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OPINION

A future for transgenic livestock

John Clark and Bruce Whitelaw

The techniques that are used to generate transgenic livestock are inefficient and expensive. This, coupled with the fact that most agriculturally relevant traits are complex and controlled by more than one gene, has restricted the use of transgenic technology. New methods for modifying the genome will underpin a resurgence of research using transgenic livestock. This will not only increase our understanding of basic biology in commercial species, but might also lead to the generation of animals that are more resistant to infectious disease.

Transgenic animals carry a segment of foreign DNA — the transgene — that is inserted into their germline and is inherited in a Mendelian fashion. The production of the first transgenic livestock was reported in 1985 (REF. 1) and much has happened in the intervening years (TIMELINE). The technique used then was pronuclear injection[2], which allowed only random introduction of new DNA sequences into the genome. More recently, nuclear transfer techniques have been adapted to allow more precise modifications of the genome, such as the disruption of specific endogenous genes[3,4].

Although transgenic livestock have had a high profile, the practical use of these animals has been limited and restricted to medical applications, such as producing pharmaceutical proteins in milk, rather than the agricultural applications that were originally envisioned. Nonetheless, there have been considerable efforts to improve the transgenic technology that was first developed to advance livestock production. Many proof-of-principle studies have been carried out, but the commercial application of this technology is still non-existent. Here we discuss the reasons for this disappointing outcome. We contrast transgenic strategies that have been used to improve performance with the tried and tested selective breeding regimes that have been used during the past 70 years. We propose that two recent developments are set to stimulate a resurgence of interest in the generation and use of transgenic livestock. First, lentivirus vectors offer the possibility of producing transgenic livestock far more efficiently and cost effectively[5,6]. Second, by combining the use of these new vectors with the rapidly developing methods that are based on RNA interference (RNAi) to suppress the expression of specific genes[7,8], we anticipate the development of innovative techniques that will further our understanding of gene function in livestock species and potentially generate farm animals that are less susceptible to infectious disease.

Selective genetic improvement

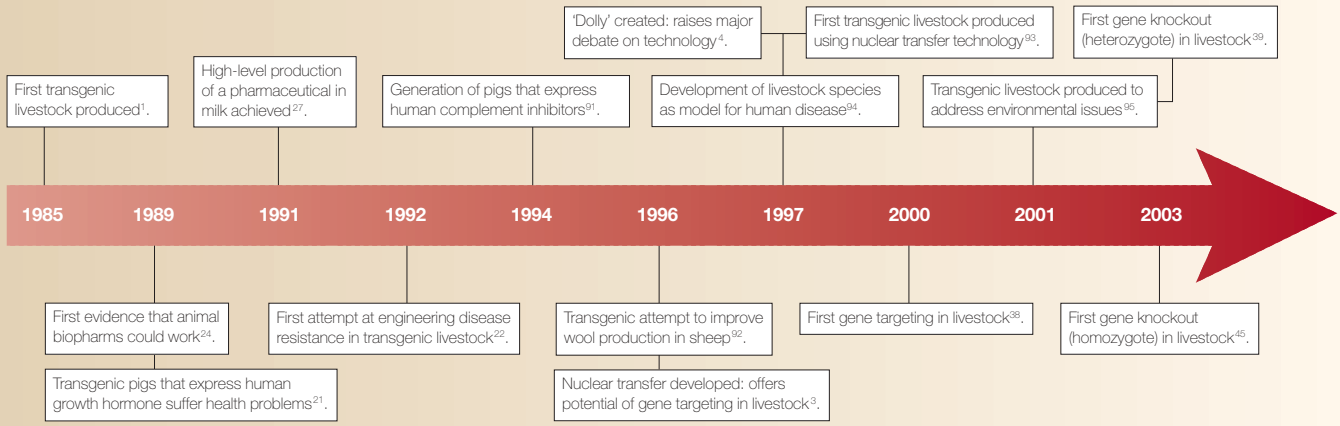
Ever since animals were first domesticated a few thousand years ago we have been indirectly genetically modifying these species for our own purposes through selection. To a large extent, the differences between today's livestock and their progenitors are testament to how successful this programme of selective improvement has been. Until relatively recently this form of genetic improvement was carried out without any knowledge of the mechanisms underlying it. Animals were selected on the basis of their observable phenotype. With the advent of molecular tools that enable the genetic nature of a desirable trait to be determined, a directed approach to the genetic improvement of livestock has been possible.

Animal breeding. Breeding based on conventional selection has been the mainstay of livestock genetic improvement for more than 70 years, and it still is today. Most agricultural production traits such as body weight or milk yield are quantitative: for any given trait there is a continuous range of values that are represented in each population. Although individual traits show only modest rates of response to selection (0.5–3.0% per year), the changes are permanent, cumulative and can, over many years, achieve large increases in production efficiency (TABLE 1). For example, continuous selection for growth rate in chickens bred for their meat has produced birds that are now four times heavier than those bred to lay eggs[9]. However, simple selection for the improvement of one specific trait is uncommon: several traits are usually combined into an overall economic merit SELECTION INDEX[10]. Sophisticated statistical and computing tools now enhance conventional genetic selection, nevertheless traits such as fertility and disease resistance remain difficult to measure and improve.

Marker-assisted selection. Unfortunately, phenotype is an imperfect predictor of the breeding value of an individual because, for example, it could be gender specific or manifest after the selection phase (the age at which selection decisions are made) in the breeding life of an animal. Also, phenotype is poor at resolving negative associations between genes that are caused, for example, by epistasis, in which the activity of one gene locus is negatively modulated by another. Selection on the basis of DNA markers offers a way round some of these limitations, as DNA markers can be tested at any age and can be measured in either gender.

