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REVIEW

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Tissue-specific RNA interference in post-implantation mouse embryos using directional electroporation and whole embryo culture

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Abstract In mammals, embryonic development is more difficult to analyze than in non-mammalian species because this development occurs *in utero*. Interestingly, whole embryo culture allows the normal development of mouse post-implantation embryos for up to 2 days *in vitro*. One limitation of this technology has been the difficulty of performing loss-of-gene function studies in this system. RNA interference (RNAi), whereby double-stranded RNA molecules suppress the expression of complementary genes, has rapidly become a widely used tool for gene function analyses. We have combined the technologies of mouse whole embryo culture and RNAi to allow the molecular dissection of developmental processes. Here, we review the manipulation by topical injection followed by directional electroporation of endoribonuclease-prepared siRNA to demonstrate that this technology may be useful to knock down genes in a tissue- and region-specific manner in several organs of the developing mouse embryo.

Key words RNA interference · endoribonuclease-prepared short interfering RNAs · whole embryo culture · *in vivo* electroporation

Introduction

In recent years, the complete genomes of several organisms have been sequenced, including the genome of the mouse (Waterston et al., 2002). While this is a remarkable accomplishment, it is only the first step toward a better understanding of the genome. One of the challenges for the coming years will be the functional analyses of each single gene encoded by the genome of a given species.

In the mouse, the classical analysis of gene function is performed through targeting of a specific gene by homologous recombination in embryonic stem (ES) cells (Capecchi, 1989). These ES cells are subsequently injected into blastocysts, and chimeric offspring is checked for germline transmission. Successful germline transmission will then allow obtaining of animals deficient in the gene of interest. Careful phenotypic analyses of these animals can then reveal the function(s) of the knocked-out gene. Because this approach is very time consuming and cost intensive, it would be a difficult undertaking to study all genes of the mouse genome in this way. Furthermore, the amount of information gained by classical gene knock-outs is often very limited, especially when the gene of interest is essential during embryonic development. Moreover, gene redundancy and compensatory mechanisms often prevent the appearance of observable phenotypes, hence requiring the production of double- or triple-knock-out organisms.

To circumvent early embryonic lethality of essential genes, the Cre/loxP system has been developed to allow conditional gene knock-outs in mice (reviewed in Lewandoski, 2001; Kuhn and Schwenk, 2003). In this system, two lines have to be generated and crossed. The first line carries the gene of interest that is flanked by the recognition target sites for Cre recombinase (loxP). The second line expresses the Cre recombinase from a promoter of choice. To avoid nonspecific effects of the selection cassette used to identify ES cells that have integrated the targeting construct, a second recombinase system is often used to remove the selection cassette after clone validation (Rodriguez et al., 2000). After expression of the Cre recombinase, the loxP-flanked gene will be excised, thereby generating a conditional knock-out.

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While these are very sophisticated and powerful methods for gene function analysis in the mouse, they are even more time consuming and even more expensive than classical knock-outs, making this method impractical for large-scale gene function analyses.

Recently, gene knock-down technology employing RNA interference (RNAi) has become available to mammalian cells in general (Elbashir et al., 2001a), and to mouse genetics in particular (Calegari et al., 2002; Hasuwa et al., 2002; Lewis et al., 2002; McCaffrey et al., 2002; Mellitzer et al., 2002). In RNAi experiments, gene loss-of-function analysis is possible because double-stranded RNA (dsRNA) that is homologous to the mRNA of the gene of interest triggers the specific degradation of this mRNA. Because long dsRNAs trigger an interferon response in most mammalian cells (Stark et al., 1998), the discovery that short interfering RNAs (siRNAs), which do not trigger this response, mediate RNAi (Elbashir et al., 2001b) was an important step toward the general use of RNAi in mammalian cells. Compared to the knock-out technology, loss-of-gene function analyses via RNAi is much less labor intensive and for that reason also more cost effective. In addition, simultaneous silencing of gene expression can easily be achieved by using a mixture of different siRNAs targeting different genes (C. Echeverri, personal communication) in order to prevent compensatory mechanisms due to the existence of redundancy.

In order to extend loss-of-gene function analyses via RNAi to mammalian embryos, systems are required that allow embryo manipulations and development. An important technological advance in this sense has been the establishment of mouse whole embryo culture (Cockroft, 1990). This technique allows the normal development of mouse post-implantation embryos for up to 2 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).

In this review, we describe the combination of (1) mouse whole embryo culture technology, (2) embryo manipulation through topical injection and directional electroporation, and (3) RNAi technology. This combination presents a powerful, fast, and economical platform to study gene function during mouse development.

RNA interference

The finding of RNAi has endowed genetic research with a new tool that facilitates gene function studies in somatic cells. RNAi was discovered in the nematode *C. elegans* and in plants (Fire et al., 1998; Jorgensen et al., 1998), where it was shown that dsRNA molecules suppress the expression of genes with complementary sequences. In these organisms, introduction of dsRNA of typical sizes around 500–1000 bp by either transgene expression, injection, or feeding was shown to trigger specific gene silencing. In *C. elegans*, loss-of-function studies using RNAi have been applied on a genomic scale and nearly all genes of this animal have been knocked down and analyzed (Gonczy et al., 2000; Kamath et al., 2003).

