15 RNA interference in postimplantation mouse embryos

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Introduction

Sequencing of whole genomes has provided new perspectives into the blueprints of diverse organisms, including the genome of the mouse (Waterston et al., 2002). Although the complete sequence is now available, the estimation of total gene number encoded by the mouse genome is ranging approximately from 25,000 to 50,000 (Okazaki et al., 2002). This uncertainty about the functional units within the genome highlights the importance of a detailed analysis of the encoded genes.

A significant step toward a better understanding of the genome has been the development of large-scale gene expression analysis tools utilizing DNA microarrays (Bono et al., 2003). This technology allows the generation of gene expression profiles that can give important clues for the interpretation of biological processes. However, the obtained data do not directly address the function of individual genes. Rather, they present a snapshot of global gene expression changes. While this is a very useful parameter for understanding the genome, it is not very useful for studying detailed phenotypic changes after gene ablation.

About 15 years ago gene function analysis became available in the mouse through the development of gene knock-out technology (Capecchi, 1989). In this approach genes are targeted in embryonic stem (ES) cells through homologous recombination. The manipulated ES cells are subsequently injected into blastocysts, and chimeric offspring are checked for germline transmission. Successful germline transmission allows the production of animals deficient in the gene of interest. Careful phenotypic analyses of these animals can then disclose the function(s) of the knocked-out gene. This approach is very time consuming and cost intensive, and it would be a difficult undertaking to study all genes of the mouse genome using this technology. In addition, the amount of information gained by classical gene knock-outs is often limited, especially when the gene of interest reveals an embryonic lethal phenotype. Furthermore, gene redundancy and compensatory mechanisms often prevent the appearance of observable phenotypes, hence requiring the production of double or triple knock-out organisms.

Conditional knock-out technologies have been developed to circumvent early lethal phenotypes (reviewed in Lewandoski, 2001; Kuhn and Schwenk, 2003). In particular, the Cre/loxP system has been developed to allow the detailed temporal and spatial analyses of gene function in the mouse. In such studies, standard gene-targeting techniques are used to produce a mouse in which a region of a gene of interest is flanked by recognition target sites (loxP) of the Cre recombinase. By crossing such a mouse line to a mouse line that expresses Cre recombinase in a tissue-specific manner, progeny are produced in which the conditional allele is inactivated only in those cells that express Cre. While the power of this approach is evident, it has the disadvantage of being even more expensive and labour intensive than classical knock-outs.

The realization that RNA interference (RNAi) is a useful tool for mammalian gene function studies has had a big impact on molecular biology. The rapid acceptance of this technology in the research community resulted primarily from the ease of use of RNAi technology and the strong need for a reliable method to down-regulate individual genes to elucidate their function. RNAi is not limited to mammalian cells grown in tissue culture, but can also be used for gene knock-down studies in whole organisms, including gene silencing in the mouse. Specifically, RNA interference (RNAi) has become available to mammalian cells in general (Elbashir et al., 2001a), and to mouse genetics in particular (McCaffrey et al., 2002; Lewis et al., 2002; Calegari et al., 2002; Mellitzer et al., 2002; Hasuwa et al., 2002). RNA interference (RNAi) is a cellular mechanism that can be used to down-regulate the expression of genes through the destruction of the cognate mRNA. Because RNAi-mediated gene regulation occurs after the mRNA has been generated from the DNA template, this mechanism is also referred to as post-transcriptional gene silencing. Compared to the knock-out technology, loss of gene function analyses via RNAi are more cost effective and less labour intensive. Furthermore, simultaneous silencing of gene expression can be achieved by using a mixture of different siRNAs targeting different genes in order to circumvent compensatory mechanisms due to the existence of redundancy. It should be noted, however, that RNAi experiments produce gene knock-downs, not knock-outs.

