Selective disruption of neuregulin-1 function in vertebrate embryos using ribozyme-tRNA transgenes

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SUMMARY

The products of the neuregulin-1 gene constitute a set of polypeptide growth factors whose signalling through the ErbB receptors is essential to the growth and differentiation of many cell types in culture. Although studies with neuregulin-1 mutant mice have demonstrated that these growth factors are also essential regulators of cellular differentiation in vivo, the mid-embryonic death of these mutants precludes an analysis of hypothesized neuregulin-1 roles in later aspects of development. To circumvent this early lethality, we have pursued a ribozyme-based strategy for the perturbation of neuregulin-1 function in developing chick embryos. Early administration of a retrovirus carrying neuregulin-1 hammerhead-type ribozymes to blastoderm-stage embryos leads to an embryonic lethal phenotype that results from the failure of ventricular trabeculation in the developing heart, a faithful phenocopy of the mouse neuregulin-1 mutations. Later, more localized delivery of the ribozyme to the developing retina inhibits both the differentiation of retinal ganglion cell neurons and the proliferation of the neuroepithelial cells from which they derive. These results suggest that neuregulin-1 promotes both muscle cell differentiation in the heart and neuronal differentiation in the central nervous system. In addition, they demonstrate the utility of hammerhead ribozymes as a simple, effective and easily adaptable method of conditional gene inactivation in vertebrates.

Key words: Neuregulin, Ribozyme-tRNA transgene, Selective gene inactivation, Retina, Chick

INTRODUCTION

Signalling by polypeptide growth factors and their cognate receptors orchestrates many aspects of multicellular development, including the proliferation of stem cells and their progeny, the adoption of specific cell fates, and the patterning of tissues and organs. Within the developing nervous system, the neuregulins (NRGs) are a growth factor family of particular interest. The prototype member of this family, NRG-1, comprises a set of alternatively spliced products that are also referred to as glial growth factor (GGF), neu differentiation factor (NDF), heregulin (HRG) and acetylcholine receptor inducing activity (ARIA) (Falls et al., 1993; Holmes et al., 1992; Lemke and Brockes, 1984; Marchionni et al., 1993; Wen et al., 1992). This surfeit of names reflects the diverse bioactivities of NRG-1s in vitro – as glial growth factor (GGF), neu differentiation factor (NDF), heregulin (HRG) and acetylcholine receptor inducing activity (ARIA) (Falls et al., 1993; Holmes et al., 1992; Lemke and Brockes, 1984; Marchionni et al., 1993; Wen et al., 1992). This surfeit of names reflects the diverse bioactivities of NRG-1s in vitro – as glial growth factor (GGF), neu differentiation factor (NDF), heregulin (HRG) and acetylcholine receptor inducing activity (ARIA) (Falls et al., 1993; Holmes et al., 1992; Lemke and Brockes, 1984; Marchionni et al., 1993; Wen et al., 1992).
exception of ErbB3, each of these mutations leads to an embryonic lethal phenotype at 10.5 days of development, due to the aborted differentiation of cardiac muscle. The heart ventricles of NRG-1, ErbB2, and ErbB4 mutant mouse embryos all lack trabeculae, the interlinked finger-like projections of cardiac muscle that are essential for embryonic blood circulation. In addition to this pronounced effect on cardiac development, the mutants also exhibit defects in neural development, which have been most extensively analyzed in the developing hindbrain (rhombencephalon) and peripheral nervous system. Analysis of the NRG-1 and ErbB2 mutants demonstrates that these genes are required for the survival of many of the cranial neural crest derivatives of rhomboemeres 2, 4 and 6, including those that give rise to the neurons of cranial sensory ganglia (Lee et al., 1995; Meyer and Birchmeier, 1995). In contrast, ErbB4 expression in the intervening rhomboemeres 3 and 5 is required for the proper targeting of sensory and motor axons to and from the cranial ganglia and the hindbrain (Gassmann et al., 1995). ErbB3 is not essential for cardiac trabeculation, but is required for normal Schwann cell development, since mice lacking this receptor are devoid of these cells in their peripheral nerves (Rietmacher et al., 1997).

