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Expediting target identification and validation through RNAi

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RNA interference (RNAi) is an efficient post-transcriptional gene silencing mechanism that is induced by double-stranded RNA. Applications of RNAi have gained increasing attention since the groundbreaking discovery that small interfering RNA (siRNA) molecules can be used to inhibit gene expression in mammalian cells in a sequence-specific manner. Numerous meetings have recently been held in this field, but the organiser from EF International succeeded in bringing some of the leading academic scientists and company researchers together in London to present and discuss exciting new results. Major topics covered in the meeting included the recent progress in understanding the basic mechanism of RNAi, genome-wide RNAi-based screens for target discovery, and approaches to use RNAi for target validation in cell culture and in animal models. In addition, borders and caveats of the technology, such as off-target effects and a possible induction of the interferon response by siRNA, have been discussed intensively. The use of siRNAs can be regarded as a highly potent strategy to identify and validate new targets for therapeutic interventions against cancer, viral infections, chronic pain and other diseases. Finally, siRNAs themselves hold the promise to become therapeutic agents in the near future.

Keywords: cancer, infectious diseases, pain, RNA interference, small interfering RNAs, target identification, target validation

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1. Basics of RNAi

Chairman C Echeverri (Cenix Bioscience GmbH, Germany) opened the meeting by giving an introduction to the field of RNA interference (RNAi) as a highly efficient gene silencing phenomenon. RNAi was first described in *Caenorhabditis elegans* in 1998, and since then it has been employed by researchers working with the nematode or other lower eukaryotes such as *Drosophila melanogaster*. In these organisms, long double-stranded RNA (dsRNA) molecules can be used to silence gene expression in a sequence-specific manner. The dsRNAs are intracellularly processed by an RNase called dicer to give 21 – 23mer small interfering RNAs (siRNAs), which are subsequently incorporated into a protein complex referred to as the RNA-induced silencing complex (RISC). After the unwinding of the duplex RNA, the antisense strand presumably guides RISC to its target RNA, which is finally cleaved and further degraded by nucleases.

The use of RNAi in mammalian cells has been hampered for a long time by the fact that long dsRNAs induce the interferon response, which results in strong changes of gene expression and finally leads to apoptosis. These problems have been circumvented by T Tuschl's discovery that the tiny siRNAs can be used to obtain sequence-specific gene silencing in mammalian cells in the absence of an interferon response [1]. This finding triggered an enormous number of studies employing siRNAs as a tool to knock down gene expression and to study the resulting loss-of-function phenotypes.

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The year 2002 was characterised by unlimited optimism bringing many researchers and companies into this new field. The first successful applications of siRNA treatment in mice were published, and progress made in the field was elected to be the breakthrough of the year by *Science* magazine. In 2003, new, challenging tasks were addressed, such as large scale or even genome-wide screens with dsRNA or siRNA libraries, or the application of RNAi for medically relevant animal models. However, some critical findings have been published which indicate nonspecific side effects after siRNA treatment due to off-target regulation or induction of the interferon response. These topics were intensively discussed throughout the RNAi meeting in London.

J Martinez from the Tuschl lab (Rockefeller University, USA) presented recent progress in the understanding of the basic RNAi mechanism. Degradation of the target RNA has been demonstrated to begin with a single cleavage, the cleavage site being 10 nucleotides from the 5' end of the antisense strand. Furthermore, RISC was isolated from HeLa cells with biotinylated siRNA duplexes. A preliminary analysis of the purified complex revealed that human RISC contains two members of the argonaute protein family. The purified RISC can now be used to perform kinetic experiments and to analyse substrate requirements in more detail. Further experiments with modified siRNAs containing 4-thio uridine are in progress in order to identify specific interactions between RISC proteins and the siRNA by crosslinking.

2. RNAi-based genomic screens

Two types of RNAi-based screens to identify new therapeutic targets by the analysis of loss-of-function phenotypes can be distinguished: genome-wide screens are usually carried out with model organisms, whereas focused screens to address the functional role of a certain class of genes can be performed with human cells. C Echeverri (Cenix Bioscience GmbH) presented a genome-wide screen with long dsRNA molecules in *C. elegans*, which led to the discovery of genes that are involved in early embryogenesis. Homologues of these genes in the human genome are potential new targets for cancer therapy. Large scale screens in mammalian cells are more challenging, as expensive siRNAs have to be used and not all of these molecules silence target gene expression. Cenix, therefore, developed a new, experimentally tested algorithm to increase the hit rate for efficient siRNAs.

B Baum (Ludwig Institute for Cancer Research, UCL branch, UK) reported on a genome-wide screen in *D. melanogaster* to identify genes that are involved in cell morphology. Silencing of numerous genes resulted in interesting morphological phenotypes, and several genes could be grouped to define new pathways. A similar approach was presented by M Sunders (Devgen NV, Belgium) to identify genes involved in insulin resistance in type II diabetes and in obesity by genome-wide screening in *C. elegans*. Results from these screening campaigns have subsequently been validated in mammalian systems. Likewise, lead compounds that were identified by high-throughput screens with human cell lines could be further analysed in *C. elegans*. RNAi-based screens with model organisms are especially valuable because human orthologues exist for approximately two-thirds of the relevant genes.

