowed some farming to flourish at the borders of the tsetse domains. The development of vaccines against trypanosomiasis has shown to be ineffective, due to the ability of the trypanosomes to change their surface proteins by antigenic variation (Aksoy *et al*. 2003). Transgenesis appears to be a promising technique to control the disease. However, unlike most of its fellow insects, the tsetse fly is viviparous, which presents an additional complication for gene therapy. The adult female produces a single egg at a time that hatches and develops *in utero*; the larva is not deposited until the third-instar phase, and pupates shortly thereafter. Thus, gene transfer into eggs is not possible. However, the tsetse fly has developed symbiotic relationships with maternally-transmitted bacteria, and it is within these symbionts that transgenesis can succeed. One of these symbionts, *Sodalis glossinidius*, has been effectively transformed with a transgene encoding the protein Attacin, which inhibits trypanosome establishment in the fly without affecting its fecundity or longevity (Hu and Aksoy 2005). A population of tsetse flies reconstituted with the modified symbiont could be established in zones affected by Trypanosome, eventually replacing the wild type population.

Blowfly strike is a serious disease in sheep caused by the Australian sheep blowfly (*Lucilia cuprina*): here the "parasite" carried by the fly is her own larvae. The female fly lays her eggs on damp and protected areas, especially under the sheep's tail. The larvae lacerate the skin and produce sores, then tunnel into the host's tissue causing irritating lesions. Bacterial infections follow this stage, and if untreated, the sheep can develop septicaemia and die. In South Africa, one of the largest wool producing countries in the world, between 2% and 15% of wool sheep are affected by blowfly strike annually, resulting in wool loss and a reduction in wool quality (MOSA). Pacific countries like Australia and New Zealand are also affected, costing the sheep industry millions of dollars per year. The Sheep blowfly is also a well-known vector for anthrax, a disease carried by sheep, goats and cattle, and infectious to people. A number of insecticides are currently used to combat *L. cuprina*. Their widespread use has the potential of promoting the development of insecticide-resistant insect populations, but can also have implications for the quality of the animal's products, such as meat and wool which need to be monitored for the presence of residual levels of the insecticides. A traditional method of control for flystrike, used since the 1930s, is mulesing. The procedure involves surgical removal of strips of wool-bearing wrinkled skin from around the tail of the sheep. Although this method has shown a very high efficiency in preventing flystrike, it poses ethical problems, since it causes a certain amount of pain and discomfort to the animal (anaesthetic or pain killers are rarely used) and wool imported from countries that still allow mulesing, such as Australia, has recently been boycotted by other countries. All this has increased the interest in biological methods to control the blowfly. One of the methods utilised dates back as far as 1950 and is known as the sterile insect technique (SIT). It involves raising large numbers of insects which are sterilised using radioactivity before being released. Their mating with the wild insects reduces the reproductive potential of the wild population and so causes a reduction in the wild population in the subsequent generation. If enough sterile males continue to be released for a sufficient time, the target population will collapse, leading to its suppression, or even total elimination, over the release area (Coleman and Alphey 2004). Scott *et al*. (Scott *et al*. 2004) investigated several strategies for the application of the SIT technology in conjunction with genetic modification and egg microinjection to combat flystrike. Their aim was to develop a genetic system for controlling female viability using an inducible transgene which would result in the selective killing of all females in the absence of Tetracycline, ensuring a sterile male-only release. This inducible system was successfully verified in *Drosophila melanogaster*, and the author later developed a strategy for germ-line transformation in *L. cuprina* using a piggyBac element, which is still being improved and might equip farmers with a more effective control mechanism for flystrike in the future.

The identification and modification of host genes which are implicated in the susceptibility to infection is another strategy to prevent potentially detrimental infections. The selective breeding of sheep with a higher breech bareness score and a lower length of bare skin below the tail has been shown to drastically reduce the risk of flystrike (Scobie and O'Connell 2002; Scobie *et al*. 2007). Al-though this strategy does not directly utilise gene therapy techniques, identification of the genes involved could provide new leads for the introduction of genetic improvements associated with resistance to flystrike in sheep.