To make RNAi useful to the study of mouse development, systems are required that allow embryo manipulation without affecting developmental processes that follow the manipulation. Such a system is provided by the technology of mouse whole embryo culture (Cockroft, 1990). This technique allows the normal development of mouse postimplantation embryos for up to two days *in vitro*. Importantly, several kind of manipulations of the mouse embryo have been developed, including topical injection followed by directional electroporation (Akamatsu et al., 1999).

Here we describe the production of endoribonuclease prepared short interfering RNA (esiRNA) as a cost-effective way to generate a cocktail of different siRNAs and its use for gene knock-down studies in the postimplantation mouse embryo. We combine (i) the manipulation of mouse embryos by topical injection and directional electroporation of esiRNA with (ii) whole embryo culture, to rapidly

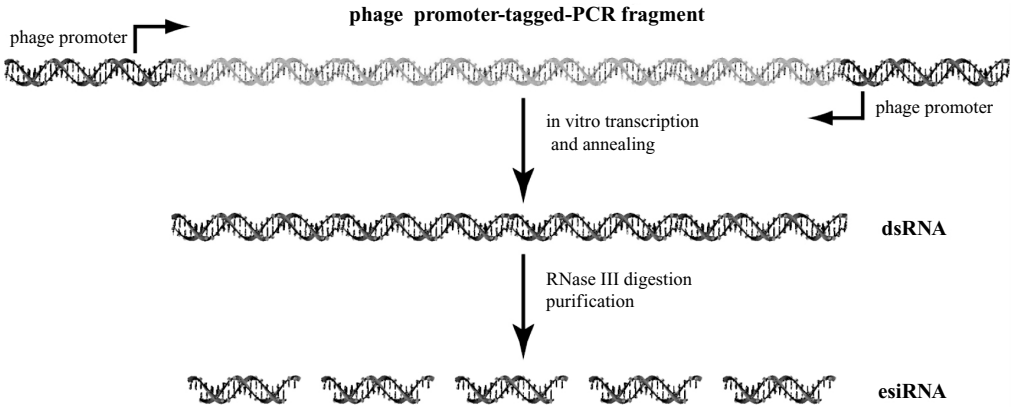

Figure 15.1. Schematic presentation of esiRNA production. Steps carried out to obtain a pool of functional siRNAs are illustrated.

analyse gene function in a tissue- and time-specific fashion in the developing mouse.

The combination provides a fast, powerful, and economical platform to analyse gene function during mouse development.

Generation of endoribonuclease-prepared siRNAs (esiRNAs)

The origin and mechanisms of RNA interference, design and synthesis have been described in depth in other chapters of this book. The present chapter highlights the generation of esiRNAs, a method that we employed to knock down genes important for mouse embryonic development. Initial RNAi experiments with siRNAs where carried out in mammalian tissue culture cells (Elbashir et al., 2001a). Soon after these reports several laboratories published the result that siRNAs could also silence gene expression in the adult (McCaffrey et al., 2002; Lewis et al., 2002; Hasuwa et al., 2002) and in the postimplantation mouse embryo (Calegari et al., 2002). As in the initial experiments in tissue culture cells most groups used reporter genes like luciferase and GFP to demonstrate the functionality of RNAi in the mouse. However, a recent report indicates that phenotypes observed via RNAi resemble phenotypes produced by classical gene knock-out through gene targeting in ES cells (Kunath et al., 2003). This study demonstrated that RNAi can be an efficient technology to study gene function in the whole animal.

Different methods have been developed for the generation of siRNA (reviewed in Kittler and Buchholz, 2003 and by Myers et al., Chapter 2 of this book). A different way to generate siRNA molecules is the enzymatic digestion of *in vitro* transcribed and annealed long double-stranded RNA (dsRNA) by an endoribonuclease like Dicer (Kawasaki et al., 2003; Myers et al., 2003), or *E.coli* RNase III (Yang et al., 2002). These enzymes bind to dsRNA and cleave them to produce endoribonuclease-prepared short interfering RNAs, or esiRNAs (Figure 15.1). The advantages of this method are that it is fast and cost effective, and that through this process a pool of different siRNAs is produced that usually contains effective silencing molecules. Therefore, screening for potent siRNAs is not necessary.