These analyses have provided important new insights into the action of NRG-1 in cardiac development and of the functional interaction of its putative receptors. Nonetheless, many of the roles that NRG-1 has been hypothesized to play in key events in neural development – e.g., in the commitment of trunk neural crest cells to particular lineages, or in the synaptic differentiation of skeletal muscle – cannot be directly assessed in the mouse NRG-1 knock-outs, since these events occur after the time at which the mouse mutants die. The in vivo importance of NRG-1 signalling in these later events and in the functioning of the mature nervous system (where it and its receptors are also expressed) therefore remains obscure.

In an attempt to circumvent the early lethality of the mouse NRG-1 mutants, we have pursued a ribozyme-based strategy for the perturbation of NRG-1 function in developing chick embryos. The gene inactivation method that we have developed relies on the ability of hammerhead ribozymes – small RNA molecules derived from plant viroids – to target unique mRNA sequences and then cleave these mRNAs in a catalytic manner (Symons, 1992). Previous work in Drosophila embryos has suggested that ribozymes may be exploited as conditional knock-out reagents for assaying gene function at selected times and places in development (Zhao and Piek, 1993). We chose the chick embryo as a model system with which to develop and test this technology in vertebrates, since this system has been widely used for studies of embryonic development and affords great advantages with regard to experimental manipulation, but is nonetheless compromised by a lack of genetic methods. We selected NRG-1 as a target for inactivation, since its role in development is of considerable interest and since the inactivation of the NRG-1 gene in the mouse provides a basis for comparison with ribozyme-generated phenotypes.

We have used an avian retrovirus, RCASBP (Boerkoel et al., 1993; Petropoulos and Hughes, 1991), to deliver hammerhead NRG-1 ribozymes, which have been embedded within a synthetic chicken tRNA expression cassette and directed against multiple independent sites in the NRG-1 mRNA. This retrovirus provides an excellent vehicle for ribozyme delivery, which is readily controllable as a function of when during embryogenesis and/or where in the embryo virus is injected. We demonstrate that early infection of blastoderm stage chick embryos with retrovirus expressing a set of inactivating NRG-1 ribozymes results in a failure of ventricular trabeculation in the heart, an exact phenocopy of the mouse NRG-1 mutants. Later, more localized expression of these same ribozymes in the developing eye, at a time at which the mouse NRG-1 mutants have already died, reveals a previously unknown role for NRG-1 in the development of retinal ganglion cell neurons.

MATERIALS AND METHODS

Constructs

All DNA templates encoding NRG-1 ribozymes were synthesized as complementary oligonucleotides (ranging in length from 47 to 55 nucleotides), which were annealed to generate PsiI sticky ends and then inserted into an engineered NsiI site next to the anticondon triplet of a synthetic chicken alanine tRNA gene (see Fig. 1) (Mezquita and Mezquita, 1992). The sequence at the insertion site is ....GCCGCATTTGCATGATG...., where the anticondon loop is underlined and the NsiI site italicized. The point mutation (A to G) in the catalytic domain of the ribozymes described in Figs 1 and 2 was introduced by PCR methods. The structures of all normal and mutated ribozyme-tRNA transgenes were confirmed by sequencing. For transfection, fragments containing either a normal or mutated ribozyme-tRNA transgene were subcloned into the NRG-1 expression vector p12.7 (a gift from Drs G. Fischbach and D. Falls). The pEGFPC1 reporter plasmid was purchased from Clontech. In order to construct RCAS/4XRZNRG, four ribozyme-tRNA minigenes (each ~170 bp in length) were clustered together in the same orientation by ligation and then subcloned in tandem into the ClaI adapter vector (Hughes et al., 1987). The use of this adapter plasmid allows the conversion of the cluster of ribozyme-tRNA minigenes into a single ClaI fragment, which is then suitable for insertion into the ClaI site of the retroviral vector RCASBP(A) (Petropoulos and Hughes, 1991), downstream of the env gene and in opposite orientation to transcription driven by the left retroviral LTR promoter.

