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Abstract

RNA interference (RNAi) provides an effective method to silence gene expression and investigate gene function. However, RNAi tools for the chicken embryo have largely been adapted from vectors designed for mammalian cells. Here we present plasmid and retroviral RNAi vectors specifically designed for optimal gene silencing in chicken cells. The vectors use a chicken U6 promoter to express RNAs modelled on microRNA30, which are embedded within chicken microRNA operon sequences to ensure optimal Drosha and Dicer processing of transcripts. The chicken U6 promoter works significantly better than promoters of mammalian origin and in combination with a microRNA operon expression cassette (MOEC), achieves up to 90% silencing of target genes. By using a MOEC, we show that it is also possible to simultaneously silence two genes with a single vector. The vectors express either RFP or GFP markers, allowing simple in vivo tracking of vector delivery. Using these plasmids, we demonstrate effective silencing of Pax3, Pax6, Nkx2.1, Nkx2.2, Notch1 and Shh in discrete regions of the chicken embryonic nervous system. The efficiency and ease of use of this RNAi system paves the way for large-scale genetic screens in the chicken embryo.
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Introduction

The chicken embryo is one of the main model systems for analysing vertebrate development (Brown et al., 2003), but simple efficient systems for reverse genetic analysis are underdeveloped. RNA interference is now widely used as a tool for the analysis of gene function, providing a quick and low cost means for silencing gene expression. In *Caenorhabditis elegans* and *Drosophila melanogaster* cells, long (>30 bp) double

stranded RNAs are routinely employed and a number of genomic scale genetic screens using RNAi have now been undertaken (reviewed in Friedman and Perrimon, 2004). In mammalian cells, long double stranded RNAs are not generally used for RNAi because they can trigger the interferon response (Stark et al., 1998). As part of this response, double stranded RNA activates PKR which in turn phosphorylates eIF2α leading to global translational arrest (Manche et al., 1992) and induces 2′,5′-oligoadenylate synthetase (OAS) which activates ribonuclease L leading to non-specific mRNA degradation (Minks et al., 1979). In contrast, short double stranded RNAs (21–23nt) trigger specific gene silencing and are now widely used (Elbashir et al., 2001). Although the interferon response was originally discovered in the chicken and chick embryos

(Isaacs and Lindenmann, 1957), it has been reported that long double stranded RNAs lead to specific gene silencing in the embryo (Pekarik et al., 2003). Perhaps even more surprising is the observation that simply electroporating a chicken embryo upregulates OAS expression yet this does not lead to the characteristic non-specific mRNA degradation. Moreover, despite inducing OAS, no developmental defects caused by the electroporation procedure have been reported (Chesnutt and Niswander, 2004). This may indicate that other components of the interferon pathway such as ribonuclease L are not active in the embryo.

Chemically synthesised short interfering RNAs (siRNAs) have also been used to efficiently silence gene expression in the chicken embryo (Hu et al., 2002). Whilst siRNAs and long double stranded RNAs may be effective, vector-based RNAi can provide stable long-term expression of siRNA and allows a marker gene to be directly linked to the siRNA expression cassette, enabling accurate in vivo tracking of silenced cells. Moreover libraries of RNAi vectors are far more suitable for distribution as a cheaply renewable resource than siRNAs or double stranded RNA.

Early attempts at vector based RNA in mammalian cells used short hairpin RNAs of varying length (Brummelkamp et al., 2002; Paddison et al., 2002) but more recently hairpins based on naturally occurring microRNAs (miRNAs) have been utilised (Zeng et al., 2002). Endogenous miRNAs, typically ~80 nucleotides in length are frequently expressed as much larger precursor transcripts known as pri-miRNAs and can be linked together in transcriptional operons (Lee et al., 2002; Hubbard et al., 2005) which are initially processed by Drosha to produce pre-miRNAs and then subsequently by Dicer to produce siRNA (Kim, 2005). For optimal excision from primary transcripts, Drosha requires unstructured flanking sequences surrounding the miRNA hairpin (Zeng and Cullen, 2005). Recent studies have shown that shRNAs are more efficient substrates than siRNAs for silencing gene expression (Siolas et al., 2005). Furthermore, synthetic miRNAs are better Dicer substrates than shRNAs and lead to greater siRNA production and gene silencing (Boden et al., 2004; Silva et al., 2005; Dickins et al., 2005). The pre-miRNA processing and target mRNA cleavage pathways are functionally coupled and RISC, which consists of DICER, TRBP and Argonaute2 in humans, displays nearly 10-fold better activity when programmed with a DICER substrate rather than an equivalent siRNA, which explains the differing efficiencies of siRNAs and shRNAs/miRNAs in gene silencing (Gregory et al., 2005). On the basis of these observations, comprehensive libraries of vectors for the human and mouse genome have now been constructed which utilise synthetic miRNA30-based hairpins to ensure optimal access to RNA interference machinery and efficient gene silencing (Silva et al., 2005).

A number of reports have described the use of the heterologous mouse U6 or human H1 promoters (Katahira and Nakamura, 2003; Bron et al., 2004; Chesnutt and Niswander, 2004; Dai et al., 2005) to drive expression of conventional short hairpin (sh)RNAs and silence gene expression in the chicken embryo by RNAi. However, the efficiency of these mammalian promoters has not been compared with the equivalent chicken promoters in vivo. Within the vertebrate U6 promoter, the proximal sequence element (PSE) has been shown to influence species specificity (Simmen et al., 1992) and within human U6 promoters, minor variations in the proximal sequence elements profoundly influence their transcriptional efficiency (Domitrovich and Kunkel, 2003). Examination of the proximal sequence elements of chicken U6 promoters with other vertebrates (Dai et al., 2005; Kudo and Sutou, 2005) reveals differences in the PSE elements and, given the sensitivity of these promoters to minor variations in the PSE, we considered that mammalian U6 promoters might not be the most suitable for RNA interference in the chicken.

Here we have generated an RNA interference system tailor-made for chickens which utilises a potent chicken U6 promoter driving expression of a modified chicken microRNA operon. The efficiency of gene silencing with this system is comparable with the best vectors available for mammalian cells and even allows dual gene silencing from a single plasmid.