Unfortunately, long dsRNAs have a strong non-specific negative effect in many vertebrate cells because they trigger an interferon response (reviewed in Stark et al., 1998). Interferon production activates two pathways in the cell that negatively affect cell proliferation. First, 2′-5′-oligoadenylate synthetase (2′-5′-OAS) is turned on, leading to mRNA degradation via activation of RNase L. Second, interferons cause the activation of dsRNA-dependent protein kinase, leading to the general inhibition of protein synthesis by phosphorylation of the translation factor eIF-2α. As a consequence, long dsRNA is not useful for specific gene silencing in most mammalian cells.

Detailed analyses of the RNAi pathway revealed that long dsRNA is processed into 20–25 bp fragments within cells by an RNase III-like endoribonuclease called Dicer (Hammond et al., 2000; Yang et al., 2000; Zamore et al., 2000; Bernstein et al., 2001). These short dsRNA fragments are called short interfering RNA (siRNA), which contain the 2-nucleotide 3′-overhang signatures of RNase III enzymes (Conrad and Rauhut, 2002). Dicer is part of a protein complex termed the RNA-induced silencing complex (RISC), which mediates target RNA cleavage (Tuschl et al., 1999). Interestingly, siRNA does not trigger a strong interferon response in mammalian cells, while efficiently and specifically acting as a silencing trigger for a corresponding gene (Elbashir et al., 2001b).

The first RNAi experiments with siRNAs were carried out in mammalian tissue culture cells (Elbashir et al., 2001a). Shortly after these experiments, several laboratories showed that siRNAs could also silence gene expression in the adult (Hasuwa et al., 2002; Lewis et al., 2002; McCaffrey et al., 2002) and in the post-implantation mouse embryo (Calegari et al., 2002). In most of these experiments exogenously expressed reporter genes were silenced, including luciferase and GFP. While it is still too early to say how effective RNAi will be in generating null phenotypes, recent results indicate that phenotypes observed via RNAi resemble phenotypes produced by classical gene knock-out through gene targeting in ES cells (Kunath et al., 2003).

Endoribonuclease-prepared short interfering RNAs

There are currently three major methods to produce siRNAs (reviewed in Kittler and Buchholz, 2003). The

most widely used methods for siRNA generation are either the chemical synthesis of two single-stranded RNAs (ssRNAs) that are subsequently annealed, or the expression of short hairpin RNAs (shRNAs) that are processed into siRNAs within the cell (Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). However, every siRNA sequence has a different potential to silence the target gene, and we currently do not understand enough about the silencing mechanism to predict the most effective siRNA for a given gene product. Hence, chemically synthesized siRNA and shRNA both have the disadvantage that each molecule has to be checked for its silencing efficiency. Furthermore, chemically synthesized siRNA is expensive and shRNA vector construction is labor intensive.

A cheap and efficient alternative for siRNA production is the enzymatic digestion of long dsRNA with purified Dicer (Kawasaki et al., 2003; Myers et al., 2003) or *E. coli* RNase III (Yang et al., 2002). The digestion of long dsRNA with these enzymes generates of a large pool of different siRNAs that are called *e*ndoribonuclease-prepared *s*hort *i*nterfering RNAs, or esiRNAs (Fig. 1). Because the digestion produces a mixture of siRNAs spanning a large region of the transcript, esiRNA typically contains siRNA molecules that will trigger an efficient and specific gene knockdown when introduced into cells. For this reason, screening for effective molecules is not necessary.

Recently, conflicting results have been published on the specificity of gene silencing with chemically synthesized siRNAs and expressed shRNAs (for a review, see Check, 2003). *Off-target* effects employing esiRNA have not yet been investigated by gene expression profiling. However, we have conducted *in silico* experiments with a large number of esiRNAs directed against different genes and have seen that less than 10% of genes contain an siRNA that matches a different gene (B. Habermann and F.B., unpublished observations). Furthermore, we have not seen cross-silencing of highly homologous genes utilizing quantitative RT-PCR experiments (F.B., unpublished observations) and Western blot analyses (Yang et al., 2002). Therefore, while data from expression profiling are not yet available, we conclude that cross-silencing of homologous genes is typically not observed when esiRNA is used.

A difference between Dicer and *E. coli* RNase III is their mode of cleavage. Dicer starts at the ends of the dsRNA and proceeds in a sequential manner (Zhang et al., 2002), whereas *E. coli* RNase III cleaves long dsRNA in a more or less random fashion (Apirion and Miczak, 1993; Yang et al., 2002), leading to a more complex mixture of siRNAs. Another difference is the ease of purification of the enzymes and their activity in *in vitro* reactions. Dicer is relatively difficult to express and purify as a recombinant protein, and its activity is very low compared to *E. coli* RNase III. In contrast,

