RNA interference (RNAi) is a technology developed after the recent discovery of well-conserved cellular processes that induce posttranscriptional gene silencing triggered by small fragments of double-stranded RNA. This technique is rapidly developing into a promising tool used for functional genetics and therapeutic applications. We focus here on the aspects concerning RNAi mechanism, applications in mammals, and construct design and delivery. We summarize some therapeutic applications in general and speculate on the relevance in cardiovascular medicine.

1. INTRODUCTION

An ancient process for defense against viral infections and transposons, and in higher developed organisms an endogenous process that regulates gene expression, triggered by double-stranded RNA (dsRNA) was recently revealed (for reviews see Carrington and Ambros, 2003; Hammond et al., 2001; Sharp, 2001). This discovery presented the scientific community with a powerful tool for sequence-specific silencing of gene expression: RNA interference (RNAi). RNAi is a technique in which any gene of which the sequence is known can be silenced in a
sequence-specific way by the introduction of complementary dsRNA. This newly developed technique is now widely used to conduct functional genetic studies in a fast, easy, and inexpensive way. Using the right means of transfection, the constructs can be built into the genome. However, as an advantage over genetically engineered knockout organisms, where genes are systemically and permanently knocked out, RNAi permits the spatial and temporal silencing of genes in specific tissues or organs. It also allows specific knock-down genes in human cells and in animals larger and less easily bred than mice.

Compared to the related gene-specific inhibition using “antisense” technique, ribozymes and DNAzymes, RNAi proved to be far more efficient. It can therefore be used at much lower concentrations with fewer side effects (Grunweller et al., 2003; Scherer and Rossi, 2003).

The final goal to develop this technique into therapeutic applications has advanced quickly to the point where the production of intruding viruses is successfully inhibited (Joost Haasnoot et al., 2003), specific genes are shut down in cultured cells, and genes are knocked down in vivo in mice and rats (Backman et al., 2003; Hommel et al., 2003).

2. RNAi: GENERAL MECHANISMS

The main characteristic of RNAi is the sequence-specific silencing of genes by dsRNA (elaborately reviewed by Agrawal et al., 2003; Tijsterman et al., 2002). The silencing process of RNAi is initiated by the introduction of dsRNA into the cell (Fig. 1). This dsRNA is either produced within the cell or artificially transfected into the cell. An endogenous enzyme called Dicer processes the RNA hybrid into 21–23 base pairs fragments. Dicer is an RNase III-like enzyme that completes the fragments with a 2-nucleotide overhang at the 3′ end and a phosphate at the 5′ end (Bernstein et al., 2001). These modifications seem to be crucial for successful RNAi (Nykanen et al., 2001). The obtained small dsRNA fragments are called small interfering RNAs (siRNAs) and are the pivotal elements in the interfering process.

Carrier units of Dicer present the siRNAs to another endogenous enzyme complex called RNA-induced silencing complex (RISC). In the presence of ATP, RISC incorporates the siRNA, unwinding the hybrid in the process (Nykanen et al., 2001). The antisense strand, that is complementary to mRNA present in the cell, is bound to the enzyme complex; the sense strand is degraded. The RISC has become active and with the antisense RNA fragment as a probe, it recognizes the homologous sequences in circulating mRNA in the cell. The thus captured mRNA is then endonucleolytically degraded by the digesting units of RISC (Nykanen et al., 2001), leading to downregulation of protein translation and consequently of the expression of the homologous gene. After cleavage of the target mRNA, the RISC complex may be recycled for another round of RNAi.

The silencing of the gene is sequence-specific as the sequence of the siRNA must be the exact complement of the target mRNA. A single mismatch appears to dramatically impede the efficiency of the silencing (Elbashir et al., 2001).
Next to silencing at this posttranscriptional level, RNAi is thought to act on transcriptional level as it can hamper the actual translation of the mRNA molecule at the ribosome (Wightman et al., 1993). On the level of transcription, it is reported that the siRNA when complexed with RITS (RNA-induced initiation of transcriptional gene silencing) complex can alter conformation of the genomic DNA by methylation (Wassenegger et al., 1994) or condensation of chromatin and consequential silencing of specific chromosomal loci (Pal-Bhadra et al., 2002; Verdel et al., 2004).
The finding of this principle was soon translated into techniques to create loss-of-function phenotypes for functional genetic studies and for the development of new therapeutic techniques.

