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Comparative genomic analysis of non-coding sequences and the application of RNA interference tools for bovine functional genomics

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Abstract. Non-coding (nc) RNAs are important regulators of developmental genes, and essential for the modification of cellular DNA and chromatin through a process known as RNA interference (RNAi). The mediators of RNAi can be in the form of short double stranded (ds) RNAs, micro (mi) RNAs or small interfering (si) RNAs. miRNAs are involved in a translation repression pathway that inhibits protein translation in mRNA targets. Comparative genomic screens have revealed conserved regulatory non-coding sequences, which assist to predict the function of endogenous miRNAs. Only a few comparative studies include bovine genomic sequence, and RNAi has yet to be applied in bovine genome functional screens. siRNAs target homologous mRNAs for degradation, and thereby, silence specific genes. The use of synthetic siRNAs facilitates the elucidation of gene pathways by specific gene knockdown. A survey of the literature identifies a small number of reports using RNAi to examine immune pathways in bovine cell lines; however, they do not target genes involved in specific production traits. Applications of RNAi to elucidate bovine immune pathways for relevant bacterial and parasite diseases are yet to be reported. The inhibition of viral replication using RNAi has been demonstrated with bovine RNA viruses such as pestivirus and foot and mouth disease virus signifying the potential of RNAi as an antiviral therapeutic. RNAi approaches combined with genome data for protozoan parasites, insects and nematodes, will expedite the identification of novel targets for the treatment and prevention of economically important parasitic infections. This review will examine the approaches used in mammalian RNAi research, the current status of its applications to livestock systems and will discuss potential applications in beef cattle programs.

Additional keywords: bovine genome, non-coding RNAs, RNA interference.

Introduction

The traditional view of biology describes the flow of genetic information from DNA through RNA to protein where genes generally code for proteins that fulfil structural, catalytic and regulatory functions in all living cells. However, due to the large extent of non-protein-coding RNA (ncRNA) transcription in higher eukaryotes (97–98% of the human genome) and the range of genetic phenomena that are RNA-directed, this traditional view of the structure of genetic regulatory systems in animals and plants is now being challenged (Jasinska and Krzyzosiak 2004; Mattick 2003; Morey and Avner 2004). ncRNA dominates the genomic output of higher organisms, and has been shown to control chromosome architecture, messenger RNA (mRNA) turnover and the developmental timing of protein expression, and it may also regulate transcription and alternative splicing (Mattick 2003; Morey and Avner 2004; Munroe 2004).

Several classes of ncRNA molecules have been identified and have been shown to be involved in crucial functional roles in eukaryotic cells. For instance, a large class of small microRNAs (miRNAs), 21–25 nucleotides (nt) in length, are involved in 2 separate but overlapping pathways that regulate gene expression. The first is a degradative mechanism that destroys RNA corresponding to duplicated or foreign gene sequences, in a process mediated by small interfering RNAs (siRNAs) (Caplen *et al*. 2001). This mechanism was termed RNA interference (RNAi) and has been widely exploited by biologists as a gene knockdown tool since it was first demonstrated that effective gene silencing could be achieved using double stranded RNA (dsRNA) in the nematode *Caenorhabditis elegans* (Fire *et al*. 1998). The second regulatory pathway, called translation repression, was also first identified in the nematode and utilises endogenous miRNAs to inhibit translation of their target mRNAs (Pasquinelli *et al*. 2000). It is now evident that siRNAs

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interact with DNA to induce heterochromatin formation and DNA methylation extending their role in the regulation of gene expression to include transcriptional gene silencing (Volpe *et al*. 2002). The abundance of ncRNA transcripts detected in genomes indicates that there are RNA regulatory pathways yet to be uncovered (Cerutti 2003; Frazer *et al*. 2004). Thus, ncRNA molecules have a fundamental role both in regulating gene expression and in protecting the genome from mobile genetic elements. The exploitation of siRNA pathways is providing biologists with powerful new functional genomic tools (Kawasaki and Taira 2004; Lippman and Martienssen 2004; Silva *et al*. 2004).

The major goal of livestock genomics is to map and identify genes involved with economically important traits and disease susceptibility and resistance. Mammalian comparative genomic analyses of conserved ncRNAs are uncovering potential regulatory sequences. Very few RNAi approaches have been applied to study ncRNA transcription or the activity of specific bovine genes (Goodwin *et al*. 2004; Paradis *et al*. 2005). Bovine coding DNA microarrays have been used to identify candidate genes associated with particular phenotypic traits and if combined with RNAi, and such studies could determine the downstream effects of specific gene knockdown on the expression of other genes (Lewin 2003). Methods such as microarray tiling could also be applied to identify up-regulated RNA transcripts involved in specific bovine gene pathways (Johnson *et al*. 2005). Selective breeding in the beef industry now also relies on DNA testing to detect variation (or single nucleotide polymorphisms, SNPs) in particular genes. Thus, candidate genes and gene regulatory pathways can be validated in RNAi knockdown experiments to confirm their suitability as new targets for genetic testing and phenotype selection.

Disease pathogenesis studies in mammalian species have concentrated on elucidating receptor signalling to improve the understanding of the immune pathways associated with host susceptibility and resistance (Werling and Jungi 2003; White *et al*. 2003*a*). RNAi tools have been exploited in *in vitro* gene knockdown experiments to determine the molecular basis of these pathways (Oshiumi *et al*. 2003; Uehara *et al*. 2005). Although this research has applications in bovine immunology, the use of RNAi to study differential cattle breed susceptibility to bacterial or parasite infection has yet to be undertaken. RNAi is an effective anti-RNA-virus knockdown method in human and bovine cells (Chen *et al*. 2004; Gitlin *et al*. 2002). Additionally, RNAi has been used as a tool to determine protozoal, tick, nematode and insect gene function (Aljamali *et al*. 2003; Boutros *et al*. 2004; Kamath and Ahringer 2003; McRobert and McConkey 2002). Bioinformatics and RNAi tools, combined with available genome sequence data (*Plasmodium* and *Theileria* protozoan species, *Drosophila* fruitfly, *C. elegans* nematode), will enable the identification of novel gene targets to develop new interventions to control important agricultural parasites such as ticks and worms (Adams *et al*. 2000; Chalfie 1998; Gardner *et al*. 2002; Knox 2004). RNAi also has the potential to elucidate both host and parasite gene pathways thereby improving our current understanding of the interaction between host and parasite.

This review will describe: (i) the current understanding of mammalian RNAi pathways and a summary of RNAi experimental approaches; (ii) the processes used for the identification of ncRNAs and the application of RNAi tools in mammalian functional genomic studies; (iii) the analysis of mammalian disease pathogenicity and host immunity including: anti-viral RNAi, bacterial and parasite host pathogenicity, and the application of RNAi to target infectious parasitic organisms; and (iv) the experimental methods currently used for the effective delivery of RNAi to mammalian systems.

Within the last 3 sections outlined above, this review will examine the relevant mammalian RNAi research approaches and discuss how these can be further exploited for the benefit of beef cattle research programs.

Molecular basis of RNAi

Background of RNAi

RNAi is an evolutionary ancient method of genome defence in many organisms. It is a way to protect the genome against invasion by viruses, mobile genetic elements such as transposable elements and repetitive genes, which produce aberrant RNA or dsRNA in the host cell when they become active (Zamore 2002). The initial observations of a gene silencing mechanism were first made in plants in the 1980s, when attempts to deepen the violet hue of petunias led to the appearance of white flowers (van der Krol *et al*. 1990). Table 1 provides a description of RNA molecules and terms used to describe silencing pathways discussed here. It was not until 1998 that the term RNAi was used to describe this phenomenon in the nematode worm *C. elegans*, when it was demonstrated that dsRNA was able to direct the degradation of mRNA in a sequence specific manner (Fire *et al*. 1998). This mechanism was then linked to the phenomena previously described in plants and fungi, initially thought to be mediated by sense or anti-sense mechanisms (Romano and Macino 1992; van der Krol *et al*. 1990). Similar dsRNA silencing experiments were simultaneously demonstrated in trypanosome parasites and flies (Kennerdell and Carthew 1998; Ngo *et al*. 1998). Crucial to this process is the RNA-directed nuclease, Dicer, which recognises and digests dsRNA into short dsRNAs (21–25 nt), and which was initially discovered in *Drosophila* (Hammond *et al*. 2000). Dicer was thus shown to be the mediator of dsRNA cleavage and mammalian homologues were subsequently described (Nicholson and Nicholson 2002).

Mammalian RNAi pathways

The mammalian RNAi pathway differs from that of *C. elegans*, plants and fungi, which employ RNA dependent RNA polymerases to amplify the RNAi response and which can also elicit specific RNAi effects following the introduction of long dsRNAs (Fire *et al*. 1998; Schwarz *et al*. 2002; Stein *et al*. 2003). In contrast, the silencing of specific genes by RNAi has proven difficult in mammalian systems. This is because of the dsRNA induction of the cellular interferon pathway, which leads to non-specific inhibition of protein synthesis and the degradation of RNA by RNase L (Elbashir *et al*. 2001*a*). It was subsequently demonstrated that short (21–25 nt) siRNAs can induce gene silencing without non-specific inhibition of gene expression in cultured mammalian cells (Elbashir *et al*. 2001*a*). In the RNAi pathway, exogenous or endogenous siRNAs (produced by Dicer) are incorporated into a multi-protein RNA-induced-silencing complex (RISC) that unwinds the duplex siRNA, leaving the antisense strand to guide the RISC to its homologous target mRNA for cleavage (Fig. 1) (Elbashir *et al*. 2001*a*; Schwarz *et al*. 2002).

Small endogenous miRNAs were also initially discovered in *C. elegans* where the small temporal (st) RNAs were found to be essential regulators of the timing of development of *C. elegans* (Pasquinelli *et al*. 2000). Potential homologues of several miRNA genes were identified in the *Drosophila* and human genomes and their evolutionary conservation implied that this class of ncRNAs has regulatory functions that are broader than temporal regulation (Lau *et al*. 2001). The miRNAs originate from long precursors (pri-miRNA) that, in animals, are cleaved by the Drosha nuclease in the nucleus to give pre-miRNAs that are about 70 nucleotides long, with a characteristic hairpin structure (Lee *et al*. 2003) (Fig. 1). Following export to the cytoplasm, these long precursors are cleaved by Dicer and the miRNA is incorporated into RISC which either leads to translation repression or triggers mRNA degradation via the RNAi pathway (Fig. 1) (Doench and Sharp 2004). The number of miRNAs that bind to the target mRNA is also thought to determine the degree of translational repression (John *et al*. 2004). Comparative genome analyses have described intronic and exonic miRNAs in human and mouse genomes (Rodriguez *et al*. 2004; Weber 2005). Weber (2005) has suggested that the criteria for defining miRNAs include the evidence of a cluster of miRNAs, in the same orientation, and not separated by a transcription unit or a miRNA in the opposite orientation.

