

Review Article

Transplacental RNAi: Deciphering Gene Function in the Postimplantation-Staged Embryo

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RNAi offers the opportunity to examine the role in postimplantation development of genes that cause preimplantation lethality and to create allelic series of targeted embryos. We have delivered constitutively expressed short hairpin (sh) RNAs to pregnant mice during the early postimplantation period of development and observed gene knockdown and defects that phenocopy the null embryo. We have silenced genes that have not yet been “knocked out” in the mouse (*geminin* and *Wnt8b*), those required during earlier cleavage stages of development (*nanog*), and genes required at implantation (*Bmp4*, *Bmp7*) singly and in combination (*Bmp4* + *Bmp7*), and obtained unique phenotypes. We have also determined a role in postimplantation development of two transcripts identified in a differential display RT-PCR screen of genes induced in ES cells by noggin exposure, *Aggf1* and an EST (GenBank AK008955). Systemic delivery of shRNAs provides a valuable approach to gene silencing in the embryo.

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INTRODUCTION

With the sequencing of the mouse genome [1], there has been tremendous interest in teasing out the function of “every” gene. In the mouse, gene targeting using homologous recombination in embryonic stem cells (ESC) has provided a unique opportunity to probe gene function in development [2], and a number of powerful techniques have been developed to target genes in temporal or tissue specific ways. Unfortunately, these are time consuming and often require the development of multiple strains of mice, which then must be mated to obtain the desired cell-type specific gene targeting. The recent application of posttranscriptional gene silencing using RNA interference (RNAi) to silence target genes has been an efficient way to study gene function initially in *C elegans* and plants, later in mammalian cells in culture, and recently in embryos.

RNAi is a powerful alternative to traditional gene targeting using homologous recombination in ES cells, large scale mutagenesis, ribozymes, morpholinos, oligonucleotides, and so forth, for many reasons. Among these are simplicity in the design of the targeting construct, efficiency, and high throughput (reviewed in [3]). In addition, RNAi offers the ability to target specific exons/specific sequences within a gene [4], to study gene dosage phenotypes, to target multiple (redundant) genes, to target multiple members of a regulatory pathway, and to produce graded levels of knockdown

analogous to allelic series, which is particularly useful in analyzing the effects of genes that have “threshold” effects rather than acting as binary on-off switches. In addition, RNAi may be particularly useful to avoid the confounding genetic background effects common to gene targeting using the limited number of “germ line” ESC lines, and finally, many other species (eg, rat) can be employed.

Relatively few studies have employed RNAi to study gene function in the developing embryo. RNAi has been electroporated [5, 6] or microinjected into oocytes or early zygotes [7–11], siRNA-transfected ES cells have been used to create germ line transgenic RNAi mice [12], or all ES embryos have been generated using tetraploid aggregation of RNAi-targeted ESC [13]. Delivery, particularly to postimplantation-staged embryos, continues to be a major limitation in the widespread application of this important technology.

Information regarding the prenatal delivery of plasmid DNA (pDNA) comes largely from the gene therapy field where in utero gene targeting/therapy has been proposed as a method to treat diseases that affect the developing embryo [14], which may ultimately be the most effective means to treat genetic defects. Various routes of pDNA delivery have been attempted for fetal “gene therapy” including direct injection of the fetus [15–17], injection into the placenta or umbilical cord [18, 19], injection into the amniotic cavity [20, 21], or the yolk sac [21],

typically resulting in the limited transduction of the embryo.

Intravascular delivery of naked DNA is increasingly recognized as a preferred route to deliver nucleic acids to target tissues [22] because of its simplicity and effectiveness and because high levels of transgene expression can be achieved and sustained (eg, [23]). However, it has required either high-pressure delivery to produce extravasation [24] or a tourniquet to keep the pDNA in place [23]. Tail vein injection has been employed to silence genes in neonatal [24], and adult mice [25–28]. Based on these reports, we have recently delivered shRNAs to pregnant mice and have observed gene silencing and additional six genes that play important roles in organogenesis of the early embryo.

MATERIALS AND METHODS

Development of targeting constructs

We developed a targeting construct that would allow us to deliver a single plasmid containing a small hairpin RNA (driven by the constitutively active H1 or U6 promoter) and a fluorochrome reporter driven by the CMV promoter (Figure 1). The vector backbone is the pCS2 plasmid (from David Turner), which contains two multiple cloning sites (MCS) for insertion of a DsRED and shRNA cassettes. A BamHI/XbaI fragment that contains the entire DsRed coding region was removed from pDsRed2-1 (Clontech) and ligated downstream of the CMV promoter in the first MCS. The H1 (GenBank AF191547) or the U6 (GenBank X06980) promoter was amplified in PCR with specific primers and SV129 mouse genomic DNA was then ligated into the second MCS. Gene-specific shRNAs were designed to target *Aggf1* (BC052410), *Bmp4* (GenBank X56848), *Bmp7* (NM007557), *geminin* (AF068780), *nanog* (AY278951), *Wnt8b* (NM011720), and *Est1* (AK008955). Each shRNA is a ligated downstream of the H1 or U6 promoter to yield the final expression plasmid. All sequences are included in the supplemental data.

In addition to confirming that the plasmid reached the embryonic compartment (DsRed fluorescence), controls include empty plasmid (pRed) and hairpins containing three nucleotide substitutions (scrambled hairpins) that correspond to no known mRNA. Blast analysis confirms unique targeting of the hairpin and that no genes are targeted by the scrambled hairpin. We monitor target gene expression using PCR and at the protein level by Western blot or immunohistochemistry when an antibody is available. It is also important to monitor additional members of the signaling pathway, compensatory genes, irrelevant genes, and genes downstream of the target. We also monitor the interferon response gene *Oas1* (GenBank AF466823) [29] to determine if our construct elicits a nonspecific response.

Tail vein injections

These are carried out in mice as we have described previously [30]. Pregnant females or neonates are placed in a conical tube (open at the tip for air flow). A small hole is also drilled

into the cap to accommodate the tail. Mice are warmed for 5 minutes using a heat lamp and heating pad, then shRNA expression plasmids (10 μ g) diluted in Ringer's solution are injected into the tail vein. We use a 23-G needle and a volume of 200–300 μ L using a slow steady pressure, usually over 10–20 seconds for pregnant mice.