3. RNAi IN MAMMALIAN CELLS

Although the silencing by dsRNA seems to be a general phenomenon in all living cells, from plants to mammals, there are certain restrictions to consider when working with mammalian cells: In nematode worms and plants, the RNAi is distributed all through the organism and even to the next generation, when applied locally, administered in the food, or injected (Palauqui et al., 1997; Vionnet and Baulcombe, 1997). In mammals it seems that RNAi is not spread systematically, but is only expressed transiently in the cells to which the dsRNA was applied.

The introduction of long molecules of dsRNA in mammalian cells initiates an interferon response, inducing nonspecific inhibition of protein synthesis (Grant et al., 1995; Jacobs and Langland, 1996), and cytotoxic reactions leading to cell death (Der et al., 1997). dsRNA fragments of 19–23 base pairs seem to be able to induce RNAi without provoking programmed death of the host cells. However, short dsRNAs are short-lived and have relatively low transfection efficiency. Furthermore, longer fragments seem to be more effective than short RNA particles, because they are more efficiently processed into more different siRNAs.

The convenient method of introducing small dsRNA fragments into the cell by hairpin-expressing plasmids (Fig. 2) can overcome these disadvantages (Kawasaki and Taira, 2003; Yu et al., 2002). The plasmid vector contains an expression cassette with an RNA polymerase promoter. This promoter initiates the transcription of a 19–23 nucleotide DNA sequence coding for a specific gene. This sequence is followed by a short (7–9 nucleotides) spacer and the same sequence in the antisense direction (inverted repeat). When transcribed into a single-stranded palindromic RNA, it will fold back into a hairpin structure, thus forming a short hairpin RNA (shRNA): a 19–23 base pair dsRNA with a loop at its end. This shRNA will then be a substrate for Dicer, and acquire the 3′ two-nucleotide overhang and a phosphate group at the 5′ end. Hairpin-expressing plasmids appear to produce a longer lasting RNAi effect but it is still time-limited. Additionally, the transfection rate of plasmid vectors is about 80% at the most, therefore not giving complete silencing of the gene of interest.

In our laboratory we are now cloning hairpin-expressing constructs into viral vectors. Retroviruses, adenoviruses, and lentiviruses penetrate into a wide range of target cells at a 100% efficiency rate. In the case of retroviruses (Liu et al., 2004), adeno-associated viruses (Hommel et al., 2003), and lentiviruses (Scherr et al., 2003; Van Den Haute et al., 2003), the expression cassette can be stably incorporated into the genome. This may expand the possibilities of RNAi in producing stable knock-down mammals. However, before applying these virus constructs to living mammals, probable immunological obstacles related to viral infections and,
FIGURE 2. Construction of siRNA by means of hairpin-expressing vectors. A 19 nucleotide DNA sequence is designed in homology to the gene of interest. A construct is created with this sequence followed by a short spacer and the same sequence in the antisense direction (inverted repeat) and a poly-T fragment. This construct is ligated into an expression vector containing a RNA polymerase III promoter. Transfection of the vector into eukaryotic cells initiates transcription of the construct into single-stranded RNA (ssRNA). The palindromic nature of the ssRNA leads to back folding of the ssRNA into shRNA. Dicer processes shRNA into functional siRNA. (See Color Plate 12.)

in the case of retro- and lentiviruses, the risk of insertional mutagenesis (Trono, 2003) may have to be overcome first.

The primary effects of RNAi should be monitored at the level of mRNA. Real-time PCR, which measures amounts of mRNA of a certain gene in tissue samples, is an excellent way of determining the effect of RNAi (Sluijter et al.,
However, the ultimate goal of RNAi is the silencing of the gene at the protein level. A limiting factor might be the turnover rate of the protein. In case a protein has an \textit{in vivo} turnover rate of several weeks, little or no silencing might be observed at the protein level.