In summary, the RNAi pathway induced by the introduction of dsRNAs was described before endogenous miRNA/siRNAs were discovered. The proteins and associated functions involved in RNA silencing pathways continue to be elucidated and defined across both plant and animal taxa, which will in turn improve the understanding of these pathways. The current putative processes involved in both miRNA and siRNA endogenous post-transcriptional silencing pathways as well as a putative pathway for nuclear or transcriptional silencing (see nuclear-RISC) are summarised in Figure 1.

Gene knockdown RNAi experiments

While research into the identification and function of conserved regulatory miRNAs with comparative genomics approaches will continue, gene silencing experiments can be

Table 1. Description of RNA molecules and terms used in this review

RNA molecules	Enzymes and processes
siRNA: small interfering RNAs. dsRNA fragments 21–25 nucleotides in length. siRNAs are products of Dicer cleavage and are the mediators of mRNA degradation in RNAi. siRNAs can result from endogenous pathways or can be synthesised and introduced in gene knockdown studies.	Dicer: belongs to the RNase III family of dsRNA specific nucleases. Dicer is responsible for the processing of long dsRNA, shRNAs and pre-miRNAs into siRNAs and miRNAs.
miRNA: micro RNAs. miRNAs are products of Dicer cleavage of nuclear exported hairpin structure pre-miRNAs. miRNAs are the mediators of translational repression and can also be synthetically introduced.	Drosha: nuclear RNase III enzyme that processes primary miRNAs to produce precursor miRNAs which are exported into the cytoplasm.
stRNAs: small temporal RNAs are miRNAs that regulate the timing of gene expression during development.	RISC: RNA-induced silencing complex. A nuclease complex composed of proteins thought to mediate both mRNA degradation and translational repression.
Pri-miRNA: primary miRNAs are transcribed from DNA in the nucleus and are digested by Drosha to produce pre-miRNAs.	mRNA: degradation/RNA interference pathway. One of the siRNA strands is incorporated into RISC and this strand guides RISC to perfectly complementary mRNAs and cleaves them resulting in their degradation (post-transcriptional gene silencing).
Pre-miRNA: precursor miRNA have a shRNA structure of about 70 nucleotides, are exported from the nucleus into the cytoplasm and are digested by Dicer to produce mature miRNAs or siRNAs.	Translational repression: the RISC incorporating a mature miRNA strand with imperfect complementarity binds to several sites in 3′ untranslated regions of coding genes which leads to a decrease in protein without a decrease in mRNA levels. The number of miRNA-RISC complexes bound to target mRNAs may determine the level of translational repression.
shRNA: short hairpin RNA. Either synthetically produced or expressed by vectors or pre-miRNAs produced by endogenous pathways which are recognised and digested by Dicer to produce siRNAs.	NRISC: nuclear-RISC — a putative RISC that via siRNA and/or miRNAs can interact with DNA to induce heterochromatin formation and DNA methylation via a transcriptional gene-silencing pathway.
ncRNA: non-coding RNA. Transcriptional products that do not code for proteins. miRNAs are a class of ncRNAs.	

undertaken by creating small inhibitory RNA molecules to suppress the expression of specific genes. Inhibitory siRNAs/miRNAs can be introduced into cells either as short duplex RNA oligonucleotides or as the expressed products originating from plasmid or viral vectors. Short oligonucleotides in the form of siRNAs or short hairpin RNAs (shRNAs) can be produced synthetically or transcribed from PCR products and/or digested by recombinant Dicer and are introduced into cells using lipid based delivery transfection reagents (Dykxhoorn *et al*. 2003; Elbashir *et al*. 2001*a*). shRNAs are recognised by cellular Dicer and processed into siRNAs. Plasmids or viral vectors engineered to express siRNAs or shRNAs transcribed from specific RNA polymerase promoters are introduced into the cells using standard transfection and transduction processes (Brummelkamp *et al*. 2002; Xia *et al*. 2002). Vectors expressing a shRNA consist of a sense sequence about 21 bases long followed by a 6–8 base non-complementary (antisense) sequence (Dykxhoorn *et al*. 2003; Rice *et al*. 2005). In most instances the above approaches are used to investigate gene function. Considerable research effort towards the use of RNAi as a therapeutic treatment for viral infection, cancer and inherited genetic disorders is also underway (see review by Gong *et al*. 2005). The delivery of RNAi is further discussed in the last section of this review.

Previous approaches for gene-specific inhibition relied upon difficult and expensive procedures such as homologous recombination, or targeted mutagenesis with limited applicability across different species (Nagy and Rossant 1996). In comparison with these traditional approaches as well as anti-sense and ribozyme methods, RNAi technology has been shown to be more specific, more sensitive, easier to

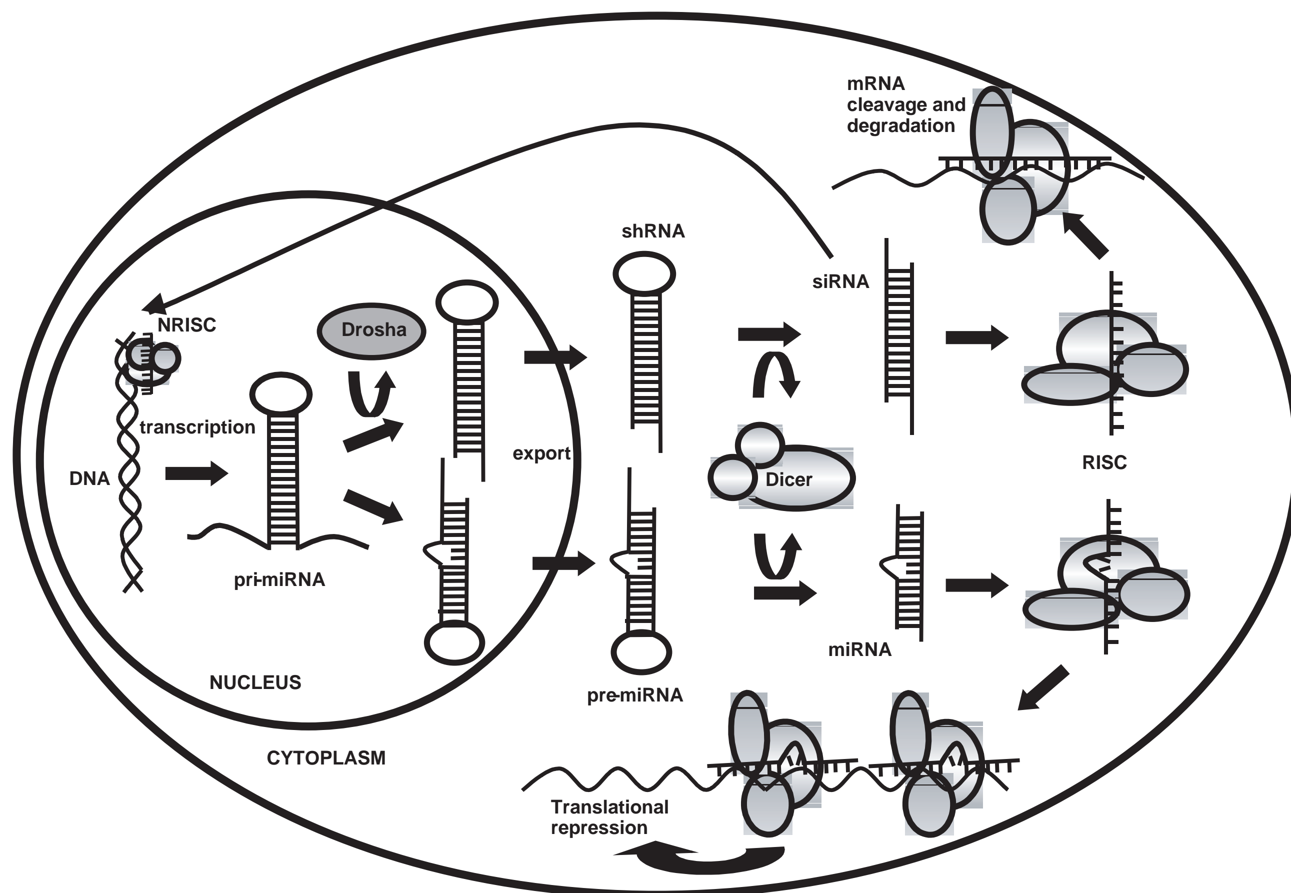


Figure 1. Model of current mammalian endogenous RNAi pathways. MicroRNAs (miRNA) are produced from long precursors (pri-miRNA) which are cleaved by Drosha to generate about 70 nucleotide-long pre-miRNAs with a characteristic hairpin structure. Following export into the cytoplasm, the long precursors are cleaved by Dicer and the short RNAs (21–25 nt) are incorporated into the RNA-induced-silencing-complex (RISC), which unwinds the duplex RNA. For mRNA degradation, the antisense strand guides the RISC to its homologous target mRNA for cleavage and degradation of the mRNA. For translational repression, the mature miRNA-RISC complex binds to the 3′ regions with incomplete complimentarity (represented by the 'bump' on the miRNAs in the diagram) to inhibit translation elongation of relevant mRNAs. The number of miRNAs that bind to the target mRNA determine the degree of translational inhibition. siRNA and/or miRNAs can also interact with DNA via a putative nuclear-RISC (NRISC) to induce heterochromatin formation and DNA methylation via a transcriptional gene silencing pathway. See also a detailed list of terms in Table 1.

apply and capable of eliciting a stronger inhibitory effect (Aoki *et al*. 2003; Brantl 2002; Miyagishi *et al*. 2003). In addition, siRNA libraries and siRNA/shRNA expression libraries have been generated for whole genome screens in fungi, *Drosophila* and mammalian cell lines which have identified the activity of novel genes in functional genetic pathways (Boutros *et al*. 2004; Miyagishi *et al*. 2004; Sachse *et al*. 2005). Thus, the ability to manipulate RNAi as a high throughput functional genomic method in a number of different eukaryotic species further validates the usefulness of this distinctive experimental approach.

Non-coding RNAs, RNAi genome screens and gene knockdown applications

Background — bovine genome studies

Traditionally, linkage maps based on polymorphic markers covering all bovine chromosomes laid the foundations for mapping loci affecting these quantitative trait loci (QTL) (see review by Lewin 2003). Expressed sequence tags (EST) and genome sequence data has enabled the specific linkage of SNPs to these QTLs and the identification of causative genes and mutations (Casas *et al*. 2005; Krininger *et al*. 2003). A constructed second generation human:bovine radiation hybrid map will contribute towards the analysis of mammalian chromosome evolution whereas the bovine genome sequence will provide the basis for high resolution genome comparisons (Everts-van der Wind *et al*. 2004; Murphy *et al*. 2004). It is now acknowledged that more genomic sequence is transcribed than accounted for by predicted exons and over 200 000 conserved non-coding sequences have been discovered in human:mouse genome comparisons (Frazer *et al*. 2004; Johnson *et al*. 2005). Similar screens of livestock genomes are yet to be reported.