Similarly, a ClaI fragment containing the RZNRGI56-tRNA transgene and the human placental alkaline-phosphatase-coding sequence (used as a marker) were cloned into the same viral vector to generate RCAS/RZNRGI56. Ribozyme-tRNA transgenes linked in tandem are transcribed as individual RNA transcripts due to the presence of strong transcription termination signals at the 3’ end of each transgene, which also prevent transcription of the non-coding strand of the retroviral genes.

Tissue culture and transfection

Approximately 10^6 human embryonic kidney 293T cells were seeded on to 60 mm dishes 1 day before transfection. Each dish was transfected with 1 µg of the reporter plasmid pEGFPC1 and 6 µg of the dual-expression plasmid by a polyethyleneimine-mediated DNA transfection method (Boussif et al., 1995). ~20 hours later, cells were lysed in TRI reagent (Molecular Research Center, Inc.) and total RNAs were subsequently isolated and analyzed by northern blot. Signals were recorded using a model 400 Molecular Dynamics phosphorimager. E5 chick retinal cell dissociation was performed as described (Austin et al., 1995). Incubation of 1 mM BrdU was carried out in cultures of dissected retina for 2 hours prior to trypsin treatment (Alshuler and Cepko, 1992).

Retrovirus production and injection

Viral construct transfection and subsequent harvesting of high titer
virus stocks were performed in chick embryonic fibroblasts by standard methods (Morgan and Fekete, 1996). For virus infection, specific pathogen-free White Leghorn eggs (SPAFAS, Inc.) were used. Normally, eggs were placed on their sides except for the blastoderm stage injection, when eggs were placed in standing position with the air sac on top, incubated at 38°C in a high-humidity incubator and windowed before injection. Injection was performed as described (Morgan and Fekete, 1996).

**Immunohistochemistry**

Immunofluorescence staining was carried out on frozen sections of fixed chick embryos and dissociated chick embryonic retinal cells. Primary antibodies were used at the following dilutions: NRG-1 (rabbit purified polyclonal antibody against HRG-α (C-20), Santa Cruz; 1:500), 13F4 (mouse monoclonal IgM a gift from N. Le Douarin; undiluted hybridoma supernatant), RA4 (mouse monoclonal IgG kindly provided by S. McLoon; 1:10 of hybridoma supernatant), BrdU (purified mouse monoclonal IgG, Boehringer Mannheim; 1:40). The BrdU epitope was unmasked by treatment with 0.07 M NaOH for 2 minutes. For double staining, either one of two antibodies to different viral gag proteins were used: 3C2 (mouse monoclonal IgG against a viral matrix protein p19, Developmental Studies Hybridoma Bank; undiluted supernatant), or p27 (purified rabbit polyclonal IgG against a viral capsid protein, SPAFAS, Inc.; 1:100). Fluorophore-conjugated secondary antibodies were purchased from Jackson ImmunoResearch and were used as specified by the manufacturer. Slices were mounted with diazobicyclooctane/ polyvinyl alcohol mounting medium and signals were collected by confocal scanning laser microscopy (Bio-Rad MRTC600). Images were processed with Adobe Photoshop (Adobe System).