4. CONSIDERATIONS FOR THE DESIGN OF RNAi CONSTRUCTS

To design a successful hairpin-forming construct, the sequence of the 19-nucleotide fragment should follow a few important rules:

A 19-nucleotide sequence is preferably picked from the coding region of the gene. Sequences with relatively low cytosine/guanine percentage appear to produce most successful constructs. The fragment should have a CG content of 35–55\%. Poly-T stretches in the 19-nucleotide sequence have to be avoided, for these will trigger premature termination of transcription that is initiated by RNA polymerase III promoters like U6, H1, or the modified tRNA polymerase promoter MTP.

The sequence should be 100\% complementary to the target mRNA, since a single mismatch will considerably decrease the effectiveness of the construct (Elbashir \textit{et al}., 2001). On the other hand, the sequence should have as little as possible homology to other genes to limit off-target reactions, i.e. unwanted silencing of genes that are not targeted (Jackson \textit{et al}., 2003). For the same reason, sequences from highly conserved domains in multigene families should be avoided. It is advised to run a BLAST search (see http://www.ncbi.nlm.nih.gov/BLAST) to screen for homology with other genes. If there is a substantial homology with related or unrelated genes, the sequence should be excluded.

RISC assembles favorably the siRNA strand whose 5′ end has the greater tendency to fray (Hohjoh, 2004; Schwarz \textit{et al}., 2003). This translates into a design in which the 5′ end of the antisense has a base pair that is not as tightly joined as the 3′ end. A C–G bond is thermodynamically stronger than an A–U bond. The 19-nucleotide sequence, then, should preferably begin with C or G and end with A or T or even with a mismatch (A–C, A–G, T–C, T–G).

The choice of RNA polymerase promoters appears to be critical for the effective production of shRNA. RNA polymerase III promoters like U6 and H1 appear to be strong promoters for the stable expression of shRNAs. A comparative study of RNA polymerase III promoters by Boden \textit{et al}.
(2003) showed that the modified tRNA\textsuperscript{met} derived (MTD) promoter was more effective in producing shRNA than did other polymerase III promoters such as H1 and U6, although the efficiency of the U6 promoter could be increased when extended by 27 nucleotides. Boden \textit{et al}.
(2003) also showed that a successful production of shRNA does not necessarily mean an equally successful destruction of complementary mRNA. What should be taken into account is that the U6 promoter is active in the nucleus whereas Dicer is a cytoplasm enzyme. MTD, on the other hand, is activated in the cytoplasm (Kawasaki and Taira, 2003) and therefore delivers the shRNA into the direct vicinity of Dicer, making it a more available target for processing into siRNA.
Even if these general rules are followed it seems that some designs are more efficient than others. It is therefore advised to choose several different sites (two to four) along the target mRNA sequence.

5. RNAi AS A THERAPEUTIC TOOL

The first reports have appeared of successful RNAi in mammals using various means of delivery into the cell. Several studies have used RNAi to inhibit replication of viruses such as HIV (Novina et al., 2002; Park et al., 2003), hepatitis B and C, polio, dengue, influenza A (reviewed by Joost Haasnoot et al., 2003), and the SARS associated corona virus (He et al., 2003) in cultured cells. Also, oncology has also rapidly embraced RNAi technology. Duursma and Agami (2003) showed the stable suppression of oncogenic K-Ras in tumor cells by RNAi.

Next to this, RNAi appeared to be successful in repressing viruses in live mammals: McCaffrey et al. (2002) showed an in vivo inhibition of reproduction of hepatitis C virus proteins in adult mice by intravenously injecting plasmids promoting siRNA production. Zhang et al. (2003) showed in vivo knock-down of gene expression in rat brain tumor. Tumorigenecity was stably suppressed in mice by Brummelkamp et al. (2003) and tyroxine hydroxilase gene was locally knocked down in the midbrain of adult mice (Backman et al., 2003; Hommel et al., 2003). At the same time, the first transgenic siRNA expressing knock-down mice and rats have been reported (Hasuwa et al., 2002; Hommel et al., 2003).

