MEETING REVIEW

RNAi at Oxford

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The third annual RNAi conference, *RNAi2008: Functions and Applications of Non-coding RNAs*, held at St Anne's College, Oxford, UK (13-14 March 2008), afforded the opportunity for participants from both academia and industry to convene and discuss recent work on the molecular mechanisms of RNA-based gene silencing as well as recent advances in harnessing RNAi for research and therapeutics. The conference featured a stimulating series of talks from experts in the field, and research posters were displayed for the full duration of the conference. In addition, researchers had the chance to learn about latest commercial products through a trade exhibition, featuring companies including Exiqon, Thermo Scientific, Integrated DNA Technologies, Open Biosystems, Polyplus-transfection, Eurogentec, Amaxa, Panomics and Applied Biosystems.

THE MECHANISMS OF SILENCING

Active RISC (RNA-Induced Silencing Complex) is composed of single-stranded siRNA bound to an endonucleolytically active Argonaute protein, which is capable of capturing and silencing a native mRNA that complements the single-stranded siRNA. In many systems, endogenous siRNA are generated from a longer double-stranded RNA precursor by the ribonuclease action of Dicer. However, James Parker (University of Oxford, UK) highlighted that some PIWI domain proteins may bind small RNAs generated by Dicer independent mechanisms, and that structural variations in the 5' phosphate binding motif of the PIWI domain might dictate some aspects of small RNA binding specificity. This observation is further supported by the recent observation of 5'nt specificity of *Arabidopsis* Argonaute proteins (Mi et al, 2008).

Addressing the question of how RISC finds its target, Renée Schroeder (Max F Perutz Laboratories, Austria) used *in vitro* and *in vivo* assays to show that accessibility of the target site is directly linked to the efficiency with which it cleaves mRNA, demonstrating that RISC is unable to unfold structured RNA. Furthermore, she presented results suggesting that RISC interacts with single-stranded RNA in a non-specific manner and promotes annealing of the 5' 'seed' region of the siRNA (nucleotides 2-7 or 2-8) to the target mRNA. Ian Sudbery (The Wellcome Trust Sanger Institute) further emphasised the role of the 'seed' in understanding siRNA off-target effects. It will be of interest to understand the loading and distribution of small RNAs between Argonaute proteins in relation to how 5' and 3' regions of siRNA contribute to non-specific silencing in specific transfection conditions.

GENE SILENCING IN EPIGENETICS

The importance of non-coding RNAs (ncRNAs) in the control of epigenetic regulation has been well characterised in the case of Xist-induced X-chromosome inactivation in female cells. Denise Barlow (Viennna, Austria) emphasised the increasing importance of macro ncRNAs in the regulation of imprinted gene clusters. Using the Igf2r imprinted cluster, which is silenced by a macro ncRNA known as Air, she observed that repressive histone modifications (H3-K9m3 and H4-K20m3) on the silent parental Igf2r gene were localised to discrete regions in *cis* to Air transcription. Moreover, using a new embryonic stem cell *in vitro* differentiation model that recapitulates the onset of Igf2r silencing, she carried out truncation analysis on Air which indicates that transcription through the adjacent promoter was necessary for some aspects of silencing, and suggested a model of transcriptional interference.

piRNAs IN GERMLINE DEVELOPMENT

Piwi proteins are a subfamily of Argonaute proteins which are specifically expressed in germ cells in vertebrates,

where they bind piRNA and may modify chromatin structure. The *RNAi2008* keynote speaker, René Ketting, demonstrated expression of the zebrafish PIWI proteins, ZIWI and ZILI, in both the female and male gonads. The maternally transmitted ZIWI was localised only in the cytoplasm whereas ZILI could be found in the nucleus as well. He created PIWI null mutants and observed that lack of ZIWI resulted in loss of germ cells due to apoptosis after three weeks, whereas lack of ZILI led to a gradual, non-apoptotic loss of germ cells due to a block of germ cell differentiation. Although most piRNAs are derived from transposable elements (TEs), PIWI mutants did not experience increased activation of TEs. Deborah Bourc'his (Paris, France) further stressed the importance of silencing of TEs in the mammalian germ line by transcriptional and post-transcriptional mechanisms. She showed that transcriptional suppression occurs by the regulatory action of Dnmt3L, which stimulates DNA methylation by Dnmt3A and Dnmt3B. Dnmt3L null mutant mice lost spermatogonia and had wide-spread DNA methylation defects as well as reactivation of hypomethylated retrotransposons. Post-translational suppression includes RNA editing by the murine PIWI proteins MILI, MIWI and MIWI2, as well as degradation by RNAi. MIWI2 mutants displayed inappropriate activation of TEs, meiotic-progression defects in early prophase of meiosis I and progressive loss of germ cells with age. These studies suggest that mammalian PIWI proteins and piRNAs are important in gene silencing by regulating TEs through multiple mechanisms.

miRNA EXPRESSION IN DISEASE

Many reports indicate that transcription and/or maturation of miRNAs may be significantly altered in the disease state. Measuring these changes in activity therefore gives a deeper molecular understanding and potential therapeutic applications.

