

## MEETING REVIEW

### RNAi2013: RNAi at Oxford

Laura A Mulcahy and David RF Carter\*

Oxford Brookes University, Faculty of Health and Life Sciences, Department of Biological and Medical Sciences. Gypsy Lane, Oxford, OX3 0BP, UK

\*Correspondence to: David Carter, Email: [dcarter@brookes.ac.uk](mailto:dcarter@brookes.ac.uk), Tel: +44 1865 484216, Fax: +44 1865 483242

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The eighth annual RNAi international conference and exhibition, RNAi2013 was hosted at St Hilda's College, Oxford, UK (19-21 March 2013), and provided a platform for congregation of researchers with both academic and industrial backgrounds to share and discuss their most recent work in the fast advancing field of RNAi. RNAi has been recognised as a fundamental method for functional genomic investigations and has great potential as a therapeutic intervention for several human diseases. RNA-induced gene expression inhibition mechanisms were discussed for both the benefit of research and clinical therapeutics. The conference conveyed an impressive series of presentations given by national and international RNAi research leaders. Additionally, research posters were exhibited for the entirety of the conference. Furthermore, technology workshops were provided by Sigma-Aldrich, Eupheria Biotech GmbH and Carl Zeiss enabling conference attendees to learn about their most advanced RNAi expertise. These companies also participated in a trade exhibition along with Exiqon to promote the latest commercial RNAi products.

#### RNAi DEVELOPMENTS

Professor Kaz Taira (University of Tokyo, Japan) was invited as a special guest speaker and opened the conference with a presentation describing the recent discovery that siRNA strand antagonism is the major cause of reduced siRNA potency when compared with the potency of shRNA. RNAi activity of siRNA is reduced compared to shRNA due to the sense RNA strand negatively regulating RNAi. By modifying the relative sense and antisense components of duplex siRNA during expression, improved potency of siRNA in target gene RNAi was achieved (Jin et al, 2012). Furthermore, Taira identified DEAD-box helicase 3 (DDX3) using a short hairpin RNA-expression library, as a fundamental component of the RNAi pathway. DDX3 was found to co-localise with Argonaut2 (Kasim et al, 2013).

Dr Laure-Alix Clerbaux (Université catholique de Louvain) elucidated the mechanism used by cells to maintain cholesterol

metabolism. Clerbaux explained that the primary transcript of sterol regulatory element binding protein (SREBP) 2 contains not only the genetic code for a sterol sensing transcription factor which promotes transcription of numerous genes involved in cholesterol and fatty acid synthesis, but also holds the highly conserved intronic miR-33. miR-33 was shown to target and successfully down-regulate activity of the cholesterol export pump, ABCA1, which considerably reduced cellular cholesterol export and hence increased cellular cholesterol concentration. The SREBP is triggered during low cellular cholesterol levels; it was revealed therefore, that miR-33 interacts with the gene in which it is located to maintain normal cellular cholesterol levels (Gerin et al, 2010).

#### NANOPARTICLE DELIVERY OF RNAi THERAPEUTICS

Due to their sensitivity to enzymatic degradation, large negative charge, high molecular weight, and rapid plasma/renal clearance, ncRNA therapeutics are notoriously difficult to deliver into mammalian cells. Hence, successful delivery of ncRNA is a great challenge within the RNAi therapeutic field. In an attempt to overcome these issues, numerous non-viral nanoparticles administered by systemic intravenous injection have been developed recently to enable therapeutic use of synthetic ncRNA for a number of RNAi applications.

Dr Klaus Giese (Silence Therapeutics, Germany) described Atu027 which employs a novel method for small interfering RNA (siRNA) delivery involving siRNA cationically complexed with liposomal nanoparticles which specifically down regulate protein kinase N3 gene expression in the vascular endothelium. This protein target has promising effects in terms of inhibition of tumour progression through lymph node metastasis and angiogenesis. Phase I clinical trial results are promising. Atu027 is well tolerated in patients with advanced solid tumours; plasma samples showed dose related increase in circulating siRNA antisense strand levels (Strumberg et al, 2012).

