Chapter 2. Development of RNA interference for genomic studies and therapeutic applications

2.1 Introduction.

RNA interference (RNAi) is an exciting new technology that involves the use of small interfering double stranded RNA (siRNA). RNAi is a protective innate response observed in a wide variety of organisms, and is a feature of all eukaryotes. At the cellular level, the detection of a double stranded RNA molecule triggers the specific degradation of the mRNA of an exact sequence to an expressed gene, leading to loss of phenotype. This technology has been developed as the method of choice to produce gene knockouts for high throughput functional genomic studies in a number of species, opening the way to utilize this for studies in the chicken. As well as genomic studies, RNAi has potential for therapeutic applications. For example, siRNA molecules against essential viral genes have been shown to reduce viral loads in cell culture. Gene specific therapeutics for cancer and auto-immune diseases are also being investigated and show promise in *in vitro* systems. A major obstacle to overcome in the development of RNAi therapeutics is efficient delivery of siRNA's into target cells *in vivo*. This has been dramatically advanced by CSIRO's discovery that small hairpin loop RNAs (shRNAs), transcribed from DNA elicit RNAi in vertebrate animal cells. This now enables DNA delivery systems such as plasmids, bacterial and viral vectors to be developed for targeted delivery of shRNAs to animals for therapeutic RNAi use.

A major focus of the project work undertaken in Strategy 2 has been the development of DNA delivery systems to express shRNAs in chicken cells. Foremost to this work was the identification and characterisation of the chicken polymerase III family of promoters for optimised expression of RNAi molecules. This was central to subsequent project work that applied RNAi technology to functional genomic studies linked with Strategy 1 and antiviral therapeutic development for chicken anaemia virus (CAV). Following a review of the RNAi work in March 2007, the major focus of project work was directed to the potential to use RNAi to control expression of genes with important production benefits, in particular genes that determine sex differentiation and genes negatively regulating muscle development. This work has been very successful and has lead to two patent applications and a collaborative project with researches at the University of Melbourne to confirm the role of the *DMRT1* gene in male sex determination in chickens. This work was published in *Nature*.

Outcomes

1. RNAi developed for routine *in vitro* and *in ovo* use as a gene knock-down tool to study the function of genes identified by genomic studies, including those responsible for sex-determination and muscle development (see Strategy 1).

    - 4 publications

        i. Wise T, Schafer D, Lambeth L, Tyack S, Bruce M, Moore R, Doran T (2007) Characterisation and application of a chicken U6 promoter for expression of small hairpin RNAs. *Animal Biotechnology* 18: 156-163.

        ii. Bannister SC, Wise TG, Cahill DM, Doran TJ (2007) Comparison of chicken 7SK and U6 RNA polymerase III promoters for short hairpin RNA expression. *BMC Biotechnology* 7: 79-88.

        iii. Hinton TM, Wise TG, Cottee PA, Doran TJ (2008) RNA loop structures are important for efficient processing of short hairpin RNAs for gene silencing. *RNAi and Gene Silencing* 4: 295-301.

        iv. Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH The conserved avian Z-linked gene DMRT1 is required for male sex determination in the chicken embryo. *Nature*. 461: 267-271.

2. RNAi developed as an anti-viral agent (e.g. CAV)

    - 1 publication

        i. Hinton TM, Doran TJ (2008) Inhibition of chicken anaemia virus replication using multiple short-hairpin RNA. *Antiviral Research* 80: 143-149.

        ii.

3. Generation of IP and potential commercial products identified via proof-of-concept.

    - 2 patent applications

        i. Commonwealth Scientific and Industrial Research Organisation, Australian Poultry CRC Pty Ltd
        International Patent Application PCT/AU2008/000835
        Entitled: "Modulating production traits in avians"

        ii. Commonwealth Scientific and Industrial Research Organisation, Australian Poultry CRC Pty Ltd
        United States of America Provisional Patent Application 61/138235
        Entitled: "Methods of modulating the sex of avians"

2.2 Results

2.2.1 Outcome - RNAi developed for routine in vitro and in ovo use as a gene knock-down tool to study the function of genes identified by genomic studies, including those responsible for sex-determination and muscle development (see Strategy 1).

2.2.1.1 Characterisation and application of a chicken U6 promoter for expression of small hairpin RNAs.

The naturally occurring cellular process of RNA interference (RNAi) is used to induce sequence specific gene knockdown to control gene expression. The conserved RNAi pathway involves the processing of double stranded RNA (dsRNA) duplexes into 21-23 nucleotide (nt) molecules known as small interfering RNAs (siRNA) to initiate gene suppression (Fire 1999; Hannon 2002). This intrinsic cellular process has been exploited for the extensive analysis of gene function in plants, invertebrates, and more recently mammalian cells. Since the initial discovery of RNAi in animals (Fire *et al.* 1998), the use of homologous long dsRNA has been effectively used in lower eukaryotes as a method to study gene function. However, in higher order eukaryotes such as vertebrates, the cellular processing of long dsRNA can induce an interferon (IFN) mediated antiviral defence mechanism that ultimately leads to non-specific translational shutdown and apoptosis. This non-specific cellular activity can be circumvented by the direct transfection of *in vitro* synthesised siRNAs of up to 30 nucleotides (nt) in length into mammalian cells. These short molecules do not activate the IFN response, but can induce reliable and efficient transient knockdown of target genes. Since this discovery, the development of DNA-based vectors for expression of short hairpin RNA (shRNA) molecules that are processed within the cell to produce active siRNA molecules has progressed rapidly.

DNA-based expression of shRNA offers some advantages over *in vitro* synthesised siRNA. Vector construction is less expensive compared to the chemical synthesis of siRNA, selection of transfected cells is possible via antibiotic markers and the option of inducible shRNA transcription is also available. Vectors for shRNA expression have been engineered using both plasmid and viral-based systems that often utilize promoters from a small subclass of pol III promoters, known as type 3, to drive the expression of shRNA. Promoters of this subclass are often preferred because they naturally direct the synthesis of small, highly abundant non-coding RNA transcripts, do not contain intragenic control regions and have defined termination sequences consisting of 4-5 thymidines (Ts).

The U6 small nuclear RNA (snRNA) promoters are the best studied type III pol III promoters commonly used in RNAi expression vectors. They contain promoter element sequence motifs located within enhancer and core regions. The core region comprises a proximal sequence element (PSE) and a TATA-like element, and the enhancer region, also known as the distal sequence element (DSE), consists of an octamer motif (OCT) and an SPH element. The human U6 gene encodes a 107 nt snRNA and from the estimated 200 copies in the human genome nine full-length loci have been identified. These nine loci are dispersed throughout the genome and five were found to have associated promoter regions, each displaying differential activities. Recently, Kudo and Sutou (2005) identified four full-length U6 genes in the chicken genome, with recognisable pol III promoter element sequences upstream. We too have independently identified the same chicken U6 promoters and report here a comparison of their use in shRNA expression vectors for RNAi.

Methods

Characterisation of chicken U6 promoters

Chicken U6 snRNA sequences were identified by comparison of the full-length human U6 snRNA sequence to the chicken whole genome sequence using Megablast (http://www.ncbi.nlm.nih.gov/genome/seq/GgaBlast.html) with default parameters. Identified sequences for each were checked for repeat elements using the pre-masked chicken genome (http://www.repeatmasker.org/). Based on the presence of pol III promoter element sequence motifs, four predicted U6 promoter sequences were identified (cU6-1, cU6-2, cU6-3 and cU6-4). Chicken genomic DNA was isolated from whole blood using a Wizard Genomic DNA purification kit (Promega) for use as template in PCR. Each promoter was amplified by PCR using primers TD135 and TD139 (cU6-1), TD152 / TD174 and TD139 (cU6-2), TD176 and TD72 (cU6-3) and TD175 and TD139 for cU6-4 (sequences shown in Table 2.1). All oligonucleotides were obtained from GeneWorks Pty. Ltd. (Adelaide, Australia). PCR products were obtained for cU6-1, cU6-3 and cU6-4 and each were ligated into pGEM-T Easy and sequenced.

Expression vector construction and shRNA target sites

The cloned chicken U6 promoters were used as templates to construct EGFP shRNA expression plasmids using a one-step PCR approach. PCR for the construction of four plasmids used the primer pairs TD135 and TD148 (cU6-1), TD135 and TD143 (cU6-1v), TD176 and TD196 (cU6-3) or TD175 and TD195 (cU6-4). The reverse primers in each PCR were designed to comprise the last 20 nt of each promoter sequence, EGFP shRNA sense, loop, and EGFP shRNA antisense sequence (Table 2.1) and were HPLC purifed to ensure full length. The EGFP siRNA sequence was from Kim & Rossi (2003).

All three PCR products for shRNA expression constructs were ligated into pGEM-T Easy (Promega) and sequenced. A chU6-1v irrelevant control plasmid was also constructed. Forward primer TD135 was paired with reverse primer TD149 comprising the last 20 nt of the chU6-1 promoter and all other irrelevant shRNA components. The PCR product was ligated into pGEM-T Easy and sequenced.

A mouse U6 EGFP shRNA construct was produced using one-step PCR with p*Silencer* 1.0-U6 siRNA Expression Vector (Ambion) as template. Universal primer M13 Forward was paired with reverse primer TD134, comprising the last 20 nt of the mouse promoter, and all other EGFP shRNA components. The PCR product was ligated into pGEM-T Easy and sequenced.

Cell culture and transfection

DF-1 (ATCC CRL-12203, chicken fibroblast) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5g/l glucose, 1.5g/l sodium bicarbonate, 10% foetal calf serum (FCS), 2mM L-glutamine supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Vero (ATCC CCL-81, African green monkey kidney) cells were cultured in Eagle's minimal essential medium (EMEM) medium containing 1.5g/l sodium bicarbonate,10% fetal calf serum (FCS), 2 mM glutamine, 10 mM HEPES, supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured in humidified atmosphere containing 5% CO_2 at 37°C and were grown to approximately 90% confluence on either 24-well plates (Nunc) for Flow Cytometry or on 8-well chamber slides (Lab-Tek) for fluorescence microscopy. Cotransfection with 500 ng of plasmid DNA (shRNA plasmids and/or pEGFP-N1 (Clonetech), was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions and was completed in triplicate for each transfection.

EGFP knockdown assays

EGFP expression was monitored at 48-hours post-transfection using fluorescence microscopy (Leica DMLB). Cells to be analysed by flow cytometry were trypsinized, washed in PBSA, resuspended in 200 μL of 0.01% sodium azide and 2% FCS in PBSA and analysed using a FACScalibur (Becton Dickinson) flow cytometer. Data analysis was performed using CELLQuest software (Becton Dickinson).

shRNA detection

Detection of EGFP shRNAs (shEGFP) was carried out using an RNAse protection assay on extracts enriched for small RNAs isolated from transfected Vero cells using the *mir*Vana microRNA (miRNA) Isolation Kit (Ambion). An RNA oligonucleotide complementary to the EGFP shRNA sequence (LL91) was end labelled with [gamma-^{32}P] ATP using the *mir*Vana Probe & Marker Kit (Ambion) and hybridised to the enriched RNAs using the *mir*Vana miRNA Detection Kit (Ambion). The RNA fragments were then separated by electrophoresis on a 15% denaturing polyacrylamide/ 8 M Urea gel and detected by autoradiography.