Because research in our laboratory has focused on the early postimplantation period of development, we have typically delivered targeting constructs at E6.5 and autopsied embryos 24 h to 72 h later. We have also carried out limited studies at midgestation when the placental barrier is most robust, as well as on E17.5 when the barrier thins and delivery should be more complete. We examine the extent of DsRed expression in all embryos using epifluorescence, followed by scanning electron microscopy (SEM), sectioning, immunohistochemistry, Western blotting, and/or PCR. DsRed is typically expressed throughout the early embryo, without a preference for a particular tissue type.

Tissue analysis

Pregnant females and neonatal mice are sacrificed by cervical dislocation followed by rapid dissection of embryos and tissues. Embryos are dissected from the decidua and images are captured using a Leitz-inverted fluorescence microscope to determine the extent of DsRed expression. Embryos are then either embedded in OCT for frozen sectioning or placed in Trizol for RNA/protein extraction. For SEM or whole mount immunohistochemistry (IHC), embryos are fixed in 1% glutaraldehyde (SEM) or 2% paraformaldehyde (IHC), then stored at 4° prior to additional processing.

For scanning electron microscopy, embryos are dehydrated through graded alcohols, washed twice in hexamethyl disilazane (HMDS), oriented on SEM stubs, and sputter-coated with gold palladium. They are viewed and photographed using an Amray 1910 scanning electron microscope.

Sectioning

Unfixed sections are cut to determine the pattern of expression of DsRed and cell type specific markers using immunohistochemistry. Embryos are embedded in OCT and frozen in hexane cooled over an acetone-dry ice slurry. Sections are cut at 10 μ m using a microm cryostat and collected onto slides.

Immunohistochemistry

Frozen sections or entire embryos are fixed, blocked extensively, followed by primary antibody overnight. The *geminin* (sc-13015) and *BMP4* (sc-6896) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif, USA); the *nanog* antibody from Kamiya Biomedical Company (Seattle, Wash, USA). Whole mount immunohistochemistry was carried out following [29]. *Geminin* and *nanog* primary antibodies were used at 1 : 100, *BMP4* at 1 : 50. Secondary antibody-HRP (1 : 200, Jackson Immunoresearch Laboratories,

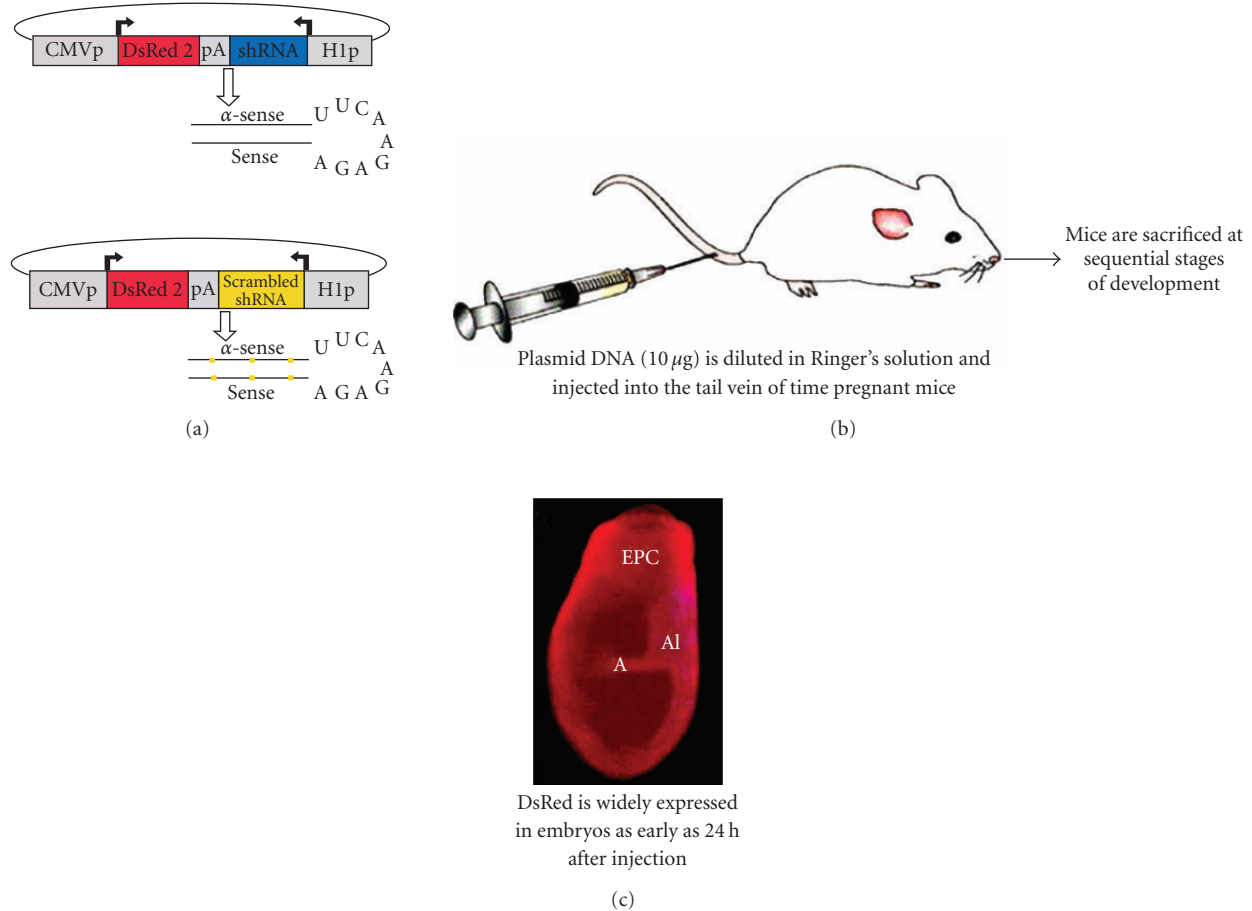


FIGURE 1: (a) shRNA expression plasmids were constructed using the pCS2 plasmid as the backbone. The DsRed 2.1 coding region was removed from the pDsRed2-1 vector (Clontech) and cloned downstream of the CMV promoter in the MCSII. The mouse H1 promoter (1040–1215 nt) of the RNaseP/PARP2 promoter, GenBank accession AF191547, was PCR-amplified from genomic DNA and cloned into MCSII. Gene-specific shRNAs (blue region) or scrambled shRNAs (yellow) are then ligated downstream of the H1 promoter. (b) Tail vein injections were carried out in pregnant mice as we have done previously (29). (c) Embryos are dissected from the uterus, and decidua and membranes are removed. Transmitted light and fluorescence images of embryos are captured using a Leitz-inverted fluorescence microscope to determine the extent of DsRed expression and to examine their morphology. A: amnion, AI: allantois, EPC: ectoplacental cone.