During the 1990s there was a concerted effort to physically map regions of the genome that control production traits and to define QUANTITATIVE TRAIT LOCI (QTLs). The idea behind this was that it would allow selection on the basis of an animal's genotype rather than its phenotype — a process known as marker-assisted selection[11]. This was seen as a prelude to isolating the genes that underpin quantitative traits. The initial low-resolution maps[12] that are available, with one marker per million bases, are now being refined by breeding studies[13,14]. As more complete DNA sequences become available for some of these species over the next few years this process will undoubtedly accelerate.

Timeline | **Landmark events in transgenic livestock research**



These initial mapping efforts initiated a more widespread hunt for the QTLs that underpin agricultural traits. Initially there was an intense debate about how useful this would be, because conventional genetic selection assumes that an infinite number of unlinked genes that have small effects control the production traits[15]. The problem was that to identify QTLs, the genes that underlie them must have moderate or large effects. Initial QTL mapping efforts used experimental crosses between breeds that were known to show large phenotypic differences — so-called 'extreme' crosses. For example, a cross between Chinese Meishan and European Large White pigs, which show marked differences in their fatness and litter size, has been used to search for pig QTLs[16]. However, although it was shown that QTLs could be identified in farm animals, it was predicted that QTLs such as those for fatness and litter size would have already been fixed for the desirable alleles in breeding populations and so there would be no variation for the breeder to select[17]. Interestingly, some QTLs that have been identified, including the fat QTL on pig chromosome 4 (REF. 17), also segregate in commercial populations and are not fixed. QTLs have been identified for several livestock species and marker-assisted selection is now used in commercial livestock breeding programmes alongside conventional selection[18].

Table 1 | **Rates of compound genetic response***

Trait	Coefficient of variation (%)	Heritability (h^2)	Genetic response (%)
Cattle			
Growth rate	10	40	1.4
Leanness	5	30	0.5
Milk yield	15	25	1.5
Sheep			
Growth rate	15	15	1.4
Leanness	5	30	0.9
Litter size	30	10	2.1
Pigs			
Growth rate	7	30	2.7
Leanness	4	30	1.6
Litter size	25	10	3.0
Chickens			
Growth rate	7	20	3.2
Leanness	5	20	2.2
Egg production	10	8	2.1

*These are the possible predicted year-on-year genetic response rates for individual traits for the principal livestock species. The genetic response rate is a predicted measure of the rate of change in a quantitative trait in response to selection rates — it is dependent on the coefficient of variation, which is a measure of the difference between individuals in a population, and heritability, which is an estimate of the proportion of a trait that can be inherited. Adapted with permission from REF. 10 © (1984) Longman Group UK Ltd.

Transgenic livestock

Although robust and successful, conventional breeding is limited, because animals produced by mating selected individuals are a genetic mixture of their parents. Unknown or undesirable traits can inadvertently be co-selected. In addition, only those genetic loci that are present in the parents can be selected, which limits the range and extent of genetic improvement. Gene addition through the use of transgenic technology has the potential to overcome these limitations.

Pro-nuclear injection. Since the production of the first genetically modified livestock was reported in 1985 (REF. 1) there has been a series of new developments in the field (TIMELINE). Pro-nuclear injection, a technique that was developed in the mouse[2], involves the direct introduction of a DNA construct into one of the two PRO-NUCLEI of the fertilized egg. This was the technique used to produce the early transgenic livestock (FIG. 1a). However, the efficiency of this method is low and usually only 3–5% of the animals born as a result carry the transgene[19].

The first attempts to genetically modify livestock owed much to pioneering experiments in mice, in which the introduction of a growth hormone gene markedly increased the growth rate and final size of the animals[20]. By contrast, initial attempts to apply the same approaches in livestock were not as successful. Transgenic pigs carrying human growth

hormone genes had only a slightly enhanced growth rate and reduced levels of fat, and these animals suffered from widespread deleterious effects, including susceptibility to stress, lameness and reduced fertility[21]. Attempts to use transgenic techniques to improve livestock resistance to viral infection were also unsuccessful[22].

So, in terms of modifying livestock for agricultural purposes, many of the early expectations were not realized. Several factors were responsible for this lack of success: the two main problems were the difficulties of modifying very complex traits that are controlled by several genes and of INTROGRESSING transgenes into large populations[23]. In contrast to the undoubted efficacy of conventional genetic selection, which delivers sustained improvements year-on-year,transgenic strategies for genetic improvement have simply not delivered. Explicitly put, no transgenic livestock have been generated that were deemed worthy of incorporation into livestock breeding regimes.

However, new uses of transgenic livestock, particularly in human medicine, have continued to attract research funding. One such use was the expression of proteins with potential therapeutic applications in the milk of livestock species, with a view to developing these transgenic livestock as 'biopharms'[24–26]. In some cases very high levels of expression have been achieved. For example 'Tracy' the transgenic sheep produced more than 30 g/l of human protease inhibitor α1-antitrypsin in her milk[27]. Large amounts of this protein are needed to treat people who have emphysema and can potentially alleviate some of the symptoms that are associated with cystic fibrosis, and large-scale expression by transgenic biopharms is perhaps the only way α1-antitrypsin can be produced cost effectively (see also the article by Ma *et al.* in this issue). Nevertheless, even though this use of transgenic livestock has been in development for over a decade, at present only one protein (antithrombin-III) produced in this way is in late clinical trials. Although the biopharming approach seems feasible, the financial commitment required during the protracted development phase has halted many attempts at commercial exploitation. Over the past few years several commercial ventures have withdrawn from transgenic biopharming for various, usually financial, reasons. So, even though much of the groundwork has been done it is unclear what the future holds for this use of transgenic livestock.

