Functional gene analysis by RNA interference (RNAi)

Anne Rieger, Isabelle Guillemin, Hans Gerd Nothwang
Abt. Tierphysiologie, Universität Kaiserslautern, D-67653 Kaiserslautern, email: nothwang@rhrk.uni-kl.de

It is estimated that the human genome encompasses 30 000-50 000 protein coding genes with the function of most being unknown. Therefore, efficient methods are needed that allow for rapid and high throughput functional gene analysis. One of the most successful approach towards this goal consists in the loss-of-function analysis, where a specific gene is selectively knocked-out and the consequences of this silencing analysed on various levels. This often gives a strong clue to the likely physiological role of the respective gene. Recently, RNAi has been shown to be a very potent method for this approach, since it is highly specific, robust, and fast. Furthermore, latest development turned this approach into a low cost technique.

The importance of functional gene analysis

The analysis of the almost completely sequenced euchromatic part of the human genome revealed the presence of 30 000-50 000 protein encoding genes [1]. A similar gene number is also predicted for important model organisms such as mouse and rat, and even simpler organisms such as the fruitfly *Drosophila melanogaster* or the nematode *Caenorhabditis elegans* harbour still 14 000 and 19 000 genes, respectively. The precise function of most genes is thereby still unknown and one of the most challenging tasks in fundamental and biomedical research in unravelling the functions of these genes. This is not only important for a better understanding of the molecular basis of life but also for a better understanding of the pathogenesis of many diseases like tumour, blindness, deafness, or neurodegenerative disorders like Parkinson or Alzheimer disease. An improved prevention and/or treatment will crucially depend on our knowledge of their molecular basis.

Homologous recombination and antisense desoxynucleotides: two classical methods for genes silencing

Important contributions to our understanding of gene functions often come from knock-out experiments, where a specific gene is by one way or the other artificially silenced in an organism. The consequences of the loss of the encoded protein often indicate where and how a given gene product is involved in a physiological pathway. Up to now, mainly two techniques were used for this approach. One is based on the replacement of a specific wild-type gene by a mutated and often non-functional allele on the genomic level by means of homologous recombination (review in [2]. Examples are the many knock-out mice that have provided invaluable insight into many gene functions. This technique, however, is relatively time consuming (>1 year). Another approach which yields much faster results makes use of antisense oligodesoxynucleotides (review in [3]. Upon uptake into cells, these probes block the gene expression either on the transcriptional or translational level. However fast and simple this method is, there are two drawbacks. One disadvantage consists in the unpredictable efficiency of a given antisense oligodesoxynucleotide in gene silencing, the other difficulty are side effects often observed. Both effects hamper the interpretation of an experiment to a significant extent and preclude high throughput analyses.

RNA-interference

During the last few years, an amazing but attractive alternative to the above mentioned techniques was established, the so-called RNA interference or RNAi. RNAi describes a phenomenon first described in the nematode *Caenorhabditis elegans* where application of long double stranded RNA (dsRNA) led to a potent, long lasting and specific silencing of selected genes [4]. Further analyses revealed that small dsRNA of 21-25 bp (small interfering RNA=siRNA) derived from the long dsRNAs interact with a protein complex to form the RNA-induced silencing complex (RISC) (review in [5]. This complex possesses nuclease activity and digests mRNA containing a base pair sequence identical to that in the siRNA. Thus, the siRNA serves as a target sequence that allows RISC to recognise specific mRNAs und to prevent their translation by digesting them. This recognition is highly specific, already one to two mismatches between mRNA and siRNA prevent the targeting of the mRNA for degradation. Initially mainly analysed in the nematode and the fruitfly, RNAi rapidly proved to be a rather ubiquitous process also present in unicellular organisms like *Neurospora* but also in plants and recently, it was shown to exist in humans, too. In humans, however, a trick had to be applied, since the presence of long dsRNA as used in the nematode and the fruitfly to initiate RNAi, cause in human cells the activation of non-specific defence mechanisms leading often to cell death. Tuschl and coworkers succeeded in overcoming this problem by transfecting mammalian cells directly with short (21bp) RNA duplexes [6]. The obtained inhibition is very specific as illustrated by RNAi experiments using the enhanced fluorescence protein (EGFP) as marker (Fig. 1). When COS7 cells are transfected with an expression vector for EGFP, pEGFP-C1 (Clontech) alone or with siRNA directed against the EGFP mRNA sequence ($siRNA_{GFP}$), a clear difference in numbers of fluorescence labelled cells can be observed after 24 h (Fig. 1A,B). In average, a reduction of 90% occurred but

