

RNA interference-based technology: what role in animal agriculture?

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Abstract. Animal agriculture faces a broad array of challenges, ranging from disease threats to adverse environmental conditions, while attempting to increase productivity using fewer resources. RNA interference (RNAi) is a biological phenomenon with the potential to provide novel solutions to some of these challenges. Discovered just 20 years ago, the mechanisms underlying RNAi are now well described in plants and animals. Intracellular double-stranded RNA triggers a conserved response that leads to cleavage and degradation of complementary mRNA strands, thereby preventing production of the corresponding protein product. RNAi can be naturally induced by expression of endogenous microRNA, which are critical in the regulation of protein synthesis, providing a mechanism for rapid adaptation of physiological function. This endogenous pathway can be co-opted for targeted RNAi either through delivery of exogenous small interfering RNA (siRNA) into target cells or by transgenic expression of short hairpin RNA (shRNA). Potentially valuable RNAi targets for livestock include endogenous genes such as developmental regulators, transcripts involved in adaptations to new physiological states, immune response mediators, and also exogenous genes such as those encoded by viruses. RNAi approaches have shown promise in cell culture and rodent models as well as some livestock studies, but technical and market barriers still need to be addressed before commercial applications of RNAi in animal agriculture can be realised. Key challenges for exogenous delivery of siRNA include appropriate formulation for physical delivery, internal transport and eventual cellular uptake of the siRNA; additionally, rigorous safety and residue studies in target species will be necessary for siRNA delivery nanoparticles currently under evaluation. However, genomic incorporation of shRNA can overcome these issues, but optimal promoters to drive shRNA expression are needed, and genetic engineering may attract more resistance from consumers than the use of exogenous siRNA. Despite these hurdles, the convergence of greater understanding of RNAi mechanisms, detailed descriptions of regulatory processes in animal development and disease, and breakthroughs in synthetic chemistry and genome engineering has created exciting possibilities for using RNAi to enhance the sustainability of animal agriculture.

Additional keywords: gene silencing, livestock, pharmacology, RNAi.

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Introduction

Animal science occupies a unique position at the interface of basic biology, medicine, and agriculture. Indeed, research and commercial application of technologies ranging from quantitative and genomic genetic selection programs, artificial insemination, embryo transfer, and various uses of endocrine and pharmaceutical products have contributed to the dramatically increased resource efficiency of animal agriculture over the past 5–7 decades (Capper 2011). Although potential risks must be evaluated for each novel technology, applications of RNA interference (RNAi) hold substantial promise for addressing both existing and potential threats to the sustainability of global animal agriculture. Our aim in this review is to introduce a broader group of animal scientists to the fundamental mechanics and broad utility of RNAi, and also to introduce to those in the RNAi field the many potential applications of this technology in animal agriculture.

What is RNA interference?

RNAi is a key intracellular mechanism regulating the function of cells, and in turn, organisms. This process generated substantial excitement upon its discovery in the late 1990s (Fire *et al.* 1998), resulting in a Nobel Prize for its co-discoverers in 2006 (the shortest period ever from discovery to award). RNAi is believed to have emerged as an anti-viral defence system whereby double-stranded RNA used as viral genetic code is recognised and cleaved, destroying the message (Obbard *et al.* 2009). However, this toolkit was adapted over evolutionary time to contribute to the regulation of eukaryotic protein synthesis. Both plant and animal genomes encode a variety of short RNA strands, typically referred to as micro RNA (miRNA), that are not destined for translation but as a secondary level of transcriptional control (Iwakawa and Tomari 2015). Pri-miRNA are produced in the nucleus, generally as hairpin structures present in mRNA introns. The

microprocessor complex comprised of two proteins, the RNase III enzyme Droscha and the double-stranded RNA binding protein Pasha, excise the pre-miRNA hairpin from the mRNA. Pre-miRNA are transported into the cytoplasm by a nucleocytoplasmic transport factor exportin 5. Once in the cytoplasm another RNase III enzyme, dicer, removes the loop structure from the pre-miRNA hairpin producing a short double-stranded mature miRNA. The miRNA then associates with multiprotein RNA induced silencing complex (RISC), which comprises argonaute 2, dicer, and the human immunodeficiency virus transactivating response RNA-binding protein. The guide strand is integrated into the RISC. The remaining strand, the passenger strand, is degraded as a RISC complex substrate. The RISC with a bound miRNA can then recognise and bind complementary mRNA, leading to formation of double-stranded RNA. At this point, the RISC cleaves the target mRNA at the position facing nucleotides 10 and 11 of the miRNA. If the sequence is not fully complementary, RISC can still silence target genes by recruiting additional effector proteins, which induce translational repression and/or mRNA decay in a manner independent of endonucleolytic cleavage (Chapman and Carrington 2007). Short binding sequences complementary to individual miRNA are often found on an array of different mRNA targets, allowing for transcription of a single miRNA to suppress translation of a whole class of proteins. This process therefore provides a rapid, responsive method for a cell to downregulate entire enzyme or signalling/response pathways (Inui *et al.* 2010; Dumortier *et al.* 2013).

Small interfering RNA (siRNA), when discussed in the context of higher animals, generally refers to synthetic molecules that mimic the effects of miRNA, although the potential sequences targeted by siRNA are not limited to those targeted by genomic miRNA. Synthetic siRNA is typically comprised of double-stranded RNA (dsRNA) molecules 20–25 base pairs in length with 3' overhangs on each strand (Elbashir *et al.* 2001). Another class of molecules that can induce RNAi is shRNA, which are single RNA strands that are self-complementary, resulting in the formation of hairpin-shaped structures like those formed by miRNA (Paddison *et al.* 2002).

Unfortunately, siRNA binding to target sequences is not so specific that off-target mRNA knockdown is impossible. Partial complementation of either the 'guide strand', (which corresponds to the antisense strand) or the passenger strand (corresponding to the sense strand) can result in off-target mRNA cleavage (Matveeva 2013). Furthermore, imperfect pairing can also lead to translational repression even if the mRNA is not degraded (Saxena *et al.* 2003). However, perfect complementarity and specificity does not guarantee efficacy; the stability of the siRNA-mRNA complex and the structure of the mRNA must be appropriate to allow for degradation of the transcript. Despite these challenges, observed off-target silencing is often subtle compared with suppression of the intended target, and a large body of work has now detailed the characteristics of functional and specific siRNA sequences (Rettig and Behlke 2012; Matveeva 2013). Despite the need to test for off-target effects, RNAi nevertheless provides the unique opportunity to target essentially any gene product in an animal with reasonable specificity. As such, RNAi has become a standard tool in functional genomics studies, with genome-wide siRNA

and shRNA libraries readily available for several animal genomes.

