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Tissue-specific RNA Interference

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Introduction

When asked what they would consider the most important development in biology during the past decade, many would answer the discovery of RNA interference. The term RNA interference (RNAi) was coined by Fire and Mello to describe the silencing of a target gene as a result of the presence of its related RNA in double-stranded form (dsRNA). Soon after they first described RNAi in *Caenorhabditis elegans* (Fire *et al.*, 1998), the mechanism was found to be conserved in other invertebrates (Kennerdell and Carthew, 1998), and even in vertebrates (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000), although results were found to be ambiguous in zebrafish (Wargelius *et al.*, 1999; Li *et al.*, 2000; but see Oates *et al.*, 2000; Zhao *et al.*, 2001; and see below). Initial attempts in mammals were successful because very early stages of mouse embryos were used (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000). RNAi with long dsRNA in adult mice or cell lines of mammalian origin was later found to result in unspecific inhibition of protein synthesis and cell death (Dykxhoorn *et al.*, 2003; Svoboda, 2004). The reason for these problems was determined to be the induction of the interferon pathway and the activation of PKR. However, soon there was a way in which these problems of unspecific effects could be avoided. The use of dsRNA shorter than 30 nucleotides (siRNAs for short interfering RNA) was found to result in gene silencing without inducing interferon or PKR-derived unspecific effects (Elbashir *et al.*, 2001a,b; but see Bridge *et al.*, 2003; Moss and Taylor, 2003; Sledz *et al.*, 2003). This was great news for biomedical research and RNAi was making headlines as the most promising tool for drug discovery and development of therapeutics. Infectious diseases, cancer,

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Abbreviations: cDNA, coding deoxyribonucleic acid; dsRNA, double-stranded RNA; ERK, endogenously regulated kinase; ES cell, embryonic stem cell; EST, expressed sequence tag; IFN, interferon; PEG-PAA, poly(ethylene-glycol)-block-poly(aspartic acid); PKR, protein kinase R; pol II, RNA polymerase II; pol III, RNA polymerase III; RNA, ribonucleic acid; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, short interfering RNA.

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and neurodegenerative disorders were named as the fields where siRNA-based therapeutics would have an enormous impact (Paddison and Hannon, 2002; Caplen, 2003; Davidson and Paulson, 2004; Holen and Mobbs, 2004). This is certainly true, based on several proof-of-principle studies carried out by numerous biotech and pharmaceutical companies. There is only one important obstacle that remains to be overcome – the problem of siRNA delivery into target cells. Large hydrophilic molecules, such as siRNAs, cannot cross the plasma membrane of vertebrate cells on their own. Current attempts are aiming to modify siRNAs chemically to make them more membrane permeable, or to find new solutions for lipofection that are less toxic. However, these attempts will not solve the problem of directed delivery of siRNAs to distinct target cells. Certainly, the problem of targeted delivery is not unique to RNAi. Rather, the selective transport of a substance to its destination is very often the crucial step in drug development. Unfortunately, until a solution to this problem is found, siRNAs will remain a big promise for the future but fail to fulfil their proclaimed role as a widely applicable therapeutic tool.

However, there are applications of RNAi that are already living up to their promise (Hannon, 2002). RNAi has revolutionized basic research. Functional genomics has gained the technology to realize its functional aspect. The speed of whole genome sequencing and high-throughput technologies for gene expression analyses has left us with an overwhelming amount of data concerning gene expression patterns. Only the functional analysis of genes could not keep up. There were no comparable high-throughput assays for gene function. RNAi is beginning to change that. Screens using siRNA libraries covering large parts of the genome are under way or have been completed for *Caenorhabditis elegans* and *Drosophila* (for references see Caplen, 2003). For vertebrate model organisms, RNAi is bound to make a difference as well. The mouse has become the most important vertebrate model organism due to the availability of genetic tools (Lewandoski, 2001). RNAi has been shown to work in the very early stages of embryonic mouse development (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000), as well as in adult mice when siRNAs rather than long dsRNA are used (McCaffrey *et al.*, 2002). As a mammal, the mouse suffers from the disadvantage that its accessibility during embryonic development is extremely limited. Therefore, non-mammalian vertebrates have been preferred traditionally as model organisms for developmental studies. Unfortunately, they suffered from their own problems, like limited (zebrafish) or lack of (chicken embryos) genetic tools. At least for the latter, RNAi is making up for that (Pekarik *et al.*, 2003; Stoeckli, 2003). With the development of *in ovo* electroporation and *in ovo* RNAi, the chicken embryo has been placed back at the top of the list of preferred model organisms for developmental studies as both gain- and loss-of-function approaches have become feasible.