CARING FOR THE INDIVIDUAL – TREATMENT OF NON-INFECTIOUS DISEASES

Most research efforts assigned to animal health are directed towards the prevention and treatment of infectious and parasitic diseases in production animals, particularly livestock. Due to the economic nature of livestock farming, the

potential protective and therapeutic treatment options for livestock animals are mainly driven by economic considerations. In addition to the direct costs of the treatment, costs incurred through lost production from diseased animals can substantially increase the financial burden. While preventative or therapeutic measures might be necessary, the associated investment is often only justified on the larger scale of entire herds, whereas the treatment of individual animals suffering from a particular disease is often uneconomical. Thus, the practical solution is, in many instances, the replacement of diseased animals rather than their treatment. Only if an animal is of sufficiently high value, such as elite production or breeding animals, does treatment become a viable option.

A good example to illustrate the point is champion race horses. Considering the large amounts of money involved in the thoroughbred industry, such elite horses represent immense value for their owners not only during their active racing careers but also in their role as breeding stock for future champions. In this case, the costs for the treatment of a disease appear to be low in comparison to the high value of the individual animal. Thus, even relatively expensive treatments become a viable proposition. Horses are particularly prone to developing osteoarthritis, a condition which renders them unable to race and may start at an early age. Viral live recombinant vaccines have shown their potential to increase intra-articular expression of the interleukin-1 receptor antagonist, resulting in significant reduction of pain and preservation of cartilage (Frisbie and McIlwraith 2001; Frisbie *et al*. 2002).

Another promising field is livestock embryonic and adult stem cells. Pioneered in mice and humans, recent progress has resulted in the isolation and characterisation of equine (Li *et al*. 2006), bovine (Gjorret and Maddox-Hyttel 2005; Roach *et al*. 2006), and ovine (Zhu *et al*. 2007) ES-like stem cells and pig derived multipotent self-renewing adult stem cells (Price *et al*. 2006; Zhu *et al*. 2007). Thus the availability of livestock stem cells, either by themselves or in combination with genomic modification, provides exciting new avenues for the repair of disease or injury affected tissues or organs.

Although ES cells might hold the potential to become a universal tool for the repair of defective tissues, the same economic considerations will apply for diseased livestock animals. Given that stem cell technology is elaborate and expensive, only the most valuable livestock animals are likely to benefit from emerging tissue repair strategies.

The premise is entirely different for companion animals when the cost for veterinary treatments may be a minor concern compared to the emotional value. Moreover, while livestock animals rarely have the opportunity to reach their natural life expectancy, most companion animals will live into the later stages of life which are commonly associated with increasing health problems. There are many ways in which gene therapy can be used to improve the quality of life in companion animals. The direct injection of a DNA construct containing a transgene which encodes a particular protein can be used to increase the expression of the deficient protein responsible for a disease, or correct a mutated one; this is in principle identical to the mechanism of a DNA vaccine, but with no immune reaction involved. Adenoviruses are one of the most utilised vectors, mainly because of their ability to infect non-dividing cells, which makes them ideal for adult tissue treatment. Adeno-associated virus vectors containing the feline erythropoietin gene, for example, have been shown to cause a dose-related increase in the expression of the gene when inoculated in healthy cats. When used in cats affected by chronic renal failure and the associated erythropoietin-responsive anaemia - an illness which affects between 2 and 3 million cats in the US alone – it could help the cat and its owner maintain a good level of interaction and companionship for much longer periods (Beall *et al*. 2000). Experiments to test the effect of the treatment on sick animals confirmed its promise, but serious secondary effects were observed in some of the animal patients (Walker *et al*. 2005). Recombinant adenoviruses are also being studied as vectors to treat feline hyperthyroidism, a common endocrine disorder affecting elderly cats (Blackwood and Argyle 2002, 2004) and genetic disorders like Haemophilia A and B, and Von Willebrand disease (VWD) in dogs (Chao *et al*. 1999; Connelly *et al*. 1996; de Meyer *et al*. 2006).