**RESULTS**

**Expression vectors for NRG-1 ribozymes**

Based on the cDNA sequence of chicken NRG-1 (Falls et al., 1993), four regions containing the consensus hammerhead cleavage sequence GUC or GUA were chosen for designing NRG-1-specific ribozymes (Fig. 1). These ribozymes target several regions that are subject to alternative splicing in the NRG-1 primary transcript – in sequences encoding an amino terminal domain (ribozyme RZNRS89), the NRG-1 immunoglobulin-related domain (RZNRS156), the NRG-1 EGF-like domain (RZNRS438) and the external juxtamembrane region (RZNRS637). (See Lemke, 1996, for a summary of the complexity of NRG-1 mRNA splicing patterns.) It is important to note that the RNA targeted by ribozyme RZNRS438 is transcribed from an exon that encodes the first half of the NRG-1 EGF-like domain, which is essential to NRG-1 bioactivity and is present in all bioactive NRG-1 isoforms (Marchionni et al., 1993; Falls et al., 1993; Lemke, 1996). Specificity was set by the inclusion of 9-12 flanking nucleotides on both sides of the catalytic domain of the enzyme, which are complementary to sequences flanking the target sites in the NRG-1 mRNA. We chose to embed ribozyme sequences within the anticodon region of a synthetic chicken alanine tRNA gene and to express the targeting ribozyme as a ribozyme-tRNA hybrid (Cotten and Birnstiel, 1989) (Fig. 1). This expression cassette has two advantages: (1) it is a small gene that allows for non-selective
transcription of the ribozyme-tRNA by RNA polymerase III, at constitutive levels in most cells, and (2) the higher order structure of the tRNA may also protect the ribozyme from degradation by cellular nucleases.

**Assessment of the antisense effects of ribozyme-tRNA transgenes**

To distinguish ribozyme catalytic activity from simple antisense action, we engineered a single base change (A to G) in the catalytic core of all four NRG-1 ribozymes (Fig. 1). This point mutation abolishes most cleavage activity but maintains the conformation, stability and antisense sequence of the flanking arms of the hammerhead ribozymes (Ruffner et al., 1990). We then set up a cell culture transfection assay to measure the activity of these normal and mutant ribozymes in human embryonic kidney (293T) cells. The cRNA expression cassette, carrying either a normal ribozyme, a mutant ribozyme or no ribozyme, was subcloned into a NRG-1 expression vector (kindly provided by D. Falls and G. Fishbach) to generate a single plasmid dual-expression vector (Fig. 2A). Every 293T cell transfected with this plasmid therefore carries a fixed 1:1 copy ratio of ribozyme and target genes. (The actual ratio of ribozyme RNA to target mRNA is a function of the relative strength of the pol III-mediated promoter of the ribozyme-tRNA gene and the pol II-mediated CMV promoter driving the target NRG-1 gene.) Transcription of the ribozyme-tRNA cassette was oriented in the opposite direction from NRG-1 gene transcription, to exclude cis-acting ribozyme effects, and transfection efficiency was measured by cotransfecting pEGFP-C1, a green fluorescent protein (GFP) reporter plasmid. Northern analysis of total RNA isolated from transiently transfected cells demonstrated a pronounced reduction of NRG-1 mRNA levels relative to control (tRNA alone), from ~45% relative to control for RZNRG156, to ~5% relative to control for RZNRG438 (Fig. 2B). In each case, reduced NRG mRNA expression was largely due to the catalytic activity of the ribozyme (Fig. 2B). Partial antisense inhibitory effects were observed for the mRZNRG156 and mRZNRG438 A to G mutants, but their catalytically active counterparts were always superior in effect. For RZNRG89 and RZNRG637, essentially all of the measured reduction in NRG-1 mRNA levels was due to ribozyme rather than antisense action.