Results

Characterisation and isolation of chicken U6 promoters

We used the 107 nt human U6 snRNA sequence to search the chicken genome sequence for potential U6 promoters. The search identified three sequences containing a 107 nt region with 100% identity to human U6 snRNA and one sequence with 99% identity. Analysis of upstream regions of these four sequences revealed promoter elements associated with other pol III promoters. These were the same four sequences recently reported by Kudo and Sutou (2005), named cU6-1, cU6-2, cU6-3 and cU6-4. We independently designed PCR primers (Table 2.1) to amplify the four predicted promoters from genomic DNA purified from chicken blood. We could only obtain PCR products for the predicted promoters of cU6-1, cU6-3 and cU6-4 and these were cloned into pGEM-T Easy and sequenced (Fig. 2.1). Similar to Kudo and Sutou (2005) our sequences were confirmed by analysing several independent clones, which had been amplified from genomic DNA isolated from two different chickens. Our cloned promoter sequences had some minor differences outside of the pol III promoter element sequences compared to the database and for cU6-1 we cloned a variable PCR product from one of the independent chicken genomic DNA samples. This variant sequence was also confirmed by analysing several independent clones and the predicted promoter was named cU6-1v. One of the sequence variances of potential importance in cU6-1v, is a single nt substitution in the conserved PSE sequence and we therefore decided to use this promoter along with cU6-1, cU6-3 and cU6-4 in the construction of plasmids to compare expression of shRNAs.

shRNA expression vector construction and validation

Each of the predicted promoter sequences cU6-1, cU6-1v, cU6-3, cU6-4 and the mouse U6 were used as templates to construct shRNA expression vectors targeting EGFP, via a one-step PCR reaction (Fig. 2.2). Full-length amplified expression cassette products were ligated into pGEM-T Easy and then sequenced to confirm the promoters were correct. The final shRNA expression plasmids were named pcU6-1-shEGFP, pcU6-1v-shEGFP, pcU6-3-shEGFP, pcU6-4-shEGFP and pmU6-shEGFP respectively. An irrelevant control shRNA vector (pcU6-1v-irrshRNA) was also produced using the cU6-1v sequence as template. Each plasmid was constructed so that the start of each shRNA sequence was at the +1 position of the native U6 snRNA transcripts. A *Xho*I restriction enzyme site was engineered downstream of the termination signal to allow screening for full-length shRNA products inserted into pGEM-T Easy which lacks a *Xho*I site. All final shRNA expression vectors consisted of either one of the full length chicken U6 promoters or the mouse promoter, a shRNA sense sequence, a loop sequence, a shRNA antisense sequence, a termination sequence and a *Xho*I site.

Validation of each expression construct encoding shRNA sequences targeting EGFP was first conducted in Vero cells (Fig. 2.3). This cell line lacks the interferon α, β and ω genes indicating that the reduction in EGFP expression was attributable to RNAi and not a non-specific inhibition of protein translation characteristic of the IFN response triggered by exogenous dsRNA. To confirm that any observed reduction in EGFP expression was a direct result of RNAi induced by the expression of specific shRNA sequences, the transcription of these molecules was analysed in transfected Vero cells. A radiolabelled RNA probe complementary in sequence to the EGFP shRNA sequence was used in an RNAse protection assay to visualise the molecules. The mouse miRNA miR-16 probe used as a loading control for each condition produced a strong signal at the expected size for all samples. The EGFP shRNA was detected only in those samples that were transfected with the vectors containing the EGFP shRNA target sequence (Fig. 2.3C). All shRNA constructs were then tested in DF-1 cells for comparison of the chicken promoters in a homologous cell line. For each transfection condition, knockdown of EGFP was visualised by fluorescence microscopy (Fig. 2.3A) and flow cytometry was used to determine the mean fluorescence intensity (Fig. 2.3B). Results in both cell lines showed that the knockdown induced by pmU6-shEGFP was greatest compared to the four chicken plasmids. All four chicken plasmids gave a similar degree of knockdown in DF-1 cells however there was some variability in Vero cells. Interestingly, pcU6-1v-shRNA produced a greater knockdown in Vero's compared to pcU6-1-shRNA, suggesting that the variant cU6-1 promoter is stronger in the monkey cell line. Of the chicken plasmids, pcU6-3-shRNA produced the greatest degree of knockdown in the Vero cell line, and this appeared to correlate with the intensity of the shEGFP signal detected in the RNAse protection assay (Fig. 2.3C).

Discussion

The use of type 3 pol III promoters for shRNA expression in vertebrate systems has been an understandable choice because of their native function in small RNA expression. Until recently this has been limited to pol III promoters derived from human and mouse, and now Kudo & Sutou (2005) have reported the identification of chicken U6 promoters for shRNA expression. We too have independently characterised four chicken U6 promoters and used them to construct shRNA expression plasmids so that we could compare them for RNAi in a chicken cell line. Our aim was to identify which U6 promoter was best for expressing shRNA in chicken cells for future functional genomic projects. RNAi has become a standard approach to knock down gene expression for the functional study of both human and mouse genes and RNAi will become increasingly important to study the role of genes identified in genomic studies that stem from the recent completion of the chicken genome. These studies will include the continued development and application of shRNA expression vectors in chick embryo's, where the chicken is used as an important model for large-scale analysis of vertebrate development.

We cloned and sequenced three of the four predicted U6 promoters (cU6-1, cU6-3 and cU6-4) that were identified in upstream regions of sequences matching the chicken U6 snRNA gene. All contained pol III promoter element sequence motifs including the TATA-box, PSE and OCT domain, located within the enhancer and core regions. The location and spacing of these elements is similar for all known type 3 pol III promoters and their requirement for pol III activity is well documented. Within the core region of each chicken promoter sequence were the PSE element and TATA-like element. The spacing of both elements was very conserved which is consistent with previous findings with the human U6 promoters (Fig. 2.1). The importance of this spacing has been highlighted by the finding that a deletion or insertion of >2-3 bp results in drastically reduced transcription. Within the enhancer region we could define the typical OCT motif but not the SPH element. Kudo and Sutou (2005) reported that they too were unable to identify a typical SPH element for the chicken promoters, but also they could not identify the PSE element that we have defined in the predicted position upstream of the TATA-box.

The mouse U6 promoter used in the p*Silencer* 1.0-U6 siRNA Expression Vector also features these element sequences and as it has been shown to be stronger than the human U6-1 promoter *in vitro*, we used it as a benchmark for promoter comparison for shRNA expression. Similar to the report of Kudo and Sutou (2005), the chicken shRNA plasmids gave a similar degree of knockdown in a chicken cell line. However, we observed a high degree of variability in Vero cells, particularly for the plasmids containing cU6-1 and the variant cU6-1v. pcU6-1v-shRNA induced a greater knockdown compared to pcU6-1-shRNA, suggesting that the variant cU6-1 promoter is stronger in the monkey cell line. One

sequence difference of potential importance in cU6-1v, is a single nt substitution within the conserved PSE sequence. This is the only sequence difference that occurs within any of the pol III promoter sequence elements for cU6-1. There is no spacing difference between the TATA-box and PSE element, so it is therefore tempting to suggest that this change may be the reason for enhanced activity in Vero cells. Results in both DF-1 and Vero cells showed that the knockdown induced by pmU6-shEGFP was greatest compared to the four chicken shRNA plasmids. We predicted that a native chicken U6 promoter may have been more effective for shRNA expression in the DF-1 chicken cell line than the mouse U6 promoter. Kudo and Sutou (2005) also expected that their chicken U6 shRNA vectors would induce greater silencing of a target gene in chicken cells than another commercially available vector with a human U6 promoter. In their experiment this human U6 vector induced a greater silencing of the target gene and they suggest the difference might reflect the additional junction sequences between the promoters and the synthetic DNA encoding shRNAs. All of our plasmids were constructed so that the start of the shRNA sequence was at the +1 position of the native U6 snRNA transcripts for each promoter. Because the chicken promoters were not stronger than the benchmark mouse U6 promoter, we suggest that the promoter sequence and structure is a much more important factor in determining efficiency *in vitro* rather than origin alone. Therefore we would currently recommend the use of the mouse U6 promoter for shRNA expression in chicken cells for gene silencing experiments.

2.2.1.2 Comparison of chicken 7SK and U6 RNA polymerase III promoters for short hairpin RNA expression.

RNAi is a sequence-specific gene silencing mechanism initiated by 21-25 nucleotide (nt) duplexes known as small-interfering RNAs (siRNAs). siRNAs are processed from long double-stranded RNA (dsRNA) molecules by the ribonuclease III enzyme Dicer and are unwound and loaded as single-stranded RNAs into the RNA induced silencing complex (RISC). RISC silences gene expression via cleavage of messenger RNA (mRNA) transcripts complementary to the loaded siRNA sequence. RNAi-mediated silencing of specific genes in vertebrates can be harnessed by transfection of siRNA duplexes or DNA vectors which express siRNAs as short-hairpin RNAs (shRNAs). shRNAs are transcribed from these vectors as 19-29 nt inverted repeat sequences, separated by a 4-10 nt loop sequence and fold spontaneously to form hairpin structures, which are cleaved by Dicer into active siRNAs.

RNA polymerase III (pol III) type 3 promoters are most commonly used to express shRNAs, as these promoters transcribe endogenous small-nuclear RNAs (snRNAs) such as U6 and 7SK. Termination of transcription by Pol III also occurs at defined tracts of 4-5 thymidines (T_{4-5}), which can be inserted

downstream of shRNA coding sequences to ensure direct termination. Unlike type 1 and 2 promoters, pol III type 3 promoters are located entirely upstream of transcription start sites (+1) and feature characteristic promoter elements including; a TATA box beginning at around bp -20 (relative to +1), a Proximal Sequence Element (PSE) centred around bp -50 and a Distal Sequence Element (DSE) beginning around bp -240. In the human U6 and 7SK (h7SK) promoters, the DSE is comprised of at least one Octamer (OCT) motif and an *Sph*I Post-octamer Homology (SPH) domain. The DSE of the human 7SK (h7SK) also, contains an additional CACCC box enhancer located between the OCT and SPH elements. U6 promoters are the most common type used in vector-based shRNA expression systems, however, more recent approaches have preferred the use of 7SK promoters.

Given the recent completion of the chicken genome project (Hillier et al., 2004), the adaptation and use of shRNA expression systems for RNAi in the chicken will be important for ensuing functional genomics studies. However, to date, most shRNA expression systems used in chickens feature mammalian pol III promoters. Although several chicken U6 (cU6) promoters have now been characterised and shown to drive efficient shRNA-mediated RNAi activity in chickens (Kudo and Sutou, 2005; Wise et al 2007), recent work has highlighted that 7SK promoters in human (h7SK) and bovine (b7SK) can stimulate more efficient shRNA expression and RNAi activity than corresponding U6 promoters. Given that expression of the 7SK snRNA appears to be conserved across non-mammalian and mammalian vertebrates we sought to investigate whether a chicken 7SK promoter (ch7SK) would also confer greater levels of shRNA-mediated RNAi activity than the recently-characterised cU6 promoters.

Methods

Isolation of the ch7SK promoter from chicken genomic DNA

The ch7SK promoter sequence was amplified from chicken genomic DNA extracted from chicken embryo fibroblast (DF-1) cells (ATCC, CRL-12203) (Wizard® Genomic DNA purification kit, Promega), using the primers: forward (TD245): 5'- GTCCAGCCATCCACCTCCCACCAATACTTC -3' and reverse (TD237): 5'- AAAGCTACGAGCTGCCCCAA -3'. Gradient PCR was conducted using; 9.5 ng of genomic DNA, 100 ng of each primer (TD245 & TD237), 2 mM $MgCl_2$ (Qiagen), 250 µM dNTPs (Promega), 1 X PCR buffer (Qiagen) and 1 unit of *Thermus acquaticus* (*Taq*) polymerase (Promega), in a Mastercycler EP Gradient S thermocycler (Eppendorf AG). Cycle conditions were: 94°C – 5 minutes, 35 cycles of; 94°C – 1 minute, 69.4°C – 45 sec and 72°C – 1 minute, with a 5 minute final extension at 72°C.

A single PCR product of approximately 780 bp was amplified, purified using the Wizard SV PCR and Gel cleanup kit (Promega) and cloned using the pGEM®-T Easy vector cloning system (Promega). Ligations were transformed into TOP10F` *Escherichia coli* (*E. coli*) cells (Invitrogen) and plasmid DNA isolated from bacterial clones (QIAprep® Spin Miniprep Kit, Qiagen) was sequenced (Micromon DNA sequencing facility, Monash University). Sequences were compared to public sequence databases using the mega-Basic Local Alignment Search Tool (mega-BLAST) [32]. The sequences of three positive ch7SK promoter clones; pch7SK-1 (783bp), pch7SK-2 (782bp) and pch7SK-3 (782bp) were deposited into Genbank under the accession numbers, EF488955, EF488956 and EF488957 respectively.

Construction of ch7SK-shRNA expression vectors

The pch7SK-shEGFP and pch7SK-shIrr expression vectors were constructed using the one-step PCR approach (Figure 2.2 and 2.7a). The primers used were; forward primer TD269 (5'-GAGGCTCAGTGTCACGCAGA-3') and reverse primer TD267 (5'-CTCGAGTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATGAACTTCAGGGTCAGCAAAGCTACGAGCTGCCCCAA-3') (shEGFP) and TD268 (5'-CTCGAGTTCCAAAAAAGGATCTTATTTCTTCGGAGTCTCTTGAACTCCGAAGAAATAAGATCCAAAGCTACGAGCTGCCCCAA-3') (hirr). Pch7SK-shEGFP was amplified using pch7SK-3 template and pch7SK-shIrr was amplified using pch7SK-1 template.