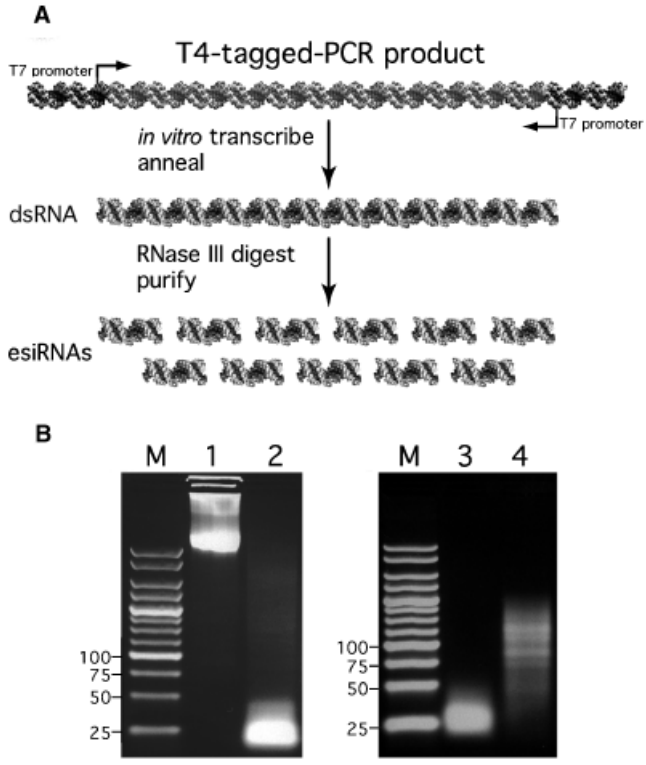

Fig. 1 Preparation of esiRNA. (**A**) Schematic flow of esiRNA production. Generated molecules and the necessary steps to produce subsequent reagents are indicated. (**B**) Agarose gels from limited digestion of dsRNA with GST-RNase III before and after purification. Lane M: molecular weight markers indicated in base pairs. Lane 1: dsRNA before digestion. Lane 2: dsRNA after RNase III digestion for 2 hr at 23°C. Lane 3: esiRNA after purification. Lane 4: concentrated dsRNA molecules >30 bp that were separated from the esiRNA fraction.

RNase III expresses well in its natural host without any obvious phenotype, and large quantities of highly active enzyme can be rapidly purified.

Complete digestion of long dsRNA with *E. coli* RNase III generates fragments of 12–15 bp in length (Apirion and Miczak, 1993; Yang et al., 2002). These fragments are too short to trigger gene silencing. However, digestion conditions can be chosen in a way that dsRNA between 15 and 30 bp in size is generated (see Fig. 1). After a simple purification step, this mixture is an efficient and specific mediator of RNAi in mammalian cells.

Embryo manipulations

Whole embryo culture

The most obvious intrinsic difficulty in studying mammalian prenatal development is the lack of a direct observation and accessibility of the object of study: the developing embryo. For this reason, in the past several

decades, great efforts have been made toward establishing reproducible techniques for the culture of whole post-implantation rodent embryos that allow normal embryonic development at the morphological, cellular, and molecular levels. The first pioneering studies date back to the 1930s, when J.S. Nicholas and D. Rudnick cultured, on plasma clots, rat embryos at the neurulation stage (Nicholas and Rudnick, 1934). If compared to the current standards of whole embryo culture, general embryonic development in these initial attempts was relatively poor; still, these experiments represent the very first promising trials in a new area of investigation.

Remarkable improvements in whole embryo culture were obtained in the 1970s, when the extensive and systematic work of D. New led to the identification of serum, obtained by immediate separation from blood cells, as the most critical constituent of the culture medium (New, 1978). At the same time, other optimizations such as a continuous oxygenation and circulation of the medium during the culture period have remarkably improved the technique of rat and mouse whole embryo culture, allowing growth and development indistinguishable from that occurring *in utero* (New, 1978; Cockroft, 1990).

The developmental stages during which whole embryo culture can be applied range from egg cylinder to about 60 pairs of somites, but the highest efficiency and reliability of this technique is obtained from early somite (E7.5–8.0 in the mouse) to organogenetic stage (E10.5–11.5; 35–40 pairs of somites), allowing normal development to continue for up to 2 days in culture. During this period of time, major processes of differentiation and organ development take place, including neurulation, somitogenesis, and development of the cardiovascular, digestive, and locomotor system.

In whole embryo culture, all organs of the embryo have been reported to develop normally, at least at the morphological level (Cockroft, 1990). Moreover, as paradigmatically shown for the onset and progression of the gradients of neurogenesis in mouse embryos grown for up to 2 days *in vitro*, whole embryo culture has been shown to support normal development not only at the morphological, but also at the cellular and molecular levels (Calegari and Huttner, 2003, and unpublished data).

Prior to whole embryo culture, embryos are freed from uterine walls, decidua capsularis, and Reichert's membrane, and are then cultured with their amnion, yolk sac (closed or open, depending on the developmental stage), and ectoplacental cone. Typically, whole embryo culture is carried out in culture tubes containing approximately 0.5–1 ml/embryo of medium, the principal constituent (50%–100%) of which is immediately centrifuged, heat-inactivated rodent serum. Sera obtained from other species, such as human or bovine sera, are also able to support development *in vitro*, although not quite as satisfactorily as rodent sera (New, 1978). Particularly important for embryonic development is the oxygenation of the medium. Depending on the developmental stage, different O_2 concentrations, from 5% at the early somite stage to 95% at the end of the organogenetic stage, are used. Continuous equilibration of the medium with the gaseous phase is obtained by placing the culture tubes on a tilted roller apparatus that is located in a common cell culture incubator. Under these conditions, a high percentage of embryos develop in culture correctly (New, 1978; Cockroft, 1990); specifically, in our hands, up to 80% of E10.5 embryos are, after 24 hr of whole embryo culture, morphologically indistinguishable from E11.5 embryos developed entirely *in utero*.

Until now, one of the most critical, time-consuming steps for whole embryo culture was the preparation of adequate amounts of immediately centrifuged rodent serum of reproducibly good quality. Fortunately, this major limitation has now been overcome by the availability of commercial rodent sera specifically for whole embryo culture and, more recently, by the characterization of serum-free synthetic media (Moore-Scott et al., 2003) that support normal embryonic development. In addition, incubators specific for whole embryo culture that provide continuous oxygenation and rotation of the culture tubes during the culture period (Osumi and Inoue, 2001) have recently become available.

