Harnessing RNA Interference Technology for Prospective Application in Animal Science

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RNA interference is an emerging scientific tool that is making inroads in medical science. However, its application in the veterinary field is still in infancy. The technology offers several potential benefits that include disease therapeutics and increasing animal productivity. In this article, we summarize the available information on RNA Interference Technology including mechanism of action, possible routes of delivery and avenues to exploit it for the benefit of animals.

KEY WORDS
RNA interference, mechanism of function, animal science, therapeutics, productivity.

INTRODUCTION
In every living cell, hereditary material called DNA carries the genetic information on genes that exist as stretches of nucleotide sequence. A gene is transcribed into messenger RNA (mRNA) depending on the cellular requirement. In a eukaryotic cell, this mRNA travels from nucleus to ribosomes in the cytoplasm, and by process of translation results in expression of its respective protein. These proteins perform vital structural and catalytic functions in the body. Alterations in the structure or expression pattern of individual proteins results in appearance of a disease. These alterations could be pathogen mediated or generated by host in response to an illness. In such a scenario, RNA interference (RNAi) could be valuable for its potential application as a

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therapeutic to silence undesirable host genes, or genes of infectious agents associated with the disease. RNA interference mediated by molecules called small interfering dsRNA (siRNA) can be used to block the production of any disease-causing protein before its actual formation, by degrading its precursor mRNA transcript. RNAi is a naturally occurring cellular process for controlling the gene expression at post transcriptional level. Probably RNAi originated as a eukaryotic defense mechanism against RNA viruses. As a defense against parasitic nucleic acids, eukaryotes might have developed this mechanism to silence integrated and invading viruses. During the course of evolution, this defense mechanism has been tailored to regulate the expression of protein-coding genes. Long double-stranded RNAs (dsRNAs) are known to silence the expression of target genes in a variety of organisms and cell types such as worms, fruit flies and plants (Fire, 1999).

RNAi technology which was discovered in 1998 as a tool to 'knock down' the expression of any gene in vertebrate cells has revolutionized the strategy to assess the function of a gene. A study involving injection of double stranded RNA (dsRNA), as opposed to the sense or anti-sense strand alone into *Caenorhabditis elegans* (a soil nematode), resulted in great interference of endogenous gene activity (Fire et al., 1998). Since this discovery, RNAi using dsRNA molecules has become widely used tool to regulate gene expression and to reveal many cellular processes in several biological systems. Its prophylactic potential has far reaching implication in virtually controlling any disease. Furthermore, the

technique has been exploited extensively in biological research to identify gene functions.

The new technology of silencing of the expression of specific genes through RNAi is likely to play a major role in the future of bioscience including veterinary science. RNAi-mediated functional genomics is allowing the study of gene function in livestock species to a great extent. The RNAi technology can be applied to an array of important areas in the field of veterinary science. Here we provide in brief, the basics of RNAi technology and discuss its potential applications relevant to animal production and health.

RNA INTERFERENCE - MECHANISM OF FUNCTION

RNAi pathways are channeled by small RNAs that include siRNAs and microRNAs (miRNAs). These induce direct sequence-specific cleavage of perfectly complementary mRNAs and translational repression. Most appreciated is posttranscriptional gene silencing (PTGS) mechanism that functions to down-regulate the expression of a specific protein by degrading the protein specific mRNA (Bumcrot, et al., 2006). Upon introduction, the long dsRNAs enter an interference (RNAi) pathway. Though the approach of introducing long dsRNA (>30 base pairs) homologous to the target gene can induce gene silencing in *C. elegans* and *D. melanogaster,* it cannot be used in mammalian cells. This is because the presence of long dsRNA in mammalian cells induces interferon (IFN) response, which results in activation of dsRNA-dependent protein kinase R (PKR) and 2',5'-oligoadenylate synthetase (OAS), leading to non-specific inhibition of protein translation and eventual cell death. This limitation can be overcome by introduction of siRNAs, chemically synthesized 21-23 base-pair double-stranded RNA molecules that suppress the target gene with high specificity, circumventing the IFN response. Owing to non-availability of precise process of predicting effective siRNA and the variable potency of silencing, it may be necessary to evaluate two or more target specific siRNAs to