However, with the efficiency of inhibition of gene expression still not 100%, and since all target cells should be transfected to obtain maximal inhibition, the way to clinical applications is still a long way ahead. Therefore, it seems that the delivery of RNAi inducing vectors is the time-limiting step toward the use of RNAi in gene therapy in humans in the near future.

The use of tissue-specific promoters may allow a more localized application to tissue-specific disorders. For instance, the Tie-2 promoter is specifically activated in endothelial tissue (Teng et al., 2002). Using this Tie-2 promoter in an expression cassette could initiate the transcription of an RNAi construct exclusively in endothelial tissue. However, one has to take into account that the RNA polymerase II-type Tie-2 promoter is less efficient in transcribing small stem-loop transcripts like shRNAs than RNA polymerase III promoters. Since arteries and the heart can be reached by catheters, it might be possible to treat vascular diseases like stenosis and atherosclerosis with RNAi inducing vectors locally at the intimal side. Additionally, RNAi vectors can be applied locally at the adventitional side of the artery in a cuff placed around the vessel during surgical intervention. In this way RNAi constructs can specifically interfere with the development of pathological processes like neo intima formation and arterial shrinkage after bypass surgery or arterio-venous shunting for dialysis patients.
6. CONCLUSION

Although the development of RNAi in the therapeutic field appears to be in the fast lane, there are still several problems to overcome before the technique can be applied to human patients. The RNAi technology, however, is so promising that it challenges scientists to work on an efficient and safe system to deliver siRNA into mammalian cells and live organisms to make the evaluation possible in clinical trials. Overall, it seems that RNAi is rapidly developing into a technology that can help us better understand and eventually treat cardiovascular diseases.

REFERENCES

Agrawal, N., Dasaradhi, P. V., Mohmmed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K., 2003, RNA interference: biology, mechanism, and applications, Microbiol. Mol. Biol. Rev. 67:657–685.

Backman, C., Zhang, Y., Hoffer, B. J., and Tomac, A. C., 2003, Short interfering RNAs (siRNAs) for reducing dopaminergic phenotypic markers, J. Neurosci. Methods 131:51–56.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J., 2001, Role for a bidentate ribonuclease in the initiation step of RNA interference, Nature 409:363–366.

Boden, D., Pusch, O., Lee, F., Tucker, L., Shank, P. R., and Ramratnam, B., 2003, Promoter choice affects the potency of HIV-1 specific RNA interference, Nucleic Acids Res. 31:5033–5038.

Brummelkamp, T. R., Bernards, R., and Agami, R., 2002, Stable suppression of tumorigenicity by virus-mediated RNA interference, Cancer Cell 2:243–247.

Carrington, J. C., and Ambros, V., 2003, Role of micro RNAs in plant and animal development, Science 301:336–338.

Der, S. D., Yang, Y. L., Weissmann, C., and Williams, B. R. G., 1997, A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis, Proc. Natl. Acad. Sci. U.S.A. 94:279–3283.

Duursma, A. M., and Agami, R., 2003, Ras interference as cancer therapy, Sem. Cancer Biol. 13:267–273.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T., 2001 (May 24), Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 411(6836):494–8.

Grant, C. E., Vasa, M. Z., and Deely, R. G., 1995, cIRF-3, a new member of the interferon regulatory factor (IRF) family that is rapidly and transiently induced by dsRNA, Nucleic Acids Res. 23:2137–2146.

Grunweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V. A., and Kurreck, J., 2003, Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-O-methyl RNA, phosphorothioates and small interfering RNA, Nucleic Acids Res. 31:3185–3193.

Hammond, S. M., Caudy, A. A., and Hannon, G. J., 2001, Post-transcriptional gene silencing by double-stranded RNA, Nat. Rev. Genet. 2:110–119.