West Grove, Pa, USA). Images are captured using a Leitz Fluovolt or DMIRB microscope then imported into Adobe Photoshop.

PCR

RNAs are extracted from embryos using the Trizol reagent (Invitrogen, Carlsbad, Calif, USA), quantified, and DNAsed. Prior to the reverse transcription (RT) reaction, RNA is subjected to 30 cycles of PCR with β -actin primers to verify that there is no genomic DNA present. RNAs ($1 \mu\text{g}$) serve as templates in RT reactions with oligo-dT primers. General PCR conditions are $94^\circ/3 \text{ m}$, $94^\circ/1 \text{ m}$, $51\text{--}63^\circ/1 \text{ m}$, and $72^\circ/2 \text{ m}$ for 25–35 cycles; however, parameters are optimized for each primer pair. The products are electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, then images are scanned into the BioRad Gel Documentation system. For quantitative analysis of gene expression, real-time

PCR is performed using the Clontech Qzyme system on a BioRad iCycler. Real-time PCR primers were designed and optimized by Clontech for use in multiplexed assays with β -actin serving as a reference gene. All reactions are performed in triplicate, and data are analyzed using the $2^{-\Delta\Delta\text{CT}}$ method.

RESULTS

We have delivered shRNA to more than 100 pregnant mice, and obtained both gene silencing and expression of the DsRed fluorochrome in embryonic tissues, persisting in postnatal mice. We have carried out a number of experiments to determine if implantation site is correlated with knockdown. In general, embryos implanted near the vagina exhibited greater knockdown than those near the ovaries. In most cases, there is knockdown and DsRed is expressed in embryos. Occasionally ($\sim 5\%$ of the injections), there is no

transfection, likely because injection itself fails due to an insufficient amount of DNA entering the circulation.

Geminin shRNA

The *geminin* gene has been both down regulated and over expressed in *Xenopus* embryos, reducing or expanding the neural ectoderm fields, respectively [31]. *Geminin* is particularly interesting because, as suggested by its name, the protein has two functions: the C-terminus functions in cell cycle progression required for differentiation; the N-terminal is involved in early neural differentiation [32]. Despite its provocative expression in the early neural ectoderm and demonstrated role in amphibian, *Drosophila*, and zebrafish development, there is not yet a knockout of *geminin* in the mouse.

When a shRNA targeted to *geminin* was delivered on E6.5, and embryos were examined one–three days later, we observed reductions in neural tissue, neural tube closure defects that typically affected the midbrain, and posterior neuropore. In early embryos, we observed abnormally expanded nodes and failure of closure of the primitive gut endoderm (Figure 2). When we examined *geminin* expression in whole mount immunohistochemistry, wild-type embryos were indistinguishable from control embryos exposed to the scrambled hairpin both in morphology and in the pattern of *geminin* protein expression in the newly induced neural ectoderm (Figures 2(a), 2(b)). *Geminin* was present at slightly higher levels in the anterior neural folds compared with the posterior region of early somite-staged control embryos (Figures 2(a), 2(b)). There was a slight *geminin* immunoreactivity in the neural ectoderm of some *geminin*-targeted embryos (Figure 2(c)); while others expressed virtually no *geminin* protein (Figure 2(d)). When semiquantitative RT-PCR was carried out on RNA isolated from individual embryos from three litters, there was some variability in knockdown in the shRNA-exposed embryos, with two embryos expressing levels similar to control, others expressing intermediate, low, or no *geminin* mRNA (Figure 3).

Although *geminin* targeting in amphibian and *Drosophila* embryos has axis patterning and neural tissue consequences, there is no information on the early expression of *geminin* or targeted deletion of the *geminin* gene in the early mouse embryo. Since it is strongly induced by *noggin*, the observed neural, node, and endoderm abnormalities are likely due to the early expression of *geminin* in these tissues.

Nanog shRNA exposure

The *nanog* gene encodes a variant homeodomain protein originally identified in ES cells, where it is required to maintain pluripotency and inhibit lineage differentiation [33]. Targeted deletion in embryos is lethal before implantation [34], but additional evidence suggested that *nanog* is expressed in germ cells and somatic tissues later in development [35]; however, its role could not be assessed due to the early lethality of null embryos. To determine the role of *nanog* in later stages of development, we have exposed

21 litters of pregnant mice to shRNA-targeted to *nanog* via tail vein injection. We have observed widespread resorption of *nanog*-targeted embryos, and in other litters we have observed abnormalities of gastrulation and neurulation. *Nanog* knockdown embryos are characterized by axis abnormalities which are present in early somite embryos, considerably earlier in development than the turning process is initiated, endoderm overgrowth, and neural tube closure defects, particularly of the midbrain neural folds. Somite segmentation is also often abnormal, and we have observed abnormalities of cell migration through the primitive streak at gastrulation. Figures 4 and 9(b) illustrate some of these malformations.

In whole mount immunohistochemistry, *nanog* protein expression is significantly reduced, particularly in the primitive streak of embryos exposed to the *nanog* shRNA (37). To correlate phenotype and knockdown, we carried out quantitative PCR on RNA from individual *nanog*-targeted embryos from an entire litter. Silencing ranged from complete in three embryos to 60% of wild-type *nanog* levels in the least severely affected embryo. The presence of phenotypic abnormalities correlates strongly with the degree of knockdown, as illustrated in Figure 4 by the largely normal appearance of the embryo from lane 6, compared with the embryo from lane 7.

Somewhat surprisingly, two *nanog*-shRNA embryos expressed slightly elevated levels of the *Oas1* gene (Figure 5, lane 15). *Bmp4* expression was robust, however, suggesting that there had not been widespread silencing of nontargeted genes. Although it is widely employed to monitor off-target effects [36], *Oas1* is expressed in muscle, brain, and connective tissue during development [37, 38]. In addition, *Oas1* plays a role in cell cycle progression [39], suggesting a need to monitor additional interferon targets in these studies.

Targeting multiple genes: Bmp4, 7RNA interference

Bmp4 has previously been shown to be required in the gastrulation-staged embryo, where it is important in mesoderm differentiation and organization of the primitive streak [40]. Later *Bmp4* plays a role in determining the boundaries of the neural ectoderm and surface ectoderm [41], with particularly high levels of BMP4 associated with regions of epidermal ectoderm differentiation.