The possibility to directly observe and manipulate mammalian embryos developing *ex utero* opens up fascinating avenues for several kinds of studies. Until now, whole embryo culture has mostly been applied to teratological and toxicological studies, in which the only manipulations of the embryo consisted of the addition of noxious or potentially dangerous compounds. However, recently, several sophisticated techniques have been successfully applied to whole embryo culture, such as cell or tissue transplantation, antibody interference, cell lineage tracing, infection with viral vectors, and electroporation of nucleic acids (Drake and Little, 1991; Tam, 1998; Inoue et al., 2000; Oback et al., 2000; Osumi and Inoue, 2001). Combining these techniques with whole embryo culture offers novel opportunities to study mammalian development at the morphological, cellular, and molecular levels.

Electroporation

Electroporation is a standard method used to deliver molecules into cells. This technique is based on electric pulses creating transient pores in the plasma membrane through which molecules can pass. Several kinds of molecules (drugs, peptides, proteins, polysaccharides, etc.) have been successfully delivered into cells by electroporation (Banga and Prausnitz, 1998), but the

most efficient and widely used application of electroporation consists in the active transfer, by an electric field, of the negatively charged DNA molecules.

In the past, attempts were made to apply electroporation conditions typically used for cultured cells to living tissues, but for reasons still not entirely clear, the bell-shaped electric pulses normally used for dissociated cells result in a degree of cell death that is incompatible with tissue integrity. Fortunately, the finding that long, square-shaped electric pulses considerably decrease the degree of cell death and tissue damage has allowed the successful application of electroporation to living tissue *in vivo*, specifically the embryonic chick neuroepithelium (Muramatsu et al., 1997). Given the availability of the mouse whole embryo culture technology, the shift from *in vivo* electroporation of chick embryos to that of mouse embryos was straightforward (Akamatsu et al., 1999).

Viral vectors were one of the first means to obtain gene transfer into post-implantation mouse embryos in culture (Oback et al., 2000). However, compared with electroporation of plasmid DNA, gene transfer via viral vectors has several disadvantages, including the relatively time-consuming preparation of viral particles, potentially toxic side effects of the viral infection, and biosafety issues (if applicable). Moreover, with *in vivo* co-electroporation, multiple genes can 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 emerges as a suitable approach for genome-wide functional screens.

Until now, *in vivo* electroporation as a means of gene transfer for functional studies has almost exclusively been applied to the developing neural tube as target tissue, both in mouse and chick embryos. The reasons for this include (1) a general high interest in developmental neuroscience, (2) the fact that key steps in neural development, such as the onset of neurogenesis, coincide with the time period during which whole embryo culture can be carried out with a high rate of success, and (3) the fact that electroporation is particularly easy and effective when nucleic acids are injected into the cavity of an organ, in this case the lumen of the neural tube (reviewed in Inoue and Krumlauf, 2001). In the latter context, it should be noted that *in vivo* electroporation is not necessarily limited to tissues facing a cavity, because this technique has also been applied to tissues lacking internal cavities such as skin (in which case nucleic acids are released in the proximity of the tissue; see below; Banga and Prausnitz, 1998). In addition, DNA molecules have successfully been delivered into target cells by direct injection into the tissue, such as muscle (Aihara and Miyazaki, 1998). Finally, the approach of introducing DNA molecules by electroporation has also been applied to adult (rather than embryonic) organisms and to cell lineage tracing studies (rather than functional studies), and may be applicable to any vertebrate, as exemplified by the use of single-cell electroporation of reporter genes in the axolotl (Echeverri and Tanaka, 2003).

Equipment for *in vivo* electroporation typically includes (1) a pneumatic pump, used to control the volume of the nucleic acid-containing fluid to be administered to the target tissue via a glass capillary, (2) a micro-manipulator, connected to the pneumatic pump, which holds the capillary and allows its fine movements, (3) a stereo microscope, (4) electrodes of the desired size and shape, depending on the size of the target tissue and the location of the target cells, and (5) a square-shaped pulse generator (see Fig. 2A).

In combination with a *topical* administration of nucleic acids, *in vivo* electroporation provides a means of *region-specific* gene transfer. Of particular relevance is the possibility to orient the electric field, which is the driving force for nucleic acid entry into the electroporated cells, with migration of the negatively charged nucleic acids from the cathode to the anode (Figs. 2B, 2C). Hence, the positioning of the electrodes will determine the region of the tissue under study that will be transfected. When nucleic acids are injected into the cavity of an organ, such as the lumen of the neural tube, their cathode-to-anode-directed migration will result in the transfection of half of the organ, with the other half serving as an internal negative control. Moreover, the use of electrodes of different shapes and sizes allows the transfection of entire tissues as well as single cells (Haas et al., 2002).

In our experimental conditions, the percentage of transfected cells in the targeted portion of the tissue, as judged from the expression of reporter plasmids, is usually between 20% and 50%. It is, however, important to mention that the transfection efficiency achieved by electroporation of nucleic acids of smaller size, such as siRNAs, is much higher than that of plasmids. In support of this, upon *in vivo* electroporation of esiRNAs directed against an endogenously expressed transgene (see below), we observed a silencing effect of transgene expression of up to 90% (Calegari et al., 2002).

After tissue- or region-specific transfection of nucleic acids using such directional *in vivo* electroporation, the whole embryo culture technology then allows one to study the effects of the introduced nuclei acids, such as the functional consequences of expression of a given transgene, on embryonic development (Fig. 2D).