The enzyme complex responsible for mediating RNA-induced silencing forms in the cytosol of the cell (Kim *et al.* 2014). Therefore, triggering this process requires the siRNA to find its way into target cells, which is no small challenge for *in vivo* application of RNAi (Rivera and Yuan 2012). Two distinct approaches have been taken to address this problem, detailed below.

Transgenic expression of RNAi-inducing molecules

The most common approach to delivery of RNAi-inducing molecules to date has been via genomic integration of shRNA constructs (Fig. 1). For detailed reviews of transgene insertion techniques, see Gama Sosa *et al.* (2010) and Maksimenko *et al.* (2013). Traditional methods of transgene insertion include the following approaches:

- *DNA microinjection.* The transgene containing the shRNA is injected into the nucleus of a fertilised egg where it is randomly inserted into the host genome. The fertilised egg is then transferred to a foster mother, and the offspring will carry the transgene if integration was successful (Gordon and Ruddle 1981).
- *Embryonic stem cell-mediated gene transfer.* Embryonic stem cells derived from early stage mouse embryos can be cultured indefinitely. The targeting vector consisting of the endogenous gene carrying the shRNA required is introduced into these cells and inserted into the genome by homologous recombination. The altered embryonic stem cells are then injected into early mouse embryos, where they mix with the endogenous cells of the embryo resulting in a chimeric animal. If the altered cells contribute to the germ cells of the mouse, progeny in a subsequent mating will inherit the shRNA expressing gene (Smithies *et al.* 1985).
- *Somatic cell nuclear transfer.* Somatic cells are maintained in culture. A targeting vector consisting of an endogenous gene carrying the shRNA required is introduced into these somatic cells and inserted into the genome by homologous recombination. The somatic cells are screened to find cells that have correctly integrated the shRNA. Oocytes are enucleated and nuclei of shRNA-integrated cells are transferred into the enucleated oocytes, and then fused with an electrical pulse. Once fused, the cells behave like typical *in vitro* fertilised embryos, and are grown in culture until the blastocyst stage, when they are transferred into recipient animals.
- *Retrovirus-mediated gene transfer.* Retroviruses have the ability to infect host cells and integrate into the genome. Therefore, they are commonly used as vectors for transgenic applications, creating a chimeric embryo with the DNA inserted randomly (Nagano *et al.* 2001).
- *Transposon-mediated gene transfer.* Transposons are mobile genetic elements that can facilitate the transposition of DNA from plasmid vectors into chromosomes. During transposition, the transposase recognises transposon-specific inverted terminal repeat sequences flanking the shRNA transgene, and moves the shRNA transgene into a random chromosomal site (Macdonald *et al.* 2012).

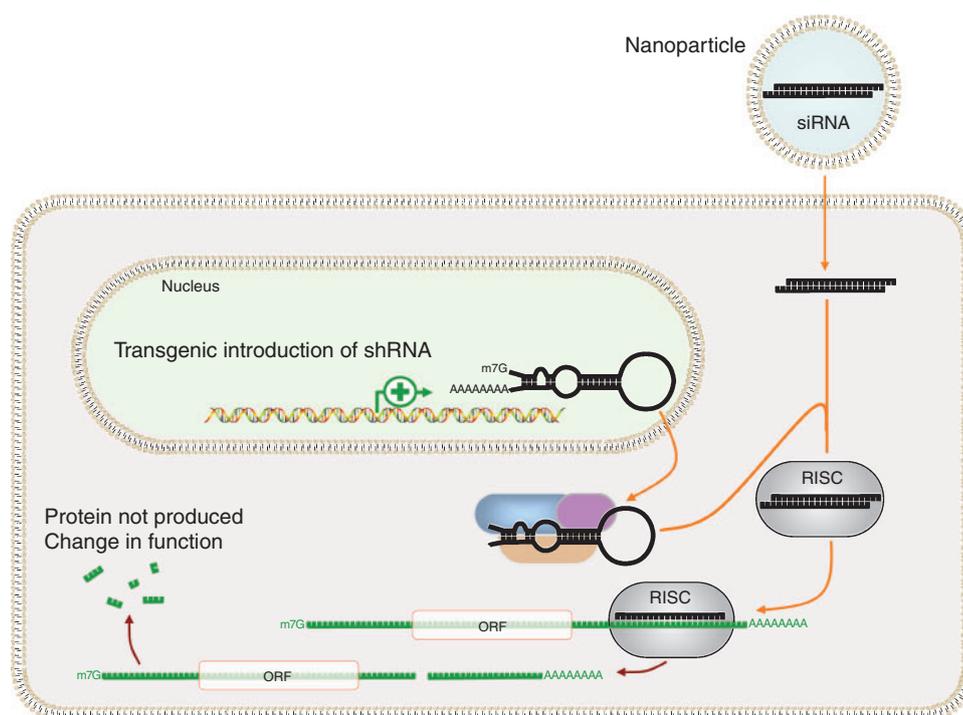


Fig. 1. Nanoparticle-based delivery of exogenous small interfering RNA (siRNA) and genetic engineering provide two distinct approaches to RNAi *in vivo*. Nanoparticles of various chemistry can be used to traffic exogenous, synthesised siRNA molecules to the target cells. After endocytosis and lysosomal release of the siRNA, it is available in the cytosol to activate the RNA-induced silencing complex (RISC). Transgenic introduction of short hairpin RNA (shRNA) sequences provides an endogenous silencing tool whose transcription is dependent on the promoters used and the genomic context of the transgene. Processing of shRNA results in a siRNA product that can also activate RISC. The activated RISC uses the antisense siRNA strand to bind to the target mRNA and subsequently cuts the strand, resulting in the mRNA being marked for destruction rather than translation. Note that silencing can be induced by mRNA cleavage in the open reading frame or in the 5' or 3' untranslated regions.

- *Sperm-mediated gene transfer.* In this method the transgene containing the shRNA is attached or inserted into the sperm head and then used to fertilise eggs (Moreira *et al.* 2007). DNA can be introduced to the sperm head in several ways including transfection (Collares *et al.* 2011), attachment by antibodies (Chang *et al.* 2002) and disruption by repeated freeze–thaw cycles or exposure to detergents (Perry *et al.* 2001).