The chicken embryo as a model organism

The chicken embryo has been used extensively as a model organism for developmental studies for decades (Stern, 2004; Wolpert, 2004). However, during the past 15 years it has lost its importance due to the lack of the appropriate genetic tools. Although there have been reports about the creation of transgenic chickens (for discussion, see Sang, 2004), this method is unlikely to be widely used in research, as

it is too cumbersome and inefficient. For largely the same reasons, and because embryonic stem cells are not available for chickens, knockout phenotypes cannot be obtained with the techniques that are used for mice. However, *in ovo* electroporation and *in ovo* RNAi make up for the lack of these traditional genetic tools (Bourikas and Stoeckli, 2003; Krull, 2004; Bourikas *et al.*, 2005).

'Gain-of-function' approaches in chicken embryos have been used for quite some time, either based on viral vectors to express a transgene of choice, or more recently, by *in ovo* electroporation (Muramatsu *et al.*, 1997). In fact, *in ovo* electroporation is an extremely efficient method of gene transfer in chicken embryos, superior to morpholino-based methods and viral vectors (Bourikas and Stoeckli, 2003; Krull, 2004).

For functional gene analysis, loss-of-function phenotypes are more commonly used than gain-of-function approaches. This is due to the fact that the endogenous expression level is often not limiting and therefore increasing the expression level during the same time window does not result in any detectable phenotype, or in contrast, the expression level of the ectopic gene may be so high that it does not reflect the physiological function of the gene. Similarly, the ectopic expression of a gene beyond the time window of its expression may not be relevant to its endogenous function.

RNAi – the method of choice for loss-of-function phenotypes in chicken embryos

We have developed *in ovo* RNAi as a means to specifically down-regulate genes of interest during the development of the embryonic chicken nervous system (Pekarik *et al.*, 2003; Stoeckli, 2003). *Figure 4.1* shows the procedure of *in ovo* RNAi. We prefer using long dsRNA for RNAi due to the high efficiency, the low cost, and its high specificity. However, other methods for RNAi have been successfully used in chicken embryos as well. Recently, it was shown that silencing neuropilin-1 by shRNA can phenocopy effects seen in the knockout mouse, which is embryonic lethal (Bron *et al.*, 2004). Those authors compared two different means of siRNA delivery, the production *in situ* from shRNA-producing constructs that were delivered by electroporation, and viral delivery. They concluded that RNAi achieved by an expression plasmid leads to rapid, but transient silencing, whereas retroviral delivery induced persistent knock-down, but with a slow onset. In addition to the U6 promoter used by Bron and colleagues (Bron *et al.*, 2004), the H1 promoter was shown to also work effectively in chicken embryos. Katahira and colleagues have transfected a hairpin construct under the control of the H1 promoter into the hindbrain of chicken embryos by *in ovo* electroporation and specifically blocked expression of Engrailed-2, as shown by *in situ* hybridization (Katahira and Nakamura, 2003).

RNAi is not restricted to the nervous system. Kaarbo and colleagues used siRNA derived from RhoA to demonstrate its role in heart development (Kaarbo *et al.*, 2003). Another group has shown the regulation of ERK-activity in the limb bud by retroviral delivery of interfering RNA molecules (Kawakami *et al.*, 2003). Interestingly, RNAi still works with rhodamine-labelled siRNAs, thus allowing for the tracking of the transfected siRNAs (Toyofuku *et al.*, 2004).