Andrei Thomas-Tikhonenko (University of Pennsylvania, USA) described a molecular model for the repression of the miR-15a/16-1 locus by the Pax5 transcription factor, which also cooperates with c-Myc to up-regulate key components of the B-cell receptor signalling pathway during B-lymphomagenesis. Interestingly, the miR-15a/16-1 locus seems to repress the expression of the oncogenic transcription factors Myb and Ets1, which in turn are needed for Pax5 function. Adam Baker (Exiqon, Denmark) detailed Exiquon's expansion into the field of cancer diagnostics, indicating novel LNA mercury microarrays that identify miRNA transcription profiles of secondary tumours, allow identification of the site of the primary tumour, and can give insight into the chemotherapy agents that might be best suited for treatment.

Thomas Thum (Würzburg, Germany) reported the exciting observation that cardiac miRNAs contribute to the transcriptional changes observed during heart failure by reactivation of fetal gene programs. Using an *in vivo* delivery model for cholesterol-bound miRNA antagonists (antagomirs), he was able to silence miR-21, a miRNA that is highly up-regulated during heart failure, in a mouse cardiac hypertrophy heart model, resulting in normalisation of many genes and graphic prevention of myocardial hypertrophy. This result generated the pertinent discussion as to whether it might also be possible to reverse tissue damage using this technology, as tissue damage is often significant before a patient presents to a clinician. The engineering of this technology into a treatment for aspects of heart disease may well illuminate therapeutic avenues for many diseases using anti-miRNA agents.

siRNAs IN THERAPY

Applications of RNAi-based gene silencing methods are promising in the treatment of many diseases. Roger Kaspar (TransDerm, USA) reported the development of a siRNA that specifically targets the keratin 6a (K6a) N171K single nucleotide mutation responsible for the dominant negative disorder pachyonychia congenita (PC). Using a PC mouse model which was obtained by engraftment of bioengineered skin equivalents derived from PC patients onto the backs of immuno-compromised mice he is currently testing various delivery methods of the siRNA to skin. Additionally he commented upon the progress of such siRNA towards clinical trials in Human subjects, and discussed the potential for topical application of siRNA. Matthew Wood (Oxford, UK) further described efforts towards an RNAi expression system for effective allele-specific silencing of target mutant repeat-expanded genes, which he used to selectively silence the mutant ataxin-7 transcript responsible for SCA7 (Spinocerebellar ataxia type 7) in cell culutre. Using this approach he was able to reduce mutant protein levels and restore the wild-type phenotype, and hopes to further validate this model *in vivo* via direct application to the retina.

siRNA DESIGN AND DELIVERY

Even though the use of RNAi is becoming established as a powerful tool to identify gene function and elucidate biological pathways, there are still some experimental challenges to face, such as the biological efficiency of the silencing RNA, its tissue-specific delivery and its specificity towards the target with low "off-target" effects. The optimisation of these processes was addressed by many of the *RNAi2008* speakers from both academic and commercial backgrounds, and their importance for the use of RNAi in research and in the clinic was repeatedly emphasised.

Improvements in siRNA action can be obtained by chemical modification. By introducing various types of 2'-modified locked nucleic acids (LNA) in both strands of the siRNA, and 1-2 nicks in the backbone of the sense strand, Jørgen Kjems (Aarhus, Denmark) devised synthetic sisiRNAs (small internally segmented interfering RNAs) that employ two 10-12nt sense strand oligos, and demonstrate enhanced serum-stability and a potent long-lasting gene-silencing effect whilst reducing the number of off-target effects. Moreover, sisiRNAs permit heavy modifications of the antisense strand which influences the pharmacokinetics of the sisiRNA significantly. Dmitry Samarsky (RXi Pharmaceuticals, USA) reported the design of another distinct class of siRNAs by RXi called rxRNA^{TM}, with improved stability and silencing characteristics. Gwen Fewell (Open Biosystems, USA) presented the newly

developed inducible genome-wide human letiviral shRNAmir library, known as TRIPZ, which allows temporal control over the gene knockdown. The pTRIPZ vector containing a TurboRFP reporter for shRNAmir expression showed high levels of induced knockdown with low basal expression.