During the 1990s another possible medical use of genetically modified animals — as a source of organs for transplantation — became the focus of research. A worldwide shortage of donor organs for transplant surgery chanelled the enthusiasm for transgenic research into ways of allowing transplantation between species (xenotranplantation). As the pig shares several anatomical and physiological features with humans it became the focus of much of this research. However, pig tissues are immunologically incompatible with humans: pig xenotransplants provoke a rapid, COMPLEMENT-based HYPERACUTE REJECTION (HAR) response that destroys the transplanted tissue[28]. Several transgenic approaches have been developed to overcome this rejection, including the production of transgenic pigs expressing human decay accelerating factor (DAF) to reduce complement activity by restricting complement 3/complement 5 activation[29]. However, serious concerns were raised about the safety of xenotransplantation when it was shown that pig RETROVIRUSES could jump species and replicate in human cells[30]. This sparked the fear that xenotransplants might lead to the creation of recombinant viruses with unknown ZOONOTIC effects. These concerns, coupled with the excitement over human stem-cell technology as a way of providing human tissue (albeit not solid organs) for transplantation, have lead to a significant reduction in the research effort into xenotransplantation during the past few years.

Gene targeting. Pro-nuclear injection enables only the random addition of genes to the germline. It does not allow the precise modification of the germline that is required for the specific deletion or modification of endogenous genes. A high proportion of transgenic lines that pro-nuclear injection generates do not efficiently express transgenes because of silencing effects at the site of integration[31]. Considering the cost of generating transgenic livestock, the ability to target transgene

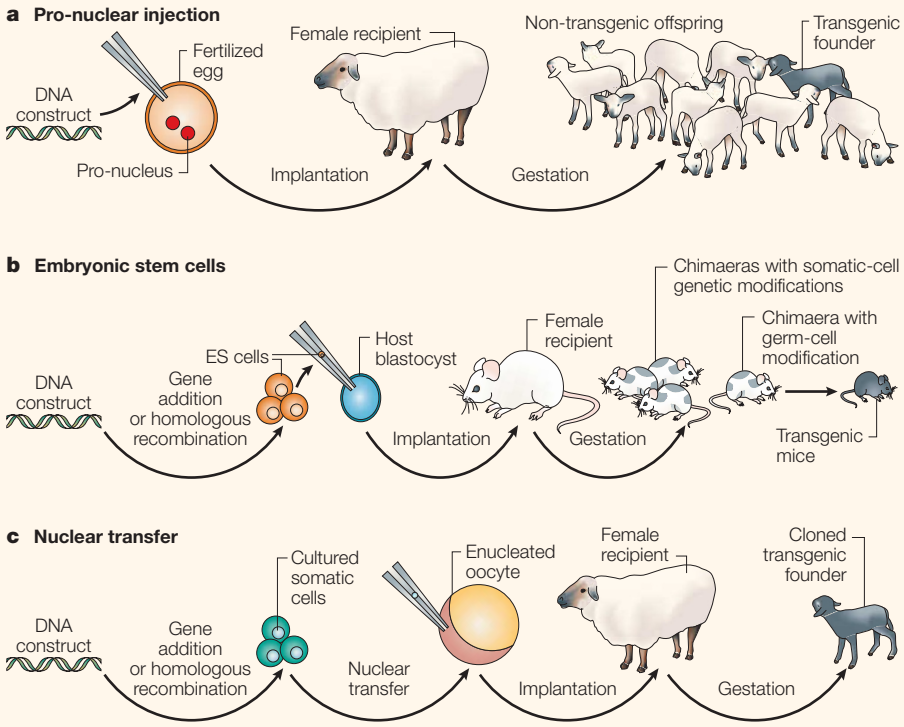


Figure 1 | **Different routes for germline modification. a** | Pro-nuclear injection involves the introduction of the DNA construct into the fertilized egg, which is then transferred to a recipient female[1,2,21,24]. Only a small proportion of the injected eggs will yield a transgenic founder animal, which is usually identified by Southern blotting after birth. **b** | Embryonic stem (ES) cells are only available in mice and, so far, this technology is limited to this species[37]. DNA manipulation occurs in the ES cells before embryo manipulation and might involve random gene addition or gene targeting[35]. The modified ES cells, which are identified by Southern blotting, are injected into a host blastocyst that will develop to form a chimaera that consists of both host and ES cells. Only chimeric mice in which the germline has arisen from the modified ES cells can become the founder of a transgenic line. **c** | Nuclear transfer[3,4] from cultured cells has been achieved in several livestock species including sheep, pigs, cattle, goats, mules and horses, as well as mice and rabbits. The genetic modifications are carried out in the cultured cells before nuclear transfer. Nuclei from the modified cells are transferred to an enucleated oocyte by cell fusion before their development in recipient animals. The process can yield several identical transgenic clones. This technology has been used to add new DNA sequences[76] and for gene-targeting strategies[38–41,44,45]. Modified with permission from REF. 90 © (2000) Kluwer Academic.

Figure 2 | **Targeted gene deletion in sheep.** Nuclear transfer from a primary sheep fibroblast, in which one copy of the prion protein gene (*PrP*) had been disrupted through gene targeting by homologous recombination, generated the lamb shown[39].

integration to selected sites that are permissive for expression would be an advantage. There are also several endogenous genes, such as the prion protein (*PrP*) and α(1,3)galactosyltransferase genes, the deletion of which is predicted to yield unique, useful phenotypes in livestock (see below).