Therapy that does not require a viral particle for its delivery would appear to be a safer option and has recently been explored. A good example is that of lupus erythematosus-like diseases in dogs. Healthy male dogs were treated with heparin sulphate to induce a systemic lupus erythematosus-like disease (SLE). Autoimmunity to heparin sulphate induces characteristic tissue damage typical for SLE, affecting predominantly skin and kidney. A therapeutic gene was constructed, containing a longer-living extracellular domain of canine CTLA-4, a protein which has been shown to ameliorate autoimmune diseases, and a delivery peptide for its transport into the target cells. After gene therapy, clinical signs of SLE were dramatically reduced, and the skin regained its normal histological features (Choi *et al*. 2005).

A particularly important class of diseases in small animals is cancer. The development of efficient anti-cancer vaccines and novel therapeutic approaches exploit tumour specific characteristics: for example, the high division rate of cancerous cells compared to cells of the surrounding tissue, and its usual localization to a distinct, highly vascularised zone. The direct intra-tumoural administration of a gene (FasL) that triggers apoptosis in dog melanomas, for example, has been shown to produce a significant reduction in tumour size (Bianco *et al*. 2003). However, most of these cancer studies are still in their initial stages and have only been validated *in vitro*. A novel strategy to boost the activity of existing anti-cancer drugs has been evaluated in feline renal epithelial cells. Transient transfection with a construct for the expression of nitroreductase significantly enhanced the toxicity of the pro-drug CB1954 for tumour cells, demonstrating the validity of the approach and encouraging further *in vivo* studies (Blackwood *et al*. 2001). Vaccination strategies based on conditionally replicative adenoviruses (CRAs), which are engineered to replicate only in the target tissue and destroy tumour cells through their cytopathic effect, have been developed *in vitro* and are currently being studied for the treatment of several types of cancer, including osteosarcoma in dogs (Le *et al*. 2006; Smith *et al*. 2006). Concurrently, non-viral DNA vaccines are under development and have already demonstrated their potential by counteracting cachexia, one of the symptoms of late stage malignant cancers in dogs. These vaccines have been shown to improve the quality of life and general health of affected animals and to increase the probability of survival following aggressive chemotherapy and radiation treatments (Draghia-Akli *et al*. 2002).

A more radical interpretation for preserving the health and presence of a beloved family pet can be seen in the efforts to clone companion animals. Initially developed in and for livestock animals, the feasibility of cloning cats and dogs has been demonstrated recently (Lee *et al*. 2005; Shin *et al*. 2002). Considering the emotional value placed on pets in developed countries, there has been strong interest from pet owners who are prepared to spend considerable amounts of money for a clone of their beloved companion animal even with the understanding that a clone is a genetic copy and not a duplication of the original animal with all its cherished characteristics (Long *et al*. 2003). While high costs and low efficiencies are currently restricting commercial success, industry recognizes the potential opportunities in this field. The creation of tailor-made pets, modelled to suit a particular group of potential owners, is another interesting possibility that cloning techniques and gene therapy offer. The American company Allerca patented in 2005 – and commercialises – specifically bred hypoallergenic cats that lack the version of the FEL D1 protein that triggers human allergic reactions (Allerca). With the help of SCNT, this profitable idea could be extended to almost any other

gene or variant which can't be found and bred by traditional methods.

PERSPECTIVE

Treatment of diseases in livestock animals on the large scale of contemporary agriculture significantly differs from medical treatments for humans which are dominated by ethical considerations. In production animals, interventions are considered affordable only if the economic benefit outweighs the costs of treatment. The majority of the methodologies and strategies to combat animal diseases outlined in this review are presently only employed in basic research or require further development, and their economic viability in agricultural production still awaits verification.