A potential disadvantage of this method that has been of concern is that the pool of different siRNAs produced may contain molecules that could induce cross-silencing (for a recent review see Check, 2003). However, published data (Yang et al., 2000) and unpublished data from our own laboratories (F. Buchholz, unpublished observations) suggest that cross-silencing of homologous genes is typically not observed. In fact, *in silico* experiments with a large number of esiRNAs directed against different genes showed that less than 10% of those contain any siRNA that matches a different gene (Henschel et al., 2004). Furthermore, we have not seen cross-silencing of highly homologous genes utilizing quantitative RT-PCR experiments (F. Buchholz, unpublished observations) and Western blot analyses (Yang et al., 2002).

Manipulation of whole postimplantation mouse embryos

Whole embryo culture

To understand the mechanisms controlling prenatal mammalian development, systems are required that allow observation and manipulation of the whole organism during its embryonic life. Because mammalian embryos develop inside the uterus, direct observation is not feasible and only a very limited kind of manipulation can be done. For this reason, for over a century great efforts have been made towards establishing reproducible techniques that allow manipulation and development *in vitro* indistinguishable at the morphological, cellular and molecular level from that occurring *in utero* (Heape, 1890; Nicholas and Rudnick, 1938; New, 1978; Cockroft, 1990).

A few days after fertilisation mammalian embryos descend the oviduct and adhere to the uterine wall (E4.5 in the mouse) in a process called implantation. Due to the reduced embryonic size and to a minor dependency on the uterine environment, preimplantation mouse embryos can be efficiently kept in culture for several days and through all stages of preimplantation development (for reviews see Biggers, 1998; Diaz-Cueto and Gerton, 2001). In contrast, the culture of postimplantation rodent embryos, which require more complex culture conditions, can be achieved only until the organogenetic stage and support normal development *in vitro* for up to two days (New, 1978; Cockroft, 1990). However, because many of the major processes of development (such as neurulation, somitogenesis and development of the cardiovascular, digestive and locomotor system) occur after implantation, the culture of postimplantation, rather than preimplantation, embryos is an attractive and powerful system towards a better understanding of the cellular and molecular processes of mammalian development.

The culture of postimplantation whole mouse embryos can be carried out from egg cylinder (E5.5) to about the 60th somite stage (E14), with the highest efficiency and reliability from early-somite (E7.5) to organogenetic stage (E11.5). Dissection and preparation of rodent embryos for culture are now very-well-established procedures, described in detail in several laboratory manuals (Cockroft, 1990; Nagy et al., 2003). According to standard protocols, embryos are freed from uterine walls and decidual tissue and then cultured with their yolk sac and

ectoplacental cone. After dissection, the culture is carried out in medium containing 50 to 100% of immediately centrifuged, heat-inactivated rodent serum, and continuous rotation and oxygenation of this medium are carried out during the whole period of culture. These conditions are known to support normal embryonic development *in vitro* for up to two days both at the morphological (New, 1978; Cockroft, 1990) and, as shown for the onset and progression of the gradients of neurogenesis, at the cellular and molecular level (Calegari and Huttner, 2003 and unpublished data).

For several decades, different kinds of manipulation were combined with whole embryo culture. In the late 1970s, the use of whole embryo culture as a system of choice for toxicological and teratogenic studies reflected the tragic episode of thalidomide, sold as a sleeping drug but inducing severe abnormalities in human foetal development (for a review see Webster et al., 1997). For a long time, the application of potentially toxic compounds remained the almost exclusive kind of manipulation of mouse embryo developing *in vitro*. However, in recent years many other techniques have been successfully applied to whole embryo culture in order to study the mechanisms of mammalian development, such as cell or tissue transplantation, antibody interference, cell lineage tracing, infection with viral vectors and electroporation of nucleic acid (Drake and Little, 1991; Tam, 1998; Inoue et al., 2000; Oback et al., 2000; Osumi and Inoue, 2001).