Random insertion of the shRNA construct into the genome can lead to problems including overloading of the RNAi machinery (Grimm *et al.* 2006), complete elimination of a required gene product, or epigenetic silencing of the construct. Newer precision genome engineering tools such as zinc finger nucleases, clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases and transcription activator-like effector nucleases (TALEN) can be used to avoid some of these problems (Gaj *et al.* 2013; Sander and Joung 2014). These technologies act as ‘molecular scissors’ creating specific double-stranded breaks at targeted locations in the genome. The transgene with flanking homologous sequences is then inserted by homologous recombination, utilising the cell’s endogenous DNA repair mechanism to repair the break. Combining precision genome engineering and

careful selection of the promoter for the construct can, in theory, address the issues with traditional transgenic methods and can also potentially target shRNA expression to specific cell types or developmental stages. These approaches will be explored in the remainder of the text.

Administration of exogenously produced RNA molecules

Exogenous siRNA have become a routinely used tool in cell culture research over the past decade. Additionally, because of the potential of RNAi for treatment of human diseases, substantial work has been carried out in rodent models to determine optimal approaches to *in vivo* administration of siRNA molecules. Early studies employed hydrodynamic intravenous administration of dsRNA to essentially flood RNA degradation processes and induce gene silencing systemically, with particularly dramatic effects in the liver (Kawakami and Hashida 2007). However, the rapid administration of large volumes into the tail vein of mice may lead to tissue damage and stress responses, and this approach is unlikely to be feasible in large animals anyway. Most current work employs nanoparticles to encapsulate siRNA (Hong and Nam 2014) or relies on chemical modification of the siRNA to slow its

degradation (Engels 2013). Upon delivery to target cells, siRNA still must enter the cell – typically through endosomes – and make its way to the cytoplasm. In addition to enhancing the stability of siRNA, current work on nanoparticle design for siRNA delivery focuses on incorporation of targeting moieties that trigger uptake by specific cell types and cleavage sites allowing for efficient dissociation of the nanoparticle in endocytic vesicles.

The relative difficulty of gene silencing with exogenous siRNA varies greatly across organs. The liver, kidneys, spleen, and phagocytic cells can be affected by many delivery formulations, with little or no targeting required (Shi *et al.* 2011). Although many other organs are difficult to target with systemic siRNA administration, there has been some success in targeting muscle tissue (Kinouchi *et al.* 2008) and adipocytes (Won *et al.* 2014). Additional target organs, including the lungs (Bitko *et al.* 2005), uterus (Brooks *et al.* 2015), mammary gland (Brock *et al.* 2014), and muscle (Koganti *et al.* 2015) can be altered by direct delivery of siRNA.

Advantages of exogenous RNAi versus the transgenic RNAi approach

Exogenous delivery has several advantages over the transgenic approach. One of these is the short-term effect of delivering the treatment; this is beneficial for transitioning between physiological states, regulation of genes specifically during development for improved physiological traits and for protection from outbreaks of infectious disease. Exogenously delivered siRNA may also be advantageous over transgenic expression to combat infectious diseases, as many viruses can mutate rapidly, potentially negating the advantage of the transgenic line. Newly emerging viruses can also be addressed more rapidly with synthetic siRNA, as once the new viral sequence is known, development of siRNA is relatively straightforward. Exogenous RNAi also avoids the detrimental effects of overexpression of transgenic shRNA, unanticipated negative consequences from prolonged downregulation of genes and does not require manipulation of the genome, making it accessible for human therapeutics and bypassing the perception issues with genetically modified organisms. Exogenous RNAi can also be rapidly adapted for multiple species, where the genome sequence is available. Similarly, exogenous RNAi can be applied on a much larger scale suitable for agricultural applications through food supplies or as plant insecticide delivery for pest control.

Potential applications of RNAi in animal agriculture

In plant agriculture, RNAi has already entered the commercial domain. Transgenic potatoes and apples utilising RNAi have been approved in the USA (Waltz 2015), and many more crops employing this technology are being developed (Koch and Kogel 2014). Although there are additional challenges for animal agriculture to utilise these tools, the advances in plant agriculture point to the potential of RNAi.

Previous reviews on potential uses of RNAi in animal agriculture were published more than 5 years ago (Wise *et al.* 2008; Long *et al.* 2010), and significant advances in our general understanding of RNAi, as well as new examples of *in vivo*

RNAi applications in livestock, have occurred during that time. We will outline a variety of opportunities for RNAi in animal agriculture while highlighting examples of successful RNAi approaches in livestock to date.

Altering developmental programs

In many agricultural species, research on fetal and early postnatal programming has revealed that even subtle differences in the environment during development can have long-term impacts on the animal that influence productivity, efficiency, and product characteristics (Reynolds *et al.* 2010). These findings demonstrate the broad potential impacts of altering gene translation during development.

Altering muscle development. Finding ways to increase overall muscle mass has long been a topic of study in animal agriculture research. One of the most studied genes relating to control of muscle mass is myostatin, which negatively regulates skeletal muscle mass by reducing protein synthesis. Long before the identification of the myostatin gene, farmers have been selecting animals with naturally occurring myostatin mutations. Both the Belgian Blue and Piedmontese cattle breeds were first established in the 1800s based on observed muscle hypertrophy phenotypes, which more than 100 years later were found to be due to mutations within the myostatin gene (Kambadur *et al.* 1997). However, during calving, these breeds have a high incidence of dystocia and Caesarean sections due to the muscle hypertrophy of their offspring.

New genome engineering tools such as TALEN and CRISPR allow scientists to make precise changes to the genome. There are multiple groups that have used these tools to produce myostatin knockout animals, including cattle (Proudfoot *et al.* 2015), goats (Ni *et al.* 2014), and fish (Dong *et al.* 2011). These knockout animals are expected to have similar muscle hypertrophy phenotypes to those breeds with naturally occurring myostatin mutations. However, precision engineering of myostatin mutations in mammals leaves the reproductive issues unresolved. Previous research shows that translation of the myostatin gene is partially regulated by endogenous microRNA, indicating that the myostatin gene is a good candidate for RNAi knockdown (Miretti *et al.* 2013). Multiple groups have now successfully produced myostatin knockdown animals, by delivery of siRNA molecules in mice (Adachi *et al.* 2010) and through transgenic integration of shRNA constructs in cattle (Tessanne *et al.* 2012) and sheep (Hu *et al.* 2013). These RNAi-based approaches offer some advantages over the precision engineered knockout animals as RNAi knocks down gene expression, but does not completely knock it out, which could lead to a more moderate muscle hypertrophy phenotype. Overall, RNAi-based approaches allow for greater customisation than gene deletion; however, the shRNA constructs used to produce myostatin knockdown in large animals to date have utilised constitutive promoters to drive abundant transcription of small RNA (Ma *et al.* 2014).