It is finally worth noting that the electrodes do not have to be in direct contact with the embryonic tissue, as long as there is electrical conductivity. This has allowed *in vivo* electroporation to be carried out directly *in utero*, either after exposure of the uterus of pregnant mice (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001) or by using an ultrasound backscatter microscope

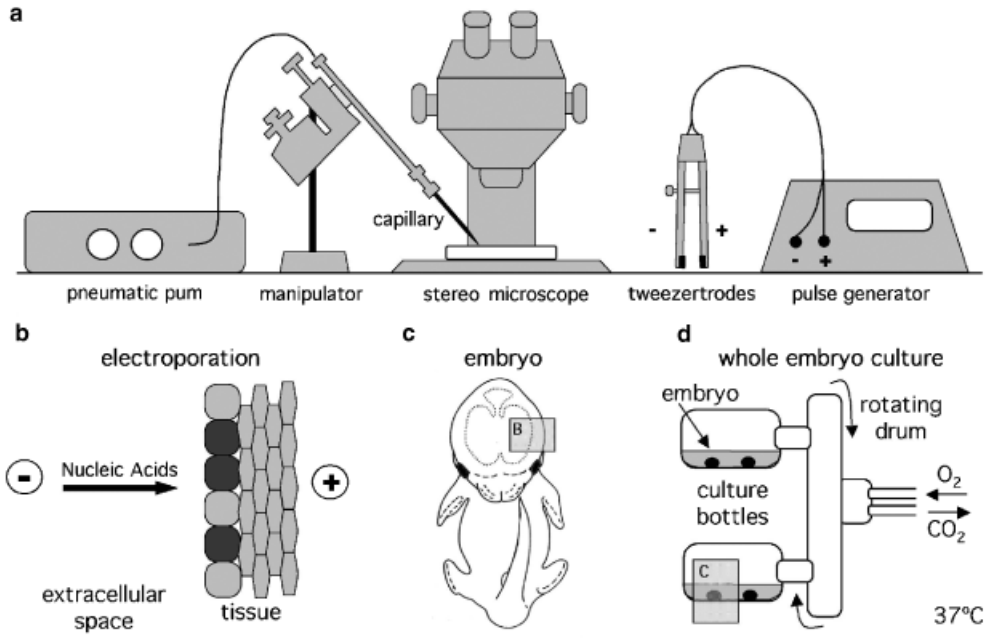

Fig. 2 Scheme recapitulating the whole embryo electroporation and culture. (**a**) Equipment used for topical administration and directional electroporation of nucleic acids. (**b**) Cartoon illustrating the cathode-to-anode migration (arrow) of nucleic acids administered into the extracellular space facing the target tissue. Darker cells represent cells having internalized the nucleic acids. (**c**) Cartoon of mouse embryo; gray box indicates the targeted region shown in (**b**); the dotted line indicates the apical (lumenal) side of the neuroepithelium. (**d**) Cartoon showing the system of whole embryo culture. Gray box indicates the embryo shown in (**c**).

(Takahashi et al., 2002). These *in utero* electroporation techniques overcome the temporal limitations of whole embryo culture (i.e., cultures for only 2 days from E7 to E12 in the mouse), thereby broadening the spectrum of application of *in vivo* electroporation to later stages of mouse embryonic development and to studies that require longer times of observation.

RNAi in post-implantation mouse embryos

The recent findings that siRNAs can be used to knock down gene expression in mammalian cells in culture (Elbashir et al., 2001a) led us to explore the use of siRNAs in the whole organism. In particular, we addressed the possibility of inducing tissue- and region-specific RNAi in mouse embryos developing in whole embryo culture (Calegari et al., 2002; Gratsch et al., 2003). As an experimental approach, we decided to use the neuroepithelium of E10 mouse embryos as a model target tissue and to investigate the use of electroporation for the delivery of esiRNAs in specific regions of the developing central nervous system.

Knocking down of gene expression by esiRNAs was first addressed for exogenous genes. Two plasmid vectors driving the constitutive expression of two reporter genes, GFP and β-galactosidase (β-gal), were co-injected, with or without β-gal-directed esiRNAs, in defined regions of the neural tube and co-electroporated into the neuroepithelium. After electroporation, embryonic development was allowed to continue for 24 hr in whole embryo culture and expression of GFP and β-gal was investigated. This showed that when a mixture of the two reporter plasmids are used alone—that is, without esiRNAs—transfected neuroepithelial cells almost always (up to 90%) expressed both reporter genes, indicating a high efficiency of co-transfection achieved by electroporation. In contrast, when a cocktail of β-gal-directed esiRNAs was added to the mixture of the reporter genes, β-gal expression was barely detectable (Fig. 3; Calegari et al., 2002).