The combination of these techniques with whole embryo culture offers a powerful system for understanding the mechanisms of mammalian prenatal development. In particular, the possibility of mediating gene transfer into target tissues of a developing organism offers a unique possibility for studies of gene function.

Gene transfer in postimplantation mouse embryos

Two methods of gene transfer have been applied in postimplantation mouse embryos viral vector-mediated gene transfer and electroporation.

One of the first means of obtaining gene transfer into postimplantation mouse embryos in culture (Figure 15.2B) involved viral vectors (Stuhlmann, 1984; Oback et al., 2000). Viral vector-mediated gene transfer as a means of delivering genes into target cells is based on the preparation of transgenic viral particles used to infect the target tissue. However, this method presents several disadvantages, such as the relatively time-consuming preparation of the viral particles, potentially toxic side effects of the viral infection, and, often, biosafety issues.

An alternative approach to mediating region-specific gene transfer into whole organisms is *in vivo* electroporation (Figure 15.2A). Electroporation, as an highly efficient means of delivering nucleic acids into target cells, is based on the capability of electric pulses to create transitory pores into the plasma membrane through which nuclei acids and other molecules such as drugs, peptides, proteins and polysaccharides can pass (Banga and Prausnitz, 1998). The electric field, in addition to "cell-poration," also induces the cathode-to-anode movement of the negatively charged nucleic acids, therefore mediating their migration into the permeabilized cells. When electroporation is applied upon injection of nucleic acids into the cavity of an organ, this allows the transfection to occur in only

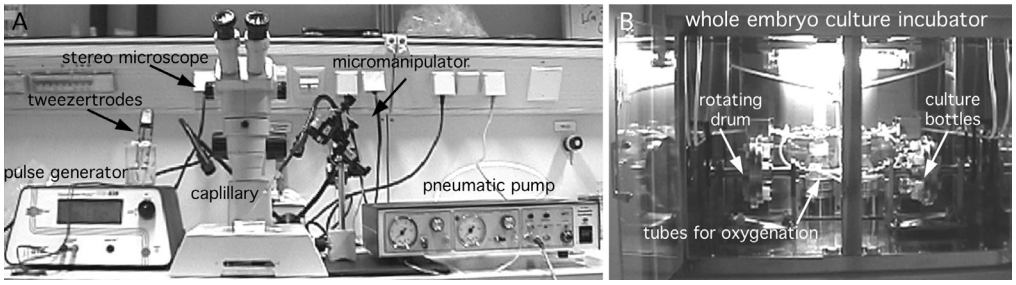

Figure 15.2. Equipment for *in vivo* electroporation and whole embryo culture. A. Picture of instruments used for *in vivo* electroporation. B. Picture of a whole embryo culture incubator (Ikemoto, Japan).

one half of the target organ, with the other, non-transfected, half serving as an internal negative control. Moreover, by using *in vivo* co-electroporation, multiple genes can easily be delivered into target cells simultaneously, with a high degree of efficiency (80–90%) (Saito and Nakatsuji, 2001; Calegari et al., 2002). Hence, electroporation of nucleic acids is emerging as a method of choice for gene function analyses (Swartz et al., 2001) and may constitute a fast and suitable approach for genome-wide functional screens. Finally, one additional advantage of electroporation is the extent of transfection of the target tissue achieved by using electrodes of the appropriate size and shape, which allows transfection to occur in entire regions, organs, tissues or even single cells of the organism (Figure 15.3). In addition, *in vivo* electroporation has also been carried out *in utero*, circumventing the limitation of whole embryo culture (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Takahashi et al., 2002), and can easily be applied to many other organisms and also to adult animals.

Possible disadvantages of *in vivo* electroporation are cell death and tissue damage caused by the electric fields. However, these side effects can be minimized by optimizing electroporation conditions such as voltage, size of the electrodes, etc. In addition to these classical methods of gene delivery, a recent report describes an alternative possibility of gene transfer into mouse embryos by tail injection of nucleic acids into pregnant mice (Gratsch et al., 2003). Although this novel approach does not allow tissue specificity, it opens the possibility for a very fast practical approach of gene transfer in developing embryos.