A number of presentations focused on the efficient delivery of siRNA into target tissues using non-viral methods. Both Jørgen Kjems and Dmitry Samarsky demonstrated the successful use of nanoparticles in RNA delivery and their potential therapeutic applications in systemic and mucosal diseases, and Amyotrophic lateral sclerosis, respectively. Ludger Altrogge (Axama, Germany) presented the Nucleofector® 96-well Shuttle® System for effective transfection of RNA into various "difficult-to-transfect" cells including primary cells in high throughput applications. He reported successful siRNA screening experiments on human umbilical vein cells and Jurkat T-lymphocytes using this technology. Stephanie Urschel (Thermofisher/Dharmacon, Germany) described the novel Accell™ siRNA delivery method which allows specific target silencing in any cell type including primary, differentiated and "difficult-to-transfect" cells. The passive delivery results in higher cell viability due to elimination of toxicity, and the lack of non-specific lipid effects may reduce off-target effects.

Of note was the quite different approach by Justin Teissie (Toulouse, France) who made use of the reversible membrane permeabilisation resulting from electric field pulse application after a local injection of polyelectrolyte to deliver siRNA into muscle and tumours. Silencing could be achieved for two weeks in muscle and a few days in tumours. However, silencing duration could be prolonged to more than two months using plasmids coding for shRNA.

At previous RNAi conferences in Oxford, George Sczakiel (Universität zu Lübeck, Germany) reported an alternative pathway for RNA uptake by phosphorothioate-stimulated internalisation of siRNA into cells via the caveosomal pathway, and the limited efficacy resulting from intracellular trapping of a major fraction of the siRNA. The importance of these observations with respect to both therapeutic siRNA delivery and interpretation of biological silencing activity using different transfection reagents is now becoming clear, (e.g. Lytle et al, 2007; Vasudevan et al, 2007). This year, Sczakiel demonstrated that ECV-304 cells, despite taking up smaller amounts of siRNA compared to SKRC-35 cells, showed target suppression; whereas there was no measurable target suppression in SKRC-35 cells. He proposed that co-localization of siRNA and the human Argonaute protein, Ago2, in the vicinity of the rough ER, relates to the extent of target suppression. Thus, siRNA is recruited from the site of trapping to the site of action in cells which show biological efficacy. Moreover, he described a cell model with a siRNA-peptide conjugate, allowing increased target inhibition which could provide tools for further mechanical and biological studies.

Barbara Demeneix (Paris, France) described the optimisation of siRNA and shRNA delivery to the neural stem cell (NSC) niche in the mouse brain, the organ with the highest number of cell types and with limited accessibility. She was able to deliver siRNA to the NSC niche in newborn mouse brain using a combination of a cationic lipid (Jetsi™) with a neutral lipid (DOPE), as well as shRNA with a hybrid construct of a cationic polymer (polyethylenimine, PEI) with CMV-H1. Moreover, making use of INTERFERin® she delivered siRNA into the neurogenic zone of adult mice. With these approaches she addressed the roles of thyroid hormone signalling in the control of neural stem cell proliferation and identified genes and receptors implicated in their control.

RNAi IN GENE FUNCTION ANALYSIS

RNAi-based gene-specific knockdowns can also provide information on temporal and spatial control of gene function during development. Ester Stoeckli (University of Zürich, Switzerland) developed *in ovo* and *ex ovo* RNAi models to study the molecular mechanisms underlying the establishment of neural circuits. She identified Sonic Hedgehog (Shh) as a guidance cue for post-commissural axons which, during early stages of neural development, is involved in cell differentiation and patterning of the dorso-ventral axis of the spinal cord. Later in development, Shh acts as a chemo-attractant in parallel to Netri-1 guiding axons towards the floor plate, the ventral midline of the spinal cord. Interestingly, as soon as the axons crossed the midline Shh becomes a chemorepellent due to a receptor switch from Patched to Hip (Hedgehog-interacting protein).

Chris Lord (London, UK) proposed the use of genome-wide RNAi approaches to identify new targets for anti-cancer drugs as well as to modify uses of existing therapeutics. David Horn (London, UK) presented a newly developed p2T7^{TAblue} Tet-on conditional RNAi vector which can be used for a systematic knockdown analysis of gene function in the parasite Trypanosomatid.

CONCLUDING REMARKS

The conference gave an opportunity to a number of speakers, poster presenters and exhibitioners from a wide range of scientific and commercial backgrounds to present their work and recent advances in RNAi research. It also created a forum for discussion of new research prospects and improvements in the applications of the technique. The fast-paced field of RNAi appears to be closer than ever to a successful therapeutic tool, but there are still technical hurdles to be overcome, and much to be understood about the role of ncRNAs. RNAi2008 inspired a great deal of excitement, and has provided a large amount of information from which attendees will benefit in many respects.

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