Genomic non-coding regions

Conserved non-coding regulatory elements can be identified in sequence alignments of multi-species comparative maps (Dubchak *et al*. 2000; Murphy *et al*. 2003). Studies including the bovine genome sequence have contributed to defining potential regulatory elements within 5′ and 3′ untranslated regions in human, bovine, porcine and rodent orthologueous genes (Larizza *et al*. 2002; Williams *et al*. 2003). Microarray tiling methods have recently been developed for genome wide screens to identify RNA modifications in human and fly genomes (Hiley *et al*. 2005; Mockler and Ecker 2005). A newly released mammalian ncRNA database reportedly contains over 800 unique experimentally studied ncRNAs with limited bovine specific entries at this stage (Pang *et al*. 2005). Identifying conserved non-coding regions in livestock genomes could identify novel regulatory elements associated with particular traits. This will lead to an improved understanding of gene expression and genetic pathways of different animal phenotypes and may establish the foundation for the development of predictive tests for relevant traits.

Mammalian miRNAs

To date, miRNA identification in mammals has concentrated on human:mouse genome comparisons (Lagos-Quintana *et al*. 2003; Weber 2005). The specific function of most miRNAs is unknown and currently there are about 220 known mammalian miRNAs (John *et al*. 2004). Microarray and bioinformatics tools have been developed to characterise tissue specific expression and the functional roles of mammalian miRNAs, which are co-transcribed with a particular coding gene (Rodriguez *et al*. 2004; Sun *et al*. 2004). For instance, Barad *et al*. (2004) developed a microarray that enabled the quantification of matching miRNAs expression profiles in labelled RNA originating from human thymus, testes and placental tissues. In addition, sequence analysis has revealed that an RNAi mechanism may be responsible for gene regulation of the Callipyge trait in sheep (Bidwell *et al*. 2004). Thus, by undertaking miRNA genome screens and by analysing untranslated regions of specific genes/alleles in bovine tissues, functional miRNAs associated with the gene regulation of particular phenotypes can be identified. As described for ncRNAs, where applicable, gene tests based on specific miRNAs may be developed to predict the regulation of expressed favourable traits.

Functional genomics — RNAi genome screens

The functional analysis of predicted genes to date has been undertaken using microarrays to identify the transcriptional profiles associated with bovine production, nutrition, reproduction, lactation and immunity (Byrne *et al*. 2005; Ishiwata *et al*. 2003; Suchyta *et al*. 2003; Tao *et al*. 2004; Ushizawa *et al*. 2005; Yao *et al*. 2004). By combining microarrays with high throughput RNAi library screens, the effect of specific gene knockdown can provide a quantitative analysis of the contribution of potentially every gene to a particular process involved in controlling development and disease. Figure 2 summarises the main approaches used for RNAi genome screens in other mammalian systems. For example, a high throughput RNAi screen of 19470 dsRNAs in cultured cells characterised 91% of the *Drosophila* genes involved in cell growth and viability (Boutros *et al*. 2004). Genome-wide siRNA or shRNA screens targeting about 15000 mouse/human genes identified new aspects of cell division and proteosome function (Kittler *et al*. 2004; Paddison *et al*. 2004*b*). These functional analyses by RNAi revealed previously unknown and evolutionarily conserved gene functions and similar applications to bovine or other livestock genomes will be equally beneficial in describing particular production traits or immune pathways.

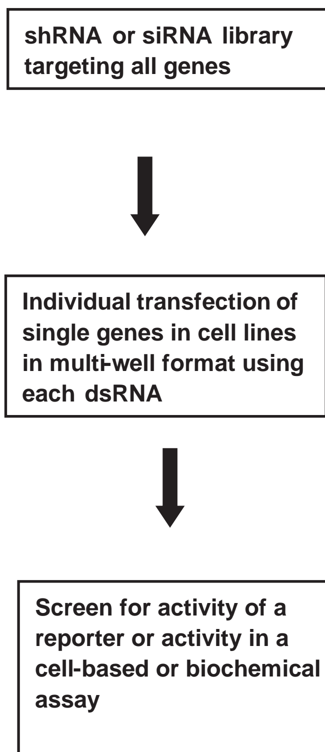
Functional genomics — RNAi gene knockdown

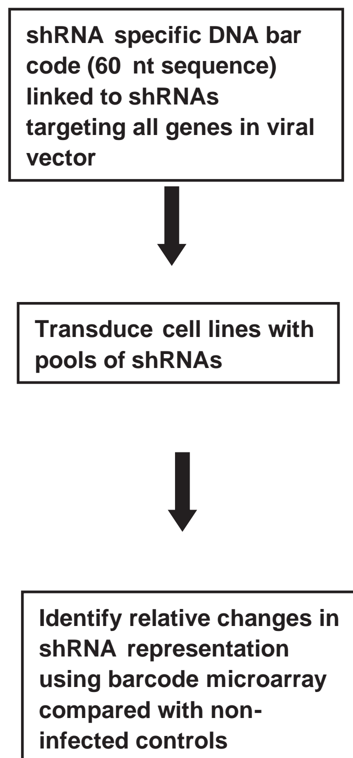
The standard technique for studying gene function is to disrupt a gene by homologous recombination; however, this

is difficult and expensive, especially in livestock species where embryonic stem cells have not been isolated (Silva *et al*. 2004). As described earlier, RNAi is relatively simple to apply to a wide variety of species in cell culture systems as a gene specific knockdown tool. Examples of specific RNAi experiments in bovine cells are summarised in Table 2. Most of the cell culture examples described are not 'bovine specific' RNAi studies *per se*, but are model systems investigating the associated functions of a particular gene involved in immune function, inflammation or mitosis. Indeed, a number of cell lines used in bovine expression studies could be further exploited in RNAi knockdown experiments, for example a bovine trophoblast cell line has been used for gene expression profiling of trophoblasts (Ushizawa *et al*. 2004). RNAi knockdown experiments in ovine cells have studied vascular smooth muscle cell migration in response to growth factors thought to be relevant in disease processes (Leung *et al*. 2004). Following the success of RNAi in human and mouse oocytes, applications of RNAi in bovine oocytes provides progress towards understanding follicular growth and development as well as early embryogenesis, see Table 2 (Donnison and Pfeffer 2004; Paradis *et al*. 2005; Yao *et al*. 2004). Furthermore, RNAi has also been successfully applied to study nuclear trafficking pathways in porcine embryos and the functional analysis of genes in porcine granulosa cells (Cabot and Prather 2003; Hirano *et al*. 2004). An example of a successful *in vivo* RNAi study silencing endogenous genes has been demonstrated in rat muscle using plasmid delivered shRNAs (Kong *et al*. 2004).

Potential applications of gene knockdown

Although there are currently limited RNAi applications to livestock, recent studies have suggested the use of specific applications of RNAi to improve bovine reproduction and to diminish calf mortality and morbidity. Joerg *et al*. (2003) suggest that RNAi could disrupt endogenous spermatogenesis to allow successful germ cell transplantation in bulls. RNAi could assist in determining the mechanism of genome activation in early developing bovine embryos, the genes involved in embryo-maternal cross-talk, and could also be applied to reduce abnormal fetal–maternal interactions and fetal loss in nuclear transfer embryos (Meirelles *et al*. 2004; Pfister-Genskow *et al*. 2005;





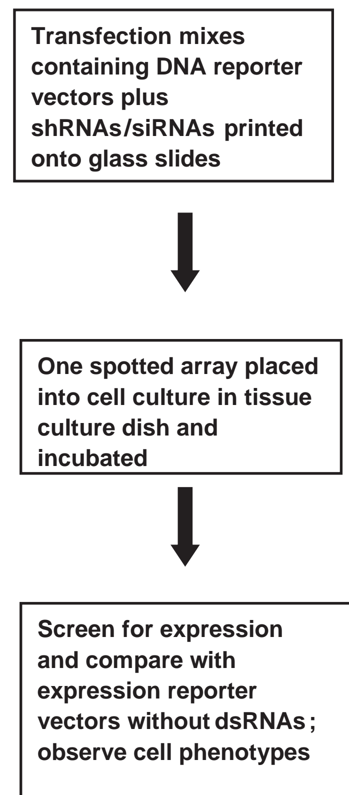


Figure 2. Summary of the current RNAi approaches used for genome functional screens. These approaches use large, genome wide siRNA or shRNA libraries to study gene expression in cell cultures for screening genes involved in particular processes or comparative phenotypes (Boutros *et al.* 2004; Paddison *et al.* 2004*b*; Silva *et al*. 2004).

Wolf *et al*. 2003). Calf mortality and morbidity can be affected by the failure of maternal immunoglobulin transfer thus RNAi may be useful towards managing the associated genetic risk factors involved (Clawson *et al*. 2004).

Most RNAi investigations in mammalian systems are targeting genes involved in cancer, autoimmune diseases, neuro-degenerative diseases and against viral replication (Gong *et al*. 2005; Tan and Yin 2004). Allele-specific silencing aims to suppress the disease gene without affecting the normal gene. The specificity of siRNAs makes this possible even for disease alleles that differ from the normal allele by only one nucleotide. Allele-specific silencing has been achieved for several neurodegenerative disease genes *in vitro* (Davidson and Paulson 2004; Miller *et al*. 2003). Therefore, RNAi technologies could be utilised to silence specific SNPs in bovine cells to further explore phenotype associated gene pathways.

Bovine pathogen interactions and RNAi in immune function

The approaches in the above section describing the identification of regulatory ncRNAs and miRNAs are also applicable in the analysis of mammalian immune pathways and disease pathogenesis. This section will further explore specific applications of RNAi to study host disease and immune susceptibility, as well as the application of RNAi as a gene function tool in the identification of novel anti-parasitic treatment candidates.

Background — host–pathogen interactions

Understanding the complex cross-talk between host and pathogen is essential to improve our understanding of infectious disease (Walduck *et al*. 2004). A number of comparative genome studies have identified SNPs or gene orthologueues associated with udder health and mastitis in cattle, bovine toll-like receptors (TLRs), bovine subspecies health and production differences (*Bos indicus v. Bos taurus*), and the susceptibility to parasites (da Mota *et al*. 2004; Goldammer *et al*. 2004; Hanotte *et al*. 2003; Park *et al*. 2004; Sonstegard *et al*. 2002; White *et al*. 2003*a*). *In silico* human and bovine genome comparative analysis enabled the development of diagnostic tuberculosis assays to discriminate vaccinated and infected individuals (Vordermeier *et al*. 1999). Comparative microarray gene expression studies have been undertaken to determine mycobacterium survival in bovine macrophages and to analyse major histocompatibility complex (MHC) haplotypes involved with susceptibility in a range of bovine diseases (Park *et al*. 2004; Tao *et al*. 2004; Weiss *et al*. 2004). These bioinformatics and microarray approaches studied host differences in response to pathogen invasion and the following section will describe how RNAi tools can be applied to further dissect the mechanisms of host immunity and disease pathogenesis.