Materials and methods

Cloning chicken U6 promoters and vector construction

Isolation of the chicken chromosome 18 U6 snRNA promoter was carried out as follows: the *Xenopus* U6 snRNA sequence was used to search the chicken EST database (Boardman et al., 2002). Surprisingly, several EST matches were found with 100% sequence identity to nucleotides 11–107 of the *Xenopus* U6 snRNA, which probably resulted from aberrant transcription of the RNA polymerase III promoter by RNA polymerase II. Based on the sequence identity, two oligonucleotides were synthesised, A + B (oligonucleotides used in cloning are detailed in Supplementary Table S1) and used to PCR amplify a fragment of the U6 snRNA gene using chicken DT40 genomic DNA. This snRNA gene fragment was radiolabelled and used to screen a gridded chicken bacterial artificial chromosome (BAC) library. This resulted in the isolation of 14 positive BACS. DNA sequence was obtained from a single BAC using oligonucleotide B and used to amplify the U6 snRNA promoter together with a 27 nucleotide U6 snRNA leader sequence using oligonucleotides C + D. Following publication of the chicken genome sequence (Hillier et al., 2004), the promoter was found to originate from chromosome 18. To create the backbone vector to accept the chicken U6 promoter, a monomeric red fluorescent protein gene (Campbell et al., 2002) was subcloned on a blunt-ended *Bam*HI–*Hin*dIII fragment into a blunt-ended *Eco*RI site in pCAGGS to generate pCAGGSRFP. The amplified promoter was subcloned into pCAGGSRFP on a *Hin*dIII–*Bam*HI fragment to generate pCAGGSSWU6. To generate the flanking sequence IV from the miRNA operon, two oligonucleotides E and F were annealed and subcloned into pCAGGSSWU6 cut with *Kpn*I and *Nhe*I. The flank V + VI sequence from the miRNA operon and RNA polymerase II terminator was generated by annealing oligonucleotides G and H and subcloning the DNA downstream of flank IV sequences on a *Sac*I–*Bam*HI fragment to generate pCAGGSSWU6FLANKIV + V.

To clone the chicken chromosome 28 U6 promoter, a chicken genomic DNA library in bacteriophage lambda was used to PCR amplify U6 promoter DNA using a T7 promoter primer and oligonucleotide I whose sequence was based on conserved regions of the U6 snRNA in human, mouse and *Xenopus* U6 promoters. A nested PCR reaction was then carried out with the product of the first reaction using the T7 promoter primer and oligonucleotide J and the PCR product was subcloned into pGEM-T Easy (Promega). The U6 promoter was then PCR amplified using oligonucleotides K and L and subcloned into the *Hin*dIII and *Bam*HI sites of pCAGGSRFP to generate pCAGGSRFPVENUU6. pRFPRNAi was generated by subcloning a *Kpn*I–*Bam*HI fragment from pCAGGSSWU6FLANKIV + V into pCAGGSRFP-VENUU6. pRFPRNAiA (Fig. 1b) was generated by annealing oligonucleotides M and N and subcloning the fragment into the *Nhe*I–*Sph*I sites of pRFPRNAi, which substitutes the *Sac*I site in pRFPRNAi for an *Afl*II site

since there is an internal *Sac*I site in the chromosome 28 U6 promoter. The mouse U6 promoter was amplified from pSILENCER U6 1.0 (Ambion) using oligonucleotides O and P and subcloned on a *Hind*III–*Bam*HI fragment into pCAGGSRFP to generate pCAGGSRFPMOUSEU6.

RCASARNAi was built by Quikchange mutagenesis of RCAS(A) with oligonucleotides Q and R which introduced a *Not*I restriction site upstream of the unique *Cla*I restriction site and generated RCAS (A) *Not*I. EGFP was then PCR amplified using oligonucleotides S and T from pEGFP-NI (Clontech) and subcloned on a *Not*I fragment into RCAS (A) *Not*I. The 5′ *Not*I site was then destroyed using Quikchange mutagenesis with oligonucleotides U and V to generate RCASARNAi. RCASBRNAi was generated by Quikchange mutagenesis of RCAS(B) with oligonucleotides Q and R which introduced a *Not*I restriction site upstream of the unique *Cla*I restriction site.

Synthetic miRNA30 Construction

The gene-specific oligonucleotides for miRNA30 like hairpin synthesis are shown in Supplementary Table S2, together with the sequences which they target. The 22 nucleotide target sequences were chosen using the design tool at www.genscript.com/ssl-bin/app/rnai. The 5′ base of the sense strand was altered in all cases so that it mismatched the guide strand base to mimic the structure found in endogenous miRNA30. Hairpins for the first miRNA cloning site were generated by PCR using 10 ng of each gene-specific oligonucleotide together with 100 ng of two generic flanking oligonucleotides W + X in a 50 µl reaction using Pfu polymerase (Promega). PCR conditions were 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 45 s at 72°C using an Eppendorf personal cycler. PCR products were gel purified, digested with *Nhe*I and *Sac*I and subcloned into pCAGGSSWU6FLANKIV + V. The miRNAs for genes were then transferred downstream of the chromosome 28 U6 promoter in pRFPRNAi on a *Kpn*I–*Bam*HI fragment to generate pRFPRNAi-GENE NAME. The miRNA active against luciferase was also transferred downstream of the mouse U6 promoter in pCAGGSMOUSEU6 on a *Kpn*I–*Bam*HI fragment. The chicken U6 promoter and miRNA expression cassette for luciferase were subcloned from pRFPRNAi-LUC into RCASARNAi on a *Not*I–*Cla*I fragment. The Pax6B MOEC was transferred to RCASBRNAi on a *Not*I–*Cla*I fragment.

The chicken chromosome 28 promoter contains an internal *Sac*I site which precluded direct cloning of miRNA hairpins. Therefore, we constructed a derivative where the *Sac*I site was replaced with an *Afl*II site to generate pRFPRNAiA (Fig. 1) and this vector was used for subsequent studies. To generate the double miRNA vector, the luciferase hairpin was firstly subcloned

into pRFPRNAiA using gene-specific oligonucleotides for luciferase together with two generic flanking oligonucleotides W and Y on an *Nhe*I–*Afl*II fragment. Gene-specific oligonucleotides for LacZ, suitable for use in the second hairpin cloning site, were used together with two generic flanking oligonucleotides Z and AA and the miRNA amplified using the conditions described above. The second miRNA was subcloned into the *Afl*II and *Sph*I sites of pRFPRNAiA, downstream of the luciferase hairpin. A single miRNA vector for LacZ was also generated using gene specific oligonucleotides and oligonucleotides W + Y and subcloning the PCR product into the *Nhe*I–*Afl*II sites of pRFPRNAiA.