To investigate whether RNAi could efficiently be applied not only to the neural tube but also to other organs of the developing mouse embryo, a similar experimental approach was used using the heart as target organ. In a series of experiments, a GFP reporter plasmid was injected into the ventricle of the beating heart of E10 mouse embryos, either alone or together with a cocktail of GFP-directed esiRNAs. After 24 hr of whole embryo culture, whole-mount analysis of embryos revealed evident GFP expression in the heart in the majority of cases when the GFP reporter plasmid had been injected alone. In contrast, when the GFP reporter plasmid was co-injected and co-electroporated with GFP-directed esiRNAs, none of the embryos showed detectable expression of GFP (data not shown). It is worth noting that the entire procedure of injection and electroporation did not have any obvious effects on the development of the heart and its functionality, apart

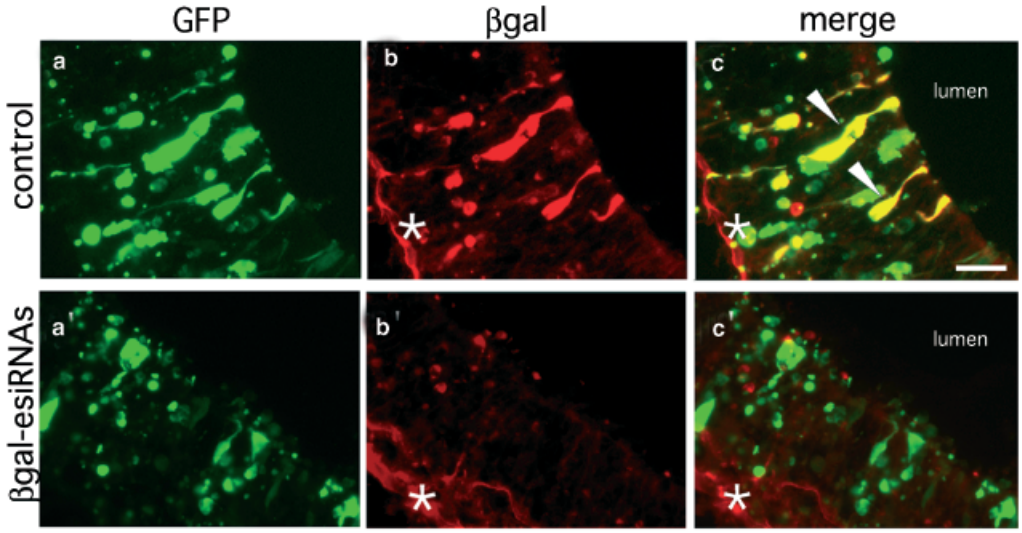

Fig. 3 RNAi in the neuroepithelium of E10 mouse embryos. E10 mouse embryos were injected into the lumen of the telencephalic neural tube with the two reporter plasmids pEGFP-N2 (for GFP) and pSVpaXD (for β-gal) either without (**a**–**c**; control) or with (**a′**–**c′**; β-gal-esiRNAs) β-gal-directed esiRNAs, followed by directional electroporation and whole embryo culture for 24 hr. Horizontal cryosections through the targeted region of the telencephalon were analyzed by double fluorescence for expression of GFP (green; a, a′) and β-gal immunoreactivity (red; b, b′). Co-expression of GFP and β-gal in neuroepithelial cells (arrowheads) appears yellow in the merge (c, c′). Note the lack of β-gal expression in neuroepithelial cells in the presence of β-gal-directed esiRNAs. Asterisks in (b), (b′), (c), and (c′) indicate signal due to the cross-reaction of the secondary antibody used to detect β-gal with the basal lamina and underlying mesenchymal cells. The scale bar in (c) represents 20 μm.

from a transient increase in cardiac frequency during the delivery of the electric pulses. Given that the cardiovascular system, like the nervous system, starts to differentiate and develop at an embryonic stage that can be studied in whole embryo culture, this technology may be particularly useful for studies of cardiovascular development. In addition, an intriguing possibility of this approach may be the delivery of siRNAs to the entire organism through injection into the heart and distribution via circulation.

We also addressed the use of esiRNAs to knock down gene expression in the ectoderm of E10 mouse embryos, which is about 2 days prior to the onset of differentiation of the skin. For this purpose, a mixture of two plasmid vectors driving the constitutive expression of two reporter genes, GFP and DsRed, together with either β-gal-directed esiRNAs (as control) or GFP-directed esiRNAs, was administered into the incubation medium in proximity to the limb bud of E10 mouse embryos, followed by directional co-electroporation into the limb bud. Embryonic development was allowed to continue for 24 hr in whole embryo culture, and expression of GFP and DsRed was investigated. When GFP and DsRed reporter genes were co-electroporated together with β-gal-directed esiRNAs, we observed, as previously shown for the neuroepithelium, almost complete co-localization of the two reporter gene products (Fig. 4, top). In contrast, when the two reporters were co-electroporated together with GFP-directed esiRNAs, the expression of GFP was almost totally prevented (Fig. 4, bottom), demonstrating the high efficiency and specificity of siRNA-mediated gene silencing. Obviously, this technology offers several interesting applications for future studies of limb development and skin differentiation.

We then investigated whether knocking down of gene expression by esiRNAs could be obtained not only for exogenous genes, delivered together with the esiRNAs, but also for endogenously expressed genes. Because RNAi will only prevent the translation of mRNA and not degrade preexisting proteins, the effect of electroporation of esiRNAs should be particularly evident for genes, the transcription of which is turned on at the stage of development at which electroporation of esiRNAs is carried out. During the development of the central nervous system, the anti-proliferative gene *TIS21* is known to be specifically expressed in neuron-generating neuroepithelial cells, with an onset of expression in the mouse telencephalon at E10 (Iacopetti et al., 1999). We therefore decided to investigate the possibility of silencing endogenous gene expression in the E10 neuroepithelium of knock-in mouse embryos expressing GFP from the TIS21 locus ($Tis21^{+/tm2(Gfp)Wbh}$ mouse line; Haubensak et al., 2004), using expression of the GFP transgene as read-out.