Dr Raymond Schiffelers (University Medical Centre Utrecht, the Netherlands) discussed biodegradable ncRNA carriers which are formulated by electrostatic interaction between ncRNA and nanoparticles. To avoid opsonisation and clearance by macrophages the particle's surface is covered by a flexible, ligand-displaying, hydrophilic polymer layer of poly(ethylene)glycol. Using this technology integrin targeted anti-angiogenic miRNAs were administered to cancerous mouse cells resulting in hindered tumour growth through inhibition of tumour vascularisation (Coimbra et al, 2012).

Professor Gilles Divita (Centre National de la Recherche Scientifique, France) described NANOVEPEP technology which involves nanoparticle self-assembly around the siRNA, aided by electrostatic and hydrophobic interactions between short amphipathic CADY peptides. NANOVEPEP technology is particularly advantageous since delivery of siRNAs into specific cell targets is possible without initiating an inflammatory response (Konate et al, 2012).

### ANTI-CANCER RNAi THERAPEUTICS

Since cancer is caused by accumulation of genetic abnormalities, nucleic acid medicines are an obvious therapeutic choice and are predicted to have the most potential for success. Many nucleic acid therapies are currently being developed; a selection of the latest RNAi associated cancer therapies were presented at RNAi2013.

Chemoresistance is a major limitation of drugs currently used to treat cancer and results in significantly reduced survival rates. Dr David Carter (Oxford Brookes University, UK) described characterisation of miRNA levels in ovarian cancer cell lines that are resistant or sensitive to cisplatin treatment. Through loss or gain of function experimentation a miRNA and coding gene pair were identified that can contribute to cisplatin resistance during carcinogenesis.

Professor Achim Aigner (University of Leipzig, Germany) demonstrated that Pim-1 activity, previously linked with poor prognosis, is fundamental to signal transduction in colon carcinoma and glioblastoma cells and is regulated by miR-15b and miR-33a. Knockdown resulted in anti-tumour effects; treated cells also became more sensitised to 5-FU (Thomas et al, 2012).

Dr Nigar Babae (University Medical Centre Utrecht, the Netherlands) presented results that identified a novel anti-angiogenic miRNA using a lentiviral miRNA expression library. Following local delivery by electroporation in a Neuro2A mouse tumour model, tumour growth rate diminished by 50% and tumour vascularisation was prevented. The identified miRNA was discovered to regulate expression of approximately 2500 genes; two of these genes were further investigated due to their role in vascularisation.

Hai-Feng Zhang (Shantou University, China and University of Alberta, Canada) showed that loss of miR-200b is possible marker of reduced survival, lymph node metastasis and advanced clinical stage in esophageal squamous cell carcinoma (ESCC). Kindlin-2 was found to participate in epigenetic repression of the tumour suppressor miR-200b by inducing CpG island hypermethylation resulting in

increased ESCC invasion and tumour formation (Yu et al, in press).

### ANTI-VIRAL RNAi THERAPEUTICS

The most difficult challenge for anti-viral drug design is the prevention of drug-resistant strains. In order to prevent such occurrences, many strategies are currently being developed; with the most sophisticated methods involving RNAi.

Dr Susanna Obad (Santaris Pharma, Denmark) described the development of miravirsin, a drug to treat chronic hepatitis C virus (HCV) infection. Miravirsin is a locked nucleic acid (LNA) and DNA mixmer oligonucleotide that targets miR-122. miR-122 acts as a liver specific host factor during HCV infection. After success in chronically HCV infected chimpanzees (Elmén et al, 2008), the first clinical trial involving miRNA inhibition was launched. Phase IIa clinical trials have recently been completed which concluded that when used to treat patients suffering with chronic HCV genotype 1 infection, miravirsin exhibited extended dose-dependent decrease in HCV RNA levels with no evidence of resistant viral strains (Janssen et al, in press). These results are promising for the development of LNA-antimiR oligonucleotides for targeting of additional miRNAs that contribute to pathogenesis in humans.