**Fig. 2.** Ribozyme delivery constructs and expression in cell culture. (A) Diagram of NRG-1-ribozyme dual expression plasmid for cell culture assay. The NRG-1 cDNA is under the control of the cytomegalovirus (CMV) promoter and contains its own polyadenylation signal. The NRG-1 ribozyme-tRNA hybrid gene is transcribed by RNA Pol III from the internal promoter of the cRNA. The two expression cassettes are set in a tail-to-tail orientation in the same plasmid and the ribozyme-tRNA gene carries a 3′ transcription termination signal, such that only trans-acting ribozyme effects should be observed. The backbone of the vector is pCDNA1/Amp (Invitrogen). (B) Total RNAs were isolated from transiently transfected human embryonic kidney 293T cells and fractionated on an 1% agarose denaturing gel. After transferring onto nylon membrane, blots were hybridized with probes against NRG-1 (top blot) or GFP (bottom blot). Radioactivity in each band was quantitated on a phosphorimager, the intensity of the NRG-1 signal relative to GFP was determined, and this ratio was then plotted relative to the ratio obtained for the NRG-1-cRNA cassette without ribozyme, whose relative signal was set to 100%. Error bars represent standard deviations of measured relative NRG-1 expression from two experiments. (C) Schematic diagram of the replication competent retrovirus used for delivery of ribozyme-tRNA transgenes to developing chick embryos. Retroviruses contained either four independently transcribed ribozyme-tRNAs (as shown) or, alternatively, the single ineffective ribozyme-tRNA RZNRG156 as a control. All ribozyme-tRNAs carry a 3′ transcription termination signal. Note that the discontinuity of DNA sequences encoding the flanking arms of the ribozyme tRNA, together with the absence of the ‘X’ nucleotide in the GUX ribozyme cut site sequence, effectively precludes ribozyme targeting of the retroviral genome itself. (D) Northern blot of cellular tRNAs and independent single ribozymetRNA transcripts in transfected cells. Total RNA from untransfected chick embryonic fibroblast cells (CEF), CEF transiently transfected with RCAS/RZNRG156 plasmid DNA, CEF transfected with RCAS/4XRZNRG plasmid DNA, and uninfected E7.5 chick eye tissue, were fractionated on a 2.5% agarose denaturing gel. After transfer to nylon membrane, the blot was hybridized with riboprobes containing RZNRG89, RZNRG156 and RZNRG637 antisense sequences. The probes detected both cellular tRNA transcripts and ribozyme-tRNA hybrids, as indicated. Note that these hybrids appear as a single band in both the single hybrid and four hybrid transfections, indicating that the four ribozyme-tRNA transgenes in RCAS/4XRZNRG are independently transcribed.
Early expression of NRG-1 ribozymes in the chick phenocopies loss-of-function NRG-1 mutations in the mouse

For expression in vivo, the RZNRG89, RZNRG156, RZNRG438 and RZNRG637 tRNA-hybrid expression cassettes were linked in tandem. As an in vivo delivery vehicle, we used an avian replication-competent retroviral vector (Petro poulos and Hughes, 1991), into which the tandemly arrayed ribozyme-tRNA genes were inserted to generate RCAS/4XRZNRG (Fig. 2C). High titer (~10^7 infectious units/ml) retroviral stocks were prepared by standard methods (Morgan and Fekete, 1996). The expression of ribozyme-tRNA transgenes was confirmed by northern blot (Fig. 2D). We found that retrovirus carrying RZNRG156, which only reduced NRG mRNA to ~45% in the 293T cell transfection assay (Fig. 2B), did not detectably perturb chick embryo development (see below), as would be predicted from the lack of developmental defects seen in mice heterozygous for NRG-1 mutations (Meyer and Birchmeier, 1995). We therefore used this relatively ineffective virus as a control for any non-specific effects on development that might be due to either retroviral infection and replication alone, or to the expression of an aberrant alanine tRNA.