RNAi in postimplantation mouse embryos

The possibility of triggering silencing of gene expression by siRNAs in mammalian cells in culture (Elbashir et al., 2001a), together with the possibility of transfering nucleic acids by topical injection and directional electroporation *in vivo*, leads us to investigate RNAi in postimplantation mouse embryos (Calegari et al., 2002; Gratsch et al., 2003). In order to address this issue different experimental approaches were designed: first, silencing of exogenous gene expression in tissues facing cavities; second, silencing of exogenous gene expression in tissues lacking cavities; third, silencing of gene expression of an endogenously expressed

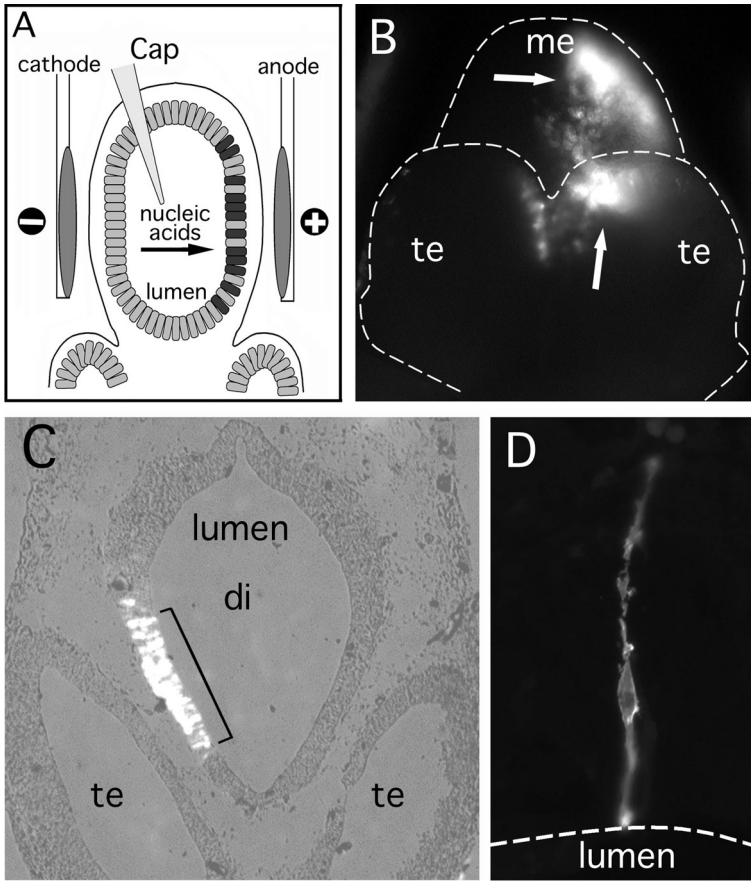

Figure 15.3. Gene transfer in postimplantation mouse embryos using directional electroporation. A. Cartoon illustrating the topical injection (Cap, capillary) of nucleic acids into the lumen of the neural tube of mouse embryo, with the electrodes in the lateral orientation, cathode-right/anode-left, and the uptake of nucleic acids into the left side of the neuroepithelium upon electroporation (darker cells). B. Frontal fluorescent view of the head of an unfixed mouse embryo (outlined by dashed lines) showing GFP expression in the left side of the dorso-lateral mesencephalon and caudal telencephalon (white region; arrows) upon injection of GFP reporter plasmid into the lumen of the anterior neural tube at E10, followed by electroporation and subsequent whole-embryo culture for 24 hours; te, telencephalic vesicles, me, mesencephalon. C. Superimposed phase contrast (grey) and fluorescence (white) micrograph of a transverse cryosection through the diencephalic (di) and telencephalinc (te) neural tube of a E10 mouse embryo upon injection into the lumen of the anterior neural tube and electroporation using a cathode-left/anode-right orientation, and subsequent 24 hours whole embryo culture. GFP expression in neuroepithelial cells of the right diencephalon (white cells) is indicated (black line). D. High magnification fluorescent micrograph of the mouse neuroepithelium (dashed line: apical, lumen facing side of the neuroepithelium) of a E10 mouse embryo showing plasma membrane localised GFP in a single neuroepithelial cell upon injection of GFP-GAP43 reporter plasmid into the lumen of the neural tube, electroporation and subsequent whole embryo culture for 24 hours.