achieve significant suppression of a target gene in a mammalian cell. The process of RNA interference pathway (Fig. 1A) encompasses various sequential steps that include: (a) The 20-25 nucleotide long dsRNAs, called small interfering RNAs (siRNAs), assembles with endoribonuclease-containing complex known as RNA-induced silencing complex (RISC); (b) RISC then activates associated helicase enzymes such as Argonaute 2 (Ago 2) to unwind the siRNAs by leaving the antisense strand (guide strand) with the RISC, while sense strand of the duplex degraded by the enzymes in the cytoplasm; (c) Eventually the RISC guides the siRNA toward its perfectly complementary sequence-specific mRNA, cleaving the target mRNA bound by the siRNA strand; (d) The cleavage of targeted mRNA subsequently leads to degradation of the cleaved mRNA transcript by cellular nucleases; (e) The activated RISC complex proliferates to destroy copies of specific mRNA targets, amplifying siRNA-induced gene silencing. Small hairpin RNA (shRNA) processed by Dicer into small interfering RNA (siRNA) follow the above pathway. Typically, transiently transfected cells in cultures show gene silencing for three to five days (Fig.1B). Similarly, long endogenous primary miRNA transcripts (pri-miRNAs) that are transcribed by RNA polymerase II in the nucleus are processed by the RNase III enzyme Drosha into ~70 nucleotide long stem-loop structures called precursor miRNAs (pre-miRNAs). The dsRNA-binding protein exportin 5 then transports the pre-miRNA into the cytoplasm, where RNase III like enzyme dicer and its associated proteins process the pre-miRNA and load the ~22 nucleotide mature miRNA into RISC. The mature miRNA recognizes target sites in the 3' untranslated region (3' UTR) of mRNAs to direct translational inhibition and subsequent mRNA degradation. Introduction of perfect duplex siRNA sequences into pri-miRNA and pre-miRNA backbones, thus generating miRNA mimics trigger the more potent PTGS pathway of mRNA cleavage upon loading into RISC (Kim and Rossi, 2007).

Though above described PTGS mechanism is the most well-known RNAi pathway, another mechanism called transcriptional gene silencing (TGS) by siRNAs has also been reported in the nuclei of mammalian cells. TGS regulates gene expression through changes in chromatin. In response to exogenous, promoter-targeting siRNAs, some level of TGS and histone methylation has been shown to occur, leading to epigenetic gene silencing (Wassenegger, 2005).

DELIVERY OF siRNAs INTO CELLS

Efficient delivery of the siRNA to its intracellular site of action is very critical, to achieve its therapeutic potential. There are two approaches currently being explored (Aagaard and Rossi, 2007). First, in RNA based approach, chemically synthesized siRNAs are delivered to target cells as preformed 21 base duplexes. Direct use of siRNA effectors is simple and result in potent gene silencing, however their effect is transient. Although effective, modulating the tissue distribution of chemically modified siRNAs is a difficult task. This approach requires repeated administering, limiting its clinical application. Second strategy involves introduction of short hairpins (shRNA) encoding DNA that produce effector siRNAs by intracellular processing of longer RNA hairpin transcripts. The latter approach is primarily based on nuclear synthesis of short hairpin RNAs (shRNAs) that are transported to the cytoplasm via the miRNA machinery and are processed into siRNAs by Dicer. DNA based systems that express shRNA have become the accepted option for DNA-based gene silencing. The minimal shRNA expression system includes a promoter (such as PolIII) followed by at least 19 nucleotides of sense (or antisense) target sequence, a 4-10 base loop, the complementary target sequence and finally a stretch of at least four to six U's as a terminator. This approach can potentially be exploited for stable use in a gene therapy.

Delivery of chemically synthesized or in vitro transcribed siRNAs into cells is generally achieved by cationic liposome based strategies.