The pch7SK-MCS-shEGFP vector was constructed from the pch7SK-MCS base-vector by ligation of complimentary annealed oligonucleotides (oligos) LL29 and LL30 as shown in Figure 2.7b. The pch7SK-MCS base vector was constructed from pUC57 by ligating a 315 bp synthetic copy of the ch7SK promoter sequence between the EcoRI and HindIII sites (Celtek Genes). The ch7SK promoter sequence was altered between bp -5 to +11 to include a 3' multi-cloning site (MCS) comprising overlapping KpnI, XhoI and EcoRI sites (Figure 2.7b). All ligations were incubated at 4°C for 48 hours and transformed into TOP10F` E. coli.

Sequence management and bioinformatics

Chicken genome sequence information was accessed through the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and viewed using the NCBI Map Viewer, *Gallus gallus* (chicken). Sequence alignments were performed using ClustalW and Clone Manager 7 software (SciEd Central).

Cell culture and transfection

Chicken DF-1 cells were maintained in 5% CO_2 at 37°C in growth media and harvested using 0.25% (w/v) trypsin-ethylenediaminetetraacetic acid (EDTA). Transfection of plasmid DNA for EGFP silencing assays was conducted in DF-1 cells grown to 80-90% confluence, in 8-well chamber slides (Nunc) for fluorescence microscopy or 24 well culture plates (Nunc) for flow cytometry. Cells were transfected with 500 ng or 1µg of each plasmid, per well, for chamber slides or 24-well plates, respectively, using Lipofectamine™2000 transfection reagent (Invitrogen). For RNA extractions, DF-1 cells were grown in 25 cm^2 culture flasks (Corning) and transfected using 12.5 µg of plasmid and 25 µl of Lipofectamine™2000.

Detection of shEGFP expression by Rnase protection assay

RNA samples enriched for small RNAs (mirVana miRNA isolation kit, Ambion) were purified from DF-1 cells 48 hours post-transfection of shEGFP expression plasmids. An RNAse protection assay (RPA) was conducted to detect expression of shEGFP using the RNA probe LL91. Gel images were transferred to Medical X-ray film over 5 days at -80°C and developed using an FPM-100A X-ray processor (FUJIFILM).

EGFP knockdown assays

EGFP expression was analysed at 60 hours post-transfection. Fluorescence microscopy was performed on duplicate co-transfections using a Leica DM LB Fluorescence Microscope (Leica Microsystems, Germany). Images were captured at 50X magnification using a Leica DC300F colour digital camera (Leica Microsystems, Germany) using Photoshop 7.0 imaging software (Adobe®). For flow cytometry, the EGFP fluorescence intensity was quantified as a mean fluorescence intensity (MFI) value for each co-transfection condition sampled in triplicate. Cells were harvested using 0.25% trypsin-EDTA, pelleted at 2000 rpm for 5 minutes, washed sequentially in cold phosphate buffered saline-A (PBSA) (Oxoid) and FACS-solution (PBSA + 1% FCS) and re-suspended in FACS-solution for sampling. Sampling and data acquisition was conducted using a FACScalibur (Becton Dickinson) fluorescence activated cell sorter and CELLQuest software (Becton Dickinson). The reduction in EGFP MFI for each co-transfection was calculated by normalising the average MFI from triplicate sampling, as a percentage of the MFI of the negative control hirr/pEGFP-N1 co-transfected cells (100% ± 4.53% (SEM)) (Figure 2.6b).

Statistics

Normalised MFI data from three independent co-transfection experiments was analysed statistically by One-way Analysis Of Variance (ANOVA) and Tukey's multiple comparisons tests (Prism, GraphPad Software). Significant difference in EGFP knockdown was accepted where $P<0.05$.

Results

Identifying the chicken 7SK promoter

RNA polymerase (pol III) type 3 promoters are characterised by the presence of gene-external promoter elements 5' of the transcription start site. To identify the ch7SK promoter we used a bioinformatics approach and scanned the chicken genome for sequences with significant (80%) homology to the chicken 7SK snRNA gene sequence (GenBank Accession Number AJ890101). We then analysed the 5' flanking regions of these sequences for the presence of pol III promoter elements. This analysis highlighted several putative 7SK pseudogenes in addition to the full-length 7SK snRNA sequence. However, putative pol III promoter elements were only present within the 5' flanking region of the full-length, ch7SK snRNA sequence, located on chicken chromosome 3 (Gga3, Contig NW_060336.1). Therefore, we reasoned that this region probably encoded the chicken homologue of the 7SK promoter.

Using PCR we amplified a 783bp region containing the putative ch7SK promoter sequence, which was cloned into pGEM®-T Easy. Sequencing of the cloned insert identified three clones; pch7SK-1 (783 bp), pch7SK-2 (782 bp) and pch7SK-3 (782 bp) with 99% homology to the first 782-783bp of the ch7SK snRNA 5' flanking region, as determined by alignment against the chicken genome. A further alignment of the last 300 bp (5' to 3') of each of these clone sequences against the h7SK and b7SK promoter sequences (Figure 2.4), identified typical pol III promoter elements in the cloned sequences including; a TATA box at bp -31 to -25, a PSE at bp -67 to -46, an OCT-1 motif at bp -222 to -215, two putative OCT-2 motifs OCT-2a at bp -138 to -132 and OCT-2b at bp -97 to -90 (not shown) and an SPH domain at bp -192 to -210. The PSE, OCT-1 and SPH elements also displayed considerable homology to published consensus sequences (Figure 2.4). The presence of these elements within the cloned 5` flanking region of the ch7SK snRNA gene sequence, suggested that this region probably encoded a functional ch7SK promoter.

The ch7SK promoter expresses shRNAs

In order to validate its function, the putative ch7SK promoter sequence was used to construct the shRNA expression vectors, pch7SK-shEGFP and pch7SK-MCS-shEGFP, designed to transcribe shRNAs targeting EGFP (shEGFP) (Figure 2.7). A third vector, pch7SK-shIrr, designed to transcribe an irrelevant shRNA (hirr) targeting an influenza virus nucleocapsid protein (NP) from the ch7SK

promoter, was also constructed as a negative control (Figure 2.7a). The function of the isolated ch7SK promoter sequence was verified by detection of shEGFP expression in DF-1 cells transfected with the pch7SK-shEGFP or pch7SK-MCS-shEGFP constructs. RNA was extracted at 48 hours post-transfection and shEGFP expression was detected using an Rnase protection assay (RPA) (Figure 2.5). As a positive control for shEGFP detection, DF-1 cells were also transfected with vectors expressing identical shEGFP sequences from pre-validated mouse U6 (pmU6-shEGFP), chicken U6-1 (pcU6-1-shEGFP), chicken U6-4 (pcU6-4-shEGFP) and chicken U6-3 (pcU6-3-shEGFP) pol III promoters.

A 19 nt band was detected in RNA samples from cells transfected with both the pch7SK-shEGFP and pch7SK-MCS-shEGFP constructs (Figure 2.5). This band corresponded with the expected size of protected shEGFP sequence as well as specific bands detected in the positive control mouse U6 (mU6) and cU6-shEGFP-transfected positive control cells (Figure 2.5). No shEGFP expression was detected in RNA samples from the pch7SK-shIrr negative control, or non-transfected cells (cells only) (Figure 2.5). These results demonstrated the isolated ch7SK promoter sequence was transcriptionally active.

The ch7SK promoter directs shRNA-mediated RNAi knockdown

To verify that the shEGFP expressed by the ch7SK promoter could direct RNAi-mediated knockdown of an EGFP reporter gene, we conducted EGFP knockdown assays by co-transfecting chicken DF-1 cells with the pch7SK-shEGFP, pch7SK-MCS-shEGFP and positive control, pmU6-shEGFP and pEZ-b7SK-shEGFP constructs, with an EGFP expression vector (pEGFP-N1) (Figure 2.6). Given that co-transfection of reporter and shRNA expression plasmids is considered to be 100% efficient for validation of specific RNAi activity, we considered any reduction in EGFP fluorescence intensity to reflect RNAi-mediated EGFP knockdown. EGFP knockdown was assessed for each co-transfection condition in duplicate using fluorescence microscopy (Figure 2.6a) and quantified using flow cytometry by sampling the mean fluorescence intensity (MFI) from triplicate co-transfections for each condition (Figure 2.6b).

In DF-1 cells co-transfected with pEGFP-N1 and either the pch7SK-shEGFP or pch7SK-MCS-shEGFP constructs, the EGFP MFI was significantly reduced to 45.19% (± 3.37%) and 47.28% (± 3.14%) respectively ($P<0.001$) (Figure 2.6b). These reductions were not found to differ significantly from the EGFP % MFI measured in the positive control pmU6-shEGFP (42.8% ± 4.67%) and pEZ-b7SK-shEGFP (45.27% ± 3.73%) co-transfected cells ($P>0.05$). Given both the mU6 and b7SK promoters are known to express functional shEGFP molecules that direct specific EGFP knockdown in DF-1 cells, this result indicated that the shEGFP molecules expressed by the ch7SK promoter could direct knockdown of EGFP by over 50% in DF-1 cells.

Comparison of ch7SK and cU6 promoter induced EGFP knockdown

We further compared the efficiency of RNAi knockdown mediated by the ch7SK promoter, to cU6 promoters; cU6-1, cU6-3 and cU6-4, by comparing the reduction in EGFP MFI induced between the pcU6-1-shEGFP, pcU6-3-shEGFP and pcU6-4-shEGFP vectors and the two ch7SK-shEGFP constructs; pch7SK-shEGFP and pch7SK-MCS-shEGFP (Figure 2.6). Fluorescence microscopy results indicated that the EGFP knockdown induced by both of the ch7SK-shEGFP constructs was comparable to that induced by pcU6-4-shEGFP and pcU6-3-shEGFP, but greater than that conferred by pcU6-1-shEGFP (Figure 2.6a). However, statistical analyses of MFI data indicated no significant difference in the reduction of EGFP MFI between the pcU6-1-shEGFP (52.93% ± 6.25%), pch7SK-shEGFP (45.19% ± 3.37%), pch7SK-MCS-shEGFP (47.28% ± 3.15%) or pcU6-3-shEGFP (39.78% ± 3.93%) transfection conditions ($P>0.05$). The pcU6-4-shEGFP co-transfected cells showed the greatest reduction in EGFP MFI to 29.05% (± 3.26%), which was significantly lower than for the pch7SK-MCS-shEGFP and pcU6-1-shEGFP co-transfected cells ($P\leq0.05$), but not significantly different to the MFI of either the pch7SK-shEGFP or pcU6-3-shEGFP-co-transfected cells ($P>0.05$). Taken together, these results indicated that neither of the ch7SK-shEGFP constructs induced more efficient RNAi-knockdown of EGFP than existing cU6-shEGFP constructs in DF-1 cells.

Discussion

The chicken is an important livestock animal and a key model for studies of vertebrate development and gene function. Thus the development of RNAi technologies adapted for use in chicken systems will be important for further annotation of the chicken genome. Although several recently characterised chicken U6 (cU6) promoters have been used to develop effective chicken-specific shRNA expression systems, 7SK promoters have been shown to direct more efficient RNAi activity than U6 promoters in mammals (Lambeth et al., 2006). Therefore, we wanted to establish whether an shRNA expression system based on the ch7SK promoter could induce more efficient RNAi activity than those based on existing cU6 promoters.

Although several 7SK pseudogenes exist in the chicken genome we could only identify a single ch7SK promoter sequence upstream of the full-length chicken 7SK snRNA sequence on chromosome 3. Single functional 7SK promoters are known to exist in the human, bovine and mouse genomes, so our findings are consistent with the presence of only a single 7SK promoter in the chicken genome. The ch7SK promoter was also found to contain typical pol III promoter elements; TATA, PSE, OCT and SPH which show positional and sequence similarities to those of the h7SK and b7SK promoters. Further, we noted that the chicken 7SK locus was flanked by homologues of the glutathione S transferase-A3 and intestinal cell kinase (MAK-related kinase) genes which are also located 5' and 3'

respectively of each of the mammalian 7SK loci. This level of synteny in the arrangement of the 7SK loci between chicken and other mammalian species, provides good evidence that the ch7SK promoter characterised in the present study is the only functional 7SK promoter in the chicken genome.