When a cocktail of shRNA targeted to *Bmp4* (exons 2 and 3) was delivered on E6.75 of gestation to pregnant mice, we observed defects of neural tube closure, allantois development, and of heart and axial rotation (Figure 6(b)) in targeted embryos. The number of primordial germ cells identified by alkaline phosphatase staining was also strikingly reduced. RT-PCR analysis of RNA obtained from individual *Bmp4* shRNA-exposed embryos from one entire litter identified only one embryo with any expression of *Bmp4* (Figure 7).

Immunohistochemical localization of BMP4 protein was carried out on sections through shRNA- and pRed (plasmid lacking the hairpin) exposed embryos, and indicated significant depletion of BMP4 in targeted embryos [30]. We also have carried out Western blotting analysis of protein isolated

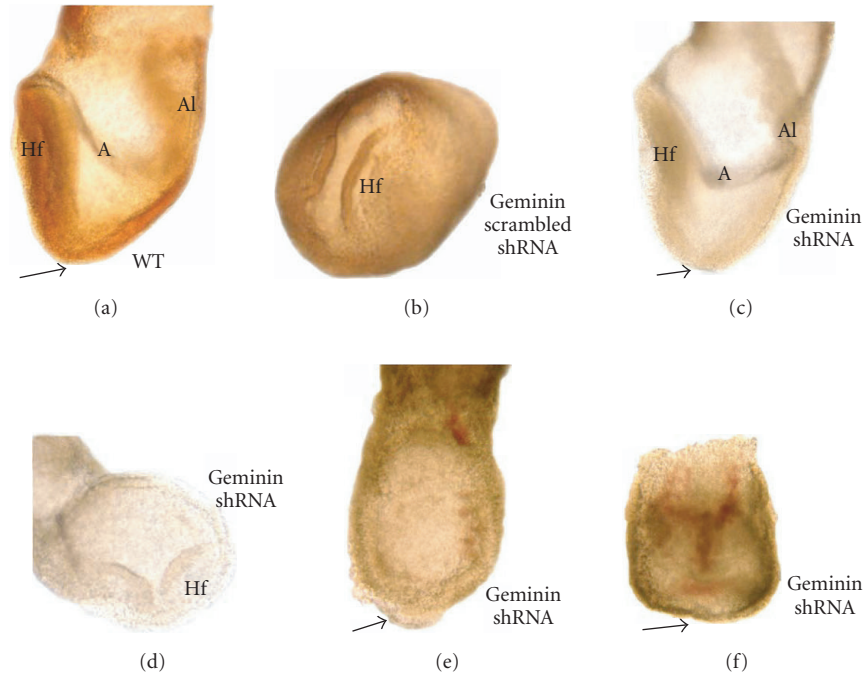


FIGURE 2: Immunohistochemical localization of geminin in control (a) and (b) embryos, and in embryos exposed on E6.5 to *geminin* shRNA (c and d). In control embryos, both wild type (a) and embryos exposed on E6.5 to a scrambled *geminin* hairpin construct (b), the expression of geminin protein was high in the neural ectoderm of the head folds, although geminin was also expressed in the posterior neural ectoderm as well (brown reaction product). There is a slight background staining of the allantois and membranes in all embryos (a)–(d). (c) and (d) Embryos were exposed to the shRNA-targeting geminin, examined and fixed on E7.5 of gestation, then immunohistochemistry to identify patterns of geminin protein expression was carried out as for (a) and (b) (secondary antibody-HRP). There is low-level geminin protein remaining in the neural ectoderm in embryo (c) less than that in embryo (d). (e) and (f) Transmitted light images of embryos exposed to the *geminin* shRNA on E6.5 and examined on E7.5. (e) Many targeted embryos exhibited axis defects, abnormal expansion of the node (arrow), and in later embryos, the endoderm of the gut tube often failed to close. (f) Occasionally, the embryonic axis appeared very flattened, and there was blood within the amniotic cavity. (a), (c), (e), and (f) are sideviews with anterior located toward the left. (b) is a dorso-lateral view, and (d) is a frontal (coronal) view. A: amnion, Al: allantois, Hf: head folds, WT: wild-type control embryo. Arrows indicate the node.

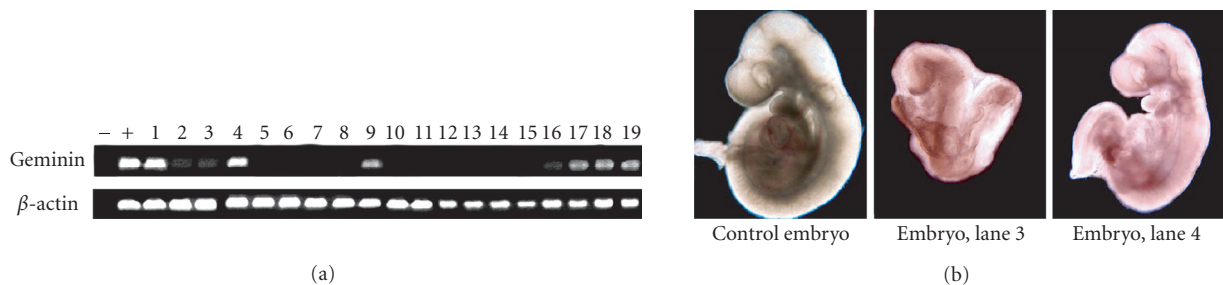


FIGURE 3: (a) Semiquantitative PCR to detect *geminin* expression in two entire litters of *geminin* shRNA- exposed embryos. Some embryos continue to express nearly normal levels of *geminin* (lanes 1, 4), while others express low (2, 3, 16), intermediate (9, 17–19), or undetectable (5–8, 10–15) levels of *geminin*. The most advanced embryos consistently expressed the highest levels of *geminin*. Two entire litters of *geminin*-targeted embryos were examined; 1–8 and 9–19. – = no RT, + = E9 embryo RNA. (b) Sideview of control and embryos expressing varying levels of *geminin*.

from individual embryos exposed to *Bmp4* shRNA, where there was a reduced expression of phospho-Smads 1/5/8, which are phosphorylated in response to BMP4,7 signaling.