As our understanding of RNAi improves, systems to tailor the knockdown of myostatin expression – in a tissue- or time-specific manner – could allow for enhanced muscle development without negative reproductive outcomes. For example, precision genome engineering could be used to place a shRNA construct

in a known 'safe harbour' in the genome, which would not disrupt transcription of any endogenous genes. These systems will likely have promoters that primarily function in postnatal muscle tissue, such as FBXO40 (Ye *et al.* 2007) or the muscle creatine kinase promoter (Trask and Billadello 1990). The muscle creatine kinase promoter has been successfully used to drive muscle-specific transgene expression in mice (Johnson *et al.* 1989; Brüning *et al.* 1998; Wang *et al.* 2008; Li *et al.* 2013). Additionally, the muscle creatine kinase promoter was used to drive production of artificial microRNA targeted at activin receptor type IIB, another regulator of muscle fibre size, in goat myoblasts *in vitro*, resulting in up to 57% protein silencing and enhanced proliferation and differentiation of myoblasts (Patel *et al.* 2014). Although tissue-specific RNAi systems for myostatin knockdown have not yet been used in livestock *in vivo*, successful knockdown of the known milk allergen β -lactoglobulin specifically in the mammary gland of cattle has been achieved (Jabed *et al.* 2012), which demonstrates the promise of such approaches.

Exogenous siRNA could also be useful for modulating development early in life. Using a chemically modified oligonucleotide intended to induce translational repression of the target gene (known as a morpholino; Morcos *et al.* 2008) Brooks *et al.* (2015) demonstrated the dramatic influence of peroxisome proliferator activator receptor gamma on elongation of the ovine conceptus between Days 7 and 14 after fertilisation. These investigators used osmotic minipumps to deliver the morpholinos into the uterus over 7 days. Although the goal of this particular study was not to influence postnatal characteristics of the offspring, it nevertheless shows the potential for targeted gene knockdown at critical prenatal stages to alter developmental physiology. Less invasive strategies for RNAi manipulation of the reproductive tract have also been investigated (Yang *et al.* 2013).

Altering sex ratios. Another major area of interest is the generation of single sex populations. In the poultry layer industry, for example, modulation of sex determination to generate only female chickens would greatly increase productivity. It would also provide an alternative to culling of non-laying male chicks, which currently presents a major welfare concern to the egg industry. Female-only hatches may also lead to increased uptake of *in ovo* delivered vaccines in the layer industry, improving control of diseases that impact egg production. Conversely, the generation of only male chicks would be of great benefit to the broiler industry as male chickens grow faster than females.

The most attractive way of stably manipulating sex is by modifying the activity of key sex-determining genes during embryonic development. In 2009 the Z-linked gene, DMRT1, was identified as a major factor required for testis development in the chicken embryo (Smith *et al.* 2009). Retrovirus-delivered RNAi targeting DMRT1 induced the gonads of genetic males (ZZ) to develop partial or complete ovaries, suggesting that it may be possible to produce genetically male chickens with female attributes. Similarly, aromatase expression is a hallmark of female development in chickens (Lambeth *et al.* 2013). By using RNAi to knockdown aromatase in the broiler industry, it may be possible to produce only male chickens.

In silkworms (*Bombyx mori*), male-only rearing has long been desired because males show better fitness, lesser food consumption, and greater silk yield (Nagaraju 2002). Using TALEN to specifically knock out the female Bmdsx gene led to 100% of the resulting females being sterile and having developmental defects, pointing to a potential use for RNAi in silk production (Xu *et al.* 2014).

Supporting transitions between physiological states

Across livestock industries, many of the most difficult health challenges occur as animals adapt to a new stage of life or environment. In all species (wild and domestic), transitioning to a new physiological state induces stress that can impair health and productivity. Examples include neonatal morbidity and mortality (Mellor and Stafford 2004), post-weaning stress in cattle (Duff and Galyean 2007) and pigs (Pluske *et al.* 1997), pregnancy toxemia in sheep (Mavrogianni and Brozos 2008), osteoporosis in laying hens (Webster 2004), transition disorders in early lactation dairy cattle (Vergara *et al.* 2014), and heat stress across species (Renaudeau *et al.* 2012). Much effort has gone into using genetics, nutrition, environmental management, and traditional pharmaceuticals to prevent these costly problems, yet many of these issues remain a thorn in the side of livestock producers.

As a tool that can precisely alter animal function, RNAi offers exciting possibilities for aiding the adaptation to a new physiological state. Furthermore, because the onset of these health challenges are predictable and they diminish once a new steady-state is achieved, the short window of efficacy for a single exogenous siRNA treatment is of limited concern. As an example of the potential use of siRNA as an adaptation aide, we will consider the case of the dairy cow transitioning into lactation.

Metabolic and infectious diseases affect more than 50% of all dairy cattle in the first 2 weeks of lactation (Vergara *et al.* 2014), manifested in a variety of clinical symptoms including ketosis, displaced abomasum, milk fever, mastitis, and metritis. Such problems not only harm the welfare of animals, but also contribute to many cows being removed from the lactating herd during early lactation, limiting the profitability and sustainability of the dairy industry. With the exception of milk fever, the incidence of these problems has not decreased in the past 25 years (USDA 2007). The reason for the high incidence of some of these disorders in early lactation remain unresolved despite decades of research. Arguably, this slow progress is largely due to the lack of efficacious tools to interrupt specific signals that may contribute to disease states during the transition to lactation.

The centrality of the liver in many of the transition cow problems provides a particularly good opportunity to employ RNAi tools, because intravenous delivery of siRNA nanoparticles consistently results in liver uptake of siRNA, often along with delivery to the spleen and kidneys (Jiang *et al.* 2011). Hypothesised roles for oxidative stress response pathways in postpartum liver dysfunction (Gessner *et al.* 2013), as an example, could be evaluated by targeting one or more pathway members through RNA silencing. If cows respond to exogenous siRNA in a manner similar to mice, it may be

possible to suppress target protein expression by >90% for at least 2 weeks after a single intravenous treatment with a siRNA nanoparticle (Love *et al.* 2010). Therefore, siRNA delivery may help to prevent maladaptive responses and subsequent diseases during the transition to lactation. Even if not successful at preventing disease, such a strategy could at least rule out proposed mechanisms underlying these disorders.