Dr Patrick Lu (Siranomics, USA) described the siRNA antidote for influenza A viruses H5N1 (avian) and H1N1 (swine) which target conserved regions of the viral genome in an effort to prevent arise of drug resistant viral mutants. Delivery of "potency enhancing motif" (PEM)-modified siRNA inhibitors increased therapeutic and prophylactic siRNA potencies. Antiviral activity was detected in mouse lungs after infection with a 10x lethal dose of H5N1.

Professor Jens Kurreck (University of Technology Berlin, Germany) described siRNA and coxsackie-adenovirus receptor (CAR) combination therapy developed to treat coxsackievirus B3 which improved heart function in the mouse myocarditis model (Werk et al, 2009; Fechner et al, 2011).

### RNAi TREATMENT FOR DISEASE

DMD-associated miRNAs (dystromiRs) are potential biomarkers for Duchenne Muscular Dystrophy (DMD). Dr Tom Roberts (University of Oxford, UK and the Scripps Research Institute, USA) showed that differing levels of dystromiRs were detected between different types of skeletal muscle (Roberts et al, 2012). Additional results suggest that dystromiRs are transported in the circulation bound to protein/lipoprotein complexes to protect them from nuclease activity.

Dr Kathia Zaleta-Rivera (Stanford University, USA) discussed allele specific oligonucleotide treatment for hypertrophic cardiomyopathy (HCM) anticipated to salvage expression of the wild-type allele enabling recovery of cardiomyocyte functionality. siRNA and short hairpin (shRNA) treatments were identified for 2 mutations associated with HCM. Models for each mutation have been developed to measure changes in cell contractility and force generation after treatment with shRNAs.

Professor Paul Holvoet (University of Leuven, Belgium) described the relationship between obesity and atherosclerosis identified by shared expression of a collection of miRNAs. The identified miRNAs were found to regulate adipocyte differentiation, oxidative stress, inflammation, and angiogenesis in adipose tissues of obese patients and vascular tissues of atherosclerosis patients. Repression of specific miRNAs was discovered to induce oxidative stress and inflammation. To complete the vicious circle, reduced levels of these miRNAs were recognised to contribute to development of obesity a condition that in itself increases the risk of development of atherosclerosis. The discovery of miRNA containing monocyte-derived micro-particles that participate in intercellular communication within and between adipose and atherosclerotic vascular tissues, were also discussed (Hulsmans et al, 2011).

### OLIGONUCLEOTIDES IN RNAi

The conference keynote speaker Dr Mike Gait (MRC Laboratory of Molecular Biology, Cambridge, UK) presented recent work involving development of peptide nucleic acids (PNA) anti-miRs which rapidly inhibit miR-122 in liver cells without the participation of transfection agents (Torres et al, 2012). Following uptake anti-miRs target miRNAs within or in relation to the endosomal compartment and strong dose dependent miR-122 inhibition has been observed for phosphorothioated oligonucleotide counterparts (Torres et al, 2011). These results are promising for the development of both diagnostic markers and anti-miR therapeutics for a wide range of genetic disorders.

Dr Dmitry Samarsky (RiboBio, China) reported the design of a sophisticated type of RNAi molecule composed of single chain oligonucleotide which has both 5' and 3' targeting regions which mediate self-dimerisation with partial complementarity. The loop containing-RNA duplex molecule has been shown to enter and activate RISC and is promising for the future design of single-stranded oligonucleotide therapies (Lapierre et al, 2011).

Dr Jonathan Watts (University of Southampton, UK) showed that combining a DNA analogue (2'F-ANA) with rigid RNA analogues (2'F-RNA and/or LNA) in siRNA duplexes increases therapeutic potency and effectively induces gene silencing through interaction with mRNA. Modified duplexes with potency equivalent to native siRNA were identified and were far less immunogenic (Deleavey et al, 2010). In addition, Watts elucidated and discussed the cause of increased binding affinity following ribonucleic sugar fluorination at the 2' position.