even a complete absence of EGFP can be observed. This reduction is not due to an increased cell death as shown in Fig. 1H. The specificity of the silencing can be demonstrated by transfecting an expression vector for the red fluorescence protein (RFP), pDsRED-N1 (Clontech) with and without $siRNA_{GFP}$. No difference in the number of red fluorescent cells is observed (Fig. 1C,D), since $siRNA_{GFP}$ is unable to target RFP mRNA for degradation. This experiment also shows that the reduced number of labelled cells after double transfection of pEGFP-C1 and $siRNA_{GFP}$ compared to pEGFP-C1 transfection alone is not due to an inefficient transfection rate using the transfection reagent Metafectene (Biontex) for double transfections. Even triple transfections can be performed with high efficiency. Cotransfection of pEGFP-C1 and pDsRED-N1 together with $siRNA_{GFP}$ resulted in a similar efficient and specific silencing of EGFP synthesis as observed after transfection of pEGFP-C1 with $siRNA_{GFP}$ alone (Fig. 1E-G). This possibility paves the avenue for experiments very difficult to perform with other gene silencing techniques. For instances, part of an endogenous gene targeted for silencing can be cloned into pDsRED-N1, whereas a mutant allele or a homologous gene can be cloned into pEGFP-C1. After triple transfection of both constructs together with a suitable siRNA against the cloned part of the endogenous gene, silencing of the endogenous gene can be monitored by the absence of red fluorescence, whereas the presence of the mutant allele or the homologous protein is demonstrated by the green fluorescence. This allows for the rapid functional analysis of specific protein domains or for gene substitution experiments.
For a longtime, siRNAs were synthesized chemically and therefore rather expensive. However, recent published protocols allow for the production of siRNAs in the own lab using simple molecular biology techniques [7;8]. One technique, for instances, make use of *in vitro* transcription from DNA oligodesoxynucleotides with an additional reverse complementary sequence to the T7 promoter.

Hairpin-mediated RNAi

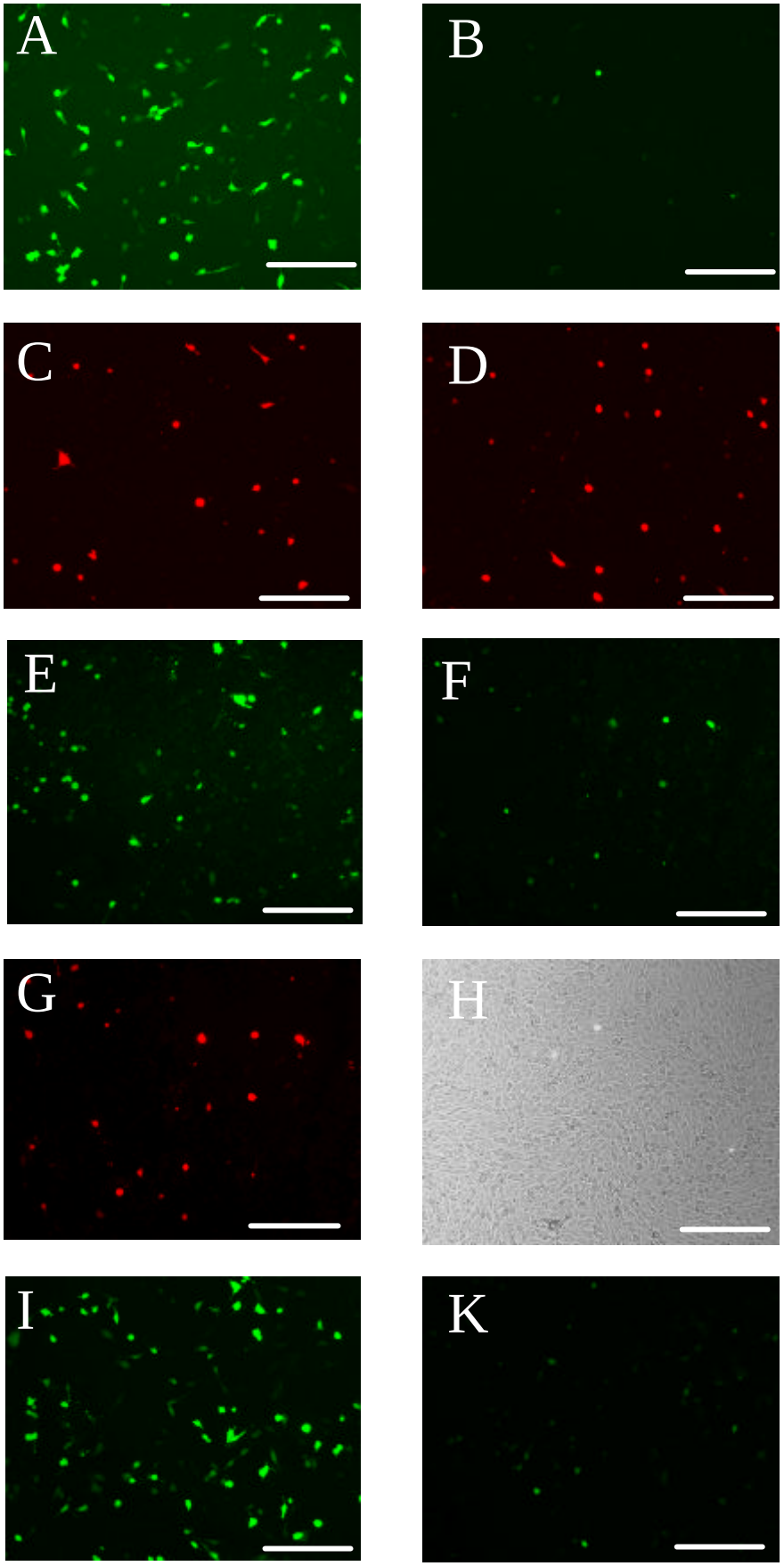
Another technique to produce siRNA is based on the use of short hairpin RNAs (shRNAs) that were shown to trigger RNAi, too [8;9]. Short (60-75 bp long) DNA oligodesoxynucleotides that form hairpins, are cloned into a plasmid under the control of the U6 promoter for RNA polymerase III. Transfection of such a plasmid causes the expression of shRNAs that induce RNAi. Double transfection of pEGFP-C1 together with a plasmid for a shRNA against EGFP mRNA under the U6 promoter results in a clear reduction of green fluorescent cells (Fig. 1I,K). The efficiency for this shRNA mediated RNAi is likely less efficiently compared to the use of double stranded siRNAs but plasmid mediated silencing has several advantages. Among them is the possibility to silence gene expression *in vivo* using a viral gene transfer system. This would allow for silencing of mutated genes and thus represent one way of gene therapy. In a pilot experiment, double transfection of a reporter gene and a plasmid for the expression of a shRNA against the reporter gene resulted in a significant silencing of the reporter gene in living mice [10].