Combating infectious disease

Infectious diseases remain a substantial problem in animal agriculture, with different but overlapping issues in intensive and extensive production systems (Perry *et al.* 2013). Despite progress in research technologies, diagnostic capabilities, and manufacturing methods, there remain many infectious diseases for which no effective vaccines exist. Furthermore, vaccination can drive the emergence of resistant and more infectious viruses, as in the case of Marek's disease virus of poultry (Witter 1997; Nair 2005). Many of the existing viral vaccines are ineffective against the prevailing strains in the field, and new vaccines have to be generated from field strains with each new outbreak, which can take months. Global availability, field compliance, effectiveness, and safety are also significant concerns.

Despite the need for new and improved vaccines, the development of vaccines is becoming more complex and expensive. There is also significant concern by the public about the use of antibiotics and other pharmaceutical products in agricultural animals, which has significant impact on control of many diseases. Ongoing work is exploring three distinct uses of RNAi to overcome these challenges to combat infectious diseases in livestock.

Directly targeting pathogens/disease agents. RNAi has an advantage over classical vaccines and small drug molecules in that it is highly selective for the pathogen and can be made to large scale very quickly (Dykxhoorn and Lieberman 2005). Entire genomes of emerging viruses can be sequenced within a day with the advent of high throughput sequencing. Once this is known, siRNA can be rapidly designed using published algorithms and can be tested *in vitro* and *in vivo* within days. By comparison, it can take months to screen the vast number of candidate small drug molecules against a new virus or to develop and test inactivated vaccines.

RNAi has been shown to inhibit a wide range of agriculturally significant pathogens both *in vitro* and *in vivo*, including foot and mouth disease virus (FMDV; Jiao *et al.* 2013; Gismondi *et al.* 2014), African swine fever virus (Keita *et al.* 2010), classical swine fever virus (Porntrakulpipat *et al.* 2010), influenza A (Stoppani *et al.* 2015), highly pathogenic avian influenza (Stewart *et al.* 2011), chicken anaemia virus (Hinton and Doran 2008), bovine viral diarrhoea virus (Lambeth *et al.* 2007), and infectious bursal disease virus (Wang *et al.* 2010).

When directly targeting a pathogen, the RNAi target site needs to be in a highly conserved region of the genome (usually within a gene essential for replication) and must take into account the three-dimensional structure of the viral genome. In the case of FMDV, the conformational folding of the RNA genome has been shown to inhibit the activity of a range of siRNA sequences (Gismondi *et al.* 2014). This has also been

demonstrated in HIV (Westerhout *et al.* 2005). Studies have also shown that to ensure complete coverage of the population and to avoid the emergence of siRNA-resistant mutants, multiple siRNA sequences should be employed (Kahana *et al.* 2004; ter Brake *et al.* 2006).

Four examples of transgenic animals expressing shRNA targeting disease agents have been published. The first example was the development of a goat that expressed shRNA targeting the prion protein that causes transmissible spongiform encephalopathies (Golding *et al.* 2006). The transgenic fetus was collected on day 81 of gestation and had a >90% reduction in prion protein abundance in the brain compared with a non-transgenic fetus at a similar stage of development. Using a similar approach to produce a viable calf expressing an anti-prion shRNA, Wongsrikeao *et al.* (2011) reported that the prion mRNA and protein levels in nervous tissue were 24% and 86% lower, respectively, compared with the Control animals. More recently, porcine reproductive and respiratory syndrome shRNA pigs were produced. These animals survived 11 days longer than the Control pigs when challenged with porcine reproductive and respiratory syndrome, with the siRNA working early in infection, similar to the vaccinated Control group (Li *et al.* 2014). In the same year, transgenic mice expressing two anti-FMDV shRNA, targeting the viral polymerase protein 3D and the non-structural protein 2B regions, showed 19–27% higher survival rates than wild type mice (Chang *et al.* 2014). In each of these examples, moderate inhibition of the disease or knockdown of the gene target was found. Although these approaches are in the early stage of development, RNAi combined with transgenic technology offers the possibility to genetically engineer livestock to promote resistance to viral infections and prion diseases.

There are multiple examples of exogenously delivered siRNA inhibiting viral infections, including important human pathogens such as influenza virus, respiratory syncytial virus, and hepatitis viruses B and C in animal models, and siRNA therapeutics targeting respiratory syncytial virus and Ebola virus have been evaluated in clinical trials (Kanasty *et al.* 2013). Few examples, however, are published for agriculturally important diseases. Inhibition of influenza virus infection has been demonstrated in both mice and chicken embryos (Tompkins *et al.* 2004; Hinton *et al.* 2014), and adenovirus constructs expressing shRNA were shown to successfully inhibit FMDV in guinea-pigs (Xu *et al.* 2012). Exogenously delivered RNAi has also been shown to be functional in aquatic species. In lower vertebrates, RNAi can be delivered as long dsRNA, as in the example where *L. vannamei* shrimp were protected from White spot syndrome virus (Robalino *et al.* 2005). Another study demonstrated protection from yellow head virus by injection of a dsRNA (Saksmerprome *et al.* 2009). *Penaeus japonicus* (the kuruma prawn) was also protected from White spot syndrome virus after siRNA injection (Xu *et al.* 2007). Exogenously delivered siRNA can be a useful therapeutic tool and should be considered as an alternative strategy in the control of livestock diseases.

Targeting disease vectors. An alternative strategy for disease control with RNAi is to target the animal or insect vector that transmits the disease. The sterile insect technique (SIT) is a common method to biologically control pest insects that

heavily damage agriculture and forestry or transmit deadly diseases to animals and humans (Whyard *et al.* 2015). In America SIT has successfully eradicated the screw worm (blow) fly that caused significant losses to the livestock industries, due to infestation of maggots in the wounds of cattle and other livestock. Similarly, in Zanzibar SIT eradicated the tsetse fly, which was responsible for cyclical transmission of the disease trypanosomiasis and annual losses estimated at US\$2 million due to loss of calves, reduced meat and milk production and disease control costs. Traditional SIT works by releasing overwhelming numbers of males sterilised by radiation. The sterile males compete with the wild males for female insects, thus reducing the next generation's population. Whyard *et al.* (2015) demonstrated that feeding sex-specific dsRNA to larvae of the dengue vector *Aedes aegypti* halted sperm production in adult males, producing fit substantially sterile males. Therefore, sterilising males by administering RNAi through food sources or by transgenics could provide an environmentally friendly, sustainable and humane approach to controlling insect pests (Whyard *et al.* 2015).