Our results clearly demonstrated that the ch7SK promoter was able to express functional shRNA molecules capable of mediating greater than 50% RNAi-knockdown of the target EGFP reporter gene. However, we found no evidence that the ch7SK promoter could direct more efficient shRNA-mediated RNAi knockdown compared to the cU6-1, cU6-3 and cU6-4 promoters, based on a lack of significant difference in the level of EGFP MFI between cells co-transfected with pch7SK-shEGFP and any of the cU6-shEGFP constructs, or between cells co-transfected with pch7SK-MCS-shEGFP and pcU6-1-shEGFP and pcU6-3-shEGFP. Although we cannot rule out that the ch7SK promoter may actually be less efficient than the cU6-4 promoter, given that our MFI data indicated the pcU6-4-shEGFP construct could direct a more significant reduction in EGFP MFI than the pch7SK-MCS-shEGFP construct, these findings indicated that in general, the efficiency of the ch7SK promoter was not greater than, but comparable to that of cU6 promoters.

Interestingly, our results contrast findings published by Lambeth et.al., (2006), who independently demonstrated that the b7SK and h7SK promoters confer more efficient shRNA expression and RNAi activity than bovine and human U6 promoters, respectively. Despite close alignment of the ch7SK, h7SK and b7SK promoters, we noted some distinct differences within the DSE or enhancer region of the ch7SK promoter, which affect the structural organisation of the ch7SK promoter in relation to its mammalian homologues. Given the structure and sequence of promoter elements within the DSE can influence maximal transcription efficiency in U6 and 7SK promoters, the variable structure of the ch7SK DSE may have an inherent impact upon its efficiency relative to U6 promoters.

Unlike the b7SK and h7SK promoters, the DSE of the ch7SK promoter does not contain a CACCC box (Figure 2.4), which appears to be a distinct feature of 7SK promoters and is reported to serve an important role in enhancing the transcriptional activity of the h7SK promoter. Interestingly, U6 promoters do not contain CACCC boxes, so it is possible that the absence of a CACCC box in the ch7SK promoter may affect its overall efficiency by reducing enhancer activity in the ch7SK DSE to a level more similar to that seen in U6 promoters. This would explain why we observed comparable levels of EGFP knockdown induced between the ch7SK and cU6 promoters. Moreover, the absence of the CACCC box from the enhancer may further indicate that the enhancer mechanism in the ch7SK promoter may be more similar to that of U6 promoters than other mammalian 7SK promoters.

A second feature of the ch7SK enhancer, distinct from mammalian 7SK promoters is the presence of a C/A substitution at position 1 (bp -222) of the ch7SK OCT-1 motif (Figure 2.4). Previous work has

shown that mutation of the OCT-1 motif in the h7SK promoter has the strongest impact on transcriptional efficiency, so it is possible that this substitution may affect the activity of the ch7SK enhancer. However, an OCT-1 sequence identical to that of the ch7SK promoter is present in the enhancer of the RNA polymerase II (pol II) promoter of the chicken U4B (cU4B) snRNA (Figure 2.8). This cU4B OCT-1 motif shows full affinity for the Octamer transcription factor (Oct-1), so it is unlikely that the ch7SK OCT-1 motif would affect promoter efficiency through a reduced ability to bind Oct-1. However, it is also known for the cU4B promoter, that optimal enhancer activity is dependent upon the presence of a downstream SPH domain adjacent to OCT-1. Interestingly, the position of the ch7SK SPH domain 4bp downstream of OCT-1 corresponds closely to that of the cU4B promoter and shows striking homology (84%) to the cU4B SPH sequence (Figure 2.8). Given this level of structural identity, it is pertinent to suggest that the enhancer mechanism of the ch7SK promoter may be analogous to that of the cU4B promoter, requiring adjacent OCT-1 and SPH domains.

Co-dependence of OCT and SPH motifs in pol III enhancer mechanisms is common to other non-mammalian vertebrate pol III promoters including the Xenopus laevis $tRNA^{sec}$ promoter. Similarly, human U6 promoters also appear to rely upon the presence of both OCT and SPH elements for efficient enhancer activity. This type of enhancer mechanism contrasts what is known about the function of the h7SK enhancer, where optimal transcription efficiency is not dependent upon the presence of an SPH domain. Based on these differences, we propose that the structure and function of the ch7SK enhancer may be less divergent from pol II and pol III promoters such as U4B and U6, than from other mammalian 7SK promoters.

In this study we have identified and isolated a functional chicken homologue of the 7SK snRNA promoter and demonstrated its ability to confer efficient shRNA expression and RNAi-knockdown of a reporter gene in a chicken cell line. We further found that the efficiency of the ch7SK promoter was similar to that of existing cU6 promoters, which contrasts previous comparisons of mammalian U6 and 7SK promoters. The ch7SK promoter is the first non-mammalian vertebrate 7SK promoter to be characterised, so this finding may reflect inherent differences in the divergence of pol III promoter activities between mammalian and non-mammalian vertebrates. This aside, our results clearly indicate that the ch7SK promoter is an efficient alternative to U6-based shRNA expression systems for inducing efficient RNAi activity in chicken cells. This and the characterisation of other chicken-specific promoters for RNAi applications will be of particular benefit to furthering functional genomics in the chicken and developmental studies which utilise the chicken as a model system.

2.2.1.3 RNA loop structures are important for efficient processing of short hairpin RNAs for gene silencing

Animal cells use RNA interference (RNAi) as a natural mechanism to regulate gene expression through the use of microRNAs (miRNAs). miRNAs are transcribed from the genome as approximately 70 nt primary miRNA transcripts (pri-miRNA), which are processed by Drosha into precursor miRNA hairpins (pre-miRNA). Exportin-5 transports the hairpin from the nucleus into the cytoplasm where the loop of the hairpin precursor is removed by the ribonuclease III enzyme Dicer, leaving the mature 22-25 nt double stranded miRNA. The miRNA guide strand loads into the RNA induced silencing complex (RISC) which directs it to the complementary messenger RNA (mRNA). This forms double stranded RNA resulting in cleavage of the target mRNA. Thousands of miRNAs are predicted to be present in every cell and many of these miRNAs are highly regulated to be tissue or cell cycle specific. The cellular RNAi mechanism has been successfully adapted to specifically silence genes of interest including viral and endogenous genes.

One method to artificially induce RNAi induced gene silencing is to express short hairpin (sh) RNAs. shRNAs consist of a silencing (si) RNA target sense sequence acting as the 5' stem, a spacer sequence which forms the loop and the anti-sense sequence forming the 3' stem. An alternative method is to mimic naturally occurring pri-miRNA structures called miRNA adapted shRNAs (shRNAmirs). Once transcribed from an expression vector these molecules enter the RNAi pathway at the Drosha step for shRNAmirs or the Dicer step for shRNAs, to be cleaved into siRNAs.

It has been shown that the shRNA loop sequence is critical for efficient mRNA silencing as the majority of the processing by Dicer occurs near the loop. Initial shRNA expression experiments showed that a 19 nt siRNA sequence and a 9 nt spacer was the most efficient and this structure has become the standard for shRNAs (Brummelkamp et al, 2002). The use of endogenous miRNA loop sequences to improve shRNA silencing has not been extensively investigated. To determine whether shRNA silencing of viral genes could be improved by the use of microRNA loop sequences, shRNAs targeting influenza A/PR/8/34 (PR8) strain Nuclear Protein (NP) mRNA and chicken anaemia virus (CAV) mRNA were designed. These shRNAs contain 19 nt siRNA target sequences with loop sequences derived from one of three native miRNAs (two chicken and one human) known to express highly in most cell types. They were compared to the original highly efficient 9 nt spacer sequence described by Brummelkamp et al, (2002).

Methods

shRNA loop design and plasmid constructs

shRNAs targeting chicken anaemia virus (CAV) mRNA were assayed for silencing against peGFP-CAV and are described elsewhere in this report. To produce pEGFP-NP, a pGEMTeasy plasmid containing a 180 bp fragment of NP was digested with *Not*I. The NP fragment was gel purified and ligated into the similarly digested pEGFP-C. The siRNA sequence targeting NP was obtained from Ge et al, (2003). NP shRNA molecules were designed to contain either the 9 nt hairpin loop sequence of Brummelkamp et al, (2002) or the microRNA loop sequences from human miR30a (miRBase ref MI0000088), chicken miR30a (miRBase ref MI0001204) and chicken miR17 (miRBase ref MI0001184) obtained from miRBase (http://microrna.sanger.ac.uk/sequences). Complementary oligonucleotides were annealed and ligated into pchU6-4. The forward oligonucleotide sequences used are shown in Table 2.2 and were obtained from Geneworks (Australia). The resulting Influenza PR8 NP shRNA constructs have been designated pshNP-OL, pshNP-mir17, pshNP-mir30agga and pshNP-mir30ahsp. The CAV shRNA constructs have been named pshVP2/3-1-OL, pshVP2/3-1-miR17, pshVP2/3-1-miR30agga, pshVP2/3-1-miR30ahsp, pshVP2/3-3-OL, pshVP2/3-3-miR17, pshVP2/3-3-miR30agga and pshVP2/3-3-miR30ahsp. All constructs were sequenced by Micromon DNA sequencing facility (Monash University, Australia)

Cells and virus

Chicken fibroblast cells (DF1: ATCC No. CRL-12203) were grown in DMEM and Madin-Darby canine kidney cells (MDCK: ATCC No. CCL-34) were grown in EMEM, both were supplemented with 10% foetal bovine serum, 2 mM glutamine, 10 mM Hepes, 1.5 g/l sodium bicarbonate, 0.01% penicillin and 0.01% streptomycin at 37 ºC with 5% CO_2 and subcultured twice weekly.
Influenza A/ PR/8/34 (PR8) strain virus stock was produced by limiting dilution passage in the allantoic cavity of 10 day old embryonated chicken eggs at 34°C for 48–72 h. Virus was passaged three times.

EGFP-fusion silencing

DF1 cells were seeded at $1.5x10^5$ cells in 24-well tissue culture plates in duplicate and grown overnight at 37 ºC with 5% CO_2. Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, USA) as per manufacturer's instructions. Briefly, 1 µg of pEGFP-NP or pEGFP-CAV and 1 µg of the relevant shRNA plasmid were mixed with 2 µl of Lipofectamine 2000 both diluted in 100 µl OPTI-MEM (Invitrogen, USA) and incubated at room temperature for 20 mins. The DNA:

lipofectamine mix was added to cells and incubated for 4 h. Cell media was replaced and incubated for 72 h. Cells were washed twice with PBS, trypsinised and washed twice with FACS wash (PBS with 1% FBS). Cells were subjected to flow cytometry and GFP silencing was analysed as a percentage of the non-silencing shRNA mean GFP (measured on FITC wavelength) fluorescence.

Influenza A-PR8 silencing

MDCK cells were transfected using Amaxa nucleofector electroporation (Amaxa Biosystems, Germany). Briefly $1.5x10^6$ MDCK cells were pelleted and resuspended in 100 µl of nucleofector T solution. Cells were transferred to a cuvette and electroporated with program T20. Cells were diluted with 500 µl of prewarmed growth media, aliquoted into 6 wells of a 24-well culture plate and incubated overnight at 37 ºC with 5% CO_2. Influenza A PR8 virus was serially diluted in viral growth media (VGM, with 0.3% BSA, 5 µg/ml trypsin and lacking FCS) and cells were infected at multiplicities of infection (MOI) of 0.01, 0.001, 0.0001 in duplicate. Cells were incubated at 37 ºC for 1 h, virus was replaced with VGM and incubated for 48 h. Supernatant was taken and used in a haemagglutination assay according to the OIE Manual. Briefly, virus solutions (50 µl) in serial two-fold dilutions in PBS were mixed with an equal volume of a 1% chicken erythrocyte suspension. After 1 h incubation at room temperature, the HA titer was estimated by the highest dilution with hemagglutination.

RNA isolation and Northern Blotting

DF1 cells were seeded and grown until 80% confluency in 25 cm tissue culture flasks (Nunc, USA). Plasmids were transfected into the DF1 cells using Lipofectamine 2000 as per manufacturers instructions. Briefly,12 µg of the relevant shRNA plasmid was mixed with 20 µl of Lipofectamine 2000 both diluted in 500 µl OPTI-MEM and incubated at room temperature for 20 mins. The DNA: lipofectamine mix was added to cells and incubated for 4 h. Cell media was replaced and the cells were incubated for a further 72 h. RNA of less than 200 nt in length was purified from transfected DF1 cell cultures using mirVana™ miRNA isolation kits (Ambion, Austin USA) and concentrated using Millipore microcon centrifugal filters (YM-30; Millipore, USA) as described by the respective manufacturers. Approximately 1 µg of low molecular weight RNA was resolved on a 7M Urea- 15% Polyacrylamide gel and transferred to a positively charged membrane (Hybond plus, Amersham Biosciences, USA) using a Trans-blot semi-dry transfer cell (BioRad, USA). The efficiency of each hairpin expression and processing was determined using a Locked Nucleic Acid (NP-LNA) probe (5' CTCCGAAGAAATAAGATCC 3') (Sigma- Proligo, USA) whereby a locked nucleic acid base was incorporated into every third nucleotide of the probe. The NP-LNA probe was end-labeled with [$^{32}\gamma$P] dATP using 10 units of OptiKinase (USB, USA) prior to their addition to a pre-hybridised Northern

blot. Hybridization was conducted overnight at 42^{o}C in 50% formamide, 0.5% SDS, 5x SSPE, 5x Denhardts solution and 100 μg ml^{-1} denatured herring sperm DNA (Roche, USA). The membrane was washed 3 times in 2x SSC, 0.1% SDS at 42^{o}C prior to overnight autoradiographic exposure. The size of the resolved RNA was determined by comparison with Ambion^{TM} Decade markers (Ambion, USA).