Conventional gene targeting of *Bmp4* results in peri-implantation lethality [40], while on a C57Bl/6 background

embryos live until approximately 26 somite stage [41], and are characterized by axis elongation abnormalities. The results of the *Bmp4* RNAi phenocopy many defects in the *Bmp4* null embryos [40, 41] including anomalies of axis formation, primordial germ-cell differentiation, and neural tube closure

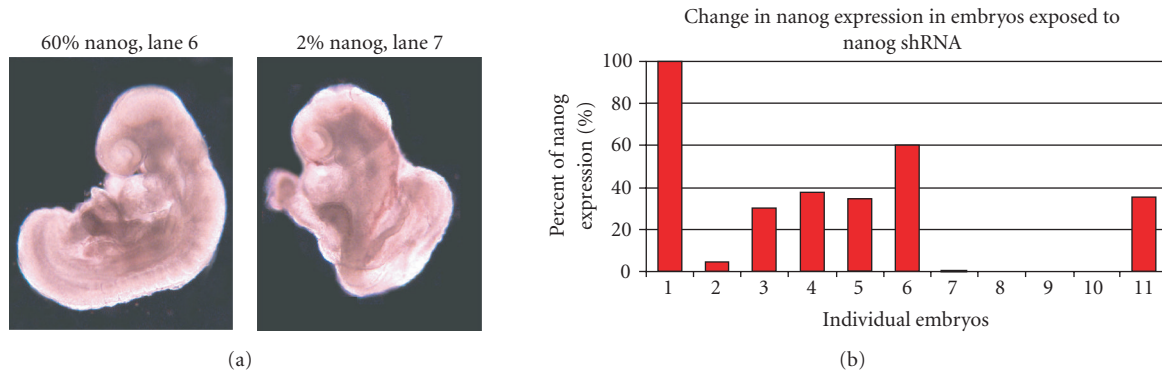


FIGURE 4: (a) Sideviews of two embryos exposed to *nanog* shRNA. Although the first embryo expressed 60% of wild-type levels of *nanog* mRNA, developmental defects are minor and include an axis abnormality and a flattened posterior neuropore. When *nanog* levels are reduced to 2% of wild type, embryos were more severely affected. The embryo in the right panel is characterized by defects of somite segmentation, neural tube closure, and abnormalities of endoderm differentiation. (b) Q-PCR analysis of *nanog* mRNA expression levels in individual embryos. Embryos were exposed on E6.5 to the *nanog* shRNA and examined on E9.0. cDNA from each embryo was run in triplicate in quantitative PCR with primers to both *nanog* and β -actin using the Clontech Qzyme system. Levels of β -actin and *nanog* expression from *nanog* shRNA-treated embryos (lanes 2–11) were compared to expression in a control embryo (lane 1). *Nanog* expression ranged from 0–60% of control levels. .

+	Wild-type				pRed		Bmp4 shRNA			Geminin shRNA				Nanog shRNA				-
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
[Gel image showing PCR products for Oas1 mRNA]																		

FIGURE 5: *Oas1* PCR. Single embryo RT reactions were subjected to 40 cycles of PCR with primers for *Oas1* mRNA (71). Mouse brain RT was used as a low-level expression positive control (+). Only 2 *nanog* shRNA embryos were positive for *Oas1* expression (one shown, lane 15). – = no cDNA control.

[30]. Many of these are also observed in embryos lacking *Bmpr1a* [42].

Because BMP proteins have overlapping functions in development, we examined the effects of knocking down multiple *Bmps* (Figure 6). We delivered a cocktail of shRNA targeted to *Bmp4* + *Bmp7*, as well as to *Bmp7* alone. The *Bmp7* shRNA embryos were the least severely affected (Figure 6(c)) with neural tube closure defects, while the *Bmp4* + *Bmp7* shRNA embryos had widely expanded neural folds, defects of rotation, failure of development of posterior structures, and ventral body wall closure defects (Figure 6(d)), a more severe phenotype than either the *Bmp4* shRNA or *Bmp7* shRNA embryos, but strikingly similar to the caudal dysgenesis and the “massive brains” reported in *Xenopus* embryos following morpholino depletion of *Bmp2*, 4, and 7 [43].

Durability of the RNAi

To determine how long knockdown could be maintained, we carried out tail vein injection of shRNA targeted to *Bmp4* on E6.5 and examined neonatal mice. On postnatal days 1–5, neonates were characterized by cystic bladders, had rudimentary testes or ovaries, and were consistently growth retarded compared with mice exposed to the pRed control (Figure 8(a)). Expression of DsRed was maintained in many tissues in both the mother (including milk) and in the

offspring (Figures 8(b), 8(c), 8(d), 8(e), and 8(f)). There were also anomalies of the subventricular neural stem cell zone (SVZ; Figures 8(b), 8(c)) which depends on noggin-BMP4 signaling [44].

Multiple targets, multiple phenotypes

Although there are considerable data available regarding the role of secreted signaling molecules in the initial events of neural induction, very little is known regarding the genes that bridge the process of neural induction and neural differentiation. To identify novel genes that mark the earliest neural ectoderm, we carried out a differential-display RT-PCR screen of genes induced in mouse embryonic stem (ES) cells by noggin protein. From this screen, we selected several transcripts that were expressed in early embryos just after induction. Based on their expression profiles, we selected several candidates for RNAi silencing. Two of these had not previously been examined during development. During the course of our work, *Aggf1* (angiogenic factor with Gpatch and FHA domains 1) was identified as an angiogenic factor mutated in human disease [45], but no information is available about its expression or role in development. Initial in situ hybridization studies indicated that *Aggf1* is expressed at high levels in the distal epiblast, especially in the posterior epiblast on E7.5. At later stages it is expressed in the neural ectoderm. shRNA