In contrast to the cellular and molecular treatment of human diseases that results in non-heritable changes, curing or preventing animal diseases may involve both transient or permanent genetic changes with the latter being passed on to future generations. Looking upon long term prospects, the introduction of permanent modifications of the genome to generate disease resistant transgenic animals appears economically more promising than curing individual animals in a somatic, non-heritable fashion. Transgenic, disease-resistant cattle, for example, are endowed with a life long disease resistance and are able to transmit the trait to their offspring, essentially preserving the acquired disease resistance for future generations. Traditional vaccination or medication, on the other hand, are typically immediate measures taken to control a disease outbreak with many animals already being infected. Vaccination, as an alternative to culling of infected animals, will only be affordable when large, economically viable animal herds are at stake. Moreover, due to similar economic constraints, prophylactic vaccination is usually reserved for high incidence diseases but not applied for diseases with rare outbreaks.

Heritable genetic changes resulting in a disease resistant or disease tolerant animal can be achieved by permanently modifying either the animal host or the pathogenic organism. The introduction of these genetic modifications is, however, labour intensive, time consuming, and costly. Transgenic animals are generated by delivering a new gene or genes to the animal genome. Random transfection and microinjection, the simplest and most straight-forward ways of achieving this, are associated with several disadvantages such as unknown copy numbers and insertion into random sites which may result in potentially deleterious interference with other genes. Site-specific gene modifications that precisely modify the target gene are expected to solve many of these problems. These modifications are, however, much more difficult to accomplish, highly inefficient, and limited by the structure and sequence of the targeted gene locus. Future methods for precisely targeting genes are likely to involve DNA recombinases such as Cre or flippase. This will not only allow for the insertion of gene sequences into well characterized loci and guarantee correct spatial and temporal expression of the transgenic protein but may also enable excision of non-essential exogenous genetic sequences such as bacterial promoters and/or drug resistance genes. Zinc finger nucleases (ZFN) are another new molecular tool which promises to dramatically increase the efficiencies for site specific modifications. They have the exciting potential to be customized for the introduction of a double strand break into any given DNA sequence. This enables the site-specific generation of double strand breaks at or close to a targeting site which was shown to substantially increase the efficiency for gene targeting by homologous recombination (HR). Gene targeting frequencies of up to 20% were reported which allows for the identification of otherwise rare HR events even in the absence of any selection scheme (Urnov *et al*. 2005). Although the generation of effective ZFNs is currently very laborious and often requires time consuming additional efforts to optimize them into highly specific molecules, ZFNs undoubtedly hold great promise for future gene therapy applications.

Another way of developing gene modifications, aimed at both human and transgenic animal applications, has emerged with the introduction of mammalian artificial chromosomes (MACs). Advantages of MACs as opposed to smaller integrated transgenes are abundant: MACs are non-integrating, non-viral gene expression systems which function like natural chromosomes; an artificial chromosome replicates independently from the rest of the chromosomes, and maintains its stability; there is virtually no size limit to the gene(s) that can be added to the MAC; the introduction of a gene into a MAC does not interrupt or alter any endogenous gene; they are not inactivated and the transgene will not be silenced after a number of generations. This new field is just emerging, but there are already promising results. In 2001, de Jong *et al* created a mouse-based MAC with an approximate size of 60 Mb, which could be readily introduced into recipient cells using ultrasound and commercially available transfection reagents (de Jong *et al*. 2001; Oberle *et al*. 2004). Their *in vivo* potential was demonstrated in a live model of murine rheumatoid arthritis, in which fibroblasts previously transfected with the MAC were locally delivered to the joints of sick rats. The modified fibroblasts were successfully engrafted and shown to correct the defective phenotype (Adriaansen *et al*. 2006). Potentially this approach could have direct applications for the arthritis treatment in elite race horses with the prospect of prolonging their race careers. Human artificial chromosomes (HACs) created by telomere truncation techniques have shown high stability in the absence of selection. It has further been demonstrated, that reducing the size of the chromosome down to 1.5 Megabases does neither affect mitotic stability nor loss rate (Mills *et al*. 1999; Spence *et al*. 2006). The feasibility of using artificial chromosomes has already been demonstrated in a number of animals such as rabbits, cattle and goats (Brem *et al*. 1996; Kuroiwa *et al*. 2002; Robl *et al*. 2003; Zhang *et al*. 2005). One of the most successful experiments was performed by introducing a HAC containing the loci for heavy- and light-chain human immunoglobulin into bovine fetal fibroblasts using microcell-mediated chromosome transfer. Fetuses and live calves were obtained later by SCNT and shown to express both immunoglobulin chains. Analysis indicated that the HAC was retained as an independent chromosome, and the proportion of cells retaining the HAC ranged from 78 to 100% with all calves expressing both human heavy and lambda genes (Kuroiwa *et al*. 2002).