Results

Influenza PR8 NP shRNA loop sequences and plasmid constructs

The loop sequences used in this study were obtained from either Brummelkamp et al. (2002) or from miRBase. We chose the human miR30a loop sequence (miR30ahsp) as miR30a based shRNAmirs express high levels of siRNAs (Boden, et al, 2004). For silencing chicken pathogens, the chicken miR30a (miRgga30a) loop sequence was also selected, as it contains two nucleotide differences to the human version. The chicken mir17 (mir17) loop sequence was selected as the native miRNA is expressed at high levels in all chicken cell types (ICGSC, 2004). A siRNA targeting Influenza NP was adapted to shRNAs containing one of the four loop sequences (Table 2.2). The resulting plasmids are referred to as pshNP-OL, pshNP-mir17, pshNP-mir30agga and pshNP-mir30ahsp. Figure 9A shows the predicted structures and ΔG values of the original shRNA structure of Brummelkamp et al, (2002) and the native microRNAs, whilst the predicted structures and ΔG values of the NP hairpins are shown in Figure 2.9B. It should be noted that 4 of the nucleotides in the Brummelkamp shRNA loop sequence are predicted to base-pair. The mir30a shRNA structure predictions and ΔG values reasonably accurately match those of the native miRNAs missing one 2 nt bulge, whilst the mir17 shRNA loop matches that predicited for the miRNA, however the stem appears quite different as it does not contain the multiple bulges. All shRNAs were under the control of the chicken U6-4 promoter (chU6-4), along with the non-silencing (pshNS) and positive (pshEGFP, Table 2.2) control described previously (Wise et al, 2007).

Chicken miR17 loop sequence decreases the ability of the shRNA to silence EGFP-fusion expression, by inhibiting processing of the hairpin to mature siRNAs.

Prior to virus silencing experiments, each NP shRNA vector was assayed for activity against EGFP-NP fusion mRNA in the chicken fibroblast cell line DF1. pshNP-OL was highly active at silencing peGFP-NP mRNA (Fig 2.10A). pshNP-miR30agga and pshNP-miR30ahsp showed a marginal increase in silencing pEGFP-NP compared to pshNP-OL (Fig. 2.10A). Inclusion of the chicken miR17 loop sequence resulted in a 3-fold decrease in GFP-NP mRNA silencing activity (Fig 2.10A).

To determine why pshNP-mir17 was less active than other constructs, small RNAs were isolated from transfected DF1 cells and analysed by Northern Blot (Fig 2.10B). This method detects both the hairpin structure and the mature siRNA using a locked nucleic acid probe directed at the NP siRNA sequence. Hairpin and mature siRNA were detected for pshNP-OL and both mir30a constructs (Fig 2.10B). The NP mir17 hairpin (pshNP-miR17) was detected, but no mature sequence was observed (Fig 2.10B). The Northern blot also shows a high level of unprocessed hairpin present from all vectors compared to the level of mature siRNAs observed (Fig 2.10B). A higher concentration of mature siRNA was present from the mir30a constructs. No bands were observed in the untransfected or non-silencing control as expected (Fig 2.10B).

shRNA silencing of viral RNA mimics the EGFP-fusion assay

The shRNA constructs were assayed for the ability to silence Influenza A strain PR8 in MDCK cells. The haemagglutination assay shows consistent results to the GFP reporter assay, highly efficient knockdown of virus replication was observed in the original loop construct however, the miR30a loop sequences increase the silencing ability of the NP siRNA at the highest concentration of virus, with the chicken miR30a loop giving the best knockdown (Fig 2.11). As expected, pshNP-mir17 was unable to inhibit viral replication efficiently.

Different loop sequences do not improve less efficient siRNA molecules

To determine if the loop sequence affected the silencing ability of other shRNA sequences, vectors expressing shRNAs with the four different loop sequences targeting chicken anaemia virus mRNA were produced. Two shRNA sequences were analysed, one highly active against EGFP-CAV fusion mRNA (pshVP2/3-1) and another less active sequence (pshVP2/3-3). The resulting constructs are referred to as pshVP2/3-1-OL, pshVP2/3-1-miR17, pshVP2/3-1-miR30agga, pshVP2/3-1-miR30ahsp, pshVP2/3-3-OL, pshVP2/3-3-miR17, pshVP2/3-3-miR30agga and pshVP2/3-3-miR30ahsp. The same non-silencing control (pshNS) and EGFP targeted shRNA (pshGFP) were utilised. Similar silencing results to that observed with the NP shRNAs sequence were obtained in the DF1 GFP reporter assay (Fig 2.12). The shRNAs containing the miR17 loop sequence were less active, whilst the chicken miR30a loop shRNAs was the most efficient (Fig 2.12 A & B). Interestingly, the different loop sequences were unable to improve the activity of shVP2/3-3, indicating that whilst loop sequences can impair hairpin processing, they are unable to improve the ability of a siRNA to silence the target gene (Fig 2.12B).

Discussion

shRNAmirs are processed by Drosha into shRNAs. shRNAs are then recognized and processed into siRNAs by Dicer. Initially shRNAmirs appeared to produce more mature siRNAs resulting in better silencing. However, McManus et al, (2002) showed that the loop sequence is the most critical region in recognition of the shRNA for processing, potentially removing the need for the longer shRNAmirs. To determine if a native miRNA loop sequence could improve shRNA processing and increase silencing, a comparison of shRNAs containing either the commonly used 9nt loop sequence from (Brummelkamp et al, 2002) and 3 endogenous miRNA loop sequences was performed.

shRNAs containing the loop sequences from the miR30a miRNAs were highly effective at silencing the GFP-fusion mRNAs and Influenza PR8. It appeared that this may be due to the presence of more mature siRNA being processed than seen with shRNAs containing the Brummelkamp et al, (2002) loop sequence. However the chicken miR17 loop sequence severely inhibited hairpin processing and silencing. This could be due to decreased Exportin-5 transport, interference with Dicer cleavage, or other cellular factors impeding maturation.

The mir17 loop sequence used in this study was selected based on the unpaired nucleotides in the predicted miRNA structure. Subsequent analysis of chicken mir17 revealed 4 additional bases extend 3' from the loop sequence, two of which are base-paired with the siRNA (miRBase; Fig 2.9A). Chicken mir17 also contains several bulges in the stem sequence not present in the shRNAs, resulting in the shRNA structure prediction looking quite different (Fig 2.9B). Little is known about the shRNA structure requirement for Dicer cleavage. However McManus et al, (2002) demonstrated that the processing of shRNAmirs was highly sensitive to modifications in structure including bulge position and loop sequence. The loss of these structures may have resulted in the loss of shRNA processing. It would be interesting to produce the NP- mir17 based shRNAmir to determine if the incorporation of the bulges and paired loop sequence restored processing. The miR30a shRNA constructs, despite missing a 2nt bulge in the stem mimic the endogenous miRNA more accurately resulting in correct recognition and processing (Fig 2.9B). Therefore it appears important to choose loop sequences that will result in an shRNA that closely mimics the endogenous miRNA structure for improved silencing.

Although chicken mir17 is highly and ubiquitously expressed in chicken cells, it is transcribed from the miR17-92 miRNA cluster which contains 6 miRNAs. One of these, mir18 is known to require a cellular protein, the nucleo-cytoplasmic shuttling protein hnRNP A1, to be processed. No similar requirement has been determined for mir17, however this could also explain why the mir17 shRNA's were not processed. If this is the case the use of a loop sequence from a regulated miRNA could result

in tissue specific or cell-cycle specific regulation of the shRNA. This level of regulation of shRNA expression would be advantageous in many circumstances.

The continuing emergence of zoonotic and highly virulent viruses has placed increased pressure on developing new vaccines and therapeutics for livestock. An alternative strategy may be to develop transgenic, disease-resistant animals that express RNAi molecules. This study indicates that endogenous miRNA loop sequences can increase the efficiency of mature siRNA production and can be derived from the species of interest, minimising the amount of foreign DNA sequence required. In the future, understanding the efficiency and specificity of miRNA loop sequences may also prove useful for delivering tissue targeted gene silencing.

2.2.1.3 In ovo modulation of production traits – myostatin and DMRT1

The work described above details an in depth analysis of development and optimisation of short hairpin RNAi delivery for applications in chickens. The next stage in the project was to develop methodologies to undertake proof-of-concept to show that RNAi silencing of the myostatin gene leads to changes in muscle development and the *DMRT1* leads to feminisation of developing male embryos. As this work progressed the focus was strongly directed towards silencing of the *DMRT1* gene and confirming the putative role of this gene in male sex development in chickens and other birds. The implications of this work would lead to a valuable commercial application for the egg laying industry. There are two clear commercial paths to impact. The first is with the breeding companies - they cull male chicks at hatch and this is both an economic and welfare issue that they would like to see improved. Even slightly increasing the ratio of females to males is of importance and value to the breeders. The second path is with the vaccine companies. Pfizer has invested into embrex technology and would like to get better uptake of embrex delivered vaccines (including new and innovative vaccines) into the layer industry. Their big problem at the moment is that the industry is reluctant to adopt the embrex platform because 50% of the injected eggs are male and are therefore culled at hatch. Pfizer are extremely interested in an approach to sway the sex ratio to females. As an added bonus, we are currently working towards embrex delivery of our RNAi molecule and Pfizer are extremely keen to develop more embrex deliverable products.

Methods

DMRT1 shRNA screening

Identification and construction of DMRT1 shRNA expression plasmids

The shRNA designer website (http://shrnadesigner.med.unc.edu) was used to identify shRNA target sites within the chicken DMRT1 gene sequence. Four shRNA target sites were identified and named relative to their start position of the 1038 bp DMRT1 gene sequence; DMRT1-208 (GACTGCCAGTGCAAGAAGT) , DMRT1-343 (GAGCCAGTTGTCAAGAAGA), DMRT1-568 (CTGTATCCTTACTATAACA) and DMRT1-694 (CTCCCAGCAACATACATGT).

Complementary olgionucleotides corresponding to DMRT1-208 (DMRT1-208T and DMRT1-208B), DMRT1-343 (DMRT1-343T and DMRT1-343B), DMRT1-568 (DMRT1-568T and DMRT1-568B) and DMRT1-694 (DMRT1-694T and DMRT1-694B) were annealed and cloned into pchU6-4 using the *Pme*I and *Sal*I restriction sites as previously described (Hinton & Doran 2008). Resulting shRNA expression plasmids were named cU6-DMRT1-208shRNA, cU6-DMRT1-343shRNA, cU6-DMRT1-568shRNA and cU6-DMRT1-694shRNA.

Analysis of DMRT1 knockdown in DF1 cells

To construct a DMRT1 reporter system, the 1038 bp DMRT1 gene sequence was PCR amplified using DMRT1-F and DMRT1-R primers. The amplified product was inserted into the *Bgl*II – *Hind*III sites of pEGFP-C using the *BamH*I – *Hind*III primer encoded restriction sites and named pEGFP-DMRT1. Briefly, the pEGFP-C vector was constructed using the pEGFP-N1 vector (Clontech). The multiple cloning site (MCS) of pEGFP-N1 was removed by *BamH*I-*Bgl*II digestion and self-ligated. Oligonucleotide pEGFP-linkerT and pEGFP-linkerB were designed to construct a new MCS (containing *Bgl*II-*Eco*RI-*Pst*I-*Eco*RV-*Hind*III-*Bam*HI) by inserting the linker into the *Not*I- *Xba*I sites downstream (3') of EGFP. The resulting construct was called pEGFP-C.