Host immunity and RNAi

Table 2 summarises bovine cell culture studies that have used siRNAs to specifically study the gene functions involved in triggering immune pathways. These studies target specific genes in order to elucidate endothelial dysfunction relevant to conditions such as hypertension, diabetes mellitus and atherosclerosis and to unravel the molecular basis of tumour necrosis factor (TNF) signalling with a view to developing novel disease therapies (Goodwin *et al*. 2004; Mawji *et al*. 2004; Zhang *et al*. 2003). The bovine cell cultures in these studies were used to model conserved mammalian immune pathways.

Cell surface or endothelial TLRs are responsible for the recognition of antigens and the subsequent initiation of appropriate immune pathways (Werling and Jungi 2003). RNAi knockdown has been used to elucidate TLR pathways in human cell lines, for example siRNAs were used to target specific genes to confirm TLR signalling and the activation of host cells in response to the stimulation by bacterial lipopolysaccharides (Oshiumi *et al*. 2003; Sasai *et al*. 2005; Uehara *et al*. 2005) In addition, Toll pathway components

Table 2. Summary of RNAi applications undertaken in bovine cells

Cells	Target gene	Significance	Reference
Bovine oocytes	Cyclin B1	Oocyte development	Paradis *et al.* (2005)
Bovine Aortic Endothelial Cells (BAEC)	Argininosuccinate synthase	Nitric oxide production — immune function	Goodwin *et al.* (2004)
BAEC	Endothelin-1	Cellular heat shock response — immune function	Mawji *et al.* (2004)
BAEC	ASK1-interacting protein (AIP1)	Apoptosis signal regulating kinase 1 (ASK1) and TNFα — immune function	Zhang *et al.* (2003)
BAEC	Integrin-linked kinase	Regulator of the endothelial phenotype and vascular — immune function	Vouret-Craviari *et al.* (2004)
BAEC	Cyclooxygenase (COX) II	Cox II gene function in inflammation — immune function	Xiuzhu *et al.* (2003)
Bovine kidney cells (MDBK)	Nestin	Assembly of filaments in mitosis	Chou *et al.* (2003)
Bovine hamster kidney-21 cells; suckling mice	Foot and mouth disease virus — viral structural protein 1	Inhibition of virus replication	Chen *et al.* (2004)

have been silenced by the injection of dsRNA directly into *Drosophila* adult flies *in vivo* to study responses to fungal and Gram-positive bacterial infections (Goto *et al*. 2003). Microarray expression analysis combined with specific RNAi knockdown could assist to delineate the TLR pathways and other immune pathways associated with the differential *Bos indicus* and *Bos taurus* responses to ectoparasite infections (Turni *et al*. 2002, 2004). Although bovine TLR homologues have been identified, research into the specific pathways induced by bovine pathogens is yet to be conducted (White *et al*. 2003*a*, 2003*b*).

Host pathogenesis and virus RNAi knockdown

As RNAi can target endogenous and exogenous mRNAs in mammalian cells, the effect of these molecules against exogenous RNA viruses in human cells was successfully demonstrated (Gitlin *et al*. 2002). siRNAs have since been applied to protect against viral infection (e.g. insect baculovirus), inhibit the expression of viral antigens (e.g. SARS associated coronavirus), suppress the transcription of viral genomes (e.g. retroviruses), block viral replication (e.g. Dengue virus), silence viral host accessory genes (e.g. host receptors for HIV), and hinder the assembly of viral particles (e.g. rotavirus) (reviewed by Tan and Yin 2004). Most of these studies aim to develop therapeutic approaches either through gene silencing or by identifying potential new drug targets (Tan and Yin 2004). *In vivo* mouse studies have demonstrated that silencing of the host's *Fas* receptor prevented liver injury caused by hepatitis virus (Song *et al*. 2003). Anti-viral RNAi has been applied to knockdown the replication of pestivirus (bovine viral diarrhoea virus) in bovine cell cultures and foot and mouth disease virus *in vitro* and *in vivo* in suckling mice (Chen *et al*. 2004; Isken *et al*. 2003). It is feasible that a RNAi therapy could be developed for the treatment of foot and mouth disease (Chen *et al*. 2004).

Host pathogenesis, bacteria and RNAi

RNAi technology is applicable to high-throughput methods permitting the investigation of the mechanisms responsible for bacterial pathogenesis. Most RNAi studies dealing with bacterial infections have targeted host epithelial binding pathways such as Toll receptors as described above. RNAi has also been used to identify host proteins such as the receptors for the internalised pneumonia–meningitis pathogen, *Streptococcus pneumoniae* and, apoptosis pathway mediators of gastric ulceration caused by *Helicobacter pylori* (Nagasako *et al*. 2003; Opitz *et al*. 2004). In addition, the *in vivo* administration of siRNAs inhibiting TNFα delayed the onset of bacterial lipopolysaccharide-induced sepsis in adult mice (Sørensen *et al*. 2003). Similar studies could be applied to study pathways associated with the establishment of bacterial mastitis and other important livestock bacterial diseases such as Johne's disease.

Parasites, insects and RNAi

Unlike viral and bacterial pathogens, eukaryotic pathogens essentially possess RNAi pathways that provide new avenues for the investigation of potential pathogen targeted drugs or treatments (Cottrell and Doering 2003). For many parasites, previous methods for gene disruption were inefficient and/or lacking (Cottrell and Doering 2003). The RNAi pathways of a number of protozoan parasites have been researched and it is apparent that as well as being distinct from mammalian and plant pathways, they also differ somewhat among related parasite species (Ullu *et al*. 2004). RNAi has been most judiciously applied to study *Trypanosoma brucei* (human sleeping sickness), and reports of other parasite species studied using RNAi as a reverse genetic tool include: *T. congolense* (bovine trypanosomosis), *Entamoeba histolytica* (human amoebiasis), *Plasmodium* spp. (malaria) and *Babesia bovis* (bovine tick fever) (Inoue *et al*. 2002; Lew *et al*. 2004; McRobert and McConkey 2002; Ngo *et al*. 1998; Vayssie *et al*. 2004). *In vivo* gene silencing has also been achieved using siRNAs to inhibit *Plasmodium berghei* specific proteases in a malaria mouse model (Mohmmed *et al*. 2003).

The advent of RNAi in *C. elegans* provides an excellent model for the study of gene function in metazoan parasites (Aboobaker and Blaxter 2003; Knox *et al*. 2003). RNAi screens of about 85% of the 19427 predicted genes in *C. elegans* have been reported (Kamath and Ahringer 2003). RNAi gene function studies have been undertaken in human filarial nematodes, blood flukes and helminths (Aboobaker and Blaxter 2003; Boyle *et al*. 2003; Hussein *et al*. 2002). Grazing ruminants are continuously exposed to nematode infection that, if uncontrolled, would restrict agricultural production and be a serious threat to animal welfare (Knox *et al*. 2003). Control is largely achieved using antihelmintics. However, producers are facing increasing problems with antihelmintic resistance and consumers are demanding better food safety with regard to drug residues in food products. RNAi tools could be applied to identify potential gene targets for the development of alternative control strategies. Reproducible RNAi effects can be produced in the free-living stages of *Haemonchus contortus* but not, as yet in parasitic stages (Knox 2004).

The fruit fly *Drosophila melanogaster* has been the traditional genetic model system for mammalian research for many years and discoveries in *Drosophila* have also contributed towards our current knowledge of animal RNAi pathways (Boutros *et al*. 2004; Clemens *et al*. 2000; Okamura *et al*. 2004). Specifically, *Drosophila* research has provided the basis for genomic and RNAi applications in other arthropod species (Sanchez-Vargas *et al*. 2004; Shaw *et al*. 2001; Tabunoki *et al*. 2004). RNAi has been applied to disrupt feeding and anticoagulation at the tick–host interface (Aljamali *et al*. 2003; Narasimhan *et al*. 2004). The control of cattle ticks (*Boophilus microplus*) is required to ensure compliance with regulatory protocols for interstate and

international livestock movement and to enhance animal welfare by reducing stress and debilitation. Historically, acaricides have been applied for tick control, however there is a need to develop less toxic treatments and perhaps target specific molecules involved in parasite pathogenicity and/or survival (Gutteridge 1997). Tick control is becoming more difficult in northern Australia due to acaricide resistance and meat quality issues resulting as a consequence of increased *Bos indicus* content in an effort to improve tick resistance. To date, there are no reports of the application of RNAi to study bovine tick–host pathogenesis. Similarly there are currently no published RNAi studies targeting other agriculturally important pests.

The genomes of number of well-researched organisms (*Plasmodium* spp. trypanosomes, *C. elegans, D. melanogaster*) are available for comparative analyses (Adams *et al*. 2000; Chalfie 1998; El-Sayed *et al*. 2000; Gardner *et al*. 2002). Comparative genomic analyses of bovine parasites with these related well-characterised species will provide insights into parasite–insect functional genomics and associated non-coding regulatory genetic elements important in host parasitism (Gunasekera *et al*. 2004; Uliel *et al*. 2004). Combined with available genome data for agricultural parasites, appropriate bioinformatic and experimental tools such as RNAi, novel targets for the treatment and prevention of bovine diseases will be identified (Ellis *et al*. 2003; Knox 2004; McCarter 2004).

RNAi delivery and therapy

Mammalian RNAi delivery systems

Figure 3 summarises the RNAi delivery options currently used for gene knockdown experiments. Recommended parameters for the design of inhibitory siRNA sequences include: selection of a target cDNA region 50–100 nucleotides downstream of the start codon, selection of a 5′AA(N19)UU target mRNA sequence, where N is any nucleotide, 50% G–C content in the target sequence, avoidance of 5′ or 3′ untranslated regions and high G–C rich areas, and confirmation of exclusive target specific sequences (i.e. <15 bases of homology with non-target cDNAs) (Elbashir *et al*. 2002). Presently custom siRNA synthesis is available online through a number of companies, including Dharmacon, QIAGEN and Ambion.

The intracellular concentration of the target RNA, the half-life of the target protein, and the intracellular concentration of the siRNA all play a part in determining the extent and duration of suppression. Excessive intracellular pools of siRNA should be avoided as a means to prolong RNAi because of the risk of targeting non-specific mRNAs. The sustained clinical benefit from the direct delivery of siRNAs would likely require continuous re-administration (Davidson and Paulson 2004). Recently Siolas *et al*. (2005) demonstrated that synthetic 29-mer shRNAs were more potent inhibitors than synthetic siRNAs (Siolas *et al*. 2005). The delivery of siRNAs is probably the fastest and easiest method currently available for producing knockdown of gene expression in cell culture by means of RNAi (Hannon and Rossi 2004).