Our own studies addressing the molecular mechanisms of axon guidance in the

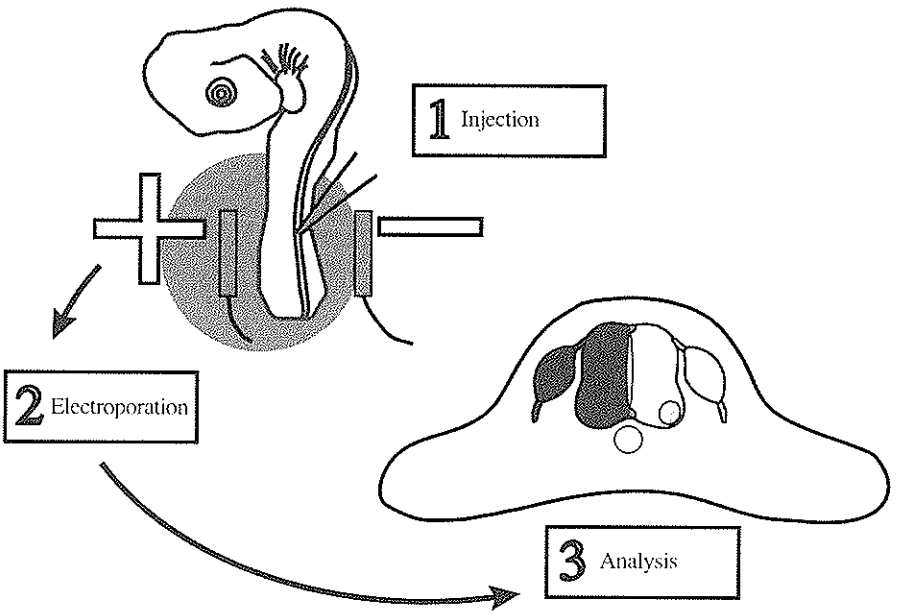

Figure 4.1. *In ovo* RNAi allows for specific silencing of the targeted gene in the developing chicken spinal cord. DsRNA in phosphate buffered saline coloured with Trypan Blue is injected into the spinal cord of a chicken embryo *in ovo* through a window cut into the eggshell. The electrodes are placed parallel to the longitudinal axis of the embryo. Transfection of the injected dsRNA is achieved by applying square-wave pulses of 10 to 26 volts, depending on the age of the embryo. According to the orientation of the electrodes, the dsRNA or co-injected plasmids encoding a tracer are taken up by cells in one half of the spinal cord. For analysis, axonal trajectories can be visualized by fluorescent tracers or by immunohistochemical methods. For details, see Pekarik *et al.*, 2003 and Stoeckli, 2003.

developing nervous system are a good example for the requirements of efficient loss-of-function approaches in the context of the entire organism. First, axon guidance can only be studied in a living embryo that is accessible for *in vivo* manipulations. Second, loss of gene functions has to be controllable both temporally and spatially in order to avoid early embryonic lethality or aberrant early aspects of neurogenesis and patterning. Third, experiments should be 'high-throughput' in order to allow for the analysis of many candidate genes; for instance, genes identified in a screen. *In ovo* RNAi fulfils all these criteria. We can access the embryo through a window in the eggshell that can be sealed after manipulations. The embryo will continue to develop normally if kept in an incubator at the appropriate temperature and humidity. We can choose the time point of gene silencing and we can restrict the area of loss of gene function by selecting the embryonic stage for our manipulation and by the site of injection and electroporation of the dsRNA. Apparently, since we work with embryos, we do not have to worry about unspecific effects induced by long dsRNA. This is a big advantage for the analysis of candidate genes identified in a screen. Many types of screens will identify candidate genes in the form of cDNA fragments or ESTs. Without the need for further cloning or knowledge of the

full-length sequence, we can turn these cDNA fragments into dsRNA (see below). Because these long dsRNA fragments can be cut into a large variety of siRNAs by an endogenous Dicer, we do not have to worry about finding functional siRNAs by computer algorithms.

RNAi in mice

Although loss- and gain-of-function phenotypes have been possible for quite some time in mice, making them the most preferred and widely used vertebrate model organism, RNAi has also been applied to mice either to provide proof of principle that RNAi works, or to apply the technique to solve specific problems associated with loss of gene function induced by traditional genetic tools (Lewandoski, 2001; Prawitt *et al.*, 2004). RNAi was first demonstrated to result in specific gene silencing in mice by knocking down genes in oocytes and early embryos (Wianny and Zernicka-Goetz, 2000). Another proof of principle was the first transgenic RNAi mouse generated by pronuclear injection of an shRNA construct under the control of the H1 promoter that silenced the expression of GFP (Hasuwa *et al.*, 2002).