After electroporation of a mixture of GFP-directed esiRNAs and whole embryo culture for 24 hr, we observed a dramatic reduction, by about 90%, of green fluorescence in the anode-facing side of the targeted telencephalon as compared to the controlateral, cathode-facing side of the neural tube. Moreover, quantification of the cells showing green fluorescence indicated

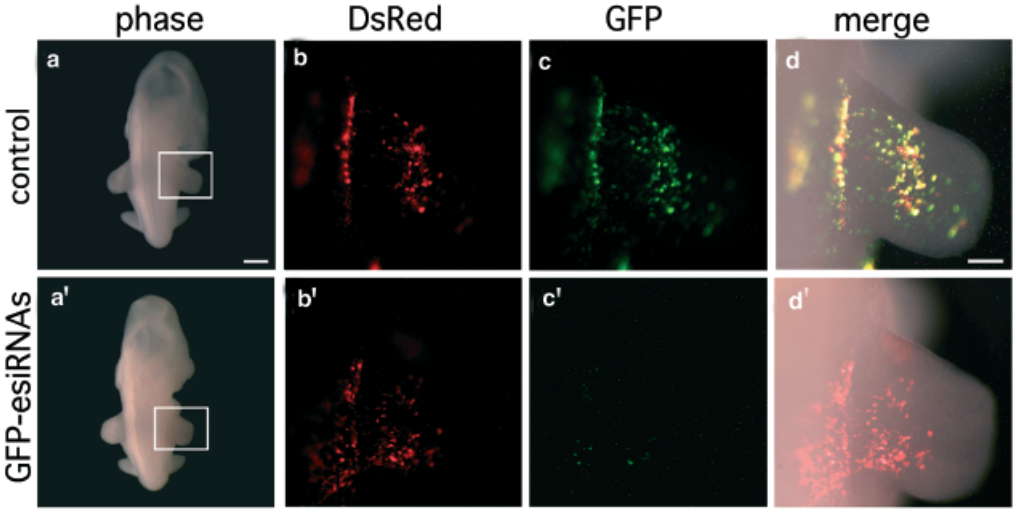

Fig. 4 RNAi in the limb bud ectoderm of E10 mouse embryos. E10 mouse embryos received, in proximity of the limb bud, the two reporter plasmids pDsRed2 (for DsRed) and pEGFP-N2 (for GFP) either with β-gal-directed esiRNAs as control (control; **a**–**d**) or with GFP-directed esiRNAs (GFP-esiRNAs; **a′**–**d′**), followed by directional electroporation and whole embryo culture for 24 hr. The whole embryos after culture (a, a′) were analyzed by direct fluorescence in the targeted region (white square boxes; a, a′) for the expression of DsRed (red; b, b′) and GFP (green; c, c′). Co-expression of DsRed and GFP in ectodermal cells appears yellow in the merge (d, d′). In (d) and (d′), the phase contrast picture of the limb bud was electronically merged with the signal of the fluorescent channels. Note the lack of GFP expression in the ectoderm electroporated in the presence of GFP-directed esiRNAs. The scale bar in (a) represents 500 μm, and in (d) represents 150 μm.

a reduction of GFP-expressing cells by about 75% as compared to the controlateral, cathode-facing side (Calegari et al., 2002). Our findings, together with reports from other laboratories showing specific knock-down of gene expression in post-implantation mouse and rat 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). The approach recently reported by O'Shea and colleagues (Gratsch et al., 2003), injection into the tail of pregnant mice, seems to offer a particularly easy possibility to silence gene expression in post-implantation embryos, as it circumvents both electroporation and whole embryo culture.

Finally, recent data show 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 endogenous genes such as *TIS21* and *prominin-1*. *In situ* hybridization reveals that 24 hr after injection into the E10 diencephalon of a wild-type mouse embryo and co-electroporation of TIS21-directed esiRNAs together with a GFP reporter plasmid, TIS21 mRNA in the targeted region (GFP-positive) of the neuroepithelium was significantly reduced as compared to the controlateral, control side of the neural tube (Fig. 5). In addition, in a similar type of experiment using esiRNAs directed against the pentaspan membrane glycoprotein prominin-1, which is concentrated on the apical plasma membrane of neuroepithelial cells (Weigmann et al., 1997), we observed a dramatic reduction of prominin-1 immunoreactivity after 24 hr of whole embryo culture (Fig. 6).

Discussion

In this review, we described the use of topical injection and directional electroporation of esiRNAs to induce region- and tissue-specific silencing of gene expression during the development of mouse post-implantation embryos in whole embryo culture. This technology offers several advantages as compared to other classical techniques of gene knock-down.

First, the present combination of techniques constitutes a practical approach to obtaining silencing of gene expression in post-implantation mouse embryos without the labor-intensive generation of genetically modified animals. In addition, it should be noted that the temporal limitation of whole embryo culture can be overcome by *in utero* electroporation (Takahashi et al., 2002). Moreover, the use of DNA templates expressing shRNAs, as an alternative to esiRNAs, would allow the extension of this technology to long-term studies of mouse development.