Unlike the expensive production of chemically or enzymatically synthesised siRNAs, large amounts of plasmid DNA vectors expressing siRNAs or shRNAs can be grown in bacteria (Dykxhoorn *et al*. 2003). DNA-directed RNAi has several advantages over the use of siRNAs and this approach has been widely adopted for potential human therapy (Rice *et al*. 2005; Zhang *et al*. 2004). Viruses possess the machinery to allow nuclear entry and access to important nuclear resident polymerases for expression. Thus, long-term gene silencing has been achieved using viral vectors to express shRNAs. For example, recombinant lentiviral vectors have been derived from HIV, FIV and equine infectious anaemia virus where the disease promoting genes have been deleted (Xia *et al*. 2002). Lentiviruses are attractive because of their ability to transduce terminally differentiated cells, and to express transgenes for a long period (Rubinson *et al*. 2003). Adeno-associated viral vectors are normally extrachromosomal (episomal) in cells after gene transfer and have also shown promise as RNAi delivery vectors. Several different serotypes of adeno-associated virus have been tested for use in different mammalian tissues and show desired properties, such as specific tropism, long-term expression, and limited immune response to the viral capsid (Lois *et al*. 2002; Shen *et al*. 2003). Comparatively, plasmid vectors are easier to construct than viral vectors; however, viral vectors are perhaps more effective for long-term silencing strategies (Zhao *et al*. 2004).

The therapeutic potential of RNAi *in vivo* has been demonstrated in mouse models using naked siRNAs, cholesterol delivered siRNAs, plasmids expressing shRNAs, and lentiviral vector delivered shRNAs (McCaffrey *et al*. 2002, 2003; Rubinson *et al*. 2003; Soutschek *et al*. 2004; Zhao *et al*. 2004). Some of the challenges in RNAi delivery include the design of conserved siRNA target sequences (particularly for viral targets), off-target–non-specific effects of the siRNAs and the delivery of siRNA/shRNAs to the appropriate cell type target and sustained expression of the introduced transcript (Downward 2004; Dykxhoorn *et al*. 2003; Jackson and Linsley 2004). RNAi relies on sequence identity between the siRNA and the target and RISC-mediated degradation of target transcripts is abolished if siRNAs carry a single base difference (Elbashir *et al*. 2001*b*). As this may have an impact on viral gene targets with high mutation rates, the stability of antiviral RNAi can be enhanced by targeting several different regions of the pathogen's genome or by targeting host genes (Gitlin *et al*. 2002; Song *et al*. 2003).

Bovine RNAi delivery systems

The delivery of siRNAs to bovine cell cultures in gene knockdown studies has been achieved with both synthetic

siRNA/shRNAs and siRNA/shRNA expression systems, Table 2 (Kaykas and Moon 2004; Paddison *et al*. 2004*a*). A number of bovine cell lines from various tissues are commercially available allowing tissue specific RNAi gene function screens. Despite the identified challenges, there are a number of researchers and commercial companies investing in the development of RNAi-based therapies (Acuity Pharmaceuticals, Alnylam Pharmaceuticals, Atugen, Benitec, CytRx, Intradigm, Nucleonics, Sirna Therapeutics). Many commercially available mammalian RNAi delivery systems will be applicable to the bovine host (Nencioni *et al*. 2004). There are also bovine specific retro- or lentiviruses which could be exploited as viral vectors in bovine RNAi studies *in vitro* and potentially *in vivo* (Li and Rossi 2005; Schmidt *et al*. 2004).

The current research investment in developing human RNAi based therapies will greatly benefit livestock researchers. Livestock that produce human therapeutic proteins in their milk, have organs suitable for xenotransplantation, or could provide resistance to diseases have been developed using nuclear transfer technologies (Denning and Priddle 2003). This can be an inefficient process, whereas lentivirus vectors expressing siRNAs to mediate gene silencing have been shown to be heritable and stable and thus provides an alternative approach for the development of transgenic animals (Dykxhoorn *et al*. 2003; Sato *et al*. 2005; Uprichard *et al*. 2005). Whether transgenic animals will be accepted by the public and by human medicine is highly debatable. This may also have an impact on the feasibility of developing DNA or virus based bovine RNAi therapies.

Conclusions

This review provides an overview of the range of RNAi methodologies widely applicable to eukaryotic and mammalian species as a potent gene knockdown tool. Given

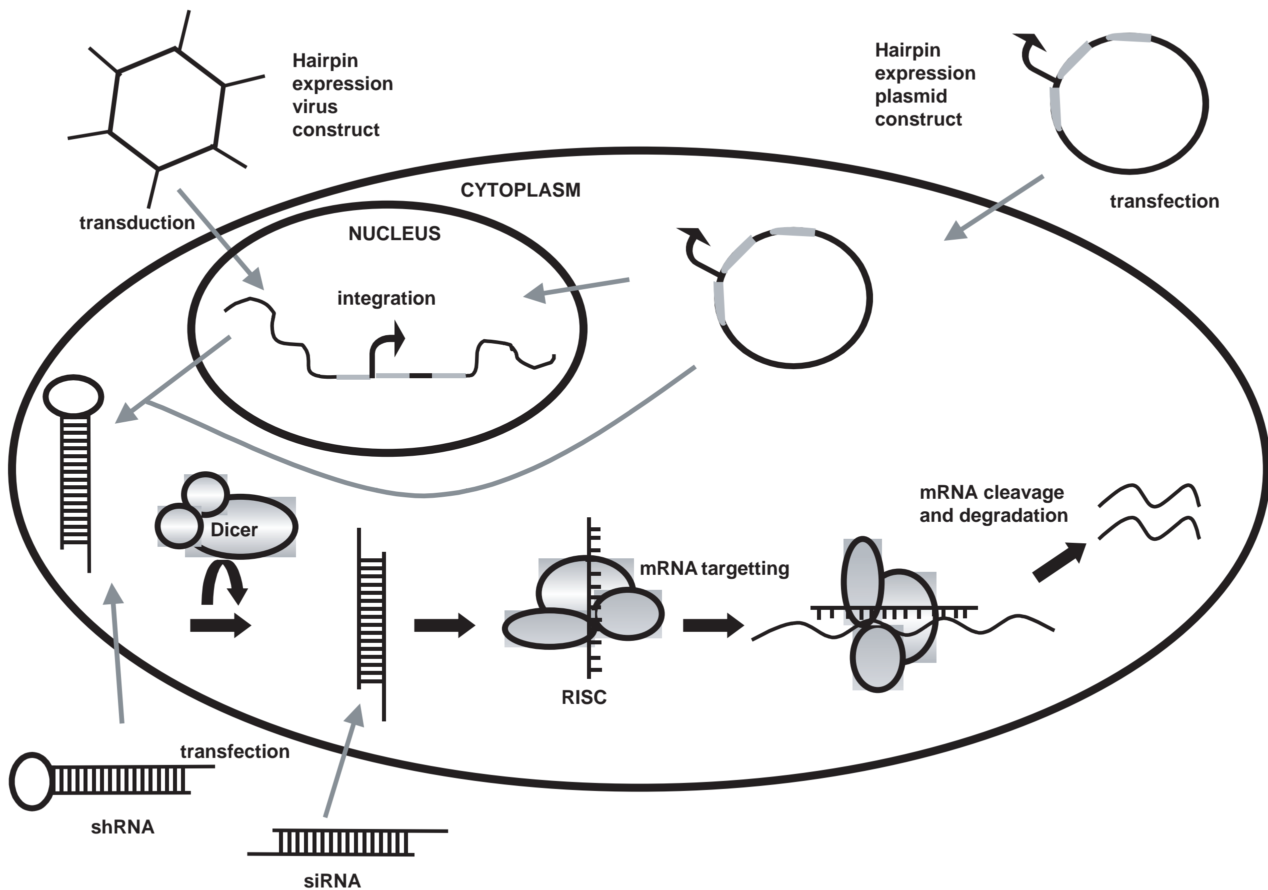


Figure 3. Summary of the delivery options for synthetic dsRNAs with cellular endogenous RNAi pathways to mediate transient and prolonged RNAi effects. Synthetic or enzyme generated shRNA and siRNAs can be transfected directly into cells for transient RNAi of targeted mRNA transcripts. Plasmids are shown to express hairpin structures via pol II or pol III promoters (arrow indicated on plasmid) and can either integrate and/or express cytoplasmic shRNAs. Virally produced shRNAs are shown to integrate into the nuclear DNA (such as lentivirus vectors). However, there are viruses which also produce shRNAs episomally (e.g. adenoviruses). The shRNAs and siRNAs are incorporated into the cellular RNAi pathways as described in Figure 2. Not shown are similar approaches used to deliver synthetic miRNAs that may induce translational repression described in Figure 1. See also the list of terms in Table 1.

the forthcoming availability of the bovine genome sequence and the accessibility of established mouse and human RNAi approaches, the basis for the wider application of RNAi in livestock studies is apparent. The next few years lead to new knowledge emanating from large scale RNAi analyses connecting the pathways that associate the bovine genome with the phenotype. It will then be possible to integrate this new knowledge with other relevant functional genomic studies to explore the dynamic nature of these interactions. Several RNAi approaches are outlined in this review. These include: the comparative analysis of functional miRNAs involved with beef quality traits and immune pathways; siRNA genome screens to isolate genes in these pathways; specific knockdown experiments in both pathogens and bovine host cells; and the development of bovine specific RNAi vectors. Research in this area will not only provide beef producers with new breeding strategies and disease therapies but also will benefit other livestock production systems and provide researchers with new insights into physiology and disease that impact on human health.

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References

Aboobaker AA, Blaxter ML (2003) Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi. Molecular and Biochemical Parasitology* **129**, 41–51. doi:10.1016/S0166-6851(03)00092-6

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, *et al*. (2000) The genome sequence of *Drosophila melanogaster. Science* **287**, 2185–2195. doi:10.1126/science.287.5461.2185

Aljamali MN, Bior AD, Sauer JR, Essenberg RC (2003) RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum. Insect Molecular Biology* **12**, 299–305. doi:10.1046/j.1365-2583.2003. 00416.x

Aoki Y, Cioca DP, Oidaira H, Kamiya J, Kiyosawa K (2003) RNA interference may be more potent than antisense RNA in human cancer cell lines. *Clinical and Experimental Pharmacology and Physiology* **30**, 96–102. doi:10.1046/j.1440-1681.2003.03801.x

Barad O, Meiri E, Avniel A, Aharonov R, Barzilai A, *et al*. (2004) MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. *Genome Research* **14**, 2486–2494. doi:10.1101/gr.2845604

Bidwell CA, Kramer LN, Perkins AC, Hadfield TS, Moody DE, Cockett NE (2004) Expression of PEG11 and PEG11AS transcripts in normal and callipyge sheep. *BMC Biology* **2**, 17. doi:10.1186/1741-7007-2-17

Boutros M, Kiger AA, Armknecht S, Kerr K, Hild M, *et al*. (2004) Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* **303**, 832–835. doi:10.1126/science.1091266

Boyle JP, Wu XJ, Shoemaker CB, Yoshino TP (2003) Using RNA interference to manipulate endogenous gene expression in *Schistosoma mansoni* sporocysts. *Molecular and Biochemical Parasitology* **128**, 205–215. doi:10.1016/S0166-6851(03)00078-1

Brantl S (2002) Antisense-RNA regulation and RNA interference. *Biochimica et Biophysica Acta* **1575**, 15–25.

Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550–553. doi:10.1126/science.1068999

Byrne KA, Wang YH, Lehnert SA, Harper GS, McWilliam SM, Bruce HL, Reverter A (2005) Gene expression profiling of muscle tissue in Brahman steers during nutritional restriction. *Journal of Animal Science* **83**, 1–12.

Cabot RA, Prather RS (2003) Cleavage stage porcine embryos may have differing developmental requirements for karyopherins alpha2 and alpha3. *Molecular Reproduction and Development* **64**, 292–301. doi:10.1002/mrd.10238

Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 9742–9747. doi:10.1073/pnas. 171251798

Casas E, White SN, Riley DG, Smith TP, Brenneman RA, *et al*. (2005) Assessment of single nucleotide polymorphisms in genes residing on chromosomes 14 and 29 for association with carcass composition traits in *Bos indicus* cattle. *Journal of Animal Science* **83**, 13–19.

Cerutti H (2003) RNA interference: traveling in the cell and gaining functions? *Trends in Genetics* **19**, 39–46. doi:10.1016/S0168-9525(02)00010-0

Chalfie M (1998) Genome sequencing. The worm revealed. *Nature* **396**, 620–621. doi:10.1038/25228

Chen W, Yan W, Du Q, Fei L, Liu M, Ni Z, Sheng Z, Zheng Z (2004) RNA interference targeting VP1 inhibits foot-and-mouth disease virus replication in BHK-21 cells and suckling mice. *Journal of Virology* **78**, 6900–6907. doi:10.1128/JVI.78.13.6900-6907.2004

Chou YH, Khuon S, Herrmann H, Goldman RD (2003) Nestin promotes the phosphorylation-dependent disassembly of vimentin intermediate filaments during mitosis. *Molecular Biology of the Cell* **14**, 1468–1478. doi:10.1091/mbc.E02-08-0545

Clawson ML, Heaton MP, Chitko-McKown CG, Fox JM, Smith TP, Snelling WM, Keele JW, Laegreid WW (2004) Beta-2-microglobulin haplotypes in U.S. beef cattle and association with failure of passive transfer in newborn calves. *Mammalian Genome* **15**, 227–236. doi:10.1007/s00335-003-2320-x

Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA, Dixon JE (2000) Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 6499–6503. doi:10.1073/pnas.110149597

Cottrell TR, Doering TL (2003) Silence of the strands: RNA interference in eukaryotic pathogens. *Trends in Microbiology* **11**, 37–43. doi:10.1016/S0966-842X(02)00004-5

da Mota AF, Sonstegard TS, Van Tassell CP, Shade LL, Matukumalli LK, *et al*. (2004) Characterization of open reading frame-expressed sequence tags generated from *Bos indicus* and *B. taurus* mammary gland cDNA libraries. *Animal Genetics* **35**, 213–219. doi:10.1111/j.1365-2052.2004.01139.x

Davidson BL, Paulson HL (2004) Molecular medicine for the brain: silencing of disease genes with RNA interference. *Lancet Neurology* **3**, 145–149. doi:10.1016/S1474-4422(04)00678-7

Denning C, Priddle H (2003) New frontiers in gene targeting and cloning: success, application and challenges in domestic animals and human embryonic stem cells. *Reproduction* **126**, 1–11. doi:10.1530/rep.0.1260001

Doench JG, Sharp PA (2004) Specificity of microRNA target selection in translational repression. *Genes and Development* **18**, 504–511. doi:10.1101/gad.1184404

Donnison M, Pfeffer PL (2004) Isolation of genes associated with developmentally competent bovine oocytes and quantitation of their levels during development. *Biology of Reproduction* **71**, 1813–1821. doi:10.1095/biolreprod.104.032367

Downward J (2004) RNA interference. *BMJ* **328**, 1245–1248. doi:10.1136/bmj.328.7450.1245

Dubchak I, Brudno M, Loots GG, Pachter L, Mayor C, Rubin EM, Frazer KA (2000) Active conservation of noncoding sequences revealed by three-way species comparisons. *Genome Research* **10**, 1304–1306. doi:10.1101/gr.142200

Dykxhoorn DM, Novina CD, Sharp PA (2003) Killing the messenger: short RNAs that silence gene expression. *Nature Reviews. Molecular Cell Biology* **4**, 457–467. doi:10.1038/nrm1129

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001*a*) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498. doi:10.1038/35078107

Elbashir SM, Harborth J, Weber K, Tuschl T (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26**, 199–213.

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T (2001*b*) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO Journal* **20**, 6877–6888. doi:10.1093/emboj/20.23.6877

Ellis JT, Morrison DA, Reichel MP (2003) Genomics and its impact on parasitology and the potential for development of new parasite control methods. *DNA and Cell Biology* **22**, 395–403. doi:10.1089/104454903767650667

El-Sayed NM, Hegde P, Quackenbush J, Melville SE, Donelson JE (2000) The African trypanosome genome. *International Journal for Parasitology* **30**, 329–345. doi:10.1016/S0020-7519(00)00015-1

Everts-van der Wind A, Kata SR, Band MR, Rebeiz M, Larkin DM, *et al*. (2004) A 1463 gene cattle–human comparative map with anchor points defined by human genome sequence coordinates. *Genome Research* **14**, 1424–1437. doi:10.1101/gr.2554404

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature* **391**, 806–811. doi:10.1038/35888

Frazer KA, Tao H, Osoegawa K, de Jong PJ, Chen X, Doherty MF, Cox DR (2004) Noncoding sequences conserved in a limited number of mammals in the SIM2 interval are frequently functional. *Genome Research* **14**, 367–372. doi:10.1101/gr.1961204

Gardner MJ, Hall N, Fung E, White O, Berriman M, *et al*. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum. Nature* **419**, 498–511. doi:10.1038/nature01097

Gitlin L, Karelsky S, Andino R (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **418**, 430–434. doi:10.1038/nature00873

Goldammer T, Kata SR, Brunner RM, Kuhn C, Weikard R, Womack JE, Schwerin M (2004) High-resolution comparative mapping between human chromosomes 4 and 8 and bovine chromosome 27 provides genes and segments serving as positional candidates for udder health in cattle. *Genomics* **84**, 696–706. doi:10.1016/j.ygeno.2003.12.003

Gong H, Liu CM, Liu DP, Liang CC (2005) The role of small RNAs in human diseases: potential troublemaker and therapeutic tools. *Medicinal Research Reviews* **25**, 361–381. doi:10.1002/med.20023

Goodwin BL, Solomonson LP, Eichler DC (2004) Argininosuccinate synthase expression is required to maintain nitric oxide production and cell viability in aortic endothelial cells. *Journal of Biological Chemistry* **279**, 18353–18360. doi:10.1074/jbc.M308160200

Goto A, Blandin S, Royet J, Reichhart JM, Levashina EA (2003) Silencing of Toll pathway components by direct injection of double-stranded RNA into *Drosophila* adult flies. *Nucleic Acids Research* **31**, 6619–6623. doi:10.1093/nar/gkg852

Gunasekera AM, Patankar S, Schug J, Eisen G, Kissinger J, Roos D, Wirth DF (2004) Widespread distribution of antisense transcripts in the *Plasmodium falciparum* genome. *Molecular and Biochemical Parasitology* **136**, 35–42. doi:10.1016/j.molbiopara.2004.02.007

Gutteridge WE (1997) Designer drugs: pipe-dreams or realities? *Parasitology* **114**, S145–S151. doi:10.1017/S0031182096008335

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296. doi:10.1038/35005107

Hannon GJ, Rossi JJ (2004) Unlocking the potential of the human genome with RNA interference. *Nature* **431**, 371–378. doi:10.1038/nature02870

Hanotte O, Ronin Y, Agaba M, Nilsson P, Gelhaus A, *et al*. (2003) Mapping of quantitative trait loci controlling trypanotolerance in a cross of tolerant West African N'Dama and susceptible East African Boran cattle. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 7443–7448. doi:10.1073/pnas.1232392100

Hiley SL, Jackman J, Babak T, Trochesset M, Morris QD, Phizicky E, Hughes TR (2005) Detection and discovery of RNA modifications using microarrays. *Nucleic Acids Research* **33**, e2. doi:10.1093/nar/gni002

Hirano T, Yamauchi N, Sato F, Soh T, Hattori MA (2004) Evaluation of RNA interference in developing porcine granulosa cells using fluorescence reporter genes. *Journal of Reproduction and Development* **50**, 599–603. doi:10.1262/jrd.50.599

Hussein AY, Kichenin K, Selkirk ME (2002) Suppression of secreted acetylcholinesterase expression in *Nippostrongylus brasiliensis* by RNA interference. *Molecular and Biochemical Parasitology* **122**, 91–94. doi:10.1016/S0166-6851(02)00068-3

Inoue N, Otsu K, Ferraro DM, Donelson JE (2002) Tetracycline-regulated RNA interference in *Trypanosoma congolense. Molecular and Biochemical Parasitology* **120**, 309–313. doi:10.1016/S0166-6851(02)00015-4

Ishiwata H, Katsuma S, Kizaki K, Patel OP, Nakano H, *et al*. (2003) Characterization of gene expression profiles in early bovine pregnancy using a custom cDNA microarray. *Molecular Reproduction and Development* **65**, 9–18. doi:10.1002/mrd.10292

Isken O, Grassmann CW, Sarisky RT, Kann M, Zhang S, Grosse F, Kao PN, Behrens SE (2003) Members of the NF90/NFAR protein group are involved in the life cycle of a positive-strand RNA virus. *EMBO Journal* **22**, 5655–5665. doi:10.1093/emboj/cdg562

Jackson AL, Linsley PS (2004) Noise amidst the silence: off-target effects of siRNAs? *Trends in Genetics* **20**, 521–524. doi:10.1016/j.tig.2004.08.006

Jasinska A, Krzyzosiak WJ (2004) Repetitive sequences that shape the human transcriptome. *FEBS Letters* **567**, 136–141. doi:10.1016/j.febslet.2004.03.109

Joerg H, Janett F, Schlatt S, Mueller S, Graphodatskaya D, Suwattana D, Asai M, Stranzinger G (2003) Germ cell transplantation in an azoospermic Klinefelter bull. *Biology of Reproduction* **69**, 1940–1944. doi:10.1095/biolreprod.103.020297

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004) Human microRNA targets. *PLoS Biology* **2**, e363. doi:10.1371/journal.pbio.0020363

Johnson JM, Edwards S, Shoemaker D, Schadt EE (2005) Dark matter in the genome: evidence of widespread transcription detected by microarray tiling experiments. *Trends in Genetics* **21**, 93–102. doi:10.1016/j.tig.2004.12.009

Kamath RS, Ahringer J (2003) Genome-wide RNAi screening in *Caenorhabditis elegans. Methods* **30**, 313–321.