Tissue culture and transfections

Chicken DF-1 cells were maintained in Dulbecco's modified Eagles medium (DMEM) + 10% foetal calf serum with 10% CO_2 at 39°C. HEK293T cells were maintained in the same growth medium with 5% CO_2 at 37°C. Cells were transiently transfected using lipofectamine 2000 (Invitrogen) and enzyme assays carried out as described previously (Williams et al., 2005). The H1 promoter-based RNA interference vector for TAP has been described previously together with GFP-TAP (Williams et al., 2005).

In ovo electroporation

White Leghorn fertilised eggs were purchased from Henry Stewart and Co. LTD (U.K.). Eggs were incubated at 38°C for 45 h (until embryos were approximately stages 10–12, according to Hamburger Hamilton (HH) staging) and windowed to reveal the embryo. Approximately 0.2–1 µl of gene-specific or control RNAi vector (1 µg/µl, resuspended in H_2O, plus 0.1% Fast green dye) was injected into the lumen of the posterior portion of the developing neural tube. When two RNAi vectors for the same gene were mixed for electroporation, the concentration of each vector was 0.5 µg/µl. Embryos were submerged in 1× PBS, and 1 mm electrodes (genetrodes, Genetronics, Inc.) 4 mm apart were placed on either side of the embryo. A current of 27 V was applied across the electrodes (6 × 10–50 ms pulses), using a TSS20 Ovodyne electroporator (Intracel). Embryos were cooled with 1 × PBS, windows covered with parafilm and Fujifilm sealon film and the eggs placed at 38°C. Embryos were dissected out either 24 h or 48 h after electroporation (approximately HH stages 19–22). For luciferase assays on electroporated embryos, HH stage 11–12 embryos were coelectroporated with various concentrations of luciferase RNAi vector together with 100 ng luciferase plasmid pGL3 (Promega) and 200 ng pcDNA-LacZ (Invitrogen). Embryos were cultured for a further 24 h and the electroporated region of each
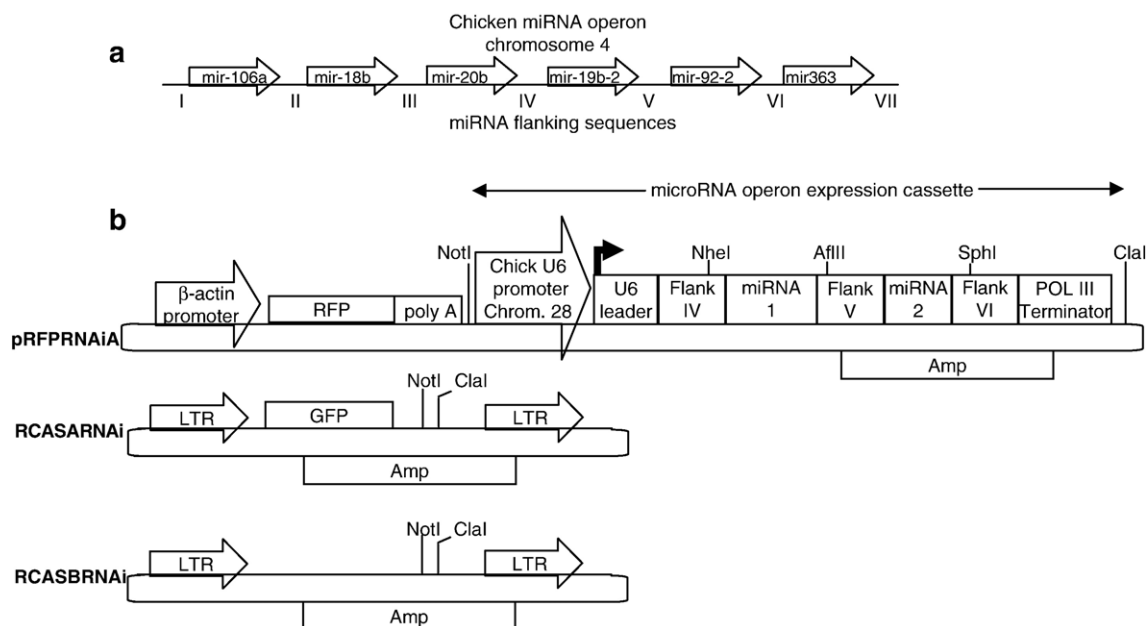

Fig. 1. RNA interference vectors for chicken cells. (a) Organisation of the pri-miRNA operon encoding miR-106a, 18b, 20b, 19b-2, 92-2 and 363. (b) Schematic of pRFPRNAiA, RCASARNAi and RCASBRNAi. The miRNA expression cassette can be transferred from pRFPRNAi to RCAS vectors using *Not*I and *Cla*I restriction enzymes.

neural tube was harvested, lysed in 80 μl of Reporter Gene Assay Lysis buffer (Roche) and luciferase activity was determined as above. The percentage luciferase knockdown was determined by comparison to the luciferase activity measured following electroporation with an RNAi vector targeting GFP. Results were normalised to the β-galactosidase activity measured in the lysate and the results presented the averages from 9 independent embryo electroporations carried out on three separate occasions. For retroviral electroporations, 1 μg/μl RCASBRNAi-Pax6B together with 0.1 μg/μl pCAGGS-RFP was coelectroporated into HH stage 12–14 embryos.

Fixation and sectioning

Embryos were dissected from the eggs, rinsed briefly in 1× PBS and fixed in 4% paraformaldehyde on ice for up to 2 h depending on the stage. They were then rinsed in 1× PBS and stored overnight in 30% sucrose (in 0.2 M phosphate buffer). Embryos were viewed under a DM5000B fluorescent microscope (Leica) to determine the RFP-positive segments, which were dissected out, frozen in optimal cutting temperature (OCT) compound (VWR International), sectioned to a thickness of 15–20 μm using a cryostat (Bright) and placed on Superfrost Plus microscope slides (VWR).

