

MEETING REVIEW

RNAi at Oxford

Martin M Fabani

Medical Research Council, Laboratory of Molecular Biology, Cambridge CB2 0QH, UK, *Email:* mfabani@mrc-lmb.cam.ac.uk, *Tel:* + 44 (0)1223 402206, *Fax:* +44 (0)1223 402070

Journal of RNAi and Gene Silencing (2007), 3(1), 220-222

© Copyright The Author

Almost half a century ago Watson and Crick proposed the central dogma of molecular biology, that genetic information flows from DNA to RNA and then to proteins. However, we now know that RNA does much more in the cell than being a mere intermediate carrier of information. Small non-coding RNAs – siRNA, microRNA and snRNA – play a vital role in the cell's homeostasis. These short RNA molecules can control events such as cellular defence against pathogen infection, post-transcriptional gene expression regulation and RNA splicing. In this second Oxford RNAi meeting, *RNAi2007: The expanding role of small RNAs*, held at St. Anne's College, Oxford, UK, a blend of participants from both industry and academia presented and discussed the latest advances on understanding the roles of these small RNA molecules as well as their applications in biology and medicine. Some of these developments and topics represented during the conference are summarized below.

REGULATION OF MICRORNA EXPRESSION

While some mammalian microRNAs are ubiquitously expressed, many are tissue-specific. It is accepted that this differential expression pattern might be due to regulation at the transcriptional level (by regulatory sequences affecting promoters or by differential expression of the genes containing the intronic microRNA sequence). Javier Martinez (Institute of Molecular Biotechnology, Vienna, Austria) proposed an alternative mechanism of regulation of microRNA expression in mammalian cells, by which a tissue-specific microRNA is regulated post-transcriptionally, possibly by the action of an inhibitor of pre-microRNA maturation. The proposal is based on the observation that although the mature miR138 is only detected in brain tissue, its pre-miR precursor is found in many other tissues. Although it is possible to speculate that this type of regulation is based on differential export of a precursor into the cytoplasm it was found that this is not the case, and that regulation is specific at a stage between cytoplasmic export and Dicer processing. It is very interesting that such pre-miRNA inhibitor is sequence-specific, since it did not appear to affect maturation of other microRNAs.

miRNAs IN DEVELOPMENT AND DISEASE

Since the discovery of the first miRNA, *lin-4*, and its putative mRNA target, *lin-14*, many advances in RNAi research have involved work in *C. elegans*. In order to increase our understanding of microRNA function, Erik Miska (University of Cambridge, Cambridge, UK) reported the first comprehensive generation and analysis of loss-of-function mutations of almost all experimentally validated microRNAs in the worm. Apart from being able to suggest a few novel miRNA functions, this study highlighted, rather surprisingly, that very few of these microRNAs knockouts displayed viability or developmental phenotypes in the propagation conditions tested. The development of multiple knockouts that tackle miRNA redundancy, or sensitive assay conditions to detect slight phenotypic alterations may be important in understanding the mechanisms by which miRNA networks control gene expression.

Following on from *RNAi2006's* Keynote lecture by Professor Ronald Plasterk, Wigard Kloosterman (Hubrecht Laboratory, Utrecht, The Netherlands) presented his latest research on miRNA expression profiles in zebrafish. Using Exiqon's LNA-based probe technology (Exiqon, Denmark), it was possible to map the expression profile in both the developing and adult zebrafish brain, highlighting the importance of such studies for providing an understanding of aspects related to development that are under microRNA regulation. In contrast to Eric Miska's approach in determination of miRNA function by generation of loss-of-function mutants, Kloosterman also presented an alternative method, based on transfection of antisense oligonucleotides. By microinjection of morpholino oligonucleotides into embryos they were able to determine the role of a particular miRNA in pancreatic islet development.

It has been shown that miRNAs also play fundamental roles in disease. In the context of lung disease, in particular asthma and COPD (chronic obstructive pulmonary disease), Mark Lindsay (Imperial College, London, UK) presented advances in the study of the relationship between

miRNA expression and lung inflammation. Detailed studies of miRNA expression profiles following activation of the innate immune response in cell and animal models made possible the identification of various miRNAs that are up-regulated upon pro-inflammatory stimulation. MiR-223 up-regulation specifically in the lung epithelium was validated by in-situ hybridisation and RT-PCR. Interestingly, this differential miRNA expression profile seems consistent with a negative feedback-type of regulation of the immune response.