While none of these applications were aimed at the generation of disease resistant or tolerant animals, we anticipate such use in the future. MACs are especially attractive in this field as they have the potential to introduce multiple genes to simultaneously target different facets of the host – pathogen interaction, maximize the stability of the disease resistance phenotype over time and confer resistance to multiple animal diseases generating animals with a broad resistance spectrum.

The successful implementation of the genetically modified host in agricultural production requires its rapid dissemination while modified parasites have to outcompete their wild type counterparts in order to have any impact on combating the disease. Transgenic animals with enhanced resistance towards diseases such as transmissible spongiform encephalopathies or mastitis currently exist only in small herds. Sophisticated breeding schemes will be necessary to disseminate the disease resistant genetics on a large scale. Moreover, production animals undergo a small but significant annual genetic gain by conventional selective breeding. Considering that the production of a transgenic animal takes a considerable amount of time, the genetic improvement through transgenesis has to be high enough to outweigh the loss in the incremental genetic gains of the contemporary animals.

How can the generation and dissemination of transgenic animals be accelerated? The modification and direct use of gametes offers the possibility for the immediate large scale dissemination of enhanced genetics without the need

to produce a sexually mature transgenic animal. In a conceptually very simple and elegant process commonly referred to as sperm-mediated transgenesis (SMT), sperm can be loaded with exogenous DNA and, when used for the fertilization, produce transgenic animals. The possibility of using sperm as the transporter of DNA, in spite of having been already suggested more than 30 years ago (Brackett *et al*. 1971), was not widely accepted until 2002, when a monoclonal antibody was used as a linker to bind the transgene to the surface of sperm cells. This method succeeded in producing viable pig and mouse offspring with integrated copies of the transgene, which were also effectively transmitted to the F1 generation (Chang *et al*. 2002). Recently, easier methods have been devised which allow the use of commercially available transfection reagents to perform sperm-mediated transgenesis in bulls (Hoelker *et al*. 2007). Similarly, spermatogonial stem cells, from which mature sperm cells will develop, can be genetically engineered and, after transplantation into the depleted testis of a recipient male, provide genetically engineered sperm for the rapid dissemination of these genetics into larger populations. The generation of transgenic offspring by this technique has been successfully demonstrated in mice (Kanatsu-Shinohara *et al*. 2006). For livestock, successful heterologous transplantation of spermatogonial stem cells has been shown for pigs (Honaramooz *et al*. 2002), goats (Honaramooz *et al*. 2003) and cattle (Herrid *et al*. 2006). Furthermore, in the case of goats the production of live offspring derived from the transplanted cells was demonstrated (Honaramooz *et al*. 2003).

A further extension of this approach is the generation of artificial gametes from ES cells. Following the genetic engineering of ES cells, the pluripotent ES cells are not transplanted for further *in vivo* differentiation but can be differentiated *in vitro* into male or female gametes. Spermatozoa produced by this process have recently been shown to be fully functional and able to fertilize oocytes and produce viable offspring in mice (Nayernia *et al*. 2006). While SMT might be limited in the repertoire of genetic modifications that can be introduced compared to the stem cell based approaches, its feasibility for livestock transgenesis has been demonstrated. Although the technologies have great potential the implementation of spermatogonial stem cell transplantation and artificial gametes for agricultural applications remains some distance in the future.