Immunohistochemistry and imaging

Slides were rinsed in 1× PBS and incubated overnight at 4°C in appropriate primary antibody solution (1:50 dilution of antibody obtained from Developmental Studies Hybridoma Bank, University of Iowa; 1:10 dilution for the Gag antibody in 1× PBS containing 0.1% Heat Inactivated Goat Serum and 0.01% Triton X100). For staining with the anti-neuron-specific β-tubulin TUJ1 (Covance), a 1:1000 dilution was used. Slides were rinsed in 1× PBS and incubated for 30 min in secondary antibody solution (1:200 dilution of FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch) in 1× PBS containing 0.1% Heat Inactivated Goat Serum/0.01% Triton X100). Secondary antibody solution was supplemented with 1:2000 dilution of TOPRO-3 iodide (Molecular Probes) for nuclear counterstaining. For BrdU labelling, 50 μl of a 0.01 M solution of BrdU (Sigma) dissolved in PBS was injected through an opening in the vitelline membrane and the amniotic sac. The egg was resealed and the embryo was incubated for an additional hour before harvesting. Sections were treated with 45 units/ml Dnase 1 (Sigma) in TBS (50 mM Tris pH 7.5, 150 mM NaCl) with 10 mM MgCl$_2$ and 10 Mm MnCl$_2$ for 60 min at 37°C to reveal the epitope. The anti-BrdU antibody was used at 1:250 dilution and the secondary antibody was Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) (1:250 dilution). Slides were rinsed in 1× PBS and mounted in Vectashield mounting media (Vector). Images were obtained using a Leica DM RE confocal microscope and a Nikon Microphot-SA.

Wholemount in situ hybridisation

Embryos were harvested 24 h after electroporation and fixed in 4% paraformaldehyde/PBS for 16 h at 4°C. Wholemount in situ hybridisation was performed as described previously (Ohyama et al., 2005) using digoxigenin (DIG)-labelled (Roche) RNA probes. DIG-labelled antisense riboprobes were generated from linearised plasmids encoding chick *Notch1* (cut with *Bam*HI and transcribed with T3 RNA polymerase (Promega)) and chick *Hes1* (cut with *Hin*dIII and transcribed with T3 RNA polymerase (Promega)). Stained embryos were photographed under a MZ12 stereomicroscope (Leica) using a Spot digital camera (Diagnostic Instruments).

Results and discussion

Construction of a microRNA operon expression cassette (MOEC)

Since synthetic miRNAs achieve significantly better silencing of genes than conventional shRNAs (Boden et al., 2004), we decided to employ them for silencing chicken genes. To achieve

efficient processing of synthetic miRNAs by both DROSHA, which preferentially excises hairpins found within unstructured regions of RNA (Zeng and Cullen, 2005) and DICER, which uses miRNAs as one of its natural substrates, we adapted a chicken miRNA operon, conserved in other vertebrates, encoding miR-106a, 18b, 20b, 19b-2, 92-2 and 363 (Hubbard et al., 2005). The unstructured flanking sequences IV, V and VI (Fig. 1a) were used to generate an expression cassette, preceded by a 27nt U6 snRNA leader sequence to ensure optimal expression (Paddison et al., 2004), into which multiple miRNAs could be inserted (Fig. 1b). Synthetic miRNAs based on human microRNA30 which has been used previously to silence genes in mammalian cells (Zeng et al., 2002) were generated for insertion in the miRNA operon expression cassette (MOEC) by a single step PCR. The synthetic miRNAs retained the base of the stem and loop sequences from miRNA30 but the remaining stem was replaced by a gene specific 22 nucleotide sequence.

Comparison of promoters for MOEC expression

To find the most appropriate promoter for driving MOEC expression, we first tested the human H1 promoter for gene silencing using existing vectors known to work well in human cells. pSUPER-TAP expresses a 19 bp stem shRNA active against the human mRNA export factor TAP, which efficiently depletes endogenous TAP in human cells and blocks mRNA export (Williams et al., 2005). When pSUPER-TAP was cotransfected into human 293T cells together with a GFP-TAP expression vector, it led to a clear reduction in the number of GFP-TAP-positive cells, compared with the control transfection using an RNAi vector active against luciferase (Fig. 2). In contrast, when the same plasmids were used in chicken DF-1 cells, we were unable to detect a convincing and reproducible knockdown of GFP-TAP expression. This indicates that the human H1 promoter does not function efficiently in chicken cells and led us to investigate alternative promoters which may be more suitable.

Chicken RNA polymerase III promoters may function more efficiently in chicken cells than mammalian promoters, therefore we cloned two different U6 promoters, located on chicken chromosomes 18 and 28. These promoters were subcloned into a pCAGGS based vector backbone which expresses monomeric red fluorescent protein as a marker for transfected cells (Fig. 1b). A MOEC containing a single hairpin based on miRNA30 active against firefly luciferase was inserted downstream of the two chicken and a single mouse U6 promoter and used to test the ability of all three promoters to silence luciferase in chicken DF-1 cells (Fig. 3a). The chicken promoter which maps to chromosome 28 consistently gave significantly higher levels of silencing compared with the chicken chromosome 18 or mouse U6 promoter, when used at lower doses, indicating that it drove the highest level of miRNA30-Luc expression. These results are consistent with a recent study which showed that the chromosome 18 chicken U6 promoter was less active than the chromosome 28 promoter (Kudo and Sutou, 2005). It is striking that the maximum knockdown of luciferase gene
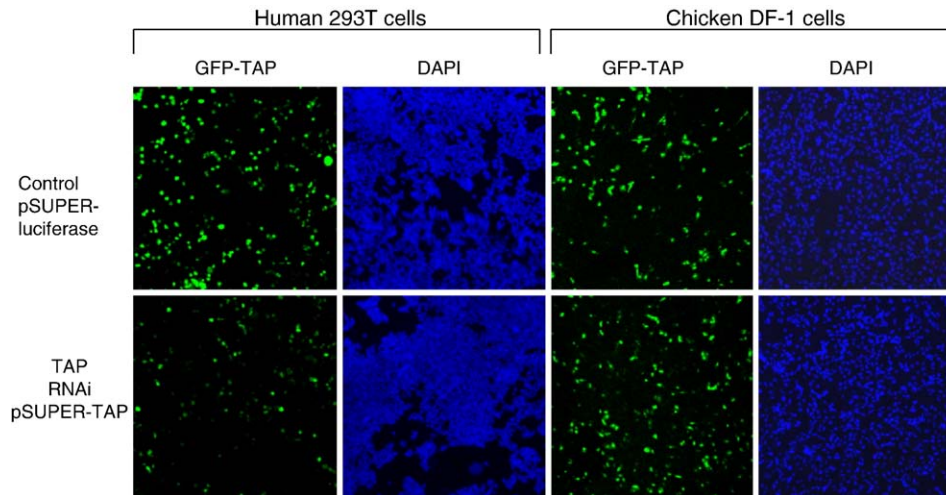
Fig. 2. The human H1 promoter does not work efficiently in chicken cells. 200 ng GFP-TAP vector (human) together with 600 ng of either pSUPER-luciferase vector (control) or pSUPER-TAP vector was transfected into either human 293T or chicken DF-1 cells. Cells were fixed 48 h post-transfection and nuclei counterstained with DAPI.

expression obtained in that study using conventional shRNAs driven from a chicken chromosome 28 U6 promoter was only 47% in chicken cells. In contrast, the vectors we describe here routinely achieve 90% silencing of luciferase expression. These differences may in part be attributed to the use of different target sequences, but probably also reflect the enhanced Drosha and Dicer processing associated with synthetic miRNAs.