Another strategy is to target genes that can decrease colonisation and transmission of the insect vector. Multiple tick genera such as *Dermacentor*, *Ixodes*, *Margaropus*, and *Rhipicephalus* from the hard bodied tick (*Ixodidae*) family and *Argas*, *Ornithodoros*, and *Otobius* from the soft tick (*Argasidae*) family are responsible for the transmission of numerous important human and agricultural pathogens (Anderson and Magnarelli 2008). These include the bacterial pathogens *Borrelia*, *Anaplasma*, *Coxiella*, *Francisella*, *Rickettsia*, and *Babesia*, as well as viruses such as the tick-borne encephalitis virus (de la Fuente and Kocan 2006). The pathogens that cause babesiosis (*B. bovis* and *B. bigemina*) and anaplasmosis (*A. marginale*) are transmitted by infestation with the cattle tick, *Rhipicephalus microplus*. This tick also has a significant economic impact on cattle production in tropical and subtropical regions of the world by reducing weight gain and milk production. Functional analyses conducted by RNAi have been performed on multiple pathogen–tick interactions to elucidate the roles of specific genes in the pathogen lifecycle within the vector (de la Fuente and Kocan 2006; de la Fuente *et al.* 2007). This approach enables rapid identification of potential pathogen colonisation or transmission-blocking tick RNAi candidates (de la Fuente *et al.* 2007). In *B. burgdorferi* colonisation of the deer tick (*Ixodes scapularis*), the repression of TROSPA expression via RNAi reduced the bacterial adherence to the tick gut *in vivo*, thereby preventing efficient colonisation of the tick vector and decreasing pathogen transmission to the host (Pal *et al.* 2004). A functional screen of salivary proteins in *I. scapularis* during infection with *Anaplasma phagocytophilum* identified significant upregulation of the gene salp16. Subsequent RNAi silencing of this salivary protein inhibited initial infection of the tick salivary gland, eliminating the ability to transmit the pathogen (Sukumaran *et al.* 2006).

Similarly, new tick-specific antigens have been identified by RNAi screens, raising the possibility of vaccination strategies against tick-borne pathogens as a cost-effective, environmentally friendly alternative to chemical control (de la Fuente *et al.* 2005, 2007). RNAi inhibition of subolesin resulted in sterile

wood ticks (*Dermacentor variabilis*) with under-developed salivary glands (de la Fuente *et al.* 2006b). Subsequently, mice vaccinated with subolesin were shown to have a 3-fold reduction in the number of *I. scapularis* nymphs infected with *A. phagocytophilum*. Subolesin-based vaccines may therefore disrupt the transmission of *A. phagocytophilum* by affecting pathogen infection and/or development in tick salivary glands (de la Fuente *et al.* 2006a).

Altering animal responses to disease. Many pathogens, particularly viruses, continually mutate, making vaccination-based control strategies difficult. New strategies to combat these diseases require an expanded knowledge of host–virus interactions as a crucial first step. Whole genome siRNA screens of infected cells have recently begun to elucidate host genes that are important in infection. In one such screening study, 21 121 pools of four chemically synthesised siRNA targeting nearly every gene in the human genome were individually transfected into a human cell line and the effect on viral replication observed (Lee *et al.* 2014). In the case of vesicular stomatitis virus that infects cattle, horses and pigs, the genome-wide siRNA screen identified 23 genes that reduced vesicular stomatitis virus replication when silenced, without affecting cell viability (Lee *et al.* 2014). Silencing one or more of these 23 host genes, which are apparently important for viral replication, may lead to a new therapeutic treatment for vesicular stomatitis virus. Similarly, FMDV is known to use host $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, and $\alpha v\beta 8$ integrins as cellular receptors to initiate infection through interactions with viral proteins. Development of transgenic suckling mice that artificially expressed αv integrin-specific miRNAs resulted in significantly higher survival rates following FMDV infection compared with their non-transgenic littermates (Du *et al.* 2014). Therefore, suppression of the host-cell αv integrin gene inhibited FMDV infection. Despite the promise of these approaches, it must be noted that life-long knockdown of host factors (e.g. through genomic introduction of shRNA) may have unanticipated negative consequences for the animal, as their function is sometimes unknown and may not be sufficiently redundant to avoid problems (Meliopoulos *et al.* 2012).

Another approach is to alter the immune response to trigger a more appropriate response to infection, which may mean driving a more aggressive response to some pathogens or limiting runaway inflammation in other pathologies (Goldsmith *et al.* 2011). Such strategies can take many forms, depending on the cell types and signalling cascades involved. One innovative approach was used to combat induced colitis in a mouse model (Peer *et al.* 2008). In this study, antibody-conjugated nanoparticles were used to target anti-cyclin D1 siRNA specifically to leukocytes. Intravenous administration of these nanoparticles dramatically improved systemic measures of health and intestinal tissue morphology, presumably by dampening the T helper 1 cytokine responses in the intestine (Peer *et al.* 2008). Similarly, knockdown of a proapoptotic gene from the Bcl-2 family, Bcl2-interacting mediator of cell death, increased sepsis survival to 90% compared with 50% survival in Control septic mice (Schwulst *et al.* 2008). In contrast to these inflammatory pathologies, hepatitis B virus interferes with pattern recognition receptor signalling, which prevents an innate immune response and allows a persistent infection to

develop. To address this condition, Han and colleagues (2011) designed siRNA that targeted hepatitis B virus genes, but also triggered pattern recognition receptors to engage the innate immune system. This approach significantly slowed viral replication *in vivo* (Han *et al.* 2011). Therefore, RNAi can be used to either suppress or amplify immune system activation.

RNAi approaches can also be used to amplify vaccine response. In one example, siRNA silencing of suppressor of cytokine signalling 1 in dendritic cells before exposure to HIV proteins led to increased antibody production as well as expansion of antigen-specific T-cell populations in mice (Song *et al.* 2006). *In vivo* studies using IL-10-silencing siRNA, co-delivered with plasmid DNA encoding for the hepatitis-B surface antigen, exhibited efficient 'switching' towards a T helper 1 immune response and increased the cytotoxic T-cell response (Singh *et al.* 2008).

Whether targeting host, pathogen, or vector, the range of RNAi targets to combat infectious disease is broad and continually expanding. RNAi therapeutics, then, have the potential to improve animal welfare and the sustainability of animal agriculture in both extensive and intensive management systems. Still, there are several technical and market challenges that must be addressed before this potential can be realised.