The successful use of genetically modified parasites which are unable to transmit animal diseases, exemplified by the transgenic mosquito Anopheles which prevents plasmodium development, depends on whether the recombinant mosquito can replace the wild type population. The development of effective mechanisms for replacement was long hampered by the fact that any genetic construct that reduces the capacity of vector species to transmit pathogens seems to have an additional fitness cost associated with it. Thus, any of these modified strains would be easily outcompeted by the wild type population and lost in a short time. A variety of possible mechanisms, each with strengths and weaknesses, was proposed to overcome this problem (for a list, see Coleman and Alphey 2004). In this light, an exciting new report of transgenic mosquitoes with increased fitness compared to their wild type siblings, due apparently to the blockage of parasite invasion of the mosquito midgut (Marrelli *et al*. 2007), is clearly encouraging and could pave the way for their use in field trials.

Nucleic acid vaccination is one of the fields which would benefit from novel advances, especially in the development of new non-viral delivery systems to achieve a more specific and effective transport of the vaccine to its target cells, thereby avoiding secondary adverse effects like the possibility of infection, as well as the development of host immunity against viral components. Special nanomaterials such as dendrimer-encapsulated nanoparticles have been shown to deliver medication directly to the affected part inside an animal's body (Scott 2005).

Undoubtedly, the greatest investment in medical research is directed towards human health. In many instances, animal research can benefit from breakthrough discoveries made in the human field. Not only are many human therapeutic strategies directly applicable to the closely related mammals, animals are also often used as model organisms to develop and prove new concepts. Some of the available gene and molecular therapies for osteoarthritis in horses, for example, are mainly the result of studies with horses as a model for humans, due to the similarities of naturally occurring osteoarthritis in both species. However, the dominant human health focus can also have adverse effects and restrict available treatment options for animals. For example, the introduction of human recombinant insulin tailored for human treatment replaced protamine zinc insulin which, until 1991, was in use for the treatment of both humans and animals, especially cats. As a result, insulin treatment of cats had to be temporarily abandoned. Fortunately, in this case a compassionate use clause for animal use was later implemented in 1997. This illustrates a major dilemma for veterinary medicine. Although many of the drugs approved for human applications would be suitable for animal use, in many instances pharmaceutical companies do not pursue their use in animals due to the additional costs involved in the approval process and lower returns compared to their human applications. The investment of pharmaceutical companies into dedicated animal drugs such as feline recombinant insulin is even less likely, as the associated costs would be much greater and unlikely to be recovered (Fenger 2001).

The ability to apply the novel gene and cell-based therapies (outlined here) to combat livestock diseases will depend on public acceptance of these technologies for food animals. Although the improvement of animal welfare is an important consumer goal and provides a strong ethical justification, the main decisive factor determining the acceptability and future prospect for the integration of the new technologies into practical farming systems will be the consumer attitude towards its risks and benefits. Only if the ethical justification can outweigh, in the opinion of the consumer, any potential risks associated with these new technologies will the application of gene and cell-based therapies for the treatment and prevention of animal disease become a valid proposition.

To enable informed decisions the scientific community needs to effectively communicate research results to the public, producers and the regulatory agencies. In one example, an active information campaign led by scientists from both universities and the private sector, resulted in a positive outcome and highlights the impact open communication can have on the general public. It resulted in the public dismissal of a Swiss initiative which aimed to make the use of transgenic animals in research illegal. More than two-thirds of the population rejected the law, even after an aggressive campaign for a ban on transgenic research animals was carried out by a diverse coalition of environmental, animal rights and political groups (Koenig 1998). The recent release of the draft risk assessment on animal cloning by the United States Food and Drug Administration (FDA), stating that food products from clones are safe for human consumption, might provide a first indication as to what extent consumer attitudes towards a controversial new technology like cloning will change in light of a low risk profile. While the risk profile of gene and cell-based therapies in livestock will depend on the specific manipulations, the objective is to reduce rather than increase the risk for the consumer. In comparison to cloning applications they have the strong ethical justification that is to improve animal health and product safety. The clear demonstration of these benefits will be important to counter present concerns and increase acceptance for these new technologies.

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