DF1 (ATCC CRL-12203, chicken fibroblast) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 4.5g/L glucose, 1.5g/L sodium bicarbonate, 10% fetal calf serum (FCS), 2mM L-glutamine and the addition of penicillin (100U/mL) and streptomycin (100mg/mL), and maintained in 5% CO_2 at 37°C . For transfection, cells were grown to 80-90% confluence, in 24-well plates (Nunc). Co-transfection was achieved using 500ng of plasmid DNA (shRNA plasmid and/or pEGFP-DMRT1) with Lipofectamine 2000 (Invitrogen) transfection reagent according to manufacturer's instructions. Flow cytometry was used to measure EGFP mean fluorescence intensity (MFI) at 48 hr post-transfection using a FACS calibur (Becton Dickinson) fluorescent activated cell sorter and CELLQuest software (Becton Dickinson). The MFI was calculated as a percentage of the non-silencing (NS) control shRNA.

Construction of RCAS-DMRT1shRNA

To facilitate insertion of the DMRT1 shRNA expression cassettes into pSlax13-EGFP (shuttle plasmid containing EGFP as described for miRNA construction earlier), a linker (annealed oligonucleotides pSlax-linkerT and pSlax-linkerB) was inserted within the *BamH*I – *Pst*I sites, adding the restriction sites *Eco*RV-*Not*I-*Bgl*II-*Xho*I. The resulting plasmid was named pSlax-EGFP-link.

The cU6-DMRT1-343shRNA plasmid was digested with *Zra*I - *Sal*I and the cU6-DMRT1-343shRNA sequence gel purified and inserted into the *Eco*RV - *Bgl*II sites of pSlax-EGFP-link to produce pSlax-EGFP-cU6-DMRT1-343shRNA.

The pSlax-EGFP-cU6-DMRT1-343shRNA construct was *Cla*I digested to release the EGFP-cU6-DMRT1-343shRNA sequence, which was then inserted into the *Cla*I site of pRCAS.BP.B to produce pRCAS-DMRT1shRNA. Virus production was achieved as per pRCAS-DMRT1miRNA.

Detection of RCAS expressed DMRT1 shRNAs

An RNase protection assay was performed to detect DMRT1 shRNA expression using the DMRT1 RNA probe. Small RNAs were isolated (mirVana miRNA isolation kit, Ambion) from a pool of nine pairs of genetic male gonads at embryonic day 10 (E10), that were blastoderm infected at embryonic day 0 (E0) with RCAS-DMRT1shRNA and shown to be EGFP positive. As controls, small RNAs were isolated from a pool of ten uninfected genetic male gonads at E10 (negative control), in addition to small RNAs isolated from RCAS-DMRT1shRNA infected DF1 cells (positive control). RNA samples were hybridised with the radiolabelled (γ-P^{32}) DMRT1 RNA probe (mirVana Probe & Marker kit, Ambion) in solution overnight at 42ºC. The DMRT1 shRNA hybridised samples and the +RNase control were RNase A/T1 treated as recommended. Samples were separated on a 15% (w/v) polyacrylamide (8M urea) gel and then exposed to Hyperfilm™ ECL (Amersham Biosciences) within an EC-AWU cassette (Fuji) and placed at -80ºC overnight. The film was developed using an X-ray processor FPM-100A (Fuji).

Confirmation of DMRT1 knockdown in ovo by qPCR

Forty fertile chicken eggs (*Gallus gallus domesticus*) obtained from SPAFAS (Woodend, Victoria) were injected intra venously at E4 with RCAS-DMRT1shRNA and twenty fertile chicken eggs with RCAS non-silencing control shRNA (RCAS-NSshRNA). At E10, gonads were individually collected and embryos genotypically sexed by PCR (as described earlier). Twenty-one genetic male gonads from RCAS-DMRT1shRNA infected embryos were obtained and divided into two groups (pool 1 (ten gonads) and pool 2 (eleven gonads)), while four genetic male gonads from RCAS-NSshRNA infected embryos were collected and pooled. Total RNA was extracted from the three pools using the mirVana miRNA isolation kit (Ambion). Purified RNA was DNase treated and then reverse-transcribed to cDNA using a Reverse Transcription Kit (Promega) according to the manufacturer's recommendations. To quantify DMRT1 knockdown, primers/probe were designed to the 1038 bp DMRT1 sequence using the Custom TaqMan Assay Design Tool (Applied Biosystems). The resulting primers/probe, qDMRT1-F, qDMRT1-probe and qDMRT1-R were used in conjunction with TaqMan® Universal PCR Master Mix (Applied Biosystems) as described by the manufacturer in a 20µL reaction containing 2µL of neat cDNA. Analysis was performed using the StepOne™ PCR cycler instrument and software v2 (Applied Biosystems).

Results

RNAi molecules were first tested *in vitro* against myostatin-EGFP fusion constructs so that effective molecules could be easily screened prior to beginning the more technically challenging *in ovo* work. We used the chicken embryo derived DF-1 cell line for the tests. This cell line was chosen because it was embryo derived and therefore of importance to our provisional patent application as it relates to the *in ovo* modulation of traits. We fused the chicken myostatin gene to EGFP in the plasmid pEGFP-C. The transcriptional fusion still expresses EGFP quite well. In this experiment we used fluorescence microscopy to visualise silencing as opposed to FACS analysis described below for the *DMRT1* gene. We made 3 shRNA plasmids targeting myostatin and co-transfected these with the fusion construct into the DF-1 cells. The plasmid expressing the td306 shRNA gave excellent silencing of the EGFP-myostatin fusion. This is an excellent RNAi molecule.

We have also tested numerous molecules *in vitro* against *DMRT1*-EGFP fusion constructs. Again, as described above, we have used the embryo derived DF1 cell line. For the *in vitro* test we constructed a gene fusion of the reporter gene EGFP and chicken *DMRT1* gene. The fusion is transcriptional and not translational and therefore EGFP still expresses quite well in tissue culture. We then made plasmids that express shRNAs that target the *DMRT1* specific part of the fusion. We then co-transfected the plasmids with the fusion construct into DF-1 cells and measured EGFP fluorescence. If the shRNAs successfully target the *DMRT1* region of the fusion and direct degradation of the fusion transcript then we would see less EGFP fluorescence. In this experiment we used FACS analysis to measure the mean fluorescence intensity of the co-transfected cells. The *DMRT1* shRNA plasmids gave a range of levels of silencing. We decided to improve on the levels of *in vitro* silencing that we observed, so using the latest computer algorithms, we designed and developed new shRNA molecules that target chicken *DMRT1* and Myostatin genes. We now have a total of 10 shRNAs for *DMRT1* and 7 shRNAs for myostatin that could be studied further both *in vitro* and *in vivo*. We constructed pCluck plasmids for expression of the shRNAs from the chicken U6-4 promoter and validated the shRNAs for target gene silencing in cultured embryonic fibroblast cells. The results for *DMRT1* and Myostatin gene silencing are shown in Figure 2.19. We have now selected the best shRNAs for the proof-of-concept *in ovo* experiments.

The next stage was to assess the myostatin and *DMRT1 in vitro* tested RNAi molecules *in ovo*. To enable us to do this, we developed the avian retroviral vector, RCASBP(B) (Replication Competent Avian Sarcoma leukosis virus, high titre Bryan Polymerase, strain B) viral vector delivery system for embryo delivery. Initially we used an EGFP reporter plasmid (pEGFP-N1) to develop skills in this technique. We do this by injecting intravenously into 4 day old embryos. The embryos are then incubated until day 10 of embryogenesis and are then screened for expression of the EGFP gene.

Figure 2.20 clearly shows EGFP fluorescence during both limb bud and organ development from an infected embryo. Now that this delivery system was working for us we were ready to deliver shRNAs to embryos.

Our best shRNA targeting *DMRT1* was shRNA343. Viruses carrying shRNA343 were used to infect day 0 chicken blastoderms, and embryogenesis was allowed to proceed until day 10. Control embryos were infected with virus carrying GFP and a scrambled non-silencing RNAi sequence. All embryos were genotypically sexed by PCR. In the chicken embryo, the gonads form on the mesonephric kidneys around day 3.5 of incubation. Sexual differentiation into testes or ovaries begins at day 6 and is normally advanced by day 10. Embryos infected with virus at day 0 showed global GFP reporter expression by day 10, including widespread expression in the urogenital system and in sectioned gonads. RNase protection assays of these day 10 gonads confirmed expression of the mature *DMRT1* knockdown siRNAs (Figure 2.22) and quantitative real time PCR confirmed silencing of the *DMRT1* gene in pooled male embryos at E4 and E10 (Figure2.21).

Treatment of genetically male (ZZ) chicken embryos with the *DMRT1* knockdown sequence (shRNA343) resulted in feminisation of the gonads by embryonic day ten. Gonadal development in embryos treated with scrambled control RNAi was normal, with bilateral testes observed in males and typical asymmetric ovarian development observed in females (n=40). In all control samples, gonadal sex matched genotypic sex. At the histological level, control females showed typical ovarian morphology. The left gonad showed a vacuolated medulla and a thickened outer cortex, where germ cells were accumulated, while the smaller right gonad showed a vacuolated medulla and no thickened cortex (Figure 2.23a and b). Control male embryos exhibited bilateral testis development, characterised by seminiferous cords in the inner medulla, enclosing germ cells, and a thin outer epithelial cell layer (Figure 2.23c, d and e).

Gonads from embryos were assessed for DMRT1 and marker gene expression. In control embryos infected with virus carrying the non-silencing scrambled miRNA, DMRT1 protein expression was not affected and gonadal histology was normal. In these control males, DMRT1 protein was uniformly expressed in the nuclei of developing Sertoli and germ cells within testis cords (Figure 2.24a). Expression was strong, bilateral (in both left and right gonads) and indistinguishable from staining in uninfected male embryos. In contrast, male embryos (ZZ) treated with two different *DMRT1* knockdown constructs showed variably reduced DMRT1 protein expression in the left gonad, disrupted testis cord formation and ectopic female gene expression. The extent of DMRT1 knockdown and testis cord disruption varied among embryos, but was more pronounced with the shRNA343 construct compared to the control construct. Gonadal DMRT1 protein expression was either greatly reduced throughout both left and right gonads (Fig.2.24b), or expression was irregular, in embryos

treated with shRNA343. Quantitative RT-PCR analysis confirmed that *DMRT1* mRNA expression was reduced by over 60% in both male and female embryos treated with miRNA563 or shRNA343 (Fig. 2.24d).

Genetic male chicken embryos treated with *DMRT1* miRNA showed ectopic activation of the robust female marker, aromatase. Aromatase enzyme is normally expressed only in female gonads, where it synthesises the oestrogen that is required for ovarian differentiation birds. Aromatase enzyme is never detected in normal male embryonic gonads. In control and DMRT1 knockdown female embryos (ZW), aromatase enzyme was strongly expressed in the medulla of both left and right gonads (Fig 2.25a). No expression was seen in male controls treated with scrambled control (Fig. 2.25b). However, in the twelve feminised males examined by immunofluorescence, aromatase was ectopically activated. In those males treated with shRNA343, both the left and right gonads showed ectopic aromatase expression (Fig. 2.25c). This finding indicates that elevated DMRT1 expression in male gonads normally suppresses aromatase and hence female development.

Overall, these results indicate that *DMRT1* plays a key role in chicken testis determination. Treatment of genetic male chicken embryos with RNAi constructs (shRNA) results in feminisation of the gonads by day ten of development. Our results support the Z dosage hypothesis for avian sex determination (Smith et al., 1999; Nanda et al., 2008). Under this hypothesis, a higher dosage of *DMRT1* initiates testicular differentiation in male embryos, activating SOX9 expression and suppressing aromatase, which is essential for female development. *DMRT1* fulfils the requirements expected of an avian master sex-determining gene. It is sex-linked, conserved on the Z sex chromosome of all birds examined, including the basal ratites (ostriches, emus, *et al*.). It is expressed exclusively in the urogenital system prior to gonadal sex differentiation in chicken embryos, with higher expression in males, and knockdown leads to feminisation. Our data provides evidence that *DMRT1* is the male sex determinant in birds.