To determine whether the chicken chromosome 28 U6 promoter also worked most efficiently in the embryo, we carried out neural tube electroporations with both chicken and mouse U6 promoters driving expression of the same
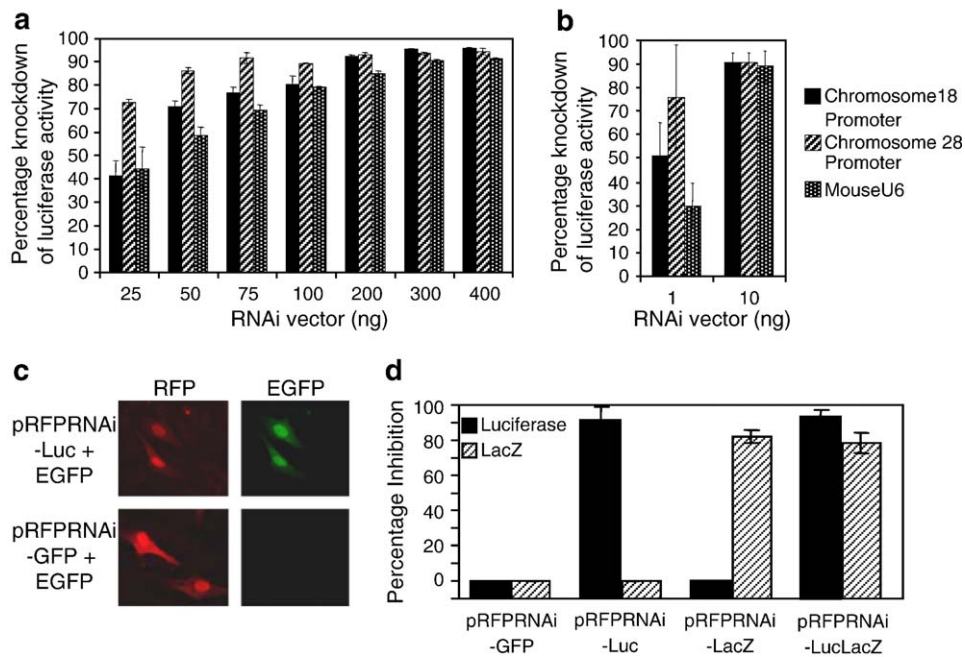

Fig. 3. RNA interference in DF-1 cells and the chicken embryo. (a) Comparison of chicken and mouse U6 promoters for silencing luciferase in DF-1 cells. Cells were transfected with the indicated amounts of RNAi vector together with 200 ng of pGL3 luciferase expression vector and 200 ng pcDNA-LacZ. An RNAi vector for GFP was used as a control (500 ng) and showed no silencing of luciferase. (b) Comparison of chicken and mouse U6 promoters for silencing luciferase in the neural tube. Embryos were electroporated with the indicated amounts of RNAi vector together with 100 ng pGL3 and 200 ng pcDNA-LacZ. (c) Silencing EGFP in DF-1 cells. Cells were transfected with 200 ng pEGFP-N1 (Clontech) together with 600 ng RNAi vector. (d) Dual silencing of LacZ and luciferase. An RNAi vector active against GFP (500 ng) was used as a control. Cells were transfected with 50 ng of lacZ expression vector, 200 ng luciferase expression vector and 500 ng of the indicated RNAi plasmids. The percentage knockdown of enzyme activities are shown for single and dual gene RNAi vectors, using the GFP RNAi vector as a control. For enzyme assays, the results represent the averages from nine independent transfections carried out on three separate occasions, the error bar showing the standard deviation. Transfection efficiencies were normalised by cotransfecting a β-galactosidase reporter, with the exception of the dual gene knockdown, where transfection efficiencies were monitored by counting RFP positive cells.

MOEC which silenced luciferase and then carried out luciferase assays on embryo lysates (Fig. 3b). At low doses of RNAi vector, the chicken chromosome 28 promoter was most efficient, yielding more than twofold better silencing than the mouse U6 promoter, whilst the chicken chromosome 18 promoter drove intermediate levels of silencing. At higher doses of RNAi vector, all three promoters led to 90% silencing of luciferase activity. In general, the levels of RNAi vector required for silencing luciferase in the embryo were considerably lower than those required for silencing in tissue culture, which may reflect the differences in the efficiency of DNA delivery using electroporation compared with lipofection. The chromosome 28 chicken U6 promoter appears to be the most potent in both tissue culture and the neural tube and was used for further experiments since in cases where suboptimal target sequences are used, silencing is likely to be greatest with maximal expression of the miRNA.

To confirm reliable expression of the RFP marker in the presence of active RNAi, we designed a vector based on pRFPRNAi to silence GFP and used this in DF-1 cells (Fig. 3c). This vector led to efficient GFP silencing, yet maintained equivalent RFP expression, indicating that the RNAi effect was specific and RFP expression was not compromised in cells exhibiting an RNAi response.

Dual gene silencing

There is a degree of redundancy in many vertebrate gene families, complicating functional analysis. To address this problem, we took advantage of the fact that within a miRNA operon, multiple genes can be targeted by several miRNAs, all expressed from a common precursor pri-miRNA. A vector based on pRFPRNAiA was constructed, carrying a miRNA against luciferase followed by a miRNA for β-galactosidase. Using this vector, we showed effective simultaneous silencing of both genes in DF1 cells; moreover, the level of silencing for each gene was comparable to that produced by vectors only encoding a single miRNA (Fig. 3d).