Attempts to perform genetic screens directly in human cells were described by T Brummelkamp (Netherlands Cancer Institute, The Netherlands), who was among the first to develop vectors that can be used to express short hairpin RNAs (shRNAs), which are rapidly processed to give siRNAs in cells. A vector library has now been employed to inactivate 50 genes that encode de-ubiquitinating enzymes [2]. With this approach, the contribution of the familial cylindromatosis tumour suppressor gene (*CYLD*) to oncogenesis could be identified. Interestingly, aspirin derivatives were found to relieve this effect and are now being tested for therapeutic intervention in patients suffering from familial cylindromatosis. T Brummelkamp and his colleagues in the laboratory of R Bernards are currently generating a genome-wide library for human genes based on shRNA-expressing vectors.

M Janitz (Max-Planck-Institute for Molecular Genetics, Germany) described the development of RNAi transfection arrays that can be used for high-throughput loss-of-function studies in mammalian cells. Numerous siRNAs are spotted at high density in gelatine solution onto glass slides and are subsequently covered with a layer of adherent cells. After addition of a transfection reagent, only the cells on top of a spot take up an siRNA, and the resulting phenotypes can be further analysed.

3. RNAi for target validation

D Samarsky (Sequitur, Inc./Invitrogen, USA) addressed the problem of nonspecific side effects induced by siRNA. He demonstrated that chemically modified siRNAs named STEALTH RNAi™ avoid induction of the interferon response and have a lower toxicity than unmodified siRNA. STEALTH RNAi has also been shown to be more stable than unmodified siRNA and to possess an improved performance to inhibit tumour growth *in vivo*.

A Santel (Atugen AG, Germany) and some other speakers, including the author of this report, who employs gene silencing strategies for target validation in pain research, noted that conventionally used single-stranded antisense oligonucleotides can be used as an independent control to confirm results that have been obtained by siRNA treatment. In this context, it should be mentioned that antisense oligonucleotides containing recently developed modified nucleotides are significantly more potent and less toxic than first generation phosphorothioate oligonucleotides. Furthermore, researchers at Atugen developed an inducible shRNA expression vector that is only active in the presence of doxycycline [3]. In an orthotopic metastatic mouse model, shRNA targeting the phosphatidylinositol 3-kinase prevented the formation of metastases without inhibiting growth of the primary tumour.

D Lewis (Mirus Corporation, USA) and his co-workers were among the first to publish *in vivo* applications of RNAi [4]. Mirus focuses on the development of non-viral technologies for nucleic acid delivery. For *in vivo* applications, siRNAs are either delivered by hydrodynamic intravascular injection or complexed by recharged polymeric particles. For the former method, a large volume containing the nucleic acid is rapidly injected into the tail vein. Knockdown of a reporter gene has been observed in multiple organs, including liver, kidney, spleen, lung and pancreas. In contrast to this rather harsh method, recharged particles allow a low-pressure intravenous injection of siRNAs. Knockdown of target gene expression has been demonstrated in the lung as a proof-of-principle.

Most of the researchers at Sirna Therapeutics, USA, have previously worked for Ribozyme Pharmaceuticals and, therefore, possess long-term experience with therapeutic applications of RNA oligonucleotides. B Polisky (Sirna Therapeutics, USA) reported on the development of highly stable and active siRNAs consisting of modified nucleotides. At present, major targets at Sirna include the internal ribosome entry site of hepatitis C viruses and the vascular endothelial growth factor, which triggers neovascularisation leading to macular degeneration.

4. Nonspecific side effects of siRNA treatment

A major concern for researchers working in the RNAi field is the possibility of nonspecific side effects due to siRNA treatment. Off-target regulation as well as induction of the interferon response has recently been reported in the literature [5,6]. This topic has, therefore, been noted by several speakers and was also intensively addressed on a panel discussion. Researchers analyse nonspecific effects of siRNA treatment by expression profiling employing DNA arrays. Participants of the meeting agreed that off-target effects and interferon responses have to be taken seriously, but in most of the experiments, nonspecific side effects of siRNA treatment were found to be negligible. Chemical modification of siRNAs might further increase their specificity.

Additional unintended effects, however, might result from the recently discovered fact that siRNAs can act as microRNAs and vice versa. Unlike siRNAs, microRNAs do not induce cleavage of the target RNA, but silence gene expression by inhibition of translation. This mechanism of action cannot be detected on the mRNA level with DNA arrays, but has to be analysed by proteomic methods.

5. Expert opinion

Within only a few years, RNAi has become a widely used and powerful technique to study loss-of-function phenotypes. It has been denoted as the most revolutionary biological tool since the invention of the polymerase chain reaction. Genome-wide RNAi-based screens in model organisms have already led to the discovery of new target genes for therapeutic interventions. Libraries for similar approaches in human cells are currently being amassed. Furthermore, several examples for successful target validation with siRNAs in cell culture and in animal models have been demonstrated. Despite the great enthusiasm about the enormous potential of the new technology, most researchers take warning reports about off-target effects and interferon responses induced by siRNAs seriously. Fortunately, these nonspecific side effects have been found to be tolerable in many cases and can be further diminished by the use of chemically modified siRNAs. Therefore, therapeutic approaches making use of RNAi can be expected to be developed soon. Several companies have already announced clinical trials to start in the near future. If researchers have learned from the mistakes made in the antisense field, RNAi will definitively become a powerful tool for functional genomics as well as for the development of therapeutics. Perhaps the hope held by many researchers working in these fields, that this technology helps to expedite drug development, will soon be fulfilled. Theoretically, an siRNA has the potential to be a single molecule for target discovery, for target validation and as a therapeutic compound.

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