Andrei Thomas-Tikhonenko (Philadelphia, USA) presented his work to investigate the complex interplay between c-Myc and the miR-17-92, which appears to have both tumour suppressor and oncogenic properties. Overexpression of Myc in p53 null colonocytes elevated miR-17-92 cluster expression, whilst retroviral transduction with a miR17-92 cluster to an extent mimicked vascularisation and growth stimulated by myc overexpression. Microarray analysis indicated that some myc-overexpressing tumours also demonstrate down regulation of miRNAs, including miR-150 and miR-195. Restoration of expression of these miRNAs in an *in vivo* selection model appeared to dramatically limit neoplastic growth relative to control miRNAs. Understanding the capacity of miRNAs for tumour suppression, and their role in oncogenic transformation is itself one of the most exciting growth areas of RNAi research.

siRNA DESIGN AND OFF-TARGET EFFECTS

siRNA design is still the subject of some debate. For example, the GC content, the presence/absence of overhangs, the accessibility of target sites and the absence of internal repeats are some of the typical parameters critical for efficient siRNA activity. Volker Patzel (Max Planck Institute, Berlin, Germany) presented work on the role of the antisense strand secondary structure for effective RNAi. Using an *in-silico* prediction algorithm he presented data that suggested that RNA structures and not the duplex free-energy profile nor the specific mRNA target are a strong influence on siRNA activity. Unstructured guide strands mediated the strongest gene silencing while those with paired ends were inactive. In addition, A to G and C to U base exchanges turned inactive into active siRNA sequences by creation of wobble base-pairs with the intended target, increasing the possible number of complementary guide strands by three orders of magnitude. The role of secondary structure in the stability of a duplex both prior to, and after, RISC loading is perhaps yet to be fully resolved.

In a different approach for evoking a strong RNAi response, this year's keynote speaker, Professor John J Rossi (Beckman Research Institute of the City of Hope, California, USA) reported on the application of Dicer-substrate siRNAs (D-siRNAs), which provide superior target suppression compared to standard siRNAs, possibly by a more direct interaction with RISC by the Dicer/siRNA complex. D-siRNAs hydrodynamically injected into mice demonstrated enhanced silencing of a luciferase target relative to 21-mer siRNAs. Given the discovery of multiple small RNA species in plant and animal cells that range

from 20-28nt in length, a more detailed characterisation of the specific cleavage activity of D-siRNAs against a target would be of some interest in understanding their enhanced efficacy over shorter chemically synthesised RNAs.

A practical aspect that needs consideration when designing RNAi studies is the minimisation of possible off-target effects. These are responsible for the loss of specificity in RNAi experiments when complementarity between an siRNA and random mRNA sequences lead to knockdown of untargeted genes. Dmitry Samarsky (ThermoFisher/Dharmacon, USA) presented the approach taken by Dharmacon for the design of efficient, sequence-specific siRNAs while diminishing off-target effects to a minimum. This approach is based on pooling rationally designed siRNAs, which include the addition of chemical modifications for 'inactivation' of passenger strands. siRNA pooling allows a low concentration of individual siRNAs – and so minimizes off-target effects of individual sequences while maintaining effective knockdown of the target mRNA.