The techniques for knocking out genes in the mouse were established during the 1980s (for example, see REF. 32; for a review, see REF. 33) and they have revolutionized modern biology, allowing the direct assessment of gene function *in vivo*[33,34]. This technology is now very sophisticated: genes can be knocked out in specific tissues[35] and single-base-pair mutations can be introduced into a selected gene[36]. Endogenous genes are targeted by homologous recombination in totipotent embryonic stem (ES) cells in culture. These cells are then reintroduced into the early embryo to colonize the tissues of the developing mouse (FIG. 1b). In a proportion of these mice the gene is knocked out in the germline and breeding from these will easily produce mouse strains that carry the knockout allele. Unfortunately, despite intensive efforts, this technology is limited to the mouse, as no germline-competent ES cells have been described for any other mammalian species[37]. The reason for this lack of success is unclear and certainly does not reflect the efforts that have been directed towards this goal for over a decade. In some quarters there is hope that by using the experiences gained from the isolation and maintenance of human ES cells new efforts to generate ruminant ES cells might be more productive.

The advent of cloning by nuclear transfer from somatic cells[3,4] offered an alternative to pro-nuclear injection and ES cells. Endogenous genes in somatic cells can be targeted by homologous recombination in much the same way that this is done in mouse ES cells. In cloning experiments, nuclei can be transferred from these targeted cells to enucleated oocytes (FIG. 1c). The first report of a cloning strategy being used to generate transgenic livestock described the replacement of the sheep collagen gene with an expression cassette designed to target expression of human factor IX to milk[38]. The second generated a lamb carrying a disruption of the *PrP* gene[39] (FIG. 2), which determines resistance to scrapie and bovine spongiform encephalopathy (BSE). Livestock that are resistant to this type of disease could be of particular importance in situations in which human therapeutic proteins, such as blood clotting factors, are produced in animals. More recently, pigs that have a deletion of the α(1,3)galactosyltransferase gene, which determines a principal cell-surface xenoepitope, have been generated[40,41]. This epitope is a primary target of a natural antibody, and so is a key determinant in HAR[42]. Animals lacking this gene do not synthesize the epitope and should have a reduced HAR response.

The few successes that have been reported for the nuclear transfer approach highlight just how technically demanding it is. The low survival rate of animals generated by nuclear transfer technology is one of the problems that must be addressed if it is to be a commercially viable strategy in the future[43]. Another problem is that the stringent selection and extended *in vitro* culture that are required for targeting somatic cells might reduce their developmental potential[44], which compounds the low efficiency of nuclear transfer. Furthermore, to achieve the phenotype both alleles must be deleted. At present, it is only possible to target one gene *in vitro* and so homozygous nulls must be generated by crossing independently generated male and female clones, or by retargeting and recloning. Recently, the generation of piglets with both copies of the α(1,3)galactosyltransferase gene knocked out has been described[45], but, unexpectedly, homozygous knockout pig fibroblasts generated by another research group seem to express low levels of the gal antigen[46].

Emerging technology

Both pro-nuclear injection and nuclear transfer are inefficient methods for modifying livestock germlines. In addition, the introgression programme that is required for these methods, which is based on repeated backcrossing, results in a loss of selection for other traits. The result is that the benefit of the transgene must substantially exceed what could be achieved by conventional selection during the introgression period, which is estimated at 10% of the overall economic merit[23]. As a consequence, these methods have primarily been used for biomedical rather than agricultural applications. For example, although it is possible to generate animals lacking a copy of the scrapie-resistance gene *PrP* it is difficult to imagine how this could be introgressed and maintained in the homozygous state in large populations. In this case homozygosity of the transgene is crucial, because studies of knockout mice show that deletion of both *PrP* copies is required to create a scrapie-resistant animal[47]. So, to protect populations of livestock from scrapie most animals would have to be homozygous. Even if this were possible, complex strategies would need to be implemented to avoid INBREEDING DEPRESSION, and such breeding programmes would certainly reduce the overall productivity of the animals. However, emerging technologies could soon revolutionize the scope and efficiency of the genetic modification of livestock. This, in turn, could allow the widespread application of transgenic technologies to modify the agriculturally significant characteristics of livestock.

There have been numerous recent developments in animal transgenesis. Some, such as sperm-mediated gene transfer, are appealing but still lack the robust nature that is needed to attract more general interest[48], particularly as there were doubts about whether this method

worked at all when it was first published[49]. Another impressive technical step forward has been the use of an artificial chromosome to genetically modify cattle[50]. However, there are still technical challenges to overcome before artificial chromosomes can be used routinely as transgene vectors. The use of chemicals to introduce mutations into the germline is another innovative approach to genetic modification. However, although *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis is a powerful experimental tool it will probably be limited to use in model species[51], especially if the regulatory authorities require that each mutation be fully characterized.

In our opinion, the most encouraging development with respect to the genetic modification of livestock is the use of viral vectors, particularly those based on lentiviruses[5,6]. These new vectors — lentivectors — seem to offer a solution to present limitations through marked increases in the efficiency of transgene delivery that should be generally applicable. We propose that combining this technology with the emerging technique of RNAi[7,8] presents new and exciting opportunities for livestock transgenesis.