transgene; and fourth, silencing of gene expression of an endogenously expressed gene.

First. Silencing of exogenous gene expression in tissues containing cavities, such as the neural tube and heart, was investigated as follows: Plasmid vectors

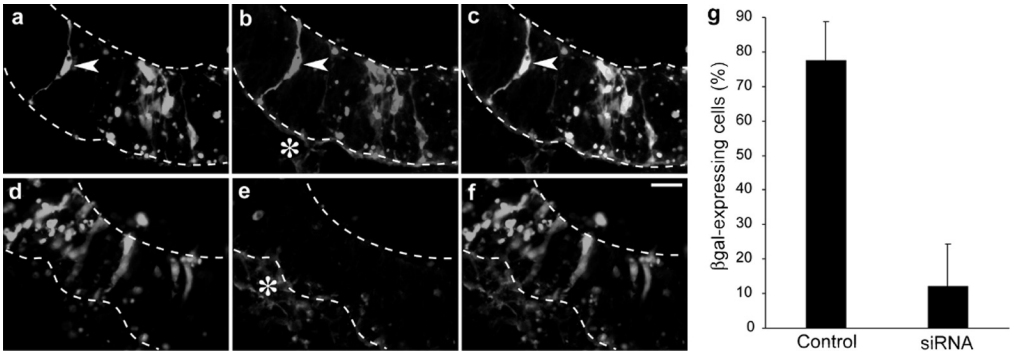

Figure 15.4. RNAi in the neuroepithelium of E10 mouse embryos. E10 mouse embryos were injected, into the lumen of the telencephalic neural tube, with the two reporter plasmids pEGFP-N2 (for GFP) and pSVpaXD (for βgal), either without (**a–c** and **g**, **Control**) or with (**d-f** and **g**, **siRNA**) βgal-directed esiRNAs, followed by directional electroporation and whole embryo culture for 24 hours. (**a-f**) Horizontal cryosections through the targeted region of the telencenphalon were analysed by double fluorescence for expression of GFP (green; **a** and **d**) and βgal immunoreactivity (red; **b** and **e**). Co-expression of GFP and βgal in neuroepithelial cells appears yellow in the merge (**c** and **f**, **arrowheads**). Note the lack of βgal expression in neuroepithelial cells in the presence of βgal-directed esiRNAs. Upper and lower dashed lines indicate the lumenal (apical) surface and basal border of the neuroepithelium, respectively. Asterisks in (**b** and **e**) indicate signal due to the cross-reaction of the secondary antibody used to detect βgal with the basal lamina and underlying mesenchymal cells. Scale bar in (**f**), 20 μm. (**g**) Quantitation of the percentage of GFP-expressing neuroepithelial cells that also express βgal without (**Control**) or with (**siRNA**) application of βgal-directed esiRNAs. Data are the mean of three embryos analyzed as in (**a-f**); bars indicate S.D. (Reprinted figure with permission from PNAS). (See color section.)

driving the constitutive expression of reporter genes were injected into the respective cavity, with or without reporter-directed esiRNAs, followed by directional electroporation and whole embryo culture for 24 hours.