Dimitry Samarsky also spoke of Dharmacon's commitment to standardisation of high-throughput applications using RNAi and the identification of the critical parameters that may distort the outcome of gene silencing studies. The data presented made clear that parameters such as assay design, use of controls, quality of reagents and experimenter experience have a significant impact on the experimental outcome. Careful implementation of guidelines like MIARE (minimum information of an RNAi experiment; <http://www.miare.org>) is of great importance in ensuring that high-throughput screens fulfil their potential in data generation.

siRNA IN THERAPY

With HIV/AIDS being a major public health problem worldwide and with millions of people currently infected, it is not surprising that much effort is put into RNAi as a new strategy for development of anti-HIV therapeutics. One such strategy is based on stable expression of antiviral shRNA by lentiviral vectors, as presented by Professor Ben Berkhout (University of Amsterdam, The Netherlands). Although these vectors provide very strong antiviral effects in the short term, viral escape remains a major problem. It was found that viruses escape not only by developing target deletions but also by creation of point mutations that alter the target RNA structure and hence the sensitivity for RNAi. One possible solution seems to be the use of multiple shRNAs that target conserved sites in essential viral genes. Professor Berkhout described successful inhibition of virus escape in cell culture using a three shRNA containing vector, but also reported problems with titre and recombination events when constructing vectors that express multiple stable hairpins from the same promoter. Methods employing alternative promoters may be required to optimize multiple hairpin delivery vectors.

Keynote speaker Professor John J Rossi also emphasised the problems associated with viral escape, and the advantages of using combinatorial approaches, for example, by combining a highly effective anti-HIV shRNA with an

aptamer directed to a virus-coded surface protein, for specific cell targeting. The cost-effective delivery of such approaches remains a major obstacle; nucleic acid based therapies possess an advantage in that they may be tailored to highly conserved viral sequences or different HIV strains, but a means of effective delivery and of satisfying the safety requirements of clinical trials (both in original form, and after minor sequence modifications) are equally important issues to address. A number of highly promising methodologies exist within the field, but a detailed clinical rationale for their application is necessary to ensure that anti-viral RNAi can benefit patients.

In a pain research setting, the targeting of the vanilloid receptor TRPV1, Jens Kurreck (Free University Berlin, Germany) presented comparative studies of various gene-targeting approaches for target validation: antisense oligonucleotides, siRNA and shRNA. A single intrathecal bolus injection of less than 1µg siRNA was sufficient to achieve a significant analgesic affect in mononeuropathic rats (compared with a still less effective twice daily, fifty-fold higher dose of phosphorothioate oligonucleotides). The study not only stressed the superior efficacy of siRNA over standard phosphorothioate oligonucleotides but then highlighted the advantages of transgenic animals expressing shRNAs for continuous target knockdown, allowing for more detailed investigation of the corresponding gene function.

Ines Royaux (Janssen Pharmaceutica NV, Belgium) described a highly efficient method for *in vitro* and *in vivo* gene silencing of TRPV1 expression using replication-defective herpes-simplex viral vectors (HSV-1), for delivery of shRNA targeting neuronal and non-neuronal cells. This study demonstrated the promise of HSV-1 vectors for the study of nociceptive processes and target validation in pain research. It also raised the interesting check that care must be taken when using vectors derived from viruses present within the patient population, that the potential for

recombination between replication deficient therapeutic viruses and wild-type strains is thoroughly understood.

siRNA TRAFFICKING AND LOCALIZATION

One of the main hurdles for a successful RNAi outcome is obtaining sufficient delivery of siRNA into the cell type of interest. Even then, delivery into cells might not be enough for a measurable biological output. Prof. Georg Sczakiel (University of Lubeck, Germany) discussed his interesting findings on cell uptake when siRNA was co-incubated with phosphorothioate oligonucleotides. The main observation was that although a relatively large number of siRNA molecules can be delivered into the cells only a minor fraction of them is available to interact with the cellular RNAi machinery. Most siRNA molecules are retained in cellular compartments from which release is minimal. It is also apparent, however, that multiple pathways for siRNA entry to cells may exist. From this perspective, efforts into development of constructs with superior compartment release properties is of great importance, but also the investigation of means to bypass sequestration uptake and provide a more biologically active route of siRNA into cells deserves greater attention.

CONCLUDING REMARKS

RNAi2007 provided a great opportunity to witness the latest advances in the RNAi area, with examples of fundamental research in the biology and mechanism of RNAi and the current development of RNAi tools for therapeutic applications. St Annes College Oxford provided an convivial atmosphere for discussion between speakers and delegates drawn from many parts of the world. We look forward to the next meeting and further advances in the understanding the biology and applications of small RNAs.

Edited by Graeme Doran