Lentivectors. Oncoretoviruses, such as Maloney murine leukaemia virus, have been used as vectors in gene therapy[52] and as transgene-delivery vehicles in livestock[53]. However, safety issues, problems with transgene expression and the constraint that oncoretroviruses only integrate into dividing cells have limited the development of this type of retroviral vector. More recently there has been considerable interest in developing REPLICATION-DEFECTIVE lentiviruses (a specialized retrovirus) as vectors (BOX 1) for gene-therapy applications[54,55], as they seem to overcome some of these limitations. Two groups recently showed that these lentivectors can efficiently introduce foreign DNA into the mouse germline[5,6]. This approach is so efficient (partially owing to the inherent ability of lentiviral DNA to integrate into the genome without a requirement for host-cell DNA replication) that 80–100% of the mouse pups born were transgenic. There is no reason why these efficiencies will not be the same for livestock. To put this in context, in previous studies using pronuclear injection about 70 sheep were required to make just 1 transgenic founder[56]. By contrast, using lentivectors in combination with *in vitro* matured and fertilized oocytes, we estimate that as few as five animals will be required!

Versatile vectors that are able to infect many vertebrate species can be generated through appropriate PSEUDOTYPING, for example, with vesicular stomatis virus G (VSV-G). So, these should be applicable to livestock species. An even more appealing aspect of these new vectors is the simplicity of their delivery. Lentivectors can be delivered by injection into the PERIVITELLINE SPACE of the fertilized egg or, after removal of the ZONA PELLUCIDA, by simply incubating DENUDED EGGS in a viral solution[5,6]. So, no specialized equipment is required, which would be another big advantage of developing the same technology for livestock species.

RNAi. Recently, a revolutionary new technology that is based on RNAi has been developed to specifically knock down gene expression[7]. RNAi has been recognized as a principal mechanism of post-transcriptional gene silencing in *Caenorhabditis elegans*, *Drosophilia* and plants[8]. RNAi is sequence-specific and works by silencing endogenous gene expression after the introduction of homologous double-stranded (ds)RNAs. The Dicer–RDE-1 (RNAi defective/argonaute-1) complex processes the exogenous dsRNA into small RNAs (guide RNAs or small interfering (si)RNAs) of 21–25 nucleotides. These siRNAs associate with RISC (the RNA-induced silencing complex) and the antisense strand then guides this complex to bind to mRNA in a sequence-specific manner. Subsequently the RISC degrades the target mRNA[57]. These siRNAs are too small to activate the mammalian interferon-mediated antiviral response that is associated with long dsRNAs, although this matter is now the subject of debate[58–60]. This technique has been used to functionally analyse genes in mammalian cells[61–63]. For example, siRNA knockdown of DNA methyltransferase-1 resulted in cell growth arrest[64], whereas knockdown of p53 prevented the p53-dependent cell arrest that is induced by ionizing radiation[65].

Gene constructs initiated and terminated at specific nucleotides using a polymerase-III promoter and designed to form a short hairpin (sh)RNA enable the stable expression of siRNA-like transcripts (BOX 2)[65–67]. Importantly, these types of construct constitutively suppress target-gene expression in transgenic mice[68].

Future horizons

There are now methods that will markedly increase the efficiency of generating transgenic livestock and knockdown the expression of specific genes. Transgenic mice carrying lentivectors that express siRNAs have recently been reported, proving that these techniques can be combined[69]. This approach (BOX 3) is an obvious one to develop for use with livestock.

Box 1 | Lentivectors

Lentiviruses are a class of retrovirus[81] that cause chronic illnesses in the host organism that they infect. Members of this group of viruses include the Visna/maedi virus of sheep, equine infectious anaemia virus (AIEV) of horses and the immunodeficiency viruses of cattle, cats and man (BIV, FIV and HIV, respectively). Like other retroviruses, lentiviruses target their host cells through their envelope proteins. They fuse with the cell membrane, and when the viral RNA is in the cytoplasm it is converted through a virus-contained reverse-transcriptase polymerase into a DNA intermediate. This DNA molecule then integrates into the host-cell genome through its long terminal repeat (LTR) sequences. A distinguishing property of lentiviruses is their ability to infect both dividing and non-dividing cells. It is this property that has promoted their development as gene-delivery vectors that are known as lentivectors.

Lentivectors[52,82,83] have been generated by the deletion of key genes that are involved in the packaging and replication of the virus from the viral genome. Only the introduction of vector DNA into a packaging cell line that has been engineered by transfection strategies to express the missing genes can produce the vector particles. For safety reasons, it is desirable to introduce into the packaging cell line each missing gene as a seperate construct, thereby reducing the likelihood of recombination events that could restore replication-competent vectors. A key feature is that the vector is otherwise transcriptionally silent and so does not activate endogenous genes that are near the site of vector integration. This is achieved by introducing mutations into the viral genome transcription control sequences to generate a self-inactivating (SIN) vector. Overall, there is a drive to reduce the number of viral sequences that are present in these vectors to further increase their safety by limiting the potential for recombination with wild-type virus.

Other modifications can be engineered. For example, to increase the host range the envelope gene is replaced, often incorporating the vesicular stomatis virus G (VSV-G) gene instead. Alternatively, elements that are thought to enhance expression can be incorporated, for example, the woodchuck hepatitis virus post-transcriptional regulatory element (WRE). These vectors have been proposed to be useful for gene therapy and transgenic applications[5,6,69,81,82].

Enhancing production characteristics. Lentivectors could be used to generate gain- or loss-of-function phenotypes in livestock. In light of the developments in RNAi technology, we have focused our discussion on the loss-of-function applications in livestock. There are several target genes that if knocked down might be expected to enhance production traits. For example, knockdown of myostatin, which is known to inhibit lean-muscle growth[70,71], could be achieved in cattle. Furthermore, after nearly 20 years work modifying the mouse genome there is now a wealth of candidate genes, the modulation of which might be expected to affect production traits in livestock. For example, deletion of the high-growth gene, identified as *Socs2* (REF. 72), is known to generate mice with increased postnatal growth. The deletion of *Socs1* in mice enhances mammary gland development[73] and, therefore, possibly milk production. As well as knocking down genes that are known to be related to production characteristics, it is also anticipated that QTL research in livestock will require the use of transgenic knockdown technology to confirm candidate gene function.