Kawasaki H, Taira K (2004) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. *Nature* **431**, 211–217. doi:10.1038/nature02889

Kaykas A, Moon RT (2004) A plasmid-based system for expressing small interfering RNA libraries in mammalian cells. *BMC Cell Biology* **5**, 16–. doi:10.1186/1471-2121-5-16

Kennerdell JR, Carthew RW (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* **95**, 1017–1026. doi:10.1016/S0092-8674(00)81725-0

Kittler R, Putz G, Pelletier L, Poser I, Heninger A-K, *et al*. (2004) An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* **432**, 1036–1040. doi:10.1038/nature03159

Knox DP (2004) Technological advances and genomics in metazoan parasites. *International Journal for Parasitology* **34**, 139–152. doi:10.1016/j.ijpara.2003.10.013

Knox DP, Redmond DL, Newlands GF, Skuce PJ, Pettit D, Smith WD (2003) The nature and prospects for gut membrane proteins as vaccine candidates for *Haemonchus contortus* and other ruminant trichostrongyloids. *International Journal for Parasitology* **33**, 1129–1137. doi:10.1016/S0020-7519(03)00167-X

Kong XC, Barzaghi P, Ruegg MA (2004) Inhibition of synapse assembly in mammalian muscle *in vivo* by RNA interference. *EMBO Reports* **5**, 183–188. doi:10.1038/sj.embor.7400065

Kringer CE, Block J, Al-Katanani YM, Rivera RM, Chase CC, Hansen PJ (2003) Differences between Brahman and Holstein cows in response to estrus synchronization, superovulation and resistance of embryos to heat shock. *Animal Reproduction Science* **78**, 13–24. doi:10.1016/S0378-4320(03)00045-9

Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T (2003) New microRNAs from mouse and human. *RNA* **9**, 175–179.

Larizza A, Makalowski W, Pesole G, Saccone C (2002) Evolutionary dynamics of mammalian mRNA untranslated regions by comparative analysis of orthologous human, artiodactyl and rodent gene pairs. *Computional Chemistry* **26**, 479–490. doi:10.1016/S0097-8485(02)00009-8

Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans. Science* **294**, 858–862. doi:10.1126/science.1065062

Lee Y, Ahn C, Han J, Choi H, Kim J, *et al*. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415–419. doi:10.1038/nature01957

Leung WC, Lawrie A, Demaries S, Massaeli H, Burry A, *et al*. (2004) Apolipoprotein D and platelet-derived growth factor-BB synergism mediates vascular smooth muscle cell migration. *Circulation Research* **95**, 179–186. doi:10.1161/01.RES.0000135482.74178.14

Lew A, Hall R, Fletcher T, Jackson L (2004) Inhibition of host cell invasion *in vitro* by *Babesia bovis* using dsRNA. In 'Program and abstracts of the 46th annual scientific meeting of the Australian Society for Parasitology'. p. 26.

Lewin HA (2003) The future of cattle genome research: the beef is here. *Cytogenetic and Genome Research* **102**, 10–15. doi:10.1159/000075718

Li MJ, Rossi JJ (2005) Lentiviral vector delivery of recombinant small interfering RNA expression cassettes. *Methods in Enzymology* **392**, 218–226.

Lippman Z, Martienssen R (2004) The role of RNA interference in heterochromatic silencing. *Nature* **431**, 364–370. doi:10.1038/nature02875

Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868–872. doi:10.1126/science.1067081

Mattick JS (2003) Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. *BioEssays* **25**, 930–939. doi:10.1002/bies.10332

Mawji IA, Robb GB, Tai SC, Marsden PA (2004) Role of the 3′-untranslated region of human endothelin-1 in vascular endothelial cells. Contribution to transcript lability and the cellular heat shock response. *Journal of Biological Chemistry* **279**, 8655–8667. doi:10.1074/jbc.M312190200

McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA (2002) RNA interference in adult mice. *Nature* **418**, 38–39. doi:10.1038/418038a

McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, Wieland SF, Marion PL, Kay MA (2003) Inhibition of hepatitis B virus in mice by RNA interference. *Nature Biotechnology* **21**, 639–644. doi:10.1038/nbt824

McCarter JP (2004) Genomic filtering: an approach to discovering novel antiparasitics. *Trends in Parasitology* **20**, 462–468. doi:10.1016/j.pt.2004.07.008

McRobert L, McConkey GA (2002) RNA interference (RNAi) inhibits growth of *Plasmodium falciparum. Molecular and Biochemical Parasitology* **119**, 273–278. doi:10.1016/S0166-6851(01)00429-7

Meirelles FV, Caetano AR, Watanabe YF, Ripamonte P, Carambula SF, Merighe GK, Garcia SM (2004) Genome activation and developmental block in bovine embryos. *Animal Reproduction Science* **82–83**, 13–20. doi:10.1016/j.anireprosci.2004.05.012

Miller VM, Xia H, Marrs GL, Gouvion CM, Lee G, Davidson BL, Paulson HL (2003) Allele-specific silencing of dominant disease genes. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 7195–7200. doi:10.1073/pnas.1231012100

Miyagishi M, Hayashi M, Taira K (2003) Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense and Nucleic Acid Drug Development* **13**, 1–7. doi:10.1089/108729003764097296

Miyagishi M, Matsumoto S, Taira K (2004) Generation of an shRNAi expression library against the whole human transcripts. *Virus Research* **102**, 117–124. doi:10.1016/j.virusres.2004.01.022

Mockler TC, Ecker JR (2005) Applications of DNA tiling arrays for whole-genome analysis. *Genomics* **85**, 1–15. doi:10.1016/j.ygeno.2004.10.005

Mohmmed A, Dasaradhi PV, Bhatnagar RK, Chauhan VS, Malhotra P (2003) *In vivo* gene silencing in *Plasmodium berghei* — a mouse malaria model. *Biochemical and Biophysical Research Communications* **309**, 506–511. doi:10.1016/j.bbrc.2003.08.027

Morey C, Avner P (2004) Employment opportunities for non-coding RNAs. *FEBS Letters* **567**, 27–34. doi:10.1016/j.febslet.2004.03.117

Munroe SH (2004) Diversity of antisense regulation in eukaryotes: multiple mechanisms, emerging patterns. *Journal of Cellular Biochemistry* **93**, 664–671. doi:10.1002/jcb.20252

Murphy WJ, Bourque G, Tesler G, Pevzner P, O'Brien SJ (2003) Reconstructing the genomic architecture of mammalian ancestors using multispecies comparative maps. *Human Genomics* **1**, 30–40.

Murphy WJ, Pevzner PA, O'Brien SJ (2004) Mammalian phylogenomics comes of age. *Trends in Genetics* **20**, 631–639.

Nagasako T, Sugiyama T, Mizushima T, Miura Y, Kato M, Asaka M (2003) Up-regulated Smad5 mediates apoptosis of gastric epithelial cells induced by *Helicobacter pylori* infection. *Journal of Biological Chemistry* **278**, 4821–4825. doi:10.1074/jbc.M211143200

Nagy A, Rossant J (1996) Targeted mutagenesis: analysis of phenotype without germ line transmission. *Journal of Clinical Investigation* **97**, 1360–1365.

Narasimhan S, Montgomery RR, DePonte K, Tschudi C, Marcantonio N, *et al*. (2004) Disruption of *Ixodes scapularis* anticoagulation by using RNA interference. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1141–1146. doi:10.1073/ pnas.0307669100

Nencioni A, Sandy P, Dillon C, Kissler S, Blume-Jensen P, Van Parijs L (2004) RNA interference for the identification of disease-associated genes. *Current Opinion in Molecular Therapeutics* **6**, 136–140.

Ngo H, Tschudi C, Gull K, Ullu E (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 14687–14692. doi:10.1073/pnas.95.25.14687

Nicholson RH, Nicholson AW (2002) Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference. *Mammalian Genome* **13**, 67–73. doi:10.1007/s00335-001-2119-6

Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes and Development* **18**, 1655–1666. doi:10.1101/gad.1210204

Opitz B, Puschel A, Schmeck B, Hocke AC, Rosseau S, Hammerschmidt S, Schumann RR, Suttorp N, Hippenstiel S (2004) Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae. Journal of Biological Chemistry* **279**, 36426–36432. doi:10.1074/ jbc.M403861200

Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T (2003) TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-beta. *Journal of Biological Chemistry* **278**, 49751–49762. doi:10.1074/jbc.M305820200

Paddison PJ, Caudy AA, Sachidanandam R, Hannon GJ (2004*a*) Short hairpin activated gene silencing in mammalian cells. *Methods in Molecular Biology* **265**, 85–100.

Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, *et al*. (2004*b*) A resource for large-scale RNA-interference-based screens in mammals. *Nature* **428**, 427–431. doi:10.1038/nature02370

Pang KC, Stephen S, Engstrom PG, Tajul-Arifin K, Chen W, Wahlestedt C, Lenhard B, Hayashizaki Y, Mattick JS (2005) RNAdb — a comprehensive mammalian noncoding RNA database. *Nucleic Acids Research* **33**, D125–D130.

Paradis F, Vigneault C, Robert C, Sirard MA (2005) RNA interference as a tool to study gene function in bovine oocytes. *Molecular Reproduction and Development* **70**, 111–121. doi:10.1002/ mrd.20193

Park YH, Joo YS, Park JY, Moon JS, Kim SH, Kwon NH, Ahn JS, Davis WC, Davies CJ (2004) Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows. *Journal of Veterinary Science* **5**, 29–39.

Pasquinelli AE, Reinhart BJ, Slack F, Martindales MQ, Kuroda MI, *et al*. (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408**, 86–89. doi:10.1038/35040556

Pfister-Genskow M, Myers C, Childs LA, Lacson JC, Patterson T, *et al*. (2005) Identification of differentially expressed genes in individual bovine preimplantation embryos produced by nuclear transfer: improper reprogramming of genes required for development. *Biology of Reproduction* **72**, 546–555. doi:10.1095/biolreprod. 104.031799

Rice RR, Muirhead AN, Harrison BT, Kassianos AJ, Sedlak PL, *et al*. (2005) Simple, robust strategies for generating DNA-directed RNA interference constructs. *Methods in Enzymology* **392**, 405–419.

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004) Identification of mammalian microRNA host genes and transcription units. *Genome Research* **14**, 1902–1910. doi:10.1101/ gr.2722704

Romano N, Macino G (1992) Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Molecular Microbiology* **6**, 3343–3353.

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, *et al*. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nature Genetics* **33**, 401–406. doi:10.1038/ng1117

Sachse C, Krausz E, Kronke A, Hannus M, Walsh A, *et al*. (2005) High-throughput RNA interference strategies for target discovery and validation by using synthetic short interfering RNAs: functional genomics investigations of biological pathways. *Methods in Enzymology* **392**, 242–277.