Silencing of endogenous genes in the chicken embryo

To demonstrate the utility of these vectors for silencing endogenous genes in the chicken embryo, we examined their ability to silence genes encoding transcription factors in the developing spinal cord by electroporation of pRFPRNAi-derived plasmids. The number of vectors constructed, target sequences and relative efficiencies are summarised in Table 1. For each gene, we constructed at least two RNAi vectors, although we found that in some cases only a single vector worked well and in some cases, vectors completely failed to silence the target gene. Electroporated cells were clearly identified by expression of RFP and in all cases the overlays of the RFP and FITC channels (staining for the endogenous protein) with the luciferase control RNAi vector showed yellow cells (Fig. 4a, left panels) indicating no effect on the expression of the endogenous genes. Pax3, Nkx2.2 and Pax6 are normally expressed in the dorsal, ventral and intermediate domains of the spinal cord, respectively (Fig. 4a). When RNAi vectors specific for the transcription factors were introduced, Pax3 protein levels were significantly reduced at 48 h using the Pax3 A vector, whilst Nkx2.2 showed a clear knockdown as early as 24 h post-electroporation using the Nkx2.2 B vector (Fig. 4a, right panels). The longer time taken to see knockdown with Pax3 compared with Nkx2.2 may reflect differences in the half lives of the proteins. For Pax6 silencing, we initially mixed two RNAi vectors targeting different sequences and observed efficient silencing as early as 24 h post-electroporation. Further electroporations using the individual Pax6 RNAi vectors revealed that the Pax6A vector may work less efficiently than the Pax6B vector, since a yellow cell was observed in overlays (Fig. 4b), yet mixing the vectors prior to electroporation did not appear to compromise the efficiency of silencing (Fig. 4a).

To establish the efficacy of the pRFPRNAi-derived plasmids in other neural tube cells, we electroporated hypothalamus and spinal cord floor plate cells with RNAi vectors for Nkx2.1 and Sonic hedgehog (Shh), respectively. Nkx2.1 gene expression was silenced in the hypothalamus at 48 h (Fig. 4d) and Shh

Table 1
Target sequences and efficiency of knockdown achieved in this study

| Vector	Target gene	Target sequence	Embryos electroporated	Embryos showing significant knockdown
pRFPRNAi Nkx2.1 A	Nkx2.1	GTCTAGGACTTGCATGTAAATA	5	5
pRFPRNAi Nkx2.1 B	Nkx2.1	ATGTGAACCTGTAGCTGTATAA	4	2
pRFPRNAi Nkx2.2 A	Nkx2.2	GCTGTTCAGACGCTGCCTTTGA	5	0
pRFPRNAi Nkx2.2 B	Nkx2.2	ACCACTTGGTACAGGCGCAGCA	8	8
pRFPRNAi Pax3 A	Pax3	TCCAAGCAGTACAGTCCATAGA	7	7
pRFPRNAi Pax3 B	Pax3	TGGACAAAGTGCCTTTCATTAT	5	0
pRFPRNAi Pax6 A	Pax6	TTTGTACACTGAAGGCATTATA	15	15
pRFPRNAi Pax6 B	Pax6	GGCACCACTTCCACAGGTCTCA	20	20
pRFPRNAi Notch1 A	Notch 1	ACACCAATGTAGTCTTCAAGAA	13	12
pRFPRNAi Notch1 B	Notch 1	GGAAGATTCTGTTGGACTGAAA	5	0
pRFPRNAi Notch1 C	Notch 1	CCGGCACGCTGATGGACGACAA	12	12
pRFPRNAi Notch1 D	Notch 1	ACGCCAACTTCAACGGCCTCAA	10	10
pRFPRNAi Notch1 E	Notch 1	ACGCCAACTTCAACGGCCTCAA	7	5
pRFPRNAi Shh A	Shh	GCTGTGAAGTAATTGGATTCAA	5	0
pRFPRNAi Shh B	Shh	ACAAGAAACTCCGAGAGATTTA	5	5
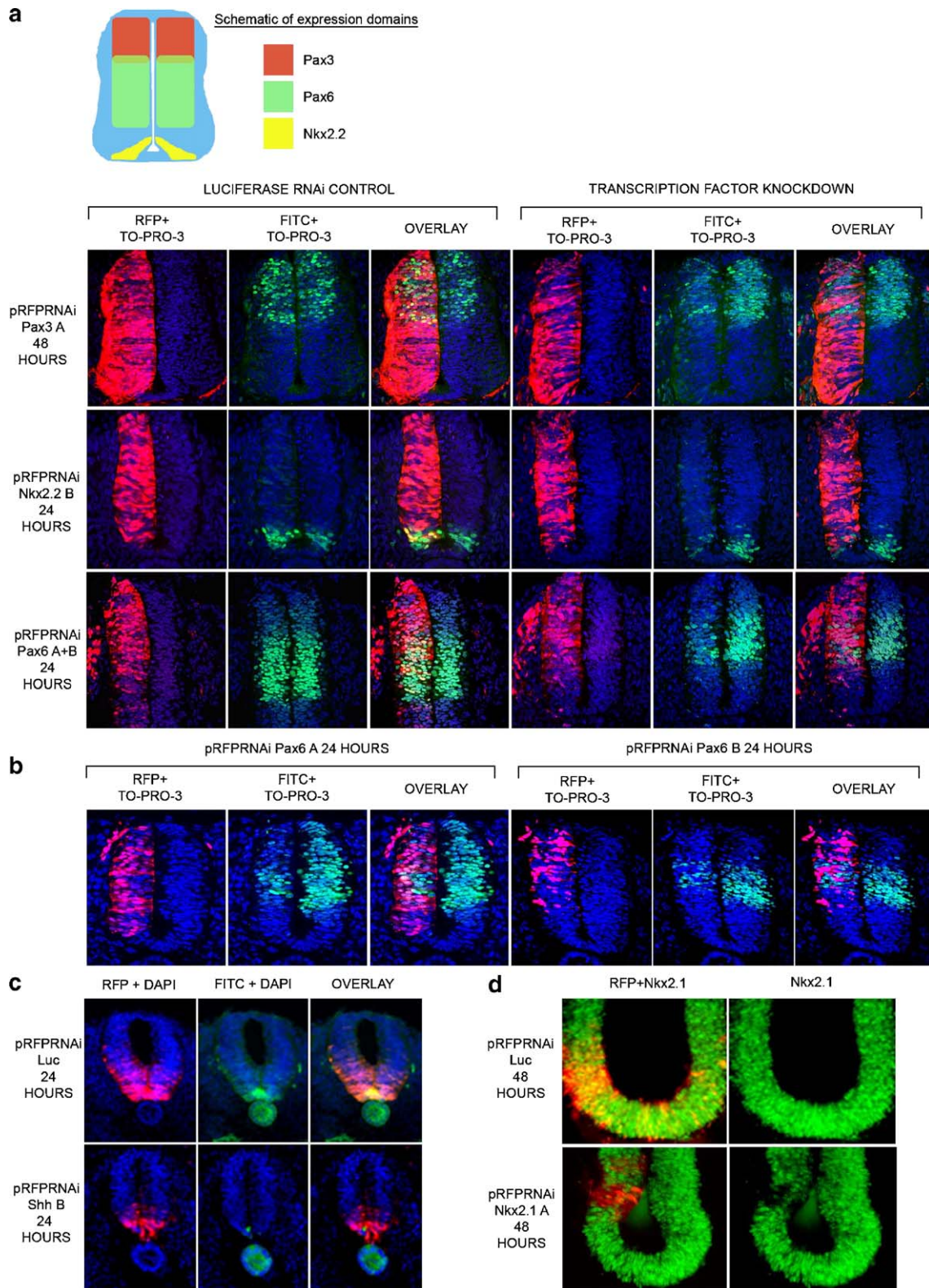
Fig. 4. Silencing of endogenous genes in the chicken embryo. (a) A schematic of the expression domains for the transcription factors is shown at the top. Knockdown of Pax3 by pRFPRNAi Pax3 A (top panels). Knockdown of Nkx2.2 by pRFPRNAi Nkx2.2 B (central panels). Knockdown of Pax6 by a mixture of pRFPRNAi Pax6 A and pRFPRNAi Pax6 B. Electroporation introduces the RNAi vector into one side of the neural tube, marked by the RFP positive cells, the other half acting as an internal control. Transcription factors are stained with FITC. (b) Knockdown of Pax6 mediated by electroporation of single vectors. Knockdown of Pax6 by pRFPRNAi Pax6 A (left panels). Knockdown of Pax6 by pRFPRNAi Pax6 B. (c) Shh knockdown in the floorplate by pRFPRNAi Shh B. (d) Nkx2.1 knockdown in the hypothalamus by pRFPRNAi Nkx2.1 A. All experiments (a–d) are controlled using the luciferase RNAi vector; in each case, the overlay image of RFP positive, electroporated cells and the FITC channel, staining for the endogenous protein, shows yellow cells demonstrating that the control vector does not silence the endogenous gene. Cell nuclei are stained using TO PRO-3.