RNAi could be a solution for the analysis of genes that are embryonic lethal when knocked out, thus preventing the establishment of a mouse line. One approach was the transfection of embryonic stem (ES) cells with a H1-shRNA construct targeting *Rasa1*. Mice resulting from these ES cells had the same phenotype as those obtained by the traditional knockout approach. This technique can only be applied to silence genes that are responsible for development of the embryo proper; placental genes will be unaffected because ES cells only give rise to the yolk sac mesoderm and to the embryo (Kunath *et al.*, 2003).

In some cases, RNAi can provide a better model for a human disease than traditional knockout mice, as demonstrated by Bai and colleagues (Bai *et al.*, 2003). *In utero* electroporation of doublecortin shRNA injected into the ventricle of rats phenocopied the neuronal migratory defects of the human disease, whereas knockout mice lacking doublecortin showed a phenotype in the hippocampus but did not have any defects in migration of cortical neurons.

In view of the development and test of therapeutic tools, different tissues and organs of postnatal mice were subjected to RNAi. *Table 4.1* provides an overview of the applications of RNAi in different species. Although it has been shown that muscle cells or the liver can be transfected with dsRNA with good efficiency by hydrodynamic infusion (up to 49%; Lewis *et al.*, 2002; Hagstrom *et al.*, 2004), this method will remain experimental. Transfer by hydrodynamic infusion refers to the injection of large volumes of saline with siRNAs with high-pressure into the tail vein (Liu *et al.*, 1999; Zhang *et al.*, 1999). This is a method that is not physiological and only applicable to well vascularized organs. Other groups have tried to target the lung via nasal instillation of siRNAs. Although they have been able to show a persistent decrease of the mRNA level of the targeted gene, the method of siRNA application caused volume-dependent emphysema (Massaro *et al.*, 2004; Zhang *et al.*, 2004). Recent attempts by Ge and colleagues to use RNAi for the treatment of infectious diseases were very promising, where they used intratracheal or intravenous administration of shRNAs to inhibit the influenza virus by targeting viral genes in a mixture with polyethyleneimine as a vehicle (Ge *et al.*, 2004).

Table 4.1. Targeted tissues by RNAi

Tissue	Species	Transfection method	References
Brain	chick, mouse	electroporation	Bai *et al.*, 2003 Katahira and Nakamura, 2003
Eye	mouse	transfection reagent	Matsuda and Cepko, 2004
Heart	chick	injection, electroporation	Kaarbo *et al.*, 2003, Zhang *et al.*, 2003 Toyofuku *et al.*, 2004
Limb	chick	injection	Kawakami *et al.*, 2003
Liver	mouse	hydrodynamic transfer	Lewis *et al.*, 2002 McCaffrey *et al.*, 2002
Lung	mouse	nasal instillation	Massaro *et al.*, 2004 Zhang *et al.*, 2004
Muscle	mouse, rat, dog, monkey	hydrodynamic transfer	Hagstrom *et al.*, 2004
Spinal cord	chick	electroporation	Pekarik *et al.*, 2003 Bron *et al.*, 2004

Generation of interfering dsRNAs

The first RNAi studies used dsRNA of several hundred basepairs. These long dsRNAs have several advantages (see below), but unfortunately, RNAi with long dsRNA is not suited to induce specific posttranscriptional gene silencing in postnatal animals due to the activation of the PKR/interferon pathway, which leads to general, sequence-independent degradation of RNA and therefore blockade of cellular protein synthesis (reviewed by Svoboda, 2004). For gene silencing during embryonic development, the use of long dsRNA is a powerful and cost-saving alternative to the time-consuming designing and testing of siRNAs. We and other groups showed that specific down-regulation of proteins in embryos and in cell lines derived from embryonic tissue is possible without affecting related proteins using long dsRNA (Pekarik *et al.*, 2003; Svoboda, 2004). For instance, the down-regulation of axonin-1 did not affect the expression levels of the related proteins NgCAM and NrCAM (*Figure 4.2*). The major advantage of long dsRNA molecules is their easy production in the lab. In contrast to chemically synthesized siRNAs, the costs for long dsRNAs are low. DsRNA can be produced by *in vitro* transcription of sense and antisense strands using a plasmid containing a partial cDNA of the gene of interest flanked by RNA polymerase promotors T3, T7, or SP6. Equal amounts of sense and antisense strands are denatured at 95°C and slowly cooled down to room temperature to allow for the annealing to dsRNA. This convenient and fast procedure allows for the analysis of the function of many genes in a short time. Neither lengthy cloning steps nor knowledge of the entire sequence of a target gene are required. Because long dsRNA is cleaved intracellularly into a variety of different siRNAs by endogenous Dicer, false negative results due to the choice of ineffective siRNAs are unlikely. This is a major advantage for the analysis of candidate genes identified in screens, where only fragments of cDNAs are obtained, and large numbers of candidates need to be analysed to pre-select the ones that will be pursued for detailed studies.