When a mixture of two reporter genes, GFP and βgal, were co-injected and co-electroporated alone, i.e. without esiRNAs, into the E10 neuroepithelium of the anterior neural tube, almost all of the transfected neuroepithelial cells expressed, with an efficiency of up to 90%, both reporter genes after 24 hours of whole embryo culture. In contrast, when the two reporter genes were co-injected and co-electroporated together with a mixture of βgal-directed esiRNAs βgal expression was barely detectable [(Figure 15.4) (Calegari et al., 2002)]. Similarly, injection and electroporation into the E10 beating heart of a GFP reporter plasmid alone, i.e. without esiRNAs, showed, in the vast majority of cases, GFP expression after 24 hours of whole embryo culture. In contrast, when GFP reporter plasmids were co-injected and co-electroporated together with GFP-directed esiRNAs, no GFP expression could be seen. These experiments revealed a very high efficiency of co-transfection *in vivo* of two reporter genes and an almost complete, specific esiRNA-mediated silencing of exogenous gene expression in cavity-facing tissues. In addition, it is worth noting that in both cases analysed, the whole procedure of injection, electroporation and whole embryo culture did not affect, to any observable extent, the normal functionality and development in culture of the target organs.

Second. Silencing of exogenous gene expression in tissues lacking cavities, such as the ectoderm, was investigated as follows: Plasmid vectors driving the constitutive expression of reporter genes were released in proximity of the tissue, with or without reporter-directed esiRNAs, followed by directional electroporation and whole embryo culture for 24 hours.

When a mixture of two reporter genes, GFP and DsRed, together with unspecific esiRNAs as control, was co-administered into the culture medium in proximity of the limb bud ectoderm and co-electroporated, almost all transfected ectodermal cells co-expressed both reporter genes after 24 hours of whole embryo culture. In contrast, when the two reporter genes were co-injected and co-electroporated together with a mixture of DsRed-directed esiRNAs, DsRed expression was barely detectable (Calegari et al., submitted). These experiments confirmed the previously shown high efficiency of co-transfection achieved by *in vivo* electroporation (Saito and Nakatsuji, 2001), and the high efficiency and specificity of esiRNA-mediated gene silencing.

Third. Silencing of gene expression for the endogenously expressed transgene GFP was investigated by injecting, into the cavity of the neural tube, a mixture of GFP-directed esiRNAs, followed by directional electroporation and whole embryo culture for 24 hours.

RNAi specifically prevents the translation of complementary mRNA and has no effect on the pre-existing protein. For this reason, the effect of esiRNAs-mediated gene silencing should be particularly evident for genes whose transcription is succeeding the delivery of the siRNA. Knowing that during neurogenesis the gene TIS21 is specifically expressed in neuron-generating neuroepithelial cells, with an onset of expression in the mouse telencephalon at E10 (Iacopetti et al., 1999), we decided to investigate the possibility of silencing endogenous gene expression in the E10 neuroepithelium of mice obtained from a knock-in line expressing GFP from the TIS21 locus ($Tis21^{+/tm2(Gfp)Wbh}$, Haubensak et al. 2004).

Upon injection of GFP-directed esiRNAs into the anterior neural tube, electroporation using a cathode right/anode left orientation, and 24-hour whole embryo culture, we observed a dramatic reduction, by about 90%, of green fluorescence in the anode-facing left side of the targeted neural tube as compared to the controlateral, cathode-facing right side (Figure 15.5). Moreover, quantification of GFP expressing cells on fixed cryosections obtained from these regions of the neuroepithelium indicated a reduction of GFP-positive cells by about 75% as compared to the controlateral side of the neural tube (Calegari et al., 2002).

Fourth. We finally investigated the possibility to induce gene silencing for a truly endogenous, non-transgenic gene by injecting and electroporating TIS21-directed esiRNAs into the anterior neural tube of wild-type E10 mouse embryos, i.e. at the onset of TIS21 expression, followed by whole-embryo culture for 24 hours.