Barriers to adoption

Despite the exciting laboratory results there are still many hurdles to overcome before RNAi therapeutics can be adopted in the field. Some are based on public perception, particularly with respect to genetically engineered animals that express RNAi molecules. Most, however, are due to the incomplete scientific understanding about how to deliver the technology. The two main methods for adoption – genome engineering and delivery of exogenous RNAi molecules – face separate hurdles.

Technology

Genome engineering. The ability to directly manipulate the DNA of agricultural animals has progressed significantly in the past several years due to the latest targeted gene editing tools including zinc fingers nucleases, TALEN and CRISPR, which enable site-specific insertion or deletion of genes and RNAi expression cassettes (Gaj *et al.* 2013; Sander and Joung 2014). Nevertheless, adoption of this technology to engineer animals expressing RNAi molecules is still in the early stages. Many barriers are still to be overcome in both the genome manipulation technology and the understanding of the RNAi mechanism (Gaj *et al.* 2013; Sander and Joung 2014).

Site-specific genome editing based on CRISPR and TALEN still requires extensive knowledge about the genome of the animal. Although most agricultural species genomes have been sequenced, identification of the role of the genes and non-coding sequences is challenging. RNAi expression cassettes are commonly inserted in the non-coding regions of the genome; however, recent evidence suggests that much of that DNA and transcribed RNA is actually important for cell function (Guttman *et al.* 2009; Wapinski and Chang 2011; ENCODE Project Consortium 2012; Picardi *et al.* 2014). Therefore, careful placement of the transgene is required. Insertion into non-specific sites is also a drawback, highlighting the need to

engineer improved specificity (Gabriel *et al.* 2011; Mali *et al.* 2013).

Initial development of genetically engineered mice expressing shRNA resulted in severe medical problems due to overexpression of the RNAi molecule, which led to dysfunction in the processing of the required endogenous miRNA (Grimm *et al.* 2006). This has also been observed in transgenic pigs expressing anti-classical swine fever virus shRNA (Dai *et al.* 2014). This can be overcome by decreasing the strength of the promoter, incorporating the shRNA into an endogenous miRNA intron or using tissue-specific or transducible promoters (Furth *et al.* 1994; Dickins *et al.* 2007).

Several siRNA have also been shown to activate an immune response through toll-like receptor activation (Sledz *et al.* 2003; Judge *et al.* 2005; Stewart *et al.* 2011). Some toll-like receptor-activating sequences have been identified; however, rigorous testing of the shRNA sequence is required before use in transgenes. Another drawback of transgenic RNAi animals is the off target effects or suppression of genes that are not the primary target (Jackson *et al.* 2003; Persengiev *et al.* 2004). The majority of the off-target gene silencing of siRNA is due to partial sequence homology, especially within the 3' untranslated region, of mRNA other than the intended target mRNA (Saxena *et al.* 2003; Scacheri *et al.* 2004). Both of these challenges also apply to synthetic RNAi delivery.

siRNA delivery. Significant research is ongoing in the use of exogenously delivered RNAi for combating a plethora of human diseases. However, due to the current cost of synthetic RNAi molecules, particularly siRNA, very little research into *in vivo* siRNA therapeutics for agricultural animals has been performed. The cost of oligonucleotides is expected to continue dropping, and the potential for large-scale synthesis for commercially viable siRNA would likely overcome current cost issues.

Beyond this concern, the same barriers that exist for human therapeutics will need to be overcome for animal agricultural applications. These challenges include degradation by serum nucleases, the inability to cross negatively charged lipid membranes, and clearance by the kidney.

To overcome these significant hurdles, potential siRNA delivery vehicles for human therapeutic applications are being extensively investigated. Amongst these are polymer-based nanoparticles, lipid-based nanoparticles, aggregation of the siRNA itself, and viral and bacterial carriers of RNAi molecules (Lee *et al.* 2013; Dong *et al.* 2014; Hong and Nam 2014). Many examples have shown sufficient promise that they are reaching clinical trials; however, these nanoparticles also have significant drawbacks (Rettig and Behlke 2012). These include targeting the appropriate tissue, clearance through the kidney, serum opsonisation and efficient release of the siRNA once in the cell. Nevertheless, the first phase III clinical trial of a systemic siRNA therapy, for the treatment of transthyretin-mediated amyloidosis, is now underway (<https://clinicaltrials.gov/ct2/show/NCT01960348>, verified 5 May 2016). This milestone provides hope that the technical challenges for RNAi therapies are surmountable and human clinical applications may finally be on the horizon.

Additional difficulties will be faced for agricultural applications. One challenge is that many of the nanoparticles under investigation require intravenous injection, which may not

be feasible for large-scale agricultural applications, such as the poultry industry, or some extensive management systems in the beef industry. In such scenarios, nasal delivery via nebulisation or delivery to the gut via feed is likely to be the preferred method. Minimal research into these delivery methods is currently ongoing, with some promising results for local effects in the lung or gastrointestinal tissue, but little evidence of systemic efficacy via these routes (Rettig and Behlke 2012). Investigation into subcutaneous injection has shown some promise with simple conjugates such as the GalNAc-siRNA (Tesz *et al.* 2011). However, nanoparticle selection for livestock will have to include consideration of not only target animal safety but also safety of the end product, which may limit the options available for animal agriculture.

Consumer and regulatory barriers

Consumer and regulatory barriers around transgenic expression of RNAi molecules. From a commercialisation standpoint, there are numerous market barriers preventing the use of RNAi-based technologies in animal agriculture. Transgenic expression of RNAi-inducing molecules in agricultural species would face the high regulatory hurdles that all transgenic animals face, which few have passed. In the United States, transgenic animals are regulated by the Center for Veterinary Medicine of the Food and Drug Administration. Under this paradigm, transgenes are treated as drugs and require investigational new drug applications when being developed for commercial use. The review process consists of seven phases (FDA 2015). The first five phases cover transgene characterisation, including assessing the makeup of the transgene, its site of integration in the animal, expression in the animal, its transmission and durability across generations, and overall phenotypic assessment of the animal. Phenotypic assessment requires submission of clinical chemistry, haematology, histopathology, and post-mortem results for multiple transgenic animals from multiple generations to determine if there are any off-target effects.