However, for knockdown experiments in postnatal animals or in cell lines, short

interfering RNAs (siRNAs) have to be used. One possibility for obtaining them is to digest long dsRNA by RNase III from *E. coli* or recombinant Dicer, thereby creating a pool of siRNAs (Calegari *et al.*, 2004). The advantage of this approach is that there is no need to find effective siRNAs from the pool of possible 23 bp-long fragments of the entire mRNA. The most common approach is, however, the chemical synthesis of siRNAs based on computer algorithms that are created to find effective siRNAs. Several companies have developed their own rules for the selection of RNA sequences, but most of them are not in the public domain. Some labs have listed guidelines for the selection of effective siRNAs on their web sites. These are freely available. There is no general consensus about the characteristics of effective siRNAs, and it is not possible to predict their effectiveness with certainty. Therefore, several siRNAs for each target gene have to be tested. Most effective silencing is achieved with a mixture of siRNAs (between 3 to 6). The use of a pool of siRNAs will allow for a decrease in the concentration of each single siRNA, and therefore decrease the chance of so-called off-target effects (see below). General rules for the selection of siRNAs predict that an A/U should be at the 5'-end of the antisense strand, and a G/C at the 5'-end of the sense strand. Furthermore, it is necessary to omit G/C-stretches of more than 9 nucleotides in length, but a string of 5 A/U in the 5'-terminal third of the antisense strand might be helpful for high efficiency (Schwarz *et al.*, 2003; Ui-Tei *et al.*, 2004).

Generation of siRNAs *in situ*

In some cases, the transient nature of RNAi is a disadvantage. In order to avoid the dilution of the effect due to cell proliferation, especially *in vitro* when dividing cells are used, siRNAs can be produced from expression vectors. These vectors produce short hairpin RNAs that are processed to siRNA molecules. Although this means a lot more benchwork, it offers the possibility to generate transgenic animals or stably expressing cell lines (Brummelkamp *et al.*, 2002; Hasuwa *et al.*, 2002). The cloning procedure consists of linking two custom-made RNA oligomers by a loop structure and placing the construct under the control of a Pol III promoter like H1 or U6 (Arendt *et al.*, 2003). These promoters are considered to drive ubiquitous expression. This can be both an advantage and a disadvantage. In many cases, a tissue-specific knockdown of the gene of interest is required. This has been achieved by using Pol II promoters. Stein and colleagues have injected a long hairpin construct under the control of the oocyte-specific Zp3 promoter into mouse oocytes, and observed in founder animals from those oocytes the expected phenotype known from the Mos knockout mouse, infertile females (Stein *et al.*, 2003).

An additional advantage of using DNA-derived silencing constructs is the possibility to combine them with inducible promoters, such as the Cre-Lox system or the Tet-on system (Matsukura *et al.*, 2003; Kasim *et al.*, 2004). For a summary of the advantages and disadvantages of the different forms of interfering RNAs, see *Table 4.2*.

Delivery of interfering RNAs

An important prerequisite for reaching efficient gene silencing is the method of delivery of the interfering RNA molecules into target cells or target tissues. Most of