As in the mouse embryo, the heart is a major site of NRG-1 expression in the early chick embryo (Fig. 3E). To test whether ribozymes can antagonize NRG-1 function during chick heart development, we injected RCAS/4XRZNRG and control RCAS/AP/RZNRG156 viruses at the blastoderm stage (~4 hours incubation). This early injection resulted in the infection of the clear majority of blastoderm cells and their progeny, as visualized with primary antibodies directed against viral gag proteins. At 44 hours postinfection, prior to cardiac trabeculation in the chick, both RCAS/4XRZNRG-infected embryos and control (RZNRG156) virus-infected embryos had developed normally and were indistinguishable from uninfected embryos. At 68 hours postinfection, however, all embryos infected with RCAS/4XRZNRG (n=16) were found to lack the normal network of yolk sac blood vessels, while this network of vessels was clearly present in 17 out of 23 RCAS/AP/RZNRG156-infected embryos. At 90 hours postinfection, an even more dramatic difference was observed, in that there was no observable heartbeat or blood flow in all RCAS/4XRZNRG-infected embryos, which were also markedly deformed. Since this time is equivalent in terms of cardiac development to the embryonic age at which the mouse NRG-1 mutants die, we analyzed histological sections of hearts from RCAS/4XRZNRG and control embryos. Myocardial trabeculae were missing from the ventricles of embryos infected with the RCAS/4XRZNRG virus (Fig. 3B), whereas ventricles from control injections were normally trabeculated, even though these control ventricles were extensively infected with RCAS/RZNRG156 (Fig. 3E). (We estimate that >70% of ventricular cells were routinely infected with the control virus in these embryos, based on anti-gag staining.) The defect in myocardial trabeculation that we have observed in these chick embryo experiments is very similar in histology, time of onset, and extent to the cardiac muscle defect seen in mice lacking either NRG-1 or its receptors ErbB2 and ErbB4 (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). In the mouse, this defect results from the loss of NRG-1 signalling, delivered from the endocardial endothelium to the myocardial muscle. Consistent with this signalling pathway, we found that NRG-1 was barely detectable inside hearts infected by RCAS/4XRZNRG (Fig. 3F), but that the expression level of the ErbB2 and ErbB4 receptors was unaffected (data not shown). We used a chick-muscle-specific marker 13F4 to demonstrate that myocardial wall development occurs normally in the RCAS/4XRZNRG-infected heart (Fig. 3D), an effect that also replicates the cardiac histology of the mouse NRG-1, ErbB2 and ErbB4 gene mutants.

Expression of NRG-1 ribozymes in the developing retina inhibits neuronal differentiation

Based on the embryonic expression patterns of NRG-1 and its ErbB receptors, it has been postulated that these molecules are regulators of neurogenesis and/or neuronal differentiation in the developing CNS (Bermingham-McDonogh et al., 1996; Corfas et al., 1995; Lai and Lemke, 1991; Meyer and Birchmeier, 1994). To test this hypothesis, we focused on the chick retina, where NRG-1 is expressed at the earliest (optic stalk and optic cup) stages of development and in which recent cell culture experiments have suggested an important role for NRG-1 in the development of retinal neurons (Bermingham-McDonogh et al., 1996). At the onset of retinal histogenesis, the optic cup is composed of undifferentiated stem cells, and the first cells to exit the cell cycle and differentiate are retinal ganglion cell (RGC) neurons. To assess the consequences of loss of NRG-1 function on the differentiation of these neurons, we injected RCAS/4XRZNRG or RCAS/RZNRG156 virus into the optic cup at stage 13-14 (E2) and repeated the injection once 12 hours later. Embryos were then incubated for an additional 96 hours. We stained retinal sections with anti-gag antibodies to identify infected cells, and then double-stained adjacent sections with the NRG-1 antibody and the monoclonal antibody RA4, which recognizes an RGC-specific antigen shortly after these cells are born (Austin et al., 1995; McLoon and Barnes, 1989).

In chick retinal regions infected by RCAS/4XRZNRG virus, the number of NRG-1-positive cells was substantially reduced, compared to RCAS/RZNRG156-infected retinal regions (see Fig. 4 middle panels). It has previously been shown that cells positive for RA4 are confined to the inner surface of the central retina, within the population of developing RGC neurons (McLoon and Barnes, 1989). At E7, this population comprises multiple cell layers with strong RA4 expression in the central retina of RCAS/RZNRG156-infected eyes (Fig. 4, upper left panel). We observed weak RA4 expression and a reduced number of cell layers in RCAS/4XRZNRG virus-infected retina (Fig. 4, upper right panel). To obtain a quantitative assessment of NRG-1 effects on RGC differentiation, we stained dissociated E5 retinal cells with a monoclonal antibody against the transcription factor Islet-1, which marks all RGC nuclei at this stage of retinal development (Austin et al., 1995). The percentage of Islet-1-positive cells was reduced from 13.0±3.6% in RCAS/RZNRG156-infected retina, which is very similar to previous observations on uninfected retina (Austin et al., 1995), to 4.9±1.2% in RCAS/4XRZNRG virus-infected retina (see Table 1).