However, lipid based delivery has the rapid liver clearance when used in vivo. Usually, siRNA are complexed with liposomes in vitro and upon treating the cells with this complex, the siRNA containing vesicles are taken up via the endosomes and then released into the cytoplasm for their effect. Though DNA-based shRNAs can be incorporated with cationic liposomes for delivery, viral vector-based shRNA systems provide an efficient platform. In this context, lentiviral vectors carrying shRNA expression system represent an efficient system as they are capable of transducing somatic as well as germ cell line.

BENEFITS OF RNAi BASED THERAPEUTICS

RNA interference opens up a plethora of possibilities for targeted therapeutic applications against several diseases (Trehan et al., 2010). Major advantages of RNAi are summarized here:

(i) RNAi offers advantage of controlling target specificity because its interactions involve Watson-Crick base pairing. Prerequisite is the knowledge of mRNA sequence of the target protein to design antisense drugs. Hypothetically, RNAi should allow antisense mediated suppression of any disease associated gene.

(ii) Other antisense technologies like antisense oligonucleotides, show short lived effect and require achieving critical concentration in nucleus. In contrast, siRNA acts in the cytosol by stable incorporation into the RISC silencing complex, thus achieving prolonged gene silencing even with small concentration.

(iii) Like a drug, its amenability to chemical synthesis and chemical modification would be useful for designing therapeutics with minimum toxicity. Moreover, unlike the chemical drugs used mostly for symptomatic therapy, siRNA can prevent the disease by blocking the expression of disease-causing protein, thus targeting the root cause of a disease.

(iv) For diseases caused by the mutation in a single allele, a specific siRNA can target the disease-causing mutation leaving the normal allele intact.

(v) RNAi therapeutics could potentially be exploited as a prophylactic agent for epidemic diseases.

(vi) Diseases manifested by viral mutations can be targeted using a mixture of mutant-specific siRNA in a single delivery.

However, our knowledge of siRNA-design, target selection and appropriate delivery system is in its infant stage. More research is necessary to identify siRNAs that are functional in sub-nanomolar amounts. This requires continued efforts of researchers, industrial partners and end users for best exploitation of the technology.

PROSPECTIVE APPLICATIONS OF RNAi IN ANIMAL SCIENCE

Recent research progress in siRNA delivery has opened a new arena for its potential therapeutic applications. Experiments using laboratory animals like mice, and primates like Chimpanzees, have demonstrated the proof of principle of safe systemic delivery of siRNA, for therapeutic purposes. Various experimented as well as potential applications of this novel technology are given here.

1) Gene function analysis

The technology of RNA interference has opened novel and simple ways to decipher the function of independent genes. In recent years, the tool has found wide application in the process of establishing the functions of several thousands of genes in a wide variety of organisms (Cullen and Arndt, 2005). Genome-wide RNAi libraries used in model organisms and mammalian cells has significantly enhanced the speed of functional genomics, dissection of signaling pathway analysis, and discovering of novel therapeutic targets, cellular processes and complex phenotypes.

RNAi know-how is contributing immensely in assigning functions to those genes for which we know the sequence, through loss-of-function analyses. RNAi screens targeting a subset of genes or genome-wide libraries are being applied in mammalian cells and animal models, to determine the function of individual genes as well as to identify interactions between genes and biological pathways. Thus, use of RNAi to study the functions of individual genes in mammalian cells has become a common practice in recent years. These studies in mammalian cells have demonstrated their potential for dissecting complex biological processes, giving a new direction to life science research.

2) Genetic diseases

Using RNAi technology, it is possible to turn off any disease manifestation due to monogenic defect, as it only requires the cleaving of one particular gene (Woodhouse, 2006). Polygenic diseases are not amenable by this process for now. However, future research might make it possible to tackle polygenic diseases, by taking each part of the disease and silencing them one by one. Examples of monogenic diseases include Overo Lethal White Foal Syndrome and epitheliogenesis imperfecta of equines. In the former, there is a lack of nerves in the distal portion of the large intestine affecting Paint and Pinto horses. In epitheliogenesis imperfecta, there is blistering of the skin and mouth epithelia and sloughing of the hooves, affecting American saddlebreds and Belgian Draft. Similarly, RNAi could be helpful in curing alpha 1-antitrypsin deficiency and exocrine pancreatic insufficiency, which are monogenic diseases of dogs (Gilbert, 2009).