Even though such modifications can now be contemplated, the manipulation of key genes will almost certainly suffer from unpredictable PLEIOTROPIC effects, reminiscent of those early problems encountered by introducing and overexpressing biologically active genes using conventional pro-nuclear injection[21]. Nevertheless, this approach will be very useful in evaluating the function of known genes and candidate genes that are identified from gene-mapping studies. However, it is unlikely that this approach will be used for livestock improvement in the near future. It will take both a better understanding of the genomes of livestock, with the anticipated increase in candidate genes to choose from, and a major practical success before transgenic technology seriously challenges genetic improvement of livestock through selection for most conventional traits.

Engineering resistance to infectious disease. Gene knockdown could also be applied to suppressing infectious pathogens, particularly viruses, by targeting the RNA of the invading agent. RNA viruses are possibly best suited to this approach, as theoretically both the genomic and the transcribed strands can be targeted, so it should be possible to interfere simultaneously with replication and expression. For example, the use of siRNAs against mRNAs from respiratory syncitial virus (RSV), a NEGATIVE-STRAND RNA VIRUS, resulted in decreased mRNA expression[74]. Over two thirds of the OFFICE INTERNATIONAL DES EPIZOOTIES (OIE) list-A pathogens are RNA viruses. These list-A pathogens include foot and mouth disease, classic swine fever and fowl plague, all of which have caused significant recent outbreaks of disease. The feasibility of this approach in general has already been tested in cells for other viruses, including human immunodeficieny virus (HIV)[63,75,76], hepatitis[77] and polio[78]. An alternative strategy would be to target host genes. In pigs, aminopeptidase N is the primary receptor for the transmissible gastroenteritis CORONAVIRUS[79]. Knocking down expression of the gene that encodes this receptor could reduce viral infectivity and enhance resistance, although this strategy might suffer from unpredictable pleiotropic effects.

We anticipate the generation of transgenic animals that constitutively express siRNAs targeting the knockdown of a pathogenic virus and/or its transcription products, thereby engineering cellular resistance to infection (FIG. 3). RNAi is sequence specific and so overexpressing siRNAs against the viral genome should not affect any host gene functions. There are many unknowns that will need to be resolved to realize such a goal. For example, what will

Glossary

COMPLEMENT SYSTEM
A protein system in the blood that, combined with the antibody response, forms a defence against cellular antigens.

CORONAVIRUS
A single virus genus, so called because of the club-shaped surface spike proteins that cause their halo- or corona-shaped appearance under the microscope. The recent high-profile outbreak of severe acute respiratory syndrome (SARS) was caused by a coronavirus.

DENUDED EGGS
Eggs that have had their zona pellucida removed by chemical or enzymatic action. These eggs can remain viable, and full development of the fetus and resulting animal is possible.

HYPERACUTE REJECTION
(HAR). An immediate immune reaction to the presence of foreign tissue that is primarily mediated by naturally occurring antibodies that activate the complement system.

INBREEDING DEPRESSION
The decrease in vigour that accompanies a programme of breeding within a restricted gene pool.

INTROGRESSION
The incorporation of a genetic locus or loci from one genotype into the gene pool of another.

NEGATIVE-STRAND RNA VIRUS
Any virus that contains a negative-strand (antisense) RNA template from which mRNA can be directly generated. An example of a negative-strand RNA virus is the influenza virus.

OFFICE INTERNATIONAL DES EPIZOOTIES
(OIE). An intergovernmental organization that was established in 1924 to collate information on significant infectious diseases. The OIE develops normative documents that relate to the rules that member countries can use to protect themselves from diseases, without setting up unjustified sanitary barriers.

PERIVITELLINE SPACE
The fluid-filled region between the surface of the fertilized egg and the fertilization membrane (zona pellucida).

PLEIOTROPIC
A gene or mutation that has many effects.

PRO-NUCLEI
Haploid nuclei that result from meiosis. The female pro-nucleus is the nucleus of the ovum prior; the male pro-nucleus is the nucleus of the sperm. The two pro-nuclei fuse in the fertilized egg.

PSEUDOTYPING
The exchange of virus surface spike proteins from one virus to another. In this way the tissue and/or cell specificity of the virus can be changed. For example, psuedotyping with the vesicularstomatis virus G (VSV-G) protein enables a virus to infect a range of cells from all livestock species, particularly the early embryo.

QUANTITATIVE TRAIT LOCI
(QTLs). Genetic loci or chromosomal regions that contribute to the variability in complex quantitative traits (such as body weight), as identified by statistical analysis. Quantitative traits are typically affected by several genes and the environment.

RETROVIRUS
An RNA-containing oncogenic virus that encodes an RNA-dependent DNA polymerase, reverse transcriptase. Human immunodeficiency virus (HIV) is an example of a retrovirus.

REPLICATION DEFECTIVE
In the context of a virus, this a copy of the virus genome that has been mutated such that it cannot replicate in a host cell. Experimentally, this is engineered by the deletion of key genes from the viral genome; the resulting vector usually remains able to undergo one round of infection. This can be the basis for a viral vector.

SELECTION INDEX
The purpose of animal breeding is to genetically improve the economic merit of livestock. Usually many traits (5–30), for example milk yield and disease resistance, are grouped under economic merit measure. Selection on these traits is weighted in a selection index rather than on profit directly.