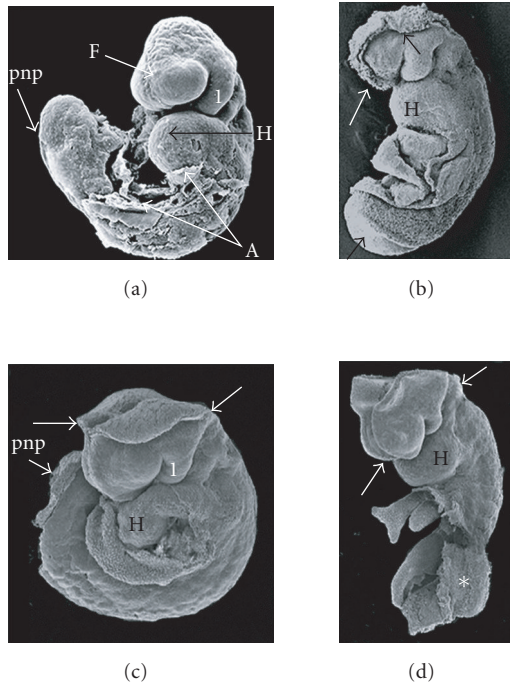


FIGURE 6: Effects of *Bmp* shRNA. (a) Scanning electron microscopy (SEM) view of a control embryo illustrating completed neural tube closure in the forebrain (F) region; the posterior neuropore (pnp) has not yet been closed. (b) *Bmp4* shRNA-exposed embryo with widely open anterior neural folds (arrows) and posterior neuropore (lower black arrow). (c) SEM view of a *Bmp7* shRNA-exposed embryo. Both the midbrain (arrows) and the posterior neuropore (pnp arrow) are widely open, but the body axis defects characteristic of *Bmp4* shRNA and *Bmp4* + 7 shRNA embryos were not present. (d) Ventrolateral SEM view of a compound *Bmp4* + *Bmp7* shRNA embryo. The cephalic neural folds are unfused (arrows) and the posterior region is rudimentary (*). I: first branchial arch, A: amnion, F: forebrain, H: heart, pnp: posterior neuropore.

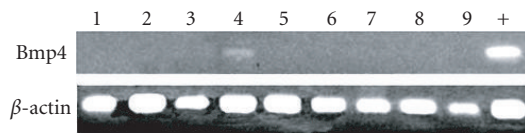


FIGURE 7: RT-PCR analysis of individual *Bmp4* shRNA-exposed embryos from one entire litter. The positive control (+) is from an embryo exposed to pRed control vector alone. Pregnant dams were injected on E6.5 and RNA extracted from embryos on E9.0.

targeted to *Aggf1* produced a lethal phenotype at E7.5. In these embryos, the ectoderm delaminated, and blood was often present within the amniotic cavity. Given its role in vessel formation, it is not surprising that we also observed implantation defects in shRNA-exposed embryos. Rare embryos that survived to E8.5 were characterized by focal hemorrhages and neural tube defects that affected midbrain and posterior neuropore (Figure 9(c)).

Est1 was identified twice in the differential display assay. Initial in situ hybridization localization studies indicate that it is expressed in the early epiblast, preconfiguring the primitive streak, in the node, later in the neural ectoderm. Targeting *Est1* produced a severe neurulation phenotype, embryos with open neural folds, defects of embryonic rotation, and differentiation of posterior structures, reminiscent of genes involved in L-R axis patterning (Figure 9(d)).

A number of Wnt family members were also identified in this screen. Because *Wnt8b* had not previously been silenced, we delivered shRNA targeted to *Wnt8b* to pregnant dams on E6.75. Resulting embryos were characterized by axis elongation defects (Figure 9(f)). These embryos also had neural tube closure anomalies and defects in closure of the endoderm.

We have delivered shRNA targeted to *Wnt8b*, *Bmp4*, *Bmp7*, *Bmp4* + *Bmp7*, *geminin*, *nanog*, and to two *Ests* identified in a differential display RT-PCR screen and observed specific targeting and unique phenotypes (Figure 9). These studies have also identified a previously unsuspected role for *nanog* in gastrulation and also in somite organization (Figures 4, 9(b)). Overall, we believe that these results are important and valid for a number of reasons. One, we have targeted multiple genes and observed unique phenotypes. These include *Bmp4* (phenocopies the *Bmp4* null embryos, as far as is possible to determine due to early lethality of the null embryos), *Bmp7* alone, *Bmp4* + *Bmp7*, *Wnt8b*, *nanog*, *Aggf1*, and *Est1*. Two, in each case where an antibody is available to the protein (BMP4, *nanog*, *geminin*) or to the downstream signal transduction cascade (PhosphoSmad1, 5, 8), we have demonstrated knockdown in “individual” embryos. Three, in cases where an antibody is not available, we have demonstrated unique phenotypes and knockdown by PCR. Four, these data also demonstrate that we can knock down multiple targets, for example, *Bmp7* and *Bmp4*, and identify an additive phenotype.

DISCUSSION

With genome-wide gene sequencing data now available, there is increased interest in systematically manipulating “all” the genes of the mouse to understand their roles in development and disease. Many new tools to manipulate gene function have been developed including ribozymes, microRNAs, DNazymes, as well as a number of methods for post-transcriptional gene silencing such as morpholinos (review [46]), antisense oligos (review [47]), and RNAi (review [3]). RNAi is typically more robust than antisense oligos or morpholinos in embryos [48, 49], and morpholinos have the additional problem that the translational start site must be known, so uncharacterized genes (such as *Ests*) cannot be targeted.

RNAi may be particularly appropriate in targeting a developmental disease such as Down’s syndrome/trisomy 21 once critical duplicated genes are identified, and may also be effective in targeting upstream pathways in metabolic disease to limit disease progression, or in silencing activating gene mutations, such as in the FGF receptor-2 which produces