Hasuwa, H., Kaseda, K., Einarsdottir, T., and Okabe, M., 2002, Small interfering RNA and gene silencing in transgenic mice and rats, Fed. Eur. Biochem. Soc. Lett. 532:227–230.

He, M. L., Zheng, B., Peng, Y., Peiris, J. S., Poon, L. L., Yuen, K. Y., Lin, M. C., Kung, H. F., and Guan, Y., 2003, Inhibition of SARS-associated coronavirus infection and replication by RNA interference, J. Am. Med. Assoc. 290:2665–2666.

Hohjoh, H., 2004, Enhancement of RNAi activity by improved siRNA duplexes, Fed. Eur. Biochem. Soc. Lett. 557:193–198.
RNA Interference

Hommel, J. D., Sears, R. M., Georgescu, D., Simmons, D. L., and DiLeone, R. J., 2003, Local gene knockdown in the brain using viral-mediated RNA interference, *Nat. Med.* 9:1539–1544.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S., 2003, Expression profiling reveals off-target gene regulation by RNAi, *Nat. Biotechnol.* 21:635–637.

Jacobs, B. L., and Langland, J. O., 1996, When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA, *Virology* 219:339–349.

Joost Haasnoot, P. C., Cupac, D., and Berkhout, B., 2003, Inhibition of virus replication by RNA interference, *J. Biomed. Sci.* 10:607–616.

Kawasaki, H., and Taira, K., 2003, Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells, *Nucleic Acids Res.* 31:700–707.

Liu, C. M., Liu, D. P., Dong, W. J., and Liang, C. C., 2004, Retrovirus vector-mediated stable gene silencing in human cell, *Biochem. Biophys. Res. Commun.* 313:716–720.

McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A., 2002, RNA interference in adult mice, *Nature* 418:38–39.

Novina, C. D., Murray, M. F., Dykxhoorn, D. M., Beresford, P. J., Riess, J., Lee, S. K., Collman, R. G., Lieberman, J., Shankar, P., and Sharp, P. A., 2002, siRNA-directed inhibition of HIV-1 infection, *Nat. Med.* 8:681–686.

Nykanen, A., Haley, B., and Zamore, P. D., 2001, ATP requirements and small interfering RNA structure in the RNA interference pathway, *Cell* 107:309–321.

Palauqui, J. C., Elmayan, T., Pollien, J. M., and Vaucheret, H., 1997, Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions, *Eur. Mol. Biol. Organ. J.* 16:4738–4745.

Park, W. S., Hayafune, M., Miyano-Kurosaki, N., and Takaku, H., 2003, Specific HIV-1 env gene silencing by small interfering RNAs in human peripheral blood mononuclear cells, *Gene Ther.* 10:2046–2050.

Scherer, L. J., and Rossi, J. J., 2003, Approaches for the sequence-specific knockdown of mRNA, *Nat. Biotechnol.* 21:1457–1465.

Scherr, M., Battmer, K., Ganster, A., and Eder, M., 2003, Modulation of gene expression by lentiviral-mediated delivery of small interfering RNA, *Cell Cycle* 2:251–257.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D., 2003, Asymmetry in the assembly of the RNAi enzyme complex, *Cell* 115:199–208.

Sharpe, P. A., 2001, RNA interference—2001, *Genes Dev.* 15:485–490.

Tijsterman, M., Ketting, R. F., and Plasterk, R. H., 2002, The genetics of RNA silencing, *Annu. Rev. Genet.* 36:489–519.
Wightman, B., Ha, I., and Ruvkun, G., 1993, Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*, Cell *75*:855–862.

Yu, J. Y., DeRuiter, S. L., and Turner, D. L., 2002, RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells, *Proc. Natl. Acad. Sci. U.S.A.* 99:6047–6052.

Zhang, Y., Boado, R. J., and Pardridge, W. M., 2003, In vivo knockdown of gene expression in brain cancer with intravenous RNAi in adult rats, *J. Gene Med.* 5:1039–1045.