SEROTYPES
A group of microorganisms including viruses that can be grouped together on the basis of serology criteria, namely the antigens than they contain.

ZONA PELLUCIDA
The outer jelly-like membrane of a fertilized egg.

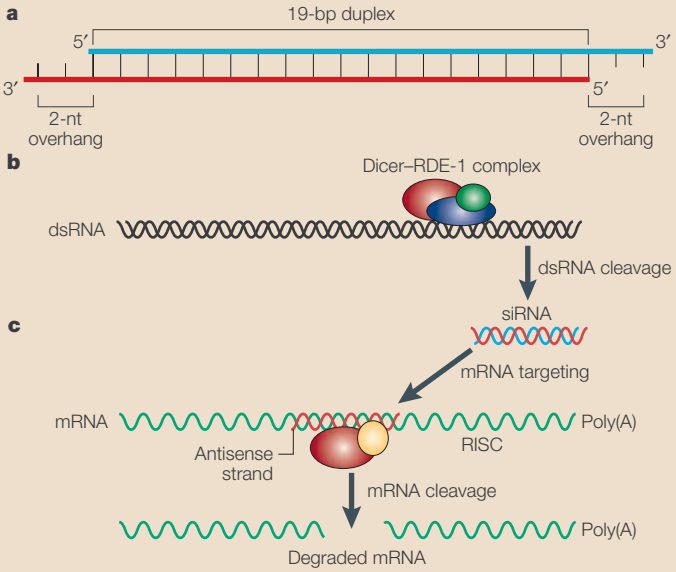
ZOONOTIC
A disease that can be transmitted from animals to humans.

Box 2 | **RNAi by siRNA**

A revolutionary new technology for gene knockdown based on RNA interference (RNAi) is being developed[7]. This is based on a phenomenon that has been recognized for some time as a principal mechanism of post-transcriptional gene silencing in *Caenorhabditis elegans*, *Drosophilia* and plants[8]. This process relies on small interfering (si)RNAs that typically consist of two 19–23 nucleotide (nt) single-stranded RNAs that are able to form a 19-bp duplex with 3′-overhangs (see figure, part a). siRNAs can be generated from long double-stranded (ds)RNA by a complex that includes the enzyme Dicer[7] (see figure, part b). Sequence-specific mRNA degradation occurs in protein–RNA complexes that are known as RNA-induced silencing complexes (RISC)[57] (see figure, part c). As an experimental tool siRNAs can be synthesized and administered to target cells in culture by transfection. This is extremely efficient and 90% knockdown can be achieved. Alternatively, stable expression can be achieved through the use of a polymerase-III promoter vector that directs expression of short hairpin (sh)RNA[65,66].

In shRNAs, two 19-nucleotide strands of an active siRNA are linked together by a few (~9) nucleotides. This structure is engineered downstream of polymerase-III promotor and upstream of a run of 5 T-residues. Transcription from the polymerase-III promoter gives rise to an shRNA terminated after the second U to generate a 2-bp UU overhang at the 3′ end. This molecule is processed by Dicer to function as an siRNA and the antisense strand is used by RISC to guide sequence-specific mRNA cleavage, so promoting mRNA degradation (see figure, part c).

The expression of shRNAs from polymerase-III promoters should allow the widespread knockdown of target genes. The challenge now is to generate tissue-restricted siRNA expression patterns and inducible gene-knockdown expression systems[88,89]. Reproduced with permission from REF. 7 © (2002) Macmillan magazines .



be the most effective shRNA structure and what level of expression will be required to suppress the replication and/or expression of a particular virus to block infection? Will it be possible to co-express siRNAs to improve efficiency, protect against different viral SEROTYPES or to cover the eventuality of escape mutants? One report indicates that double knockdown of two genes can be accomplished[80], but another indicates that there might be competition between two targets, which suggests that the RNAi machinery might be limiting[37]. In the study targeting RSV, the knockdown of viral mRNA was achieved in the absence of any effect on the full-length RNA genome[74], which indicates that in some cases the viral genome might be inaccessible owing to its association with other proteins or sequestered in cellular domains at which siRNA cannot function.

One possible concern is that, rather than being completely resistant, the genetically engineered animals could have a persistent but asymptomatic infection, shed virus and function as a reservoir for infection. It is difficult to imagine how such animals could live side by side with unprotected animals, for example, in countries where there is a slaughter policy for that particular disease. This, however, would not be a problem in countries where the disease was already endemic and where there would be clear benefits from the introduction and introgression of resistant genotypes.

If lentivectors are to have the impact we predict several significant technical hurdles need to be overcome[81,82]. One of these hurdles is the potential effect that transgene insertion can have on the expression of endogenous genes. Retroviral integration is a largely random event and so it could lead to the alteration of the expression of a gene that is at or close to the insertion site, either by direct insertional mutagenesis or through transcriptional interference from the viral terminal repeat elements. Driven by the need for safer gene-therapy vectors, the residual terminal repeat sequences in the self-inactivating (SIN) vectors that are available at present have a severely impaired transcriptional and recombination potential[83,84].

Other technical difficulties present opportunities. Founder transgenic animals might carry numerous copies of the lentivector and this will require extensive breeding to resolve lines with a single integrated copy. However,

Box 3 | **Lentivector delivery of shRNA in mice**

Recently, it has been shown that it is possible to deliver a functional short hairpin (sh)RNA into mice using a lentivector[69]. In this study, an H1-promoter-driven shRNA targeted against the gene encoding green fluorescent protein (GFP) was introduced into one of the long terminal repeats (LTRs) of a human immunodeficiency virus (HIV)-derived lentivector. Owing to the mechanism of reverse transcription, this resulted in the pro-virus containing two copies of the shRNA, one in each LTR. This vector was introduced into fertilized eggs that were generated from animals known to contain a functional GFP transgene. The reduction in GFP fluorescence was observed in early embryos as well as the pups born from these eggs. The lower pup shown in the photograph has had the expression of the GFP transgene knocked down by lentivector delivered shRNA. Reproduced with permission from REF. 69 © (2003) National Academy of Sciences .