2.2.2 Outcome - RNAi developed as an anti-viral agent (e.g. CAV)

2.2.2.1 Inhibition of chicken anaemia virus replication using multiple short-hairpin RNA.

Chicken anaemia virus (CAV) is an important worldwide problem in the poultry industry, causing acute anaemia in young chicks or subclinical infections in adult birds (Adair 2000). The subclinical infection results in immunosuppression which is particularly troubling as this leaves chickens with enhanced susceptibility to other avian pathogens and decreases the effectiveness of vaccines reducing

production efficiency. Currently vaccination against CAV uses attenuated virus in drinking water, however reversion to virulence from these strains has been seen

CAV is a member of the *gyrovirus* genus in the *Circoviridae* family of viruses that are characterized by their small, single-stranded, circular DNA genome The genome is 2.3kb from which a single 2.0kb mRNA transcript encoding the three viral proteins (VP) 1, 2 and 3 in overlapping reading frames is transcribed. Splice variants of this transcript have recently been identified, however translation of these transcripts has not been shown. VP1 is the 52kDa structural capsid protein; VP3, is a 13·8 kDa virulence factor known to induce apoptosis in transformed cell lines; and VP2, is a 28 kDa dual-specificity protein phosphatase (DSP). CAV with mutations in VP2 or VP1 have been shown to reduce or stop replication of the virus. CAV is difficult to grow in tissue culture and will only grow in transformed lymphoblastoid cell lines such as MDCC-MSB1 cells (Marek's disease transformed chicken T cell). These suspension cells are difficult to transfect and the best efficiency that has been observed is approximately 50%.

RNA interference is a naturally occurring mechanism found in both plants and animals that uses short RNA molecules (21-23 nts) to degrade or sequester mRNA resulting in specific gene suppression (Hannon 2002; Fire et al. 1998). This mechanism has been taken advantage of by artificially introducing short interfering (si)RNAs and short hairpin (sh)RNAs into cells to suppress genes of interest. Introduction of siRNAs and shRNAs targeting viral mRNA has been shown to be effective at silencing several human and animal viruses *in vitro* and *in vivo* including HIV-1, Hepatitis B, Influenza A, FMDV and BVDV.

This study showed inhibition of CAV mRNA expression in MDCC-MSB1 cells by using single or multiple shRNAs targeted to various regions of the CAV mRNA.

Methods

Cell culture and virus growth

Chicken lymphoblastoid cells, MDCC-MSB1, transformed by Marek's disease virus, were cultured at 37ºC with 5%CO_2 in a 100mm dish with RPMI1640 medium supplemented with 10% fetal bovine serum (IBL), 2 mM glutamine, 0.01% penicillin and 0.01% streptomycin. DF1 cells were grown in DMEM medium supplemented with10% fetal bovine serum (IBL), 2 mM glutamine, 0.01% penicillin and 0.01% streptomycin at 37ºC with 5%CO_2. Both cell types were subcultured twice weekly.

The Australian isolate CAV269/7 was obtained from G. Browning (School of Veterinary Scinece, The University of Melbourne) and grown in MDCC-MSB1 cells. Virus was prepared by freeze /thawing the culture three times and then clarifiying by centrifugation at 6000 *g* for 10 min. Virus was stored at -80 ºC.

pEGFP-CAV and chicken U6 promoter plasmid construction

pCAV269/7 (from Melb Vet School) was digested with *Pst*I and *BamH*I, the 935bp CAV fragment was gel purified and ligated into the similarly digested pEGFP-C1. Single chicken U6 promoters 1, 3 and 4 were amplified by PCR from previously reported plasmids (Wise et al. 2007) and ligated into pGEMTeasy. ChU6#4 was ligated via *Sph*I and *Sal*I sites on primers and plasmid whilst ChU6#1 was ligated with *BfuA*I and *Nde*I. The reverse primers also contained an extra restriction site between the two cloning sites for removal of parental vector before transforming *E.coli* cells. Oligonucleotides used are shown in Table 2.4. All restriction enzymes were obtained from either Promega or New England Biolabs.

For construction of the multi-promoter vector 3 rounds of overlapping PCR was performed on the three U6 chicken promoters. Oligonucleotides are shown in Table 2.4. The first round of PCR amplified ChU6#1, 3 and 4 individually. The second round combined the ChU6#4 and ChU6#3 DNA templates. A third round of PCR combined the ChU6#4 & 3 template from round two with the individual ChU6#1 from round 1 to produce a PCR product containing all three promoters. This product was digested with *Sph*I and *Nde*I and ligated into a similarly digested pGEMTeasy to produce pMP. Each reverse primer contained restriction sites for ligation of the shRNAs. All PCRs were performed with Platinum TaqHiFi as per manufacturers instructions (Invitrogen). All plasmids were sequenced by Micromon DNA sequencing facility (Moansh).

shRNA design and plasmid construction

Firstly siRNAs were designed based on the CAV 269/7 strain sequence (Genebank. Accession No. AF227982) using the Dharmacon siRNA Design tool (http://www.dharmacon.com). Sequences were selected to be in the ORF and contain a GC content between 30% to 64%. The first three criteria of the Taxman algorithm (Taxman et al. 2006) were then applied to the first 40 siRNAs predicted from the Dharmacon tool. Any sequences with a score of 3 or 4 then had the free-energy of the central six bases calculated using free-energy parameters for predictions of RNA duplex stability as published by Freier *et al* (1986). Six siRNA sequences that had the best Taxman score and a central duplex closest to a $\Delta G > -12.9$ kcal/mol were chosen. Complementary DNA oligonucleotides containing the siRNA followed by the loop sequence TTCAAGAGA, then the anitsense of the siRNA and followed by a PolIII

termination sequence were chemically synthesized and annealed. A 5' blunt end and 3' *Sal*I site overhang sequence were included for ligation into *Pme*I and *Sal*I digested pChU6#4 or a 5' *BfuA*I and 3' *Nde*I site overhang sequence were included to ligate into *BfuA*I and *Nde*I digested pChU6#1. A non-silencing (shNS) negative control and a positive control shRNA targeting GFP were also used the sequences have been published previously (Lambeth et al. 2005). All oligonucleotide sequences used to produce CAV shRNAs are shown in Table 2.4

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For the multipromoter plasmid, annealed shRNA oligonucleotides were ligated into either *Pme*I and *Sal*I digested pMP to be transcribed by ChU6#4, *Nco*I (nuclease treated to produce a blunt end) and *Xho*I digested pMP to be transcribed by ChU6#3, or *BfuA*I and *Nde*I digested pMP to be transcribed by ChU6#1 producing pCAV-shMW. All plasmids were sequenced by Micromon DNA sequencing facility (Moansh).

Transfection of GFP-CAV and shRNA plasmids into DFI cells

DF1 cells were seeded at $1.5x10^5$ cells in 24 well tissue culture plates and grown overnight at 37ºC with 5%CO_2. Plasmids were transfected into the DF1 cells using Lipofectamine 2000 as per manufacturers instructions (Invitrogen). Breifly1ug of pEGFP-CAV and 1ug of the relevant shRNA diluted in 100ul OPTI-MEM (Gibco) were mixed with 2ul of Lipofectamine 2000 diluted in 100ul OPTI-MEM and incubated at room temperature for 20mins. The DNA; lipofectamine mix were added to cells and incubated for 4hrs. Cell media was changed to normal growth media and incubated for 72hrs. Cells were then washed twice with PBS, trypsinised and washed twice with FACS wash (PBS with 1% FBS). Cells were subjected to flow cytometry and analysed as a percentage of the non-silencing shRNA mean FITC fluorescence.

Transfection of GFP and shRNA plasmids into MDCC-MSB1 cells

MDCC-MSB1 cells were washed twice in RPMI 1640 medium (Sigma–Aldrich), $4x10^6$ cells were resuspended in 350 µl RPMI 1640 containing 10 µg of pEGFP-N1 and 10 µg of the relevant shRNA plasmid in a microfuge tube. Transfection was performed in a 0·4 cm gap electroporation cuvette in a Gene Pulser apparatus (Bio-Rad) set at 400 V, 900 µF, ∞ resistance and extension capacitance. The cells were incubated at room temperature for 5 min, then resuspended in 1 ml warm growth medium and incubated for 4hrs before infection with CAV.

Infection of transfected MDCC-MSB1 cells

Transfected cells were counted and $1x10^6$ cells for each transfection were pelleted and infected with an MOI of 2 CAV269/7 for 1 hr at 37 ºC or mock infected with 200µl of growth medium. Cells were then transferred to 3ml warm growth medium and incubated for 72 or 96hrs.

Detection of GFP and CAV VP3 by flow cytometry

Cells for experiments with GFP alone were washed 3x in FACS wash and analysed by flow cytometry at 72hrs. At 72 or 96hrs transfected and infected cells were counted and $1x10^6$ cells were removed. Cells were pelleted and fixed in 1ml BD permeablilisation solution 2 for 20mins at room temperature (Becton Dickinson). Cells were then washed once in PBS with 0.01% Tween 20. Cells were then stained with 1/1000 mouse antiCAV VP3 and 1/500 goat anti mouse IgG1 APC conjugated antibody and analyzed by flow cytometry for GFP and APC fluorescence. Dot plots of the cells comparing FITC and APC channels were analysed and histograms of APC fluorescence of GFP transfected cells were produced. Mean fluorescence of the histograms were taken and analysed as a percentage of the non-silencing shRNA mean fluorescence.

Results

shRNA design and plasmid constructs

The single-stranded DNA genome of CAV is transcribed into a single long mRNA molecule encoding the three overlapping CAV genes. Therefore targeting any region of the coding sequence should silence the expression of all three proteins. As the genome is a small DNA strand sequence diversity amongst isolates is low. The Australian CAV269/7 strain used in the study has been shown to have 95% sequence identity to seven other sequenced isolates, whilst the other seven isolates had 98-99% identity to each other. shRNAs were therefore designed against the more highly conserved regions of the genome to give cross strain protection. As VP2 is overlapped by the entirety of VP3 and a portion of VP1 (Fig 2.13) most of the shRNAs designed targeted two of the genes, although two shRNAs targeted VP1 alone. Each shRNA was inserted into an expression vector individually, the three most active shRNAs were then cloned into pMP to determine whether individual or multiple shRNAs work most effectively. This plasmid has previously been shown to express three different shRNA molecules targeted against influenza by RNase protection assay indicating the plasmid is functional (unpublished data). The GFP-CAV fusion vector contains 935bp of the CAV genome at the 3' end of the GFP gene. This region contains the target sequence for all six of the CAV shRNAs (Fig 2.13) and is expressed as a single mRNA therefore an active shNA will result in the loss of GFP expression.

Silencing of GFP-CAV fusion transcript by single or multiple shRNAs

Each shRNA vector was first tested against the GFP-CAV fusion mRNA in DF1 cells. All of the shRNAs had some activity with the least active giving only 30% knockdown of GFP expression (Fig 2.14). shRNA VP2/3-3 appeared to be extremely effective with over 80% knockdown of GFP-CAV expression, considerably more than the shGFP positive control. This indicates that efficient inhibition of CAV replication should be possible. The three most active shRNAs VP2/3-1, VP2/3-3 and VP1-2 were cloned into pMP to produce pCAV-MW. pCAV-MW contains shVP1-2 under the control of the U6#4 promoter, shVPVP2/3-3 under the control of the U6#3 promoter and shVPVP2/3-1 under the control of the U6#1 promoter. A schematic diagram is shown in Fig 2.13. This construct was then examined for it's ability to inhibit GFP-CAV mRNA expression compared to the individual shRNAs. pCAV-MW was less efficient at silencing GFP expression than the single most active shRNA VP2/3-1 (Fig 2.15). However the knockdown was still significant at over 70%. All results are shown as a percentage of the non-silencing shRNA negative control.

MDCC-MSB1 cells have the RNAi pathway

As MDCC-MSB1 cells have not previously been used in RNA interference work and are highly transformed, it was necessary to determine whether the cells had retained the RNA interference pathway. Therefore the GFP expression vector alone or with a non-silencing shRNA or a previously published, highly active shRNA targeting GFP was electroporated into MSB1 cells. Results from both fluorescent microscopy and flow cytometry indicate MDCC-MSB1 cells do have RNAi ability (Fig 2.16). The results also indicate that the best silencing is observed at 96hrs post-transfection. This was therefore used for future experiments.