In situ hybridization on fixed slices obtained from these embryos showed that esiRNA-triggered RNAi may be achieved not only for an endogenously expressed transgene (GFP in the $Tis21^{+/tm2(Gfp)Wbh}$ mouse line), but also for TIS21 itself (Calegari et al., 2004). It is therefore conceivable that esiRNA-mediated gene

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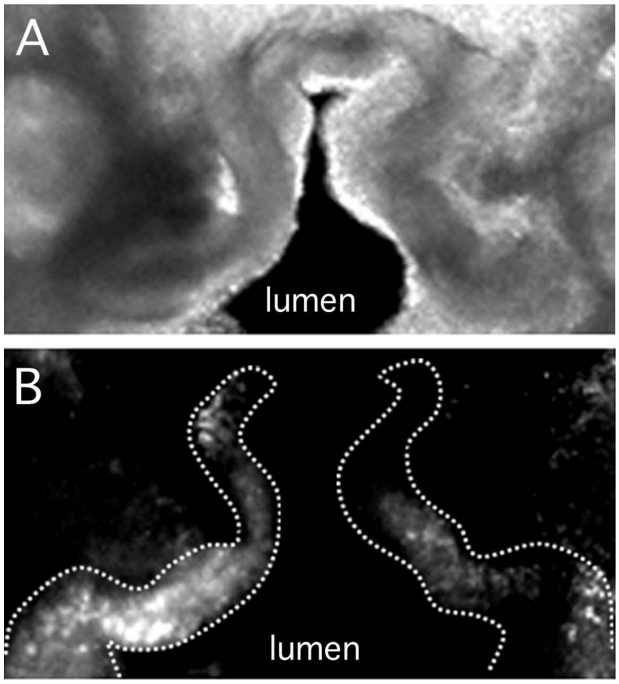

Figure 15.5. esiRNA-mediated RNAi of a gene endogenously expressed during the development of postimplantation mouse embryos. Heterozygous E10 *Tis21*$^{+/tm2(Gfp)Wbh}$ mouse embryos were injected with GFP-directed esiRNAs into the lumen of neural tube, followed by directed electroporation (lateral, cathode-right/anode-left orientation) and whole-embryo culture for 24 hours. A. Low-power dark-field micrographs of horizontal vibratome sections through the diencephalon. The left half of the brain is on the right side of the panels. B. Note the silencing of GFP expression in the selected neuroepithelium (dashed lines) in the left, anode-facing half of the embryo.

silencing may be achieved for any endogenously expressed gene by using topical injection and directional electroporation of esiRNAs.

These findings, together with reports from other laboratories showing specific knock-down of gene expression in postimplantation mouse embryos using either long dsRNAs (Mellitzer et al., 2002) or shRNAs (Gratsch et al., 2003), extend previous work showing siRNA-triggered RNAi in adult mice (Lewis et al., 2002; McCaffrey et al., 2002).

Conclusions

The use of topical injection and directional electroporation of esiRNAs to induce region- and tissue-specific silencing of gene expression during the development in whole embryo culture of postimplantation mouse embryos offers several advantages as compared to other classical techniques of gene knock-down.

The first advantage of this technique is the possibility to obtain gene knock-down without the labour-intensive and time-consuming generation of genetically modified animals. In addition, the combination of topical injection and directional electroporation (using electrodes of the appropriate size and shape) allows

easy restriction of the silencing effect only to a selected portion of organ, tissue or even single cells. Another major advantage of this approach is the possibility of using a mixture of siRNAs directed against mRNAs of different genes as an easy and rapid way of preventing the possible appearance of compensatory effects due to gene redundancy. In fact, the knock-down of multiple genes using mixtures of siRNAs may constitute a feasible approach for genome-wide functional screens in order to systematically study gene function during mammalian development.

The present approach is mainly limited by the temporal restrictions of whole embryo culture, which can be efficiently carried out only for up to two days from E7 until E12. However, it should be noted that this limitation could be overcome both by *in utero* electroporation (Takahashi et al., 2002) and by tail injection into pregnant mice (Gratsch et al., 2003). Moreover, the use of DNA templates expressing shRNAs, as alternative to esiRNAs, would allow the extension of this technology to long-term studies of mouse development.

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