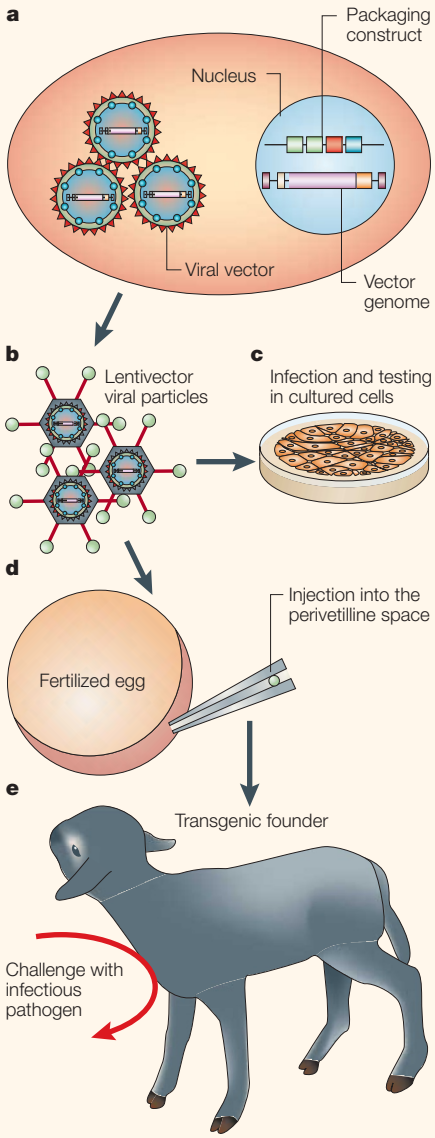


Figure 3 | **Generation of transgenic sheep using lentivectors.** Recombinant lentivector components (**a**), including a small interfering (si)RNA construct, are transfected into a packaging cell line and the packaged viral particles (**b**) are purified by centrifuging the cell supernatant. The efficiency of interference with viral replication/transcription can be tested in cell culture (**c**) before injecting the particles into the perivitelline space of a fertilized egg (**d**). After *in vitro* culture the infected early stage embryos are transferred into recipients. After gestation, founder transgenic animals are born (**e**) and they can be tested for resistance.

commercially this could be seen as an advantage as many lines will be generated from each founder animal. Lentivectors can only transport a limited amount of genetic material, probably less than 10kb. This might limit the size of transcriptional promoter and/or coding sequence that can be incorporated, and this raises issues about the level and specificity of transgene expression in livestock. Although the use of lentivectors to deliver transgenes will be restricted to relatively small genes, this does underline their potential as vectors to deliver siRNA constructs.

The genetic modification debate. The safety of products that are derived from transgenic livestock is of concern to the public and will also be a key consideration in the application of these emerging technologies. No doubt serious concerns will be raised with regard to the use of viral vectors to engineer livestock that is destined for human consumption. Lentivectors are being developed for human gene therapy[54] and so they are already undergoing extensive safety testing, particularly with respect to their replication-defective nature. Nevertheless, the public will need to be assured that this is a robust and safe technology.

The issue of 'release', in particular the inadvertent transmission of transgenes to wild varieties that has dogged the genetically modified (GM) plant debate[85] (see also the article by Stewart *et al.* in this issue) should not pose a problem for livestock. In many parts of the world there are no wild populations of agricultural species. Also, it is much easier to keep gene flow within the agricultural population in animals than it is in plants. In animals it is a matter of confining the population, whereas in plants genes can be introgressed through indirect means such as pollen transfer. In livestock, an exception to this is farmed fish, for which there are substantial and much debated risks of gene flow to wild populations[86,87].

In Europe, GM issues revolve around need and trust, and there are several non-governmental organizations leading the anti-GM debate. By contrast, there has been a greater tendency to accept GM in the United States, and in many developing countries it is issues relating to trade that are often uppermost in the public's mind. There are also keenly held ethical views about GM animals. Many people accept the benefits of using transgenic animals for the production of human therapeutic proteins, although this view is by no means universal. By contrast (and in Europe particularly), even if GM could deliver improvements in animal productivity, such as feed efficiency or food quality, this use of the technology might not be politically acceptable because of public concerns about GM organisms. Given this backdrop, if it does become possible to create GM animals that are innately resistant to diseases, such as foot and mouth or swine fever, it is difficult to gauge what the future attitude of the consumer would be. This will be a debate in which GM concerns will need to be balanced against the consequences of outbreaks of infectious diseases in livestock populations. For example, if allowing GM livestock could prevent the mass slaughter of animals and the environmental consequences associated with the disposal of millions of carcases, seen recently during the foot and mouth outbreak in the United Kingdom, it might well be seen as the lesser of two evils. Furthermore, infectious diseases in animals are seen to be increasingly relevant to human health. For example, the recent outbreak of severe acute respiratory syndrome (SARs) highlighted the problem of devastating zoonotic infections that can arise from domesticated species such as pigs and chickens. Would there be general acceptance of transgenic technology if it could be applied to engineering resistance to influenza in poultry and therefore lessen the risk of an influenza epidemic, such as the one in 1918 that killed more than 20 million people?

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