expression was silenced in the floor plate at 24 h post-electroporation (Fig. 4c), demonstrating that RNAi functional analysis can be applied to a number of different cell types in the chick neural tube.

To test the potential of these vectors in the investigation of genes involved in neuronal differentiation, we constructed vectors to silence *Notch1*. Loss of *Notch1* expression was initially assessed using in situ hybridisation (Fig. 5a, lower panels) which clearly demonstrated silencing of *Notch1* mRNA. We also examined the expression of *Hes1* a downstream target of *Notch1* signalling (Jarriault et al., 1995) and found that it was downregulated following *Notch1* silencing (Fig. 5b, lower panels). However, some residual *Hes1* expression was detected which may be due to Notch1 signalling from unelectroporated cells. Notch1 signalling has been shown to result in premature neuronal differentiation in many vertebrate species (reviewed in Lewis, 1998). A reduction of Notch1 protein in the chick neural tube is

therefore predicted to result in differentiation of the electroporated cells. This was assessed by examining the RFP distribution in neural tube sections. Electroporation of a luciferase RNAi plasmid resulted in cells expressing RFP throughout the ventricular and mantle zones of the spinal cord at 48 h post-electroporation (Fig. 5c, top left panel). In contrast, cells electroporated with constructs targeting three different sequences in the *Notch1* mRNA were predominantly located in the mantle region of the neural tube (Fig. 5c), with few remaining in the ventricular zone. Since these results were seen with RNAi vectors targeting different regions of *Notch1* mRNA, it is unlikely that they were caused by off target effects. BrdU labelling analyses indicated that the electroporated cells, expressing RFP, were not in S-phase (Fig. 5d, right panels), and antibody labelling indicated that these cells expressed the early neuronal marker TUJ1, indicating premature neuronal differentiation (Fig. 5d, left panels).
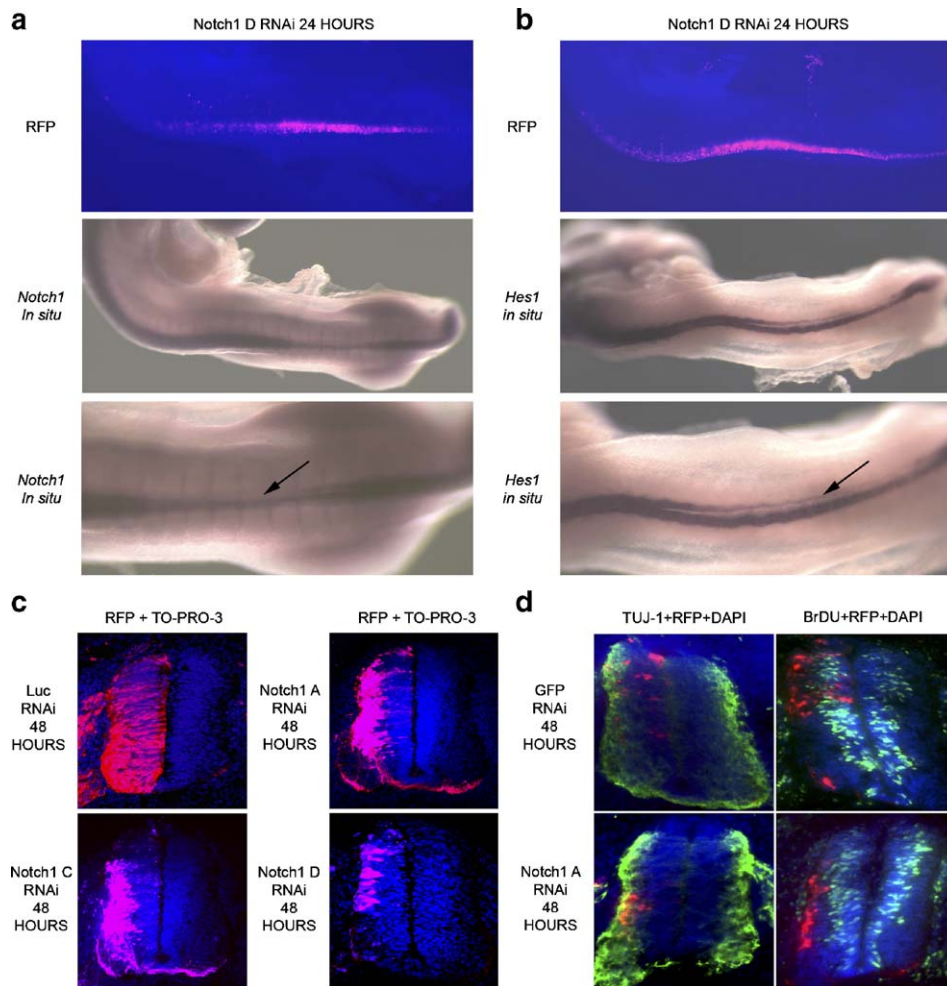