After the genotypic and phenotypic characterisation is the food/feed safety and environmental safety assessments. The food/feed safety portion involves proving that any food product for human consumption or feed product for consumption by other animals that is derived from the transgenic animal is as safe as the conventional food/feed. This portion of the assessment can be particularly onerous, as there is often little existing data on the food/feed safety of conventionally produced items, and there is little clarity on the types of studies and desired endpoints considered acceptable to meet this requirement. The environmental safety assessment is used to evaluate the risk posed if the transgenic animal were to escape into the wild. Depending on the species, this part of the assessment can be very straightforward (for most domestic livestock species for which there are no wild populations), but this assessment can be very complicated for aquatic or avian species that have wild counterparts, like farmed trout or ducks.

After the environmental assessment, the application reaches the final phase, which is claims validation. If the purpose of the transgene is to confer disease resistance, for example, data must be provided to support that claim. Claims are often framed to include a set of conditions under which the claim is made; for

example, a claim may state that transgenic animals, when maintained under appropriate husbandry conditions including adequate nutrition, appropriate vaccinations, and regular veterinary care, are resistant to the target disease.

To date, only two transgenic animal lines have made it through the United States regulatory process. The first animals approved were goats that produce recombinant therapeutic proteins in their milk, and are not approved for human consumption (Kling 2009). In November 2015, the AquAdvantage salmon became the first transgenic animal to be approved for human consumption (Ledford 2015). However, this step was approximately 20 years in the making, and all regulatory assessments, the draft Environmental Assessment, a preliminary Finding of No Significant Impact, and the public comment period were all completed by early 2013, more than 2 years before the final approval by the Food and Drug Administration. Currently, the regulatory burden for transgenic animals is extremely high, and for transgenic RNAi expression systems to be applied in agricultural animals, the regulatory process will likely need to become more streamlined.

In Australia, genetically engineered foods are regulated under the Australia New Zealand Food Standards Code (Australian Government 2014). The standard is an enforceable regulation with two provisions – mandatory pre-market approval (including a food safety assessment) and mandatory labelling requirements. Each food safety assessment is carried out on a case by case basis where the genetically engineered food is compared with a similar, commonly eaten conventional form. Assessment involves a molecular, toxicological, nutritional and compositional characterisation. If the genetic modification causes an unexpected effect in the food, such as increasing its allergenicity or toxicity, or decreases nutritional value, it will not be approved. At this point in time, animal feeding studies are not required, as it is believed they are unlikely to contribute any further useful information. So far, no safety concerns with any genetically engineered foods have been identified, similar to other national regulators. However, only plant-based products have been approved and tested, and regulations may change when Australia begins to consider genetically engineered animal products.

Consumer acceptance of RNAi transgenes in animal products is difficult to predict. There is certainly a small and vocal portion of the population that will not purchase these products and will actively campaign against them. However, most consumers in several countries buy and consume transgenic plant material; the question is whether they will view transgenic animals differently following regulatory approval. Consumers may be more or less likely to accept products produced from transgenic animals depending on the application, with a particular focus on whether from the point of view of the consumer it is the producer, the animal, or the consumer that is receiving the most benefit. Although animals with improved growth and efficiency alone are less likely to appeal to the general public, animals that are resistant to zoonotic diseases or have improved welfare are likely to be viewed more favourably (Heiman and Zilberman 2011).

Regulatory issues around exogenous delivery of RNAi molecules. The regulatory framework for exogenously delivered siRNA in animals has yet to be established, and we

Table 1. Key applications of pharmaceutical technologies used in animal agriculture – advantages and disadvantages

Technology	Key applications	Relative advantages	Disadvantages
Small molecule pharmaceuticals	Antibiotics	Well established efficacy and pharmacology	Broad impact – for example, disruption of gastrointestinal microbiota. Development of resistance. Withdrawal times
	Metabolic modifiers	Well established efficacy and pharmacology	Potential off-target effects, withdrawal times
Biologics	Vaccination	Low cost, established regulatory framework and consumer acceptance	For some viruses, strains evolve so rapidly that vaccine production cannot keep pace
Genetic engineering	Introduction of novel traits	For introducing a gene, the only option. For eliminating a gene product, this approach allows for lifetime suppression without continuous treatment	Very high upfront cost, regulatory and market constraints. Additionally, some gene deletions are lethal or impair health
	Prevention of infectious disease	Once the trait is established, there are no ongoing costs, and can be easily utilised in extensive production systems	In addition to the above issues, there is potential for pathogens to evolve mechanisms to evade the transgenic shRNA
Exogenous RNAi	Response to emerging pathogens (especially viral)	The time required to design anti-viral siRNA is short compared with vaccines, and can potentially be used after exposure	Depending on the disease, delivery to the appropriate anatomical location can be a challenge. Off-target effects with high doses
	Physiological modifiers	Lower risk of off-target effects than small molecules	Relatively short period of effectiveness (~3–20 days) following a single administration. Potential immunogenicity of siRNA formulations. Safety and clearance of any delivery agents

are not aware of any published work on consumer perceptions on the use of exogenous RNAi strategies. However, given that several genetically engineered crop varieties employing RNAi machinery have now been approved, the public health issues to consider around potential siRNA residues have been detailed (Petrick *et al.* 2013). One difference from transgenic approaches to RNAi delivery is that delivery of exogenous siRNA is unlikely to require evaluation of multiple generations of animals, as the time scale for siRNA effects are more like those of traditional pharmaceuticals. In fact, it is likely that any nanoparticles or chemical modifications necessary to deliver siRNA to target organs will raise greater concern than the siRNA itself, and nanoparticle toxicology as well as degradation and clearance kinetics will need to be determined to assess whether withdrawal times might be necessary following such treatments. These details will depend heavily on the class of nanoparticles that emerges as the best fit for livestock applications of RNAi.

Conclusions

Efforts to improve the health and productivity of livestock through genetics, nutrition, and management improvements over the past 100 years have resulted in dramatic progress in the quality and sustainability of animal-derived products. Nevertheless, continuing challenges such as shifting market demands, climate change, problematic transitions between physiological states, and emerging infectious diseases require continued efforts to meet the growing demand for these products without additional inputs. RNAi offers exciting novel approaches to these challenges (Table 1). The ability of dsRNA molecules to precisely induce the silencing of a single mRNA target, coupled with technologies to deliver the dsRNA to target cells, offers hope of developing RNAi therapeutics with few

side effects. Genetic engineering can be used to hard-wire the RNAi process, or exogenous RNAi approaches can be used, sidestepping consumer resistance to genetic engineering in food animals. However, numerous hurdles remain for RNAi in animal agriculture, including the lack of efficient techniques to deliver exogenous siRNA to certain organs, the current cost of both dsRNA and delivery nanoparticles, regulatory uncertainty, and questions about how consumers will perceive this technology.

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