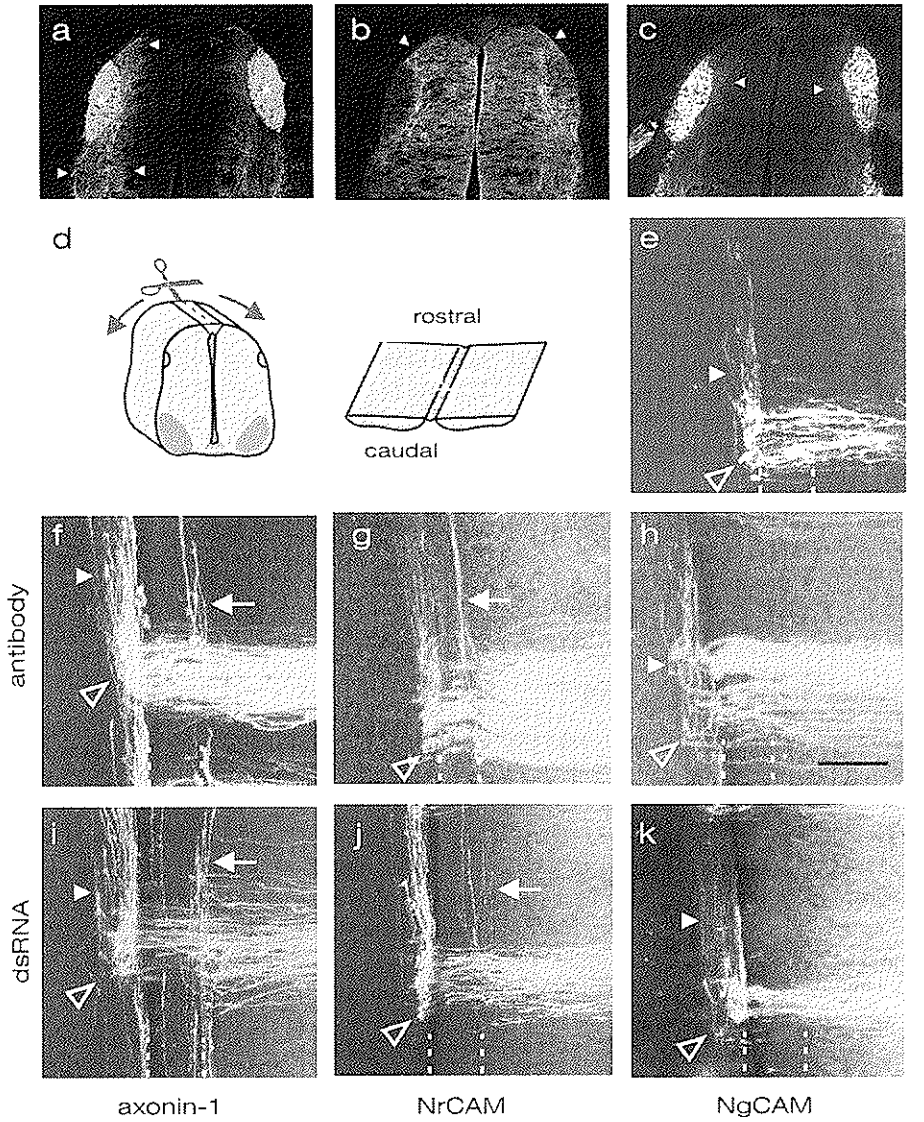

Figure 4.2. Gene silencing by *in ovo* RNAi in the spinal cord is specific for the targeted gene, in this case the cell adhesion molecule axonin-1 (a). The expression level of related cell adhesion molecules, such as NrCAM (b) and NgCAM (c), are not affected. Arrowheads and open arrowheads mark equivalent positions on the control and electroporated side, respectively. Note that the staining of the dorsal root entry zones (in a, marked by asterisk) is not weaker on the electroporated side compared to the control side because the fibres that are responsible for the axonin-1 staining in these areas are originating from the dorsal root ganglia, which were not targeted under the conditions used in this experiment.

For the analysis of the loss-of-function phenotypes, embryos were sacrificed, the spinal cord was dissected and cut open along the dorsal midline, as shown in (d). In control spinal cords (e), commissural axons cross the floor plate, the ventral midline of the spinal cord (indicated by dashed lines), and turn rostrally along the contralateral floor-plate border. In embryos treated with dsRNA derived from axonin-1 (f), NrCAM (g), or NgCAM cDNA (h), commissural axons showed distinct phenotypes that were the same as those obtained with function-blocking antibodies injected into

the time, when gene function is studied in cell lines, siRNAs are transfected with cationic lipophilic agents (Reich *et al.*, 2003; Dalby *et al.*, 2004), although both the calcium phosphate method and electroporation have also been used (Paddison *et al.*, 2002; Jiang *et al.*, 2003). *In vitro*, transfections are usually carried out in the absence of serum in the media because of RNases that might be contained in serum. Most of the lipophilic transfection reagents require serum-free conditions to work efficiently anyway. Some companies offer specific transfection reagents for siRNAs, although, due to their small size, most of the transfection methods should work well for siRNAs. For the delivery of an shRNA expression vector, the transfection technique can be selected based on the cell type to be transfected.