Perspective

Despite its novelty, RNAi has already proven to be a very fascinating technique for functional gene analysis. The advantages over other knock-out techniques such as homologous recombination or antisense probes are the ease, the quickness, and the low price, with which the experiments can be set up in a normal molecular biology lab. In addition, there are several companies providing siRNAs or kits to generate them and internet pages are available providing hints and protocols for the work with siRNAs and programs for their design (http://www.ambion.com/RNAi/index.html). Therefore, RNAis very likely keeps its promises and will become soon very popular.

Figure capture

Fig. 1. RNAi mediated silening of GFP synthesis. Expression plasmids pEGFP-C1 and pDsRED-N1 (Clontech) encoding the green or red fluoresent proteins (GFP and RFP) were transfected with or without siRNA directed against EGFP mRNA into COS7 cells using the transfection reagent **METAFECTENE** (www.biontex.com). Cells were analysed 24 h after transfection under a fluorescence microscope. (A) Transfection of 2 µg pEGFP-C1. (B) Double transfection of 2 µg pEGFP-C1 and 2 µl $siRNA_{GFP}$, generated by *in vitro* transcription and directed against EGFP mRNA [7]. (C) Transfection of 2 µg pDsRED-N1. (D) Transfection of 2 µg pDsRED-N1 and 2 µl $siRNA_{GFP}$. (E) Double transfection of 2 µg each pEGFP-C1 and pDsRED-N1. (F) Triple transfection of 2 µg each pEGFP-C1 and pDsRED-N1, and 2 µl $siRNA_{GFP}$ (due to the selected filter, only green fluorescence is monitored). (E) Triple transfection of 2 µg each pEGFP-C1 and pDsRED-N1, and 2 µl $siRNA_{GFP}$ (due to the selected filter, only red fluorescence is monitored). (H) Transmission light image of (B): no cell death after RNAi treatment. (I) Transfection of 2 µg pEGFP-C1. (I) Transfection of 2 µg pEGFP-C1 and 2 µg U6-$shRNA_{GFP}$ plasmid that causes expression of a hairpin RNA directed against EGFP mRNA and thereby induces RNAi [9]. Bars represent 200 µm in each picture.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Materials and Methods

Cos-7 cells were grown in DMEM with 10% fetal bovine serum with 1% pen/strep to near confluency, thereafter trypsinated and seeded onto glass coverslips in sterile cell culture 6-well plates. By the time of transfection (typically next day), the cells were covering about 90%-100% of the plate surface (90%-100% optical confluency, corresponds to about 70% true confluency).
10 µl of **METAFECTENE** transfection reagent was added to 100 µl of serum-free cell culture medium (DMEM) without antibiotics and mixed gently. In a separate vessel 2 µg of the DNA/RNA was mixed with 100 µl pure medium. The media containing **METAFECTENE** and DNA were combined at once and mixed gently. The mixture was allowed to stand at room temperature for 15-20 min for DNA-lipid-complexes to form.
During this time, the cells were supplied with fresh serum-comtaining medium, 2 ml per well. At the end of the incubation time, the DNA-lipid-complex mixtures were pipetted onto the cells, mixed by tilting the plates a few times and thereafter incubated at 37°C incubator under 5% CO2 for about 24h. Afterwards cells were analysed under a fluorescence microscope.

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