3) Improving productivity

RNAi could be used for suppression of endogenous genes that regulate production traits. Application of this strategy in livestock species would provide a valuable opportunity for improving economically important traits. In this pursuit, scientists have demonstrated in a mouse model that by regulating the level of myostatin, using RNAi technology, it is possible to increase muscle mass (Magee et al., 2006). This approach could potentially be applied in meat purpose livestock species. Interestingly, the RNAi allows both constitutive as well as inducible systems that provide control over regulation of a particular target. Through intensive research using tools of RNAi and its delivery into embryos, in the near future, we may see transgenic founder animals

that exhibit phenotype of enhanced food or wool/fiber production, or show resistance to disease.

4) Infectious diseases

Infectious diseases like foot-and-mouth disease (FMD) and peste-des-petits-ruminants (PPR) cause staggering loss to farmers and to a nation as a whole. Application of RNA-induced silencing of in vivo replication of such infectious agents would make a significant contribution to live stock health. Research using mouse model has shown that siRNA treatment after FMDV infection gave effective viral inhibition, resulting in high survival rates in suckling mice following challenge (Kim et al., 2008). Thus RNAi could enhance antiviral effects. RNAi based therapeutics can be applied to target the window between initial infection and virus transmission for effective disease control in a herd. Furthermore, lentiviral delivery of shRNA into the brain of scrapie-infected mice reduced the level of prion protein, extending the lifespan of treated mice (Pfeifer, 2006). Furthermore, inclusion of motif such as 5'-UGUGU- 3' at 5' end of sense strand of siRNA has been shown to have innate antiviral immune-stimulatory effect in chickens (Villanueva et al., 2011; Stewart et al., 2011). Thus use of RNAi shows considerable promise for targeting infectious diseases. Though research in this endeavor is in its infancy, we may soon witness validated RNAi strategies making a reality in livestock species, thus defending animal resources against infectious diseases.

5) Cancer

Cancer related morbidity and mortality among pet animals like dogs is a growing concern. In cancers, there will be abnormally high expression of oncogenes. Knocking down of specific oncogene expression using RNAi machinery may help in development of broad spectrum therapeutics of cancers. RNAi is potentially useful therapeutic strategy against cancer, for it enables the specific silencing of gene targets involved in cancer progression. Targeted down regulation of various genes, including oncogenes/anti-apoptotic molecules, telomerase, growth factor receptor

genes and signaling molecules, may become a suitable approach for molecular therapeutics. Using a lentiviral expression construct, targeting the RNA component of canine telomerase was shown to be effective at inhibiting telomerase, highlighting its potential application in curing canine haemangiosarcoma (Lund et al., 2008)

LIMITATIONS OF RNAi

The RNAi technology has definitely created a new wave in functional genomics and gene therapeutics. However, there are some stumbling blocks in exploiting this technology to its full potential. Major concerns are off-target effects that might affect in vivo safety, non-specific type-I interferon responses affecting body's normal protein turnover, and non-availability of effective tools for in vivo delivery. In viral infections it may be hard to ensure that RNAi cleaves every single viral RNA, thus necessitating for its repeated administration. Furthermore, if there are multiple mutations in the target sequence, it may be difficult to identify the siRNA required to inhibit the mutated gene.

CONCLUSION

RNAi technology is finding wide spread applications across various fields of biological science. It has given a new dimension to functional genomics and gene therapeutics. The value of potential applications of RNAi is being recognized in veterinary science too. Hopefully in the near future RNAi therapeutics will provide more amenable strategy to control a disease and intervene in disease pathogenesis. Continued research and development in this area will likely make the potentials into reality, thus benefitting animal health and production.

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FIGURES

Fig.1: (A) Schematic representation of mechanism of RNAi pathway. The siRNA interacts with RNA-induced silencing protein complex, to cleave target mRNA. A shRNA after action of Dicer enters a similar pathway. (B) Western blot showing inhibition of RelA protein expression in HeLa cells transfected transiently with RelA-specific siRNA; NS, nonspecific siRNA (Source: Basagoudanavar et al., 2011).