For tissue-specific delivery, *in vivo* viral vectors are the method of choice. So far, three sorts of viruses have been used successfully: retroviruses, adenoviruses, and more recently, lentiviruses. Lentiviruses are particularly useful because of their ability to infect postmitotic cells very effectively (Mochizuki *et al.*, 1998; Jacque *et al.*, 2002; Qin *et al.*, 2002; Xia *et al.*, 2002; Rubinson *et al.*, 2003; Shen *et al.*, 2003).

With the aim to use RNAi as a therapeutical tool, novel transfection agents have been developed (Kakizawa *et al.*, 2004; Minakuchi *et al.*, 2004). Kakizawa and colleagues have reported the successful uptake of siRNAs into cells via endocytosis, when the siRNA is enclosed in a nanoparticle built by association of PEG-PAA with calcium phosphate. Minakuchi and colleagues use atellocollagen, a pepsin-treated type I collagen used clinically in wound healing and vessel prosthesis, to complex the siRNA. Injection of this complex into a mouse tumour was shown to provide a prolonged release of the siRNA, with the additional advantage of nuclease resistance. Even 7 days after injection of the siRNA complex, the target gene was still silenced (Minakuchi *et al.*, 2004).

Limitations of RNAi

RNAi has been used successfully in a wide variety of species. In chicken embryos, it is certainly the method of choice for gene silencing, as 'traditional' genetic tools are not available or are extremely inefficient (Bourikas and Stoeckli, 2003; Bourikas *et al.*, 2005). In zebrafish, however, RNAi has not been a success story. Initial reports about the successful application of RNAi (Wargelius *et al.*, 1999; Li *et al.*, 2000) were soon followed by reports demonstrating unspecific effects (Oates *et al.*, 2000; Zhao *et al.*, 2001). Long dsRNA was found to have dose-dependent unspecific effects, and resulted in high rates of embryonic lethality. Higher survival rates were reported for lower concentrations of dsRNA, but at the price of no, or only marginal, differences between experimental and control-injected embryos (Mangos *et al.*, 2001; Zhao *et al.*, 2001). In contrast to these reports, Wargelius and colleagues

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the spinal cord *in ovo* (i, j, k). When axonin-1 interactions were perturbed (f and i), some commissural axons failed to cross the midline and extended along the ipsilateral floor-plate border instead (arrows). In addition, axons did not fasciculate properly, as seen in control embryos (arrowheads). The perturbation of NrCAM interactions (g and j) did not result in defasciculated growth morphology but also induced pathfinding errors. In contrast, no failures in midline crossing were seen after NgCAM loss of function (h and k), although the lack of NgCAM resulted in strong defasciculation of commissural axons. For details, see text and Pekarik *et al.*, 2003 (adapted from Pekarik *et al.*, 2003).

Table 4.2. Advantages and disadvantages of different types of interfering RNAs

dsRNA	Advantages	Disadvantages
siRNA	easy to transfect subtype-specific downregulation possible	expensive several siRNAs must be tested off-target-effects
shRNA	stably transfected cell lines possible transgenic mouse lines possible subtype-specific downregulation possible tissue-specific expression by the use of specific promotors	long cloning process several siRNAs must be tested
long dsRNA	cheap *in vivo* processed to different siRNAs genome-wide librairies (e.g. *C. elegans*)	IFN response not necessarily subtype-specific

found specific effects (besides a lot of unspecific ones) in 20 to 30% of all embryos, although the concentrations of long dsRNA that they used were 100-fold higher than the ones used in *Drosophila* (Wargelius *et al.*, 1999). Routinely, morpholinos are used to induce loss-of-function phenotypes in zebrafish, although they are much more expensive than dsRNA, and they have additional disadvantages (for a discussion and references, see Bourikas and Stoeckli, 2003). In only one publication, the morpholino phenotype could be copied by RNAi in zebrafish, when siRNAs were used for the experiments (Dodd *et al.*, 2004).

Despite these limitations, RNAi has changed biomedical research. It has enhanced the speed of functional gene analysis exponentially. It has given us new opportunities to control gene silencing, both temporally and spatially, an aspect that is particularly important for gene analysis during development. When the problem of specific targeting of siRNAs is solved, RNAi will deliver its promise to be a widely applicable therapeutic tool for diseases that are still untreatable.

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