### MINIMALISATION OF OFF-TARGET EFFECTS

One of the greatest limitations of siRNA-based gene silencing is the incidence of sequence-specific off-target effects. These adverse interactions are often not foreseen because siRNAs can induce gene silencing through association with regions of the genome with only partial complementarity. During these incidences the siRNA functions as a miRNA inhibiting gene expression by destabilising mRNA or blocking transcription. Therefore off-target interactions are a major consideration during siRNA design. Dr Michael

Hannus (Intana Bioscience GmbH, Germany) described a possible resolution which employs siPools which contain up to 60 specifically selected siRNA molecules. Within a siPool each siRNA is retained at a low concentration so off-target interactions are reduced to such a degree that they lie below the lower limit of detection.

### TECHNICAL FOCUS

In order for the ever-evolving field of RNAi to grow, the most advanced and innovative technology needs to be developed and utilised by researchers worldwide. Presentations and exhibitions by Sigma-Aldrich, Eupheria Biotech GmbH and Carl Zeiss described their latest products and/or techniques that may assist scientists in cutting-edge research.

Dr Steven Thompson (Sigma-Aldrich, UK) described CompoZr® Zinc Finger Nucleases (ZFNs) which facilitate genome manipulation through site-specific mutagenesis by generating double-strand breaks in DNA. As a result the cell's DNA repair mechanisms are exploited to include gene knockouts, integrations or modifications (Hansen et al, 2012). Dr Christina Smith (Sigma-Aldrich, USA) described a range of products for the analysis and manipulation of miRNAs.

Dr Mirko Theis (Eupheria Biotech GmbH, Germany) promoted Eupheria Biotech's latest endoribonuclease-prepared siRNA (esiRNA) products which target long non-coding transcripts for RNAi loss of function screens. These multiple silencing triggers result in efficient gene silencing which is highly target specific and has lower off-target interactions than similar methods which employ single or pooled siRNAs (Chakraborty et al, 2012).

Dr Tom Quick (Carl Zeiss, UK) presented information about their PALM MicroBeam Laser micro-dissection for isolating high-purity tissue. The specimen of interest, typically a single cell, is isolated without contact, hence contamination of the sample is prevented and neighbouring tissues/cells remain unchanged. The genetic and proteomic material of both the specimen of interest and adjoining areas are sustained enabling further DNA, RNA and protein analysis (Micke et al, 2005). This technique can also be used to isolate live cells which can be successfully re-cultured.

### POOLED shRNA SCREENS

Highly efficient, adaptable and cost effective phenotypic loss-of-function RNAi screens that employ pooled complex lentiviral-based shRNA expression libraries allow synchronized screening of multiple transcripts to accurately determine sequences that participate in specific cellular mechanisms. Continual development and optimisation of these methods of RNAi screening is essential and supports progression of the RNAi research field.

Dr Annaleen Vermeulen (Thermo Fisher Scientific, USA) explained that technical reproducibility between PCR replicates in a pooled shRNA screen are significantly improved by ensuring amplification remains within the exponential phase and that the correct quantity of genomic DNA is used to sustain the average template copies per shRNA

used during library transduction. This enabled identification of higher reproducibility of biological replicates in screens with at least 500-fold shRNA representation (Strezoska et al, 2012).

Dr Paul Diehl (Cellecra, USA) described the in-house service offered by Cellecra where pooled shRNA screens are coupled with quantitative sequencing which enables accurate depiction of hairpin levels inside cells transduced with shRNA libraries. *In vitro* “drop-out” screens which identify critical functional genes and novel drug targets fundamental to cell growth and proliferation and positive selection screens which recognise genes that participate in specific cell signalling pathways were described (Tsuji et al, 2010).

## CONCLUDING REMARKS

Results discussed at RNAi2013 are very promising and strongly suggest that many common limitations of RNAi-based therapies including successful delivery of ncRNA and reduction of off-target effects could be overcome in the foreseeable future. Additionally, RNAi-based therapies have been developed to specifically target chemoresistant cancers, DMD, HCV and influenza, amongst many others, which shows great reassurance for development of personalised medicines; attendees were encouraged to continue their work in this field in order to achieve this objective. Data presented continue to provide hope that RNAi-based therapies will revolutionise future treatment of disease. Indeed, in the words of one speaker at the conference, RNAi looks set to become the next treatment modality.

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