NRG-1 reduction decreases mitotic activity in the developing retina

Since NRG-1 appears to act both as a differentiation agent and a mitogen for many developing cells, we reasoned that it might
also stimulate division of retinal stem cells. To test this possibility, we cultured dissected retinae in the presence of 1 mM BrdU, which is incorporated into the newly synthesized DNA of dividing cells. After a 2 hour labeling period, retinae were dissociated and the number of BrdU+ cells was compared between injected RCAS/4XRZ NRG and control RCAS/RZ NRG156 samples. We observed a substantial reduction in mitotic activity in RCAS/4XRZ NRG -infected retinae compared to RCAS/RZ NRG156 -infected retinae (see Table 1).

Recently, NRG-1 has also been demonstrated to act as a survival factor to prevent apoptosis of neonatal rodent Schwann cells in vivo and in vitro (Dong et al., 1995; Pinkas-Kramarski et al., 1994; Syroid et al., 1996; Trachtenberg and Thompson, 1996), and rodent retinal neurons in vitro (Birmingham-McDonogh et al., 1996). We therefore asked whether NRG-1 reduction in the embryonic retina might result in an increase in apoptotic cell death. Using the TUNEL-labelling technique to visualize the nuclei of dying cells, we did not observe any significant differences in the number of labeled nuclei between experimental and control retinae at E6 or E7. It remains possible that NRG-1 might rescue certain retinal cells from programmed cell death at later times in development.

DISCUSSION

We have developed an efficient and flexible strategy for conditional gene inactivation in chick embryos, using retroviral delivery of a hammerhead-type ribozyme. In order to achieve high level ribozyme expression, a synthetic chicken tRNA expression cassette was designed. Its compact size also allows for the incorporation of multiple ribozymes into a single replication-competent retroviral vector whose foreign DNA cloning capacity is ~2 kb. Moreover, the secondary structure of the tRNA, into whose anticodon region the ribozyme sequence is inserted, may provide nuclease resistance to the hybrid ribozyme-tRNA in vivo. Another potentially useful Pol III expression vector, derived from the human adenovirus type 2-associated vaRNA I gene, has also recently been used for embedding and expressing ribozyme sequences (Lieber and Strauss, 1995).

One major factor hampering the application of antisense ribozymes is the accessibility of target sequences within the highly folded structure of substrate (target) mRNAs in vivo. Currently, the choice of appropriate target sequences is somewhat empirical. Extending upon earlier work of Lieber and Strauss, we have found that certain computer programs that predict RNA secondary structure based on free energy minimization parameters are, when properly applied, particularly helpful. Through a detailed comparison of the published results of multiple antisense and ribozyme applications with the predicted secondary structures of the target RNAs for these reagents, we have found that the most effective ribozymes and antisense oligonucleotides are targeted to the predicted loop or single-stranded regions of substrate.
mRNAs, and have formulated a set of rules for the rational choice of ribozyme cut sites (Zhao and Lemke, 1998). Based on the database that we assembled for the formulation of these rules, we estimate that ~90% of computationally chosen target sites will result in a reduction in target RNA levels of at least 30%.