Sanchez-Vargas I, Travanty EA, Keene KM, Franz AW, Beaty BJ, Blair CD, Olson KE (2004) RNA interference, arthropod-borne viruses and mosquitoes. *Virus Research* **102**, 65–74. doi:10.1016/ j.virusres.2004.01.017

Sasai M, Oshiumi H, Matsumoto M, Inoue N, Fujita F, Nakanishi M, Seya T (2005) Cutting Edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *Journal of Immunology* **174**, 27–30.

Sato Y, Ajiki T, Seiichiro I, Jun F, Hiroyuki Y, *et al*. (2005) Gene silencing in rat-liver and limb grafts by rapid injection of small interference RNA. *Transplantation* **79**, 240–243. doi:10.1097/ 01.TP.0000147786.52502.2F

Schmidt M, Katano H, Bossis I, Chiorini JA (2004) Cloning and characterization of a bovine adeno-associated virus. *Journal of Virology* **78**, 6509–6516. doi:10.1128/JVI.78.12.6509-6516.2004

Schwarz DS, Hutvagner G, Haley B, Zamore PD (2002) Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Molecular Cell* **10**, 537–548. doi:10.1016/ S1097-2765(02)00651-2

Shaw PJ, Salameh A, McGregor AP, Bala S, Dover GA (2001) Divergent structure and function of the bicoid gene in *Muscoidea* fly species. *Evolution and Development* **3**, 251–262. doi:10.1046/ j.1525-142x.2001.003004251.x

Shen C, Buck AK, Liu X, Winkler M, Reske SN (2003) Gene silencing by adenovirus-delivered siRNA. *FEBS Letters* **539**, 111–114. doi:10.1016/S0014-5793(03)00209-6

Silva J, Chang K, Hannon GJ, Rivas FV (2004) RNA-interference-based functional genomics in mammalian cells: reverse genetics coming of age. *Oncogene* **23**, 8401–8409. doi:10.1038/sj.onc. 1208176

Siolas D, Lerner C, Burchard J, Ge W, Linsley PS, Paddison PJ, Hannon GJ, Cleary MA (2005) Synthetic shRNAs as potent RNAi triggers. *Nature Biotechnology* **23**, 227–231. doi:10.1038/nbt1052

Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J (2003) RNA interference targeting *Fas* protects mice from fulminant hepatitis. *Nature Medicine* **9**, 347–351. doi:10.1038/nm828

Sonstegard TS, Capuco AV, White J, Van Tassell CP, Connor EE, *et al*. (2002) Analysis of bovine mammary gland EST and functional annotation of the *Bos taurus* gene index. *Mammalian Genome* **13**, 373–379. doi:10.1007/s00335-001-2145-4

Sørensen DR, Leirdal M, Sioud M (2003) Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *Journal of Molecular Biology* **327**, 761–766. doi:10.1016/S0022-2836(03)00181-5

Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, *et al*. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178. doi:10.1038/nature03121

Stein P, Svoboda P, Anger M, Schultz RM (2003) RNAi: mammalian oocytes do it without RNA-dependent RNA polymerase. *RNA* **9**, 187–192.

Suchyta SP, Sipkovsky S, Kruska R, Jeffers A, McNulty A, *et al*. (2003) Development and testing of a high-density cDNA microarray resource for cattle. *Physiological Genomics* **15**, 158–164.

Sun Y, Koo S, White N, Peralta E, Esau C, Dean NM, Perera RJ (2004) Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs. *Nucleic Acids Research* **32**, e188. doi:10.1093/nar/gnh186

Tabunoki H, Higurashi S, Ninagi O, Fujii H, Banno Y, *et al*. (2004) A carotenoid-binding protein (CBP) plays a crucial role in cocoon pigmentation of silkworm (*Bombyx mori*) larvae. *FEBS Letters* **567**, 175–178. doi:10.1016/j.febslet.2004.04.067

Tan FL, Yin JQ (2004) RNAi, a new therapeutic strategy against viral infection. *Cell Research* **14**, 460–466.

Tao W, Mallard B, Karrow N, Bridle B (2004) Construction and application of a bovine immune-endocrine cDNA microarray. *Veterinary Immunology and Immunopathology* **101**, 1–17. doi:10.1016/j.vetimm.2003.10.011

Turni C, Lee RP, Jackson LA (2002) Effect of salivary gland extracts from the tick, *Boophilus microplus*, on leucocytes from Brahman and Hereford cattle. *Parasite Immunology* **24**, 355–361. doi:10.1046/j.1365-3024.2002.00471.x

Turni C, Lee RP, Jackson LA (2004) A comparison of the immunosuppressive effects of salivary gland extracts from two laboratory strains of *Boophilus microplus. International Journal for Parasitology* **34**, 833–838. doi:10.1016/j.ijpara.2004.03.002

Uehara A, Yang S, Fujimoto Y, Fukase K, Kusumoto S, Shibata K, Sugawara S, Takada H (2005) Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. *Cellular Microbiology* **7**, 53–61. doi:10.1111/j.1462-5822.2004.00433.x

Uliel S, Liang XH, Unger R, Michaeli S (2004) Small nucleolar RNAs that guide modification in trypanosomatids: repertoire, targets, genome organisation, and unique functions. *International Journal for Parasitology* **34**, 445–454. doi:10.1016/j.ijpara.2003.10.014

Ullu E, Tschudi C, Chakraborty T (2004) RNA interference in protozoan parasites. *Cellular Microbiology* **6**, 509–519. doi:10.1111/j.1462-5822.2004.00399.x

Uprichard SL, Boyd B, Althage A, Chisari FV (2005) Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 773–778. doi:10.1073/pnas.0409028102

Ushizawa K, Herath CB, Kaneyama K, Shiojima S, Hirasawa A, *et al*. (2004) cDNA microarray analysis of bovine embryo gene expression profiles during the pre-implantation period. *Reproductive Biology and Endocrinology* **2**, 77. doi:10.1186/ 1477-7827-2-77

Ushizawa K, Takahashi T, Kaneyama K, Tokunaga T, Tsunoda Y, Hashizume K (2005) Gene expression profiles of bovine trophoblastic cell line (BT-1) analyzed by a custom cDNA microarray. *Journal of Reproduction and Development* **51**, 211–220. –

van der Krol AR, Mur LA, de Lange P, Mol JN, Stuitje AR (1990) Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect. *Plant Molecular Biology* **14**, 457–466. doi:10.1007/BF00027492

Vayssie L, Vargas M, Weber C, Guillen N (2004) Double-stranded RNA mediates homology-dependent gene silencing of gamma-tubulin in the human parasite *Entamoeba histolytica. Molecular and Biochemical Parasitology* **138**, 21–28. doi:10.1016/j.molbiopara.2004.07.005

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837. doi:10.1126/science.1074973

Vordermeier HM, Cockle PC, Whelan A, Rhodes S, Palmer N, Bakker D, Hewinson RG (1999) Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle. *Clinical and Diagnostic Laboratory Immunology* **6**, 675–682.

Vouret-Craviari V, Boulter E, Grall D, Matthews C, Van Obberghen-Schilling E (2004) ILK is required for the assembly of matrix-forming adhesions and capillary morphogenesis in endothelial cells. *Journal of Cell Science* **117**, 4559–4569. doi:10.1242/jcs.01331

Walduck A, Rudel T, Meyer TF (2004) Proteomic and gene profiling approaches to study host responses to bacterial infection. *Current Opinion in Microbiology* **7**, 33–38. doi:10.1016/j.mib.2003.12.010

Weber MJ (2005) New human and mouse microRNA genes found by homology search. *FEBS Journal* **272**, 59–73. doi:10.1111/j.1432-1033.2004.04389.x

Weiss DJ, Evanson OA, Deng M, Abrahamsen MS (2004) Sequential patterns of gene expression by bovine monocyte-derived macrophages associated with ingestion of mycobacterial organisms. *Microbial Pathogenesis* **37**, 215–224. doi:10.1016/j.micpath.2004.07.001

Werling D, Jungi TW (2003) TOLL-like receptors linking innate and adaptive immune response. *Veterinary Immunology and Immunopathology* **91**, 1–12. doi:10.1016/S0165-2427(02)00228-3

White SN, Kata SR, Womack JE (2003*a*) Comparative fine maps of bovine toll-like receptor 4 and toll-like receptor 2 regions. *Mammalian Genome* **14**, 149–155. doi:10.1007/s00335-002-2213-4

White SN, Taylor KH, Abbey CA, Gill CA, Womack JE (2003*b*) Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10364–10369. doi:10.1073/pnas.1333957100

Williams SH, Mouchel N, Harris A (2003) A comparative genomic analysis of the cow, pig, and human CFTR genes identifies potential intronic regulatory elements. *Genomics* **81**, 628–639. doi:10.1016/S0888-7543(03)00089-2

Wolf E, Arnold GJ, Bauersachs S, Beier HM, Blum H, *et al*. (2003) Embryo-maternal communication in bovine — strategies for deciphering a complex cross-talk. *Reproduction in Domestic Animals* **38**, 276–289. doi:10.1046/j.1439-0531.2003.00435.x

Xia H, Mao Q, Paulson HL, Davidson BL (2002) siRNA-mediated gene silencing *in vitro* and *in vivo. Nature Biotechnology* **20**, 1006–1010. doi:10.1038/nbt739

Xiuzhu T, Shore L, Stram Y, Michaeli SC, Brietbart HC, Shemesh M (2003) Duplexes of 21 nucleotide RNA specific for COX II mediates RNA interference in cultured bovine aortic coronary endothelial cells (BAECs). *Prostaglandins and Other Lipid Mediators* **71**, 119–129. doi:10.1016/S1098-8823(03)00043-1

Yao J, Ren X, Ireland JJ, Coussens PM, Smith TP, Smith GW (2004) Generation of a bovine oocyte cDNA library and microarray: resources for identification of genes important for follicular development and early embryogenesis. *Physiological Genomics* **19**, 84–92. doi:10.1152/physiolgenomics.00123.2004

Zamore PD (2002) Ancient pathways programmed by small RNAs. *Science* **296**, 1265–1269. doi:10.1126/science.1072457

Zhang R, He X, Liu W, Lu M, Hsieh JT, Min W (2003) AIP1 mediates TNF-alpha-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. *Journal of Clinical Investigation* **111**, 1933–1943. doi:10.1172/JCI200317790

Zhang Y, Zhang YF, Bryant J, Charles A, Boado RJ, Pardridge WM (2004) Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clinical Cancer Research* **10**, 3667–3677.

Zhao N, Zu ZX, Liu CM, Dong WJ, Liu DP, Liang CC (2004) Knockdown of mouse adult beta-globin gene expression in MEL cells by retrovirus vector-mediated RNA interference. *Molecular Biotechnology* **28**, 195–199. doi:10.1385/MB:28:3:195

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