Fig. 5. Notch1 knockdown in the neural tube. (a) Wholemount in situ hybridisation to detect expression of *Notch1* mRNA following RNAi with a Notch1 vector. Electroporated region of the neural tube is marked by RFP fluorescence (top panel). Electroporated cells show loss of *Notch1* mRNA expression (middle panel and arrow in lower panel). (b) Wholemount in situ hybridisation to detect expression of *Hes1* mRNA following RNAi with a Notch1 vector. *Hes1* mRNA is significantly reduced in the RFP-positive region of the neural tube (compare top panel with arrow in lower panel). (c) RFP distribution was examined in non-stained sections using a luciferase RNAi vector as a control. Electroporation with Notch1A, Notch1C and Notch1D RNAi vectors resulted in premature migration of RFP-positive cells towards the mantle zone of the neural tube. (d) Sections were also examined for differentiation by staining for BrdU (green) to mark proliferating neuroprogenitor cells in the ventricular zone (right panels) and TUJ-1 (green) (left panels), which stains differentiated neurons of the mantle region, using a GFP RNAi vector as control.
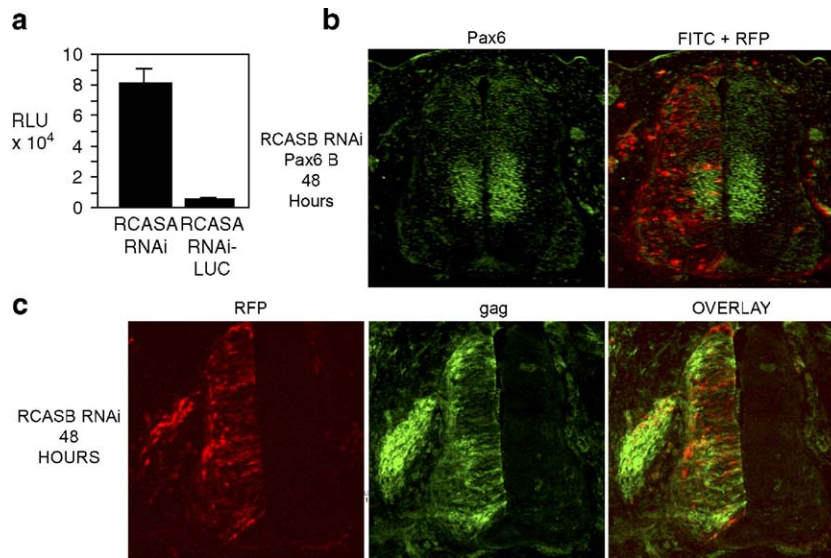
Fig. 6. RNA interference mediated by RCAS based vectors. (a) Luciferase silencing in DF-1 cells transduced with RCASARNAi vectors. Cells were transfected with the indicated vectors, cultured for 1 week, then transfected with 200 ng luciferase expression vector. (b) Silencing of Pax6 expression. Stage 12–14 embryos were electroporated with RCASBRNAi-Pax6B together with a separate RFP expression vector and examined 48 h later. Pax6 expression is shown in the left panel and RFP expression in the right panel. (c) Spread of retrovirus. Sections from the same embryos used in panel b were stained for Gag (central panel) and electroporated cells were marked by RFP expression (left panel). The RFP expression vector is more efficiently electroporated into cells than the RCAS vector hence some cells show RFP but no Gag expression.

RCAS-based vectors for RNAi

There are situations where stable long-term RNAi is desirable or vector delivery by electroporation is not feasible which led us to develop complementary retroviral vectors for RNAi. A replication competent avian splice (RCAS) vector (A envelope) was generated (RCASARNAi) which carries a GFP marker and an RCAS (B envelope) vector (RCASBRNAi) was also produced (Fig. 1b). To confirm that RCASARNAi worked, we transferred the MOEC for luciferase into RCASARNAi to generate RCASA-Luc. DF-1 cells were transfected (approximately 20% of cells) with RCASA-Luc and the cells cultured for a week. At this point, most cells were GFP positive, indicating that the virus containing the Luc MOEC still replicated efficiently in tissue culture. When the RCASA-Luc stably transduced cell population was transfected with a luciferase expression vector, luciferase expression was silenced by 90% (Fig. 6a). These data indicate that the MOEC still functions efficiently in the context of RCASARNAi and provides a means to rapidly generate a stable population of cells with a gene knockdown.

To confirm that the retroviral RNAi vectors worked in the embryo, we used RCASBRNAi, whose envelope infects the neural tube most efficiently (Homburger and Fekete, 1996), carrying hairpin B active against Pax6 (Fig. 6b). This vector led to silencing of Pax6 in the neural tube (Fig. 6b) and also showed evidence of viral spread (Fig. 6c), which indicates that the retroviral RNAi vectors can replicate in the chicken embryo and the activity of the MOEC is not compromised in the context of an RCAS vector. A range of different tissues in the chick embryo have previously been targeted using RCAS vectors which raises the possibility of carrying out loss of function RNAi studies in tissues which are not readily electroporated.

In conclusion, we have generated plasmid and retroviral vectors which allow low cost, efficient single or dual gene silencing in the chicken embryo using RNA interference. The simplicity of construction and use of these vectors coupled with their reliability should now make it possible to carry out large-scale genetic screens in the chicken using RNAi vector libraries.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.02.020.

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