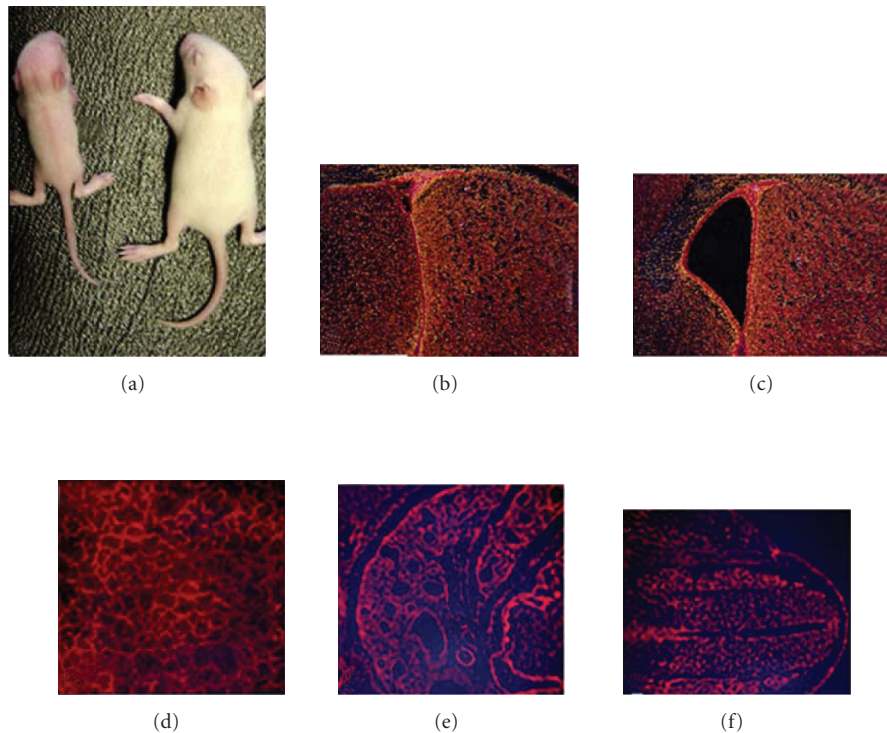


FIGURE 8: *Longevity of the RNAi*. (a) Day 10 neonatal mice (PN10) obtained from litters exposed on E6.5 to the *Bmp4* shRNA (left) or to pRed (no hairpin plasmid) control (right). *Bmp4* shRNA mice were consistently developmentally delayed and lacked testis or ovaries. (b) and (c) Coronal sections through the lateral ventricles of PN3 mice exposed on E17.5 to pRed control (b) or to *Bmp4* shRNA (c). In addition to the obvious anomalies of the subventricular zone and ventricle, neural stem cells obtained from the *Bmp4* shRNA mice fail to differentiate normally. (d)–(f) illustrate the persistent expression of DsRed in liver (d), lung (e), and spinal cord (f) in neonates exposed to *Bmp4* shRNA on E6.5.

craniosynostosis [50]. Systemic delivery will also be applicable to diseases that affect tissues with open circulations, as well as diseases in which the blood brain barrier is opened such as Duchenne muscular dystrophy, certain brain tumors, in aging, and in multiple sclerosis (review [51]).

These studies have identified unsuspected roles in development for several genes. In the case of *nanog*, which in null embryos is lethal at early cleavage stages of development, we have identified a role in gastrulation, neurulation, and in endoderm differentiation. There is not a report of a knockout of the *geminin* gene in the mouse, and it will be of particular interest to study carefully the characteristics of the neural tissue in targeted embryos, as well as the characteristics of the node. Neither is there a published report of a *Wnt8b* knockout, but many of the defects observed in this study are similar to those present in other Wnt null embryos. For example, *Wnt3a* null embryos have similar severe posterior truncations [52]. The use of RNAi directed against individual Wnt mRNAs should allow rapid analysis of specific Wnt functions. In addition, since Wnts may compensate for each other, masking functions in single-gene knockouts, combinatorial Wnt RNAi should help elucidate overlapping relationships between the Wnts. Delivery of shRNA to pregnant dams has also provided an opportunity to rapidly determine if there was a role in early embryos for novel genes identified

in a differential display RT-PCR screen. A role for *Aggfl* in later aspects of vasculogenesis was described previously [45], and given its role in vessel development, it is not surprising that targeting *Aggfl* affected the implantation process.

The ability to target multiple genes with overlapping expression and function, as in the case of *Bmp4/7* [53], is an important improvement over traditional knockouts in which mutations in multiple genes are obtained by breeding. In the future, it will be important to target multiple genes using a single plasmid containing multiple hairpins, rather than the cocktail we have employed to target *Bmp4* and *Bmp7*.

To date, study of the placental transport of plasmid DNA has come largely from attempts to deliver pDNA for in utero gene therapy, which have produced conflicting results. Thus, when pDNA complexed with liposomes was delivered by intravenous injection of pregnant mice on E2.5, 5.5, 8.5, 11.5, or 14.5, no plasmid DNA was detected in fetuses exposed on E2.5 or E5.5, while embryonic expression peaked with delivery on E8.5, compared with E11.5 or E14.5. "All" embryos treated on E8.5 expressed the plasmid, with sustained expression at 40 days postinjection [54]. However, it has also been reported that DNA-liposome complexes were trapped in the visceral endoderm prior to placenta development on E11.5 [55]. Others have also reported hemodynamic transfer of genes to the fetal compartment, however. For example,