Second, gene silencing can easily be limited to a selected (portion of an) organ by using a combination of topical injection/administration and directional electroporation of siRNAs. An important advance in gene function analyses in the mouse has been the development of conditional knock-out technology using

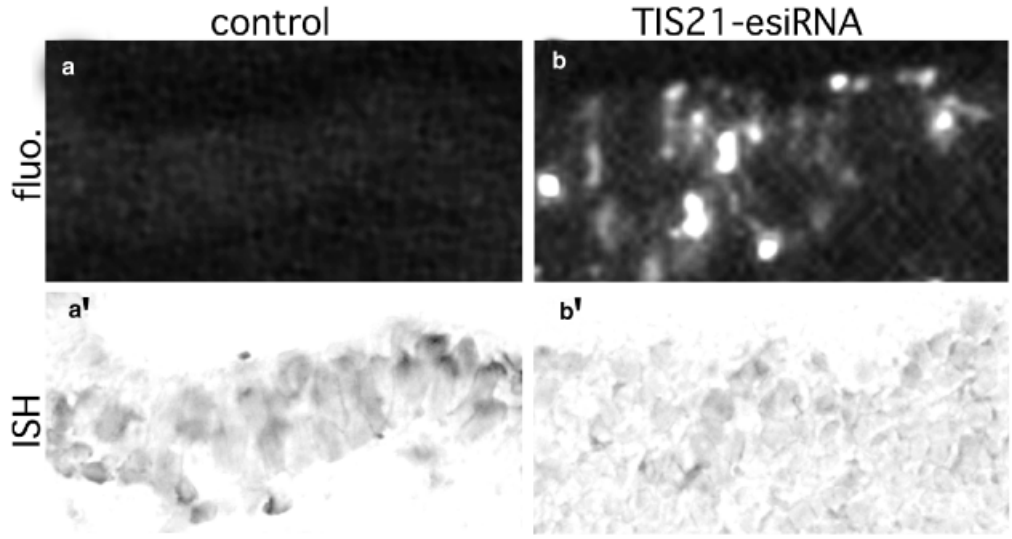

Fig. 5 Knock-down of TIS21 mRNA in the neuroepithelium. E10 wild-type mouse embryos were co-injected, into the lumen of the diencephalic neural tube, with the reporter plasmid pEGFP-N2 together with TIS21-directed esiRNAs, followed by directional electroporation and whole embryo culture for 24 hr. A horizontal cryosection through the targeted region of the diencenphalon was first analysed by fluorescence microscopy for the expression of GFP (fluo.; **a**, **b**), photographed, and then processed for *in situ* hybridization to reveal TIS21 mRNA expression (ISH; **a′**, **b′**). The transfected, GFP-positive region of the diencephalic neuroepithelium (TIS21-esiRNA; b, b′) was compared to the corresponding controlateral, untransfected, GFP-negative region (control; a, a′). At this stage of brain development, TIS21 mRNA expression is known to occur in only a subpopulation of neuroepithelial cells (a′) (Iacopetti et al., 1999). Note the reduction in TIS21 mRNA upon RNAi in the targeted region of the neurepithelium (b, b′).

recombinase systems. This technology is often also referred to as tissue-specific knock-outs. However, this term is somewhat misleading because the deletion of the gene is not determined by the tissue as such. Rather, it is the promoter that is driving the recombinase that directs loss of gene expression in cells where the promoter is active. In contrast, the electroporation of siRNAs can be made truly tissue and region specific. Therefore, RNAi in post-implantation mammalian embryos using topical injection, directional electroporation, and whole embryo culture adds another dimension to conditional mutagenesis in mice.

Third, we find it likely that the use of a mixture of siRNAs, directed against mRNAs of different genes, provides an easy and rapid way to prevent the possible appearance of compensatory effects due to gene redundancy, which often prevents the appearance of a loss-of-function phenotype. In fact, the use of a mixture of siRNAs may constitute a feasible approach for genome-wide functional screens in order to system-

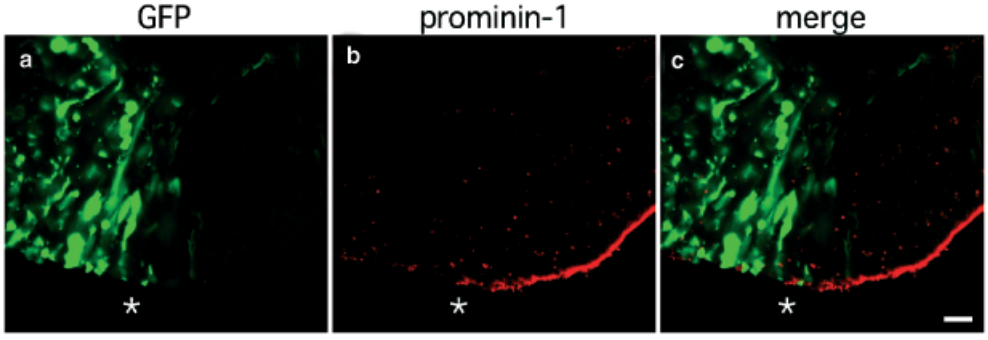

Fig. 6 Knock-down of prominin-1 in the neuroepithelium. E10 wild-type mouse embryos were co-injected, into the lumen of the telencephalic neural tube, with a GFP expression vector (pCAGGS-GFP) together with prominin-1-directed esiRNAs, followed by directional electroporation and whole embryo culture for 24 hr. A transverse cryosection through the targeted region of the telencephalon was analysed by double fluoresence microscopy for GFP expression (green; **a**, **c**), and prominin-1 immunoreactivity (red; **b**, c). Note the almost complete loss of prominin-1 immunoreactivity, which is known to be concentrated on the apical surface of the neuroepithelium (Weigmann et al., 1997), from the targeted region as revealed by GFP expression. The asterisk near the apical surface of the neuroepithelium indicates the boundary between the targeted (left) and nontargeted (right) regions. The scale bar in (c) represents 20 μm.

atically study gene function during mammalian development.

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