It is particularly significant that early expression of the NRG-1-inactivating ribozyme resulted in a failure of trabeculation during the early development of the embryonic heart in the chick, rather than a more subtle alteration of trabecular morphogenesis, since this is a spatial and temporal phenocopy of the NRG-1 mutant phenotype in the mouse (Meyer and Birchmeier, 1995). Trabeculae rapidly project into the ventricular cavity from the myocardial wall of the chick heart, between E3 and E4. This trabeculation results from the invasion of endothelial (endocardial) cells into the inner spongy layer of myocardium (muscle) (Brutsaert and Andries, 1992). In both the chick and the mouse, NRG-1 is expressed in endocardial endothelial cells, while ErbB2 and ErbB4 are expressed in myocardial cells. The more compact ErbB2/4+ outer myocardial wall, which is not in extensive contact with the NRG-1+ endocardium, is relatively normal in both chick and mouse mutant embryos deprived of NRG-1. This, together with the fact that the endocardium itself is to a first approximation normal in these mutants, suggests that NRG-1 signalling is largely paracrine. Direct cellular communication between the endocardium and the myocardium is clearly essential for the formation of myocardial trabeculae in multiple vertebrate species. The additional vascular phenotype that we observe upon NRG-1 disruption in the chick; i.e., a block to the normal formation of yolk sac blood vessels, reflects a basic difference in the organization of embryonic blood flow between mammalian and non-mammalian vertebrates, but also suggests that paracrine NRG-1/ErbB signalling may play a more general role during vertebrate angiogenesis than previously suspected (Hanahan, 1997).

Our results indicate that NRG-1 function can be selectively disrupted by ribozymes through the selection of when and where in the developing chick embryo these ribozymes are introduced. The selective disruption of NRG-1 function by either method effectively avoids the embryonic lethality associated with complete loss-of-function NRG-1 mutations, and has allowed us, for example, to demonstrate that both trophic and mitogenic activities of NRG-1 are required during early development of the retina. While the differentiation of distinct retinal cell types has been the subject of considerable descriptive research, the prior proliferation of retinal stem cells is the foundation for this process. The fact that disruption of NRG-1 function in the retina results in a reduction in neuroepithelial cell proliferation is consistent with previous demonstrations of NRG-1 mitogenic activity against several cell types, including Schwann cells and their precursors, and mammary epithelial cells. Recent work suggests that the fate of retina ganglion cells may be determined prior to the final cycle of cell division (Waid and McLoon, 1995). We have not examined the fate of other cell types besides retinal ganglion cells and it is therefore unclear if the loss of NRG-1 affects the subsequent differentiation of other retinal cell types. Since we have not observed elevated apoptosis associated with loss of NRG-1 activity in our experiments, NRG-1 may not act as a survival factor during the earliest stages of retinal neurogenesis. This is in agreement with the observation that the majority of embryonic chick retinal cells are proliferative at the times that we have analyzed (Prada et al., 1991), and have not adopted the differentiated cell fates in which survival factors generally play a critical role.

These results demonstrate that ribozyme-tRNA technology affords an effective alternative method for the analysis of gene function in organisms for which targeted gene disruption is not developed. Given appropriate delivery vehicles and the application of rules for rational choice of ribozyme cut sites (Zhao and Lemke, 1998), the methods that we describe for the NRG-1 gene in chick embryos are directly applicable to any

**Fig. 4.** Inhibition of retinal ganglion cell differentiation. Immunostaining of sections from RCAS/4XRZNRG156-infected (right panels) and RCAS/RZNRG156-infected (left panels) E7 chick retinas with antibodies to RA4 (top panels), NRG-1 (middle panels), and GAG (lower panels). In each panel, the pigment epithelium is at the bottom and the ganglion cell layer is at the top.
gene in any developing embryo. The coupling of UAS-driven ribozyme expression to Gal4-expressing *Drosophila* lines may be a particularly powerful application. In addition, the cassettes described above could be readily adapted for use in cell culture. For each of these applications, the prior cell culture assessment of both ribozyme efficacy and antisense effects by the methods outlined above (Fig. 2) is an important prerequisite for the effective application of the method.

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NRG-1 disruption by ribozymes