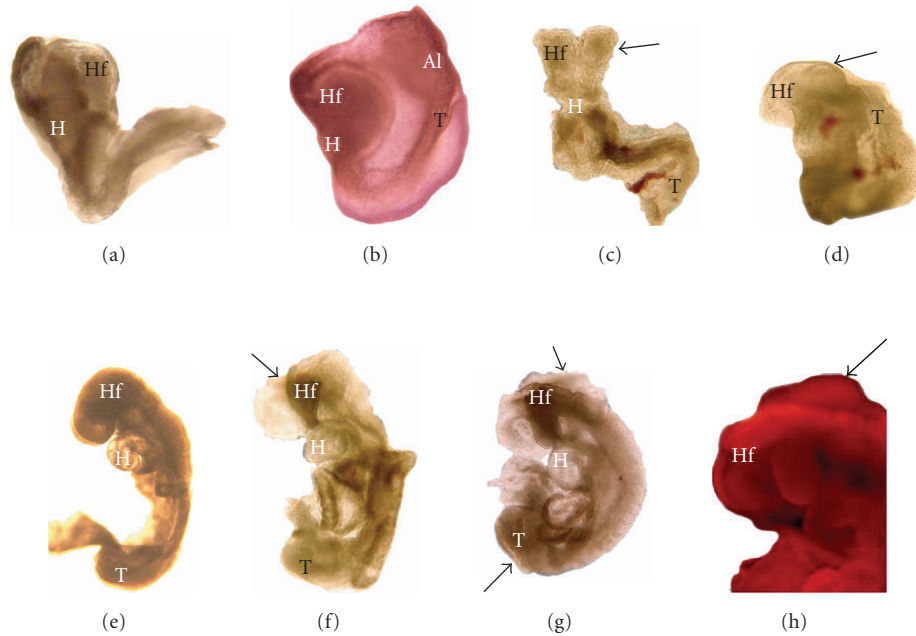


FIGURE 9: Control- and Gene-targeted embryos: *nanog*, *geminin*, *Aggf1*, and *Est1*. (a) Control embryo. Sideview of an embryo exposed to pRed plasmid (no hairpin) on E6.5 and examined on E8.5, illustrating the normal appearance of the head folds (Hf), somites, and unturned body axis. (b) *Nanog* shRNA. Embryo exposed on E6.5 to shRNA targeting *nanog*, illustrating the typical lack of development of the head folds (Hf) and posterior region in the tail bud (T). Somites have also failed to segregate normally. (c) and (d) We carried out a differential display RT-PCR screen of genes induced in D3 ESC by noggin exposure, then targeted two using tail vein injection of shRNAs. (c) *Aggf1*-targeted embryos failed to implant normally and the primitive ectoderm often delaminated into the amniotic cavity. Hemorrhages are present within the embryo; there are striking abnormalities of turning and posterior development in the rare embryo that survived to E8.5. (d) *Est1*-targeted embryo. There were anomalies of primitive streak organization in these embryos. They also often failed to turn to adopt the fetal position and exhibited abnormalities of the node. (e) pRed control. Sideview of an E8.5 pRed (no hairpin) control. This embryo is beginning the turning process, the body axis is elongated, neural folds are fused in the anterior (head fold, Hf) region, although the posterior neuropore remains open in the tail bud (T). (f) *Wnt8b*-targeted embryo illustrating the shortened axis and open neural folds typical of these embryos. (g) and (h) *Geminin* shRNA. An embryo exposed to *geminin* shRNA on E6.5 and examined on E8.5. There are very characteristic midbrain (upper arrows) and posterior neuropore (lower arrow in (g)) defects in these embryos, which exhibit widespread DsRed fluorescence (H). All embryos are oriented with anterior toward the left. Al: allantois, H: heart, Hf: head folds, T: tail bud. Arrows indicate open neural folds.

intravenous delivery of plasmid DNA to pregnant mice on E9.5 successfully immunized the fetuses against HIV-1 and influenza [56]. We have avoided carriers since liposomes are often immunogenic, are generally less effective in serum, and can be toxic to both the embryo and the pregnant female [55].

Although we have obtained widespread expression of our construct, a number of improvements and alternative approaches can be considered. It would be possible to increase the amount of DNA injected, although 5 μg plasmid DNA was optimal (saturating) and > 25 $\mu\text{g}/\text{mouse}$ was toxic [57, 58]. Other studies have shown that transfection efficiency is not determined by volume or rate, but the amount of DNA delivered, with highest expression achieved with 1000 ng/mouse (23). Given the \sim 1.6 mL blood volume of an 18 g mouse and observations that there is less degradation of pDNA in a larger volume of carrier [58], increasing the volume delivered would be an option. Rate of injection—5 seconds is better than 30 [24, 58]—could also be considered, but very rapid injection can be lethal.

Despite careful breeding, the developmental stage of individual embryos at the time of exposure to shRNAs cannot be known precisely, and may account for some of the variability in our results. Alternatives include using exo-utero surgery of midgestation embryos with injection of shRNAs and electroporation [49]. For early postimplantation stages when exo-utero surgery is not applicable, whole embryo culture presents another option [59]. Better promoters and better control of CRE expression in the early embryonic compartment will allow the development of hybrid approaches to specifically, inducibly silence gene expression in a particular tissue/cell type (eg, 61). Interestingly, the oocyte-restricted ZP3 promoter was recently employed to drive expression of dsRNA targeted to the *Mos* gene, recapitulating the null phenotype, with spontaneous parthenogenetic activation [60]. These and other recent investigations suggest that it will be possible to target RNAi to particular cells or tissues.

One drawback to tail vein injection is the loss of plasmid DNA to the female and unintended transfection of maternal

TABLE 1

Aggf1	tttacttcgaccatgacttgca gctcgtcctttctatcgaggc	ttaTttcgCccatgacttgTa gcGcgtcctGtctatcgagTc
Bmp4	cagtccatgattcttgga cctcagaaaccggtcggc	cagtAatgaGtcttgTga cAttcagaaTccggtAggc
Bmp7	tcattctccgtagtatccg ttcgacgacgctctaag	tccGtctccgCagatTcg ttcCacgaTagctctCatg
Est1	cccgaacgatattggtgga caagacgacgtgacaattcca	ccGgaacgaGatttggtgTa caCgacgaTgtgacaattcTa
Geminin	tcatgtacacggcctagcat attcctgactatccggtga	tAatgtacCggcctagAat atCctgactatAcggcga
Nanog	ttctgggaacgctcatcaatgc ggaagcagaagatgaggactgt	ttcCgggaaAcctcatcaTt ggaTgcagaCgatgTggactgt
Wnt8b	atgtacaccctgactagaaactgcagcct gtccgctgcgagcagtgccgccg	aGgtacaccAtgactagatgcagAct gGccgctAcgagTagtgccgccgA
PCR Primers		
Gene	Forward 5'-3'	Reverse 5'-3'
Bmp4	ctccaagaatcatggactg	aaagcagagctctcactggt
Geminin	gagaaaatgagttgccaaaagg	ccacagcttgaagtctgagatg
Nanog	agggtctgctactgagatgctctg	atcttctcttctcggcaag
Oas1g	atggtagtagtaataagaagc	gcatagacagtgagtagctcc
actin	ttgctgacaggatgagaaggaga	actcctgcttctgatccatct

tissues. Since the liver has an expandable circulation and is easily transfected using intravenous delivery, it is important to monitor liver function in pregnant females and neonates. Obviously, when the targeted gene is important in maternal tissues, this is a larger concern that must be constantly monitored. Additional experiments might therefore include targeting of a nonessential protein such as EGFP in the GFP mouse [61] which has no known downstream targets, nor have there been deleterious effects of EGFP cleavage products. It would be possible to mate hemizygous GFP mice to determine if there are any deleterious effects that are transmitted to the nontargeted +/+ embryos. It would also be useful to target a gene expressed only in male embryos, so that female littermates would serve as a control for off-target and/or maternal effects.

It is impractical to carry out microarray analyses of individual, targeted embryos to determine specificity of targeting, although in previous studies when the targeting construct was specific, RNAi signatures were unique and highly specific for the target gene [62, 63]. More detailed analysis can also be carried out to verify the presence of specific mRNA cleavage products using 5' RACE, PCR to identify the cleavage fragments with sequencing [64]. It has generally been assumed that early development in the embryo is incapable of mounting a full interferon response [65], yet interferon responsive genes such as *fragilis* are expressed during very early postimplantation development [66]. Since *Oas1* may have additional roles in development, monitoring other interferon-responsive genes would also be appropriate in these studies. Recent evidence also suggests that shRNA

expression can competitively inhibit endogenous miRNA function via exportin 5 [67], although inclusion of scrambled hairpin constructs should control for this effect. Much remains to be understood about this technique, particularly regarding transport, uptake, and expression in the embryos and fetuses.

Since the first transgenic mouse was developed in 1980 by pronuclear injection of DNA [68], there have been major improvements to the technological base for mouse functional genomics, and RNAi promises to be a powerful new addition to that tool set.

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