Silencing of CAV replication using shRNAs

It has been shown that some siRNAs that are highly effective against the GFP-fusion reporter assay are not effective against the target virus (Lambeth et al. 2007). This is most likely due to different folding configurations of the native virus mRNA compared to the GFP-fusion mRNA. Therefore to determine the effectiveness of the chosen shRNAs at inhibiting CAV replication an assay to determine silencing was required. Although it has been shown that MDCC-MSB1 cells have retained the RNAi pathway, the best transfection efficiency that has been obtained is approximately 50% of cells (data not shown). This is relatively low to determine if CAV has been silenced as all cells are infected with CAV. Therefore a way to distinguish infected and transfected cells was required. This was

accomplished by co-transfecting the shRNA with pEGFP-N1 (clontech). Any cells that contain GFP are expected to also contain the shRNA. A method to detect CAV infection by flow cytometry has recently been elucidated by the use of a monoclonal antibody targeting VP3 and a secondary anti-mouse antibody conjugated with APC (personnel communication). Therefore both GFP transfected and infected cells can be distinguished by flow cytometry by acquiring fluorescence from two different flourophores (GFP and APC; Fig 2.17). If a higher APC mean fluorescence is detected in GFP positive cells it indicates that more of those cells have VP3 expression indicating those cells have a productive CAV infection and therefore CAV is not silenced. Alternatively a lower APC mean fluorescence detected in GFP positive cells indicates VP3 expression is less and CAV protein expression is inhibited.

When this assay was used with pCAV-MW and the three individual shRNAs contained within, a decrease in virus protein expression was observed with all of them (Fig 2.18). Interestingly the most active was shRNA VP2/3-3 with a 60% knockdown of CAV VP3 expression, this was not the most active in the GFP-fusion reporter system. The MW was again slightly less active than the best single shRNA, however it still showed over 50% knockdown. All results are shown as a percentage of the non-silencing shRNA negative control.

Discussion

CAV is a major problem for the world-wide poultry industry due to its ability to produce sub-clinical infection and immunosuppression in vaccinated mature birds. The use of RNA interference to silence virus replication has been shown to be highly effective against many human and animal viruses both *in vitro* and *in vivo*. This study demonstrates the ability to silence CAV protein expression up to 60% in MDCC-MSB1 cells with single shRNAs targeting various regions of the genome. However, the use of one shRNA particularly against highly variable RNA viruses such as poliovirus and HIV, has allowed for the production of escape mutants. Whilst this may not be such a significant problem in the highly conserved CAV DNA genome, expression of multiple shRNAs would be more appropriate.

The best method for expressing multiple shRNAs has not been shown. Several methods have been explored including expression from multiple cassettes using the same promoter, from single vectors containing multiple promoters or as extended or long hairpin RNAs containing two siRNA sequences. This study used a plasmid construct directing the expression of three shRNA molecules from three different chicken U6 promoters. These promoters are the most active in chicken cells and therefore appropriate for use against an avian pathogen (Wise et al. 2007). The vector was shown to reduce viral protein by 50%, this is a significant decrease as the virus dose in this assay would be considerably higher than a normal environmental exposure. Interestingly expression of the three different shRNAs

from pCAV-MW showed less protection than the most active individual shRNA alone, this has been observed previously when multiple siRNAs or shRNAs are used. This may be due to pCAV-MW containing a less active shRNA molecule against the virus decreasing the overall effect. It may also be possible that the order of either the promoters, as ChU6#1 is weaker than ChU6#4 (Wise et al. 2007),or the shRNAs may have an impact. More work is required on the best delivery vector for multiple shRNAs.

This is the first time a method to detect both infected and transfected MDCC-MSB1 cells by flow cytometry has been reported. This method will be useful in the future. It is also the first study that demonstrates silencing of CAV expression is possible with single or multiple shRNA molecules. As the CAV genome sequence is highly conserved amongst strains, developing shRNAs targeting this virus should be highly efficient at cross-strain protection. It will now be interesting to determine whether these shRNAs are able to protect chickens from CAV infection.

2.2.3 Outcome - Generation of IP and potential commercial products identified via proof-of-concept.

2.2.3.1 Patents

We have two key patent applications that have resulted from our RNAi *in ovo* modulation work. The first patent is quite broad and covers claims for a range of production traits including health, muscle and sex determination. The second patent is focused on modulation of sex determination. This has become the major emphasis of our RNAi work with the greatest potential for a commercial application.

Commonwealth Scientific and Industrial Research Organisation, Australian Poultry CRC Pty Ltd
International Patent Application PCT/AU2008/000835
Entitled: "Modulating production traits in avians"

Status: National / Regional phase in selected juristictions.

Commonwealth Scientific and Industrial Research Organisation, Australian Poultry CRC Pty Ltd
United States of America Provisional Patent Application 61/138235
Entitled: "Methods of modulating the sex of avians"

Status: Full patent filed 17th December 2009

2.2.3.2 Extension to Project 03-16b: Application of genomics-based technology for the development of new health products.

In 2009, Supplemental funding was provided to 03-16b in order to provide additional data supporting the patent applications.

A major outcome of the research in this project is to now apply RNA interference (RNAi) technology to modulate production traits in poultry. We are of the firm opinion that *in ovo* modulation of production traits will happen and will be of commercial value. The production traits that we are targeting include health, muscling and sex determination. Work so far undertaken in this project has lead to the two joint Poultry CRC and CSIRO International Patent Applications detailed above.

Discussion with industry has highlighted modulation of sex determination as the major area of focus for this research (in particular, driving males to females by targeting the *DMRT1* gene). There are two clear commercial paths to impact. The first is with the breeding companies - they cull male chicks at hatch and this is both an economic and welfare issue that they would like to see improved. Even slightly increasing the ratio of females to males is of importance and value to the breeders. The second path is with the vaccine companies. Pfizer has invested into embrex technology and would like to get better uptake of embrex delivered vaccines (including new and innovative vaccines) into the layer industry. Their big problem at the moment is that the industry is reluctant to adopt the embrex platform because 50% of the injected eggs are male and are therefore culled at hatch. Pfizer are extremely interested in an approach to sway the sex ratio to females. As an added bonus, we are currently working towards embrex delivery of our RNAi molecule and Pfizer are extremely keen to develop more embrex deliverable products.

In September 2008, PCT/AU2008/000835 was subjected to an International Search Report and Written Opinion. Based on the result of this report and subsequent discussions with the Poultry CRC, CSIRO and the patent firm FB Rice & Co, it was decided that the *in ovo* delivery of our RNAi molecules should be narrowed to neat double stranded RNA (dsRNA) formulations. Such formulations are compatible with embrex injection directly to eggs. Prior art already exists for other delivery options (e.g. viral vectors) that we were already using to generate data for the filed patent. As a result of this decision we submitted a new patent entitled “Modifying chicken sex”. The application was filed on the 17th of December 2008. We undertook this extension project to employ a technician for 12 months to help us obtain vital new data and file the full patent application. The specific extra

work that was approved and carried out was to: (i) Develop neat dsRNA formulations for direct injection into embryonated eggs; (ii) Assess gene knockdown of targeted sex determination genes in embryos, in particular the dmrt1 gene; and (iii) Prepare data for inclusion in full patent application.

Results

In ovo modulation of DMRT1 gene expression in chickens

An siRNA targeted to a conserved exon of the chicken *DMRT1* gene was designed using the Ambion siRNA Target Finder tool (www.ambion.com). The chosen siRNA was designated *DMRT1*-343-siRNA (5'-GAGCCAGUUGUCAAGAAGAUU-3'). The siRNA was synthesized and obtained from Qiagen. All primer and oligo sequences are detailed in Table 2.5.

For *in ovo* delivery, the siRNA was formulated with lipoefectamine 2000 (Invitrogen) according to the manufacturer's instructions. The now complexed siRNA was then delivered *in ovo* at a dose of either 100 pmol or 200 pmol. The siRNA was injected into embryonated eggs via an intravenous (I.V.) route or directly into the amnion at embryonic day 4.5 (E4.5). For both I.V. and amnion delivery, a small opening (1cm x 1cm) was created at the top of the blunt end of the egg so as to avoid the membrane, veins and arteries, and 100 pmol or 200 pmol in a 4 ul volume was then injected directly into a vein or into the amnionic cavity using a micro-capillary pipette. Micro-capillaries of 1 mm diameter were used for injections, and their tips were pulled to a diameter of 40 microns with bevelled tip of 22.5 ^{o}. After injection, the holes in the eggs were sealed with appropriate sized parafilm squares using a heated scalpel blade. In total, 286 embryonated eggs (E4.5) were used in this experiment; Group 1: 48 eggs were used as controls and were not injected with the *DMRT1*-343-siRNA formulation; Group 2: 51 eggs were injected I.V. with 100 pmol of siRNA; Group 3: 53 eggs were injected I.V. with 200 pmol of siRNA; Group 4: 81 eggs were injected into the amnion with 100 pmol of siRNA and; Group 5: 53 eggs were injected into the amnion with 200 pmol of siRNA (Table 2.6). All embryos were incubated until day E10. At E10, all embryos were assessed for viability and then removed from the egg. Control Group 1 had an embryo viability of 100%; Group 2 had a viability of 76%; Group 3 had a viability of 94%; Group 4 had a viability of 40% and; Group 5 had a viability of 75%. A single limb bud from each embryo was removed and used in a sex determination PCR test to determine if the embryos were of male or female genotype. Lower limb buds from each embryo were collected into 50 ul of PCR digestion buffer (50 mM KCl; 10 mM Tris-HCl, pH8.3; 0.1 mg/ml gelatine; 0.45% Nonidet P-40; 0.45% Tween-20; 0.2 mg/ml proteinase K; stock stored at -20^{o}C) at room temperature and digested at 55^{o}C for a minimum of 1 h, then at 95^{o}C for 10 min to release genomic DNA. Sexing was carried out by PCR using the method of Clinton *et al.*, 2001. The PCR mix consisted of 1 ul of digestion mix, 10 X RedTaq reaction buffer (Sigma-Aldrich), $MgCl_2$ to 1.5 mM (Promega), 1 unit of

RedTaq DNA polymerase (Sigma-Aldrich) and Milli-Q water (Millipore) to a total volume of 20 ul. Reactions were carried out in a Master cycler S (Eppendorf) PCR machine. Products were run on a 1.5% 1 X Tris-borate (TBE) agarose gels.

Once the sex PCR test was complete and analysed, the embryos were definitively labelled as either being genotypically male or female. The embryos were then opened via dissection and the gonads exposed for macroscopic analysis of gonadal development. The gonadal development of all control embryos was normal as expected. Control female embryo's showed typical asymmetric development that was characterised by a large left ovary and smaller regressing right gonad. Control male embryos all had typical bilateral testes. All female embryos from the siRNA knockdown groups (Groups 2-5) had normal gonadal development. In contrast, some male embryos from the siRNA knockdown groups showed varying degrees of female-like asymmetry at the macroscopic level of the gonads. The feminisation effect of the *DMRT1*-343-siRNA was characterised by an average or small-sized right testis and a larger feminised left gonad (Table 2.7). Feminisation was observed in a small number of male embryos in Groups 2, 3 and 5 and resulted in an increase in the ratio of embryos with female-like gonads in these groups.

Gonads from both male and female embryos in each treatment groups were assessed for *DMRT1* gene expression using quantitative RT-PCR analysis. Both the female and male gonads were pooled separately from each group and RNA was extracted for cDNA synthesis and qPCR analysis. The pooled gonads were added to 1 ml of Trizol and homogenised well by pipetting and vortexing at room temperature until all gonad tissue had dissolved. 200 ul of chloroform was added and mixed well by inverting the sample for 15 sec. The sample was then incubated at room temperature for 3 min and then centrifuged at 12000 g for 15 min at 4^{o}C. The aqueous phase of the sample was then transferred to a new tube and then 500 ul of isopropanol was added and mixed well by inversion. The mix was then incubated at room temperature for 10 min and then centrifuged at 12000 g for 10 min at 4^{o}C. The supernatant was removed from the tube carefully, so as not to disturb the RNA pellet, and the pellet was then washed with 1 ml of 70% ethanol. The tube was then centrifuged at 7500 g for 5 min at 4^{o}C and the supernatant again was carefully removed and the RNA pellet was air dried at room temperature for 10 min. The RNA pellet was then resuspended in 25 ul of RNase-free water and the final concentration of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). RNA was reverse transcribed to complimentary DNA (cDNA) using the Promega Reverse Transcription kit (Promega). The reaction mix contained 1 ug of RNA, random hexamers (1 ul), dNTPs (2 ul), AMV reverse transcriptase (Promega) (0.5 ul) and nuclease free water added to a total reaction volume of 20 ul. The mix was incubated at 42^{o}C for 1 hour, followed by a 10 min incubation at 95^{o}C for enzyme inactivation.

cDNA was then used to quantify relative *DMRT1* gene expression levels in the pooled male and female gonad samples from each treatment group. qPCR primers and probes were designed using Primer Express (Applied Biosystems) software and sequences are shown in Table 2.6. PCR's were set up in 20 ul reaction volumes that contained 2 X TaqMan qRT PCR mastermix (Applied Biosystems), 1 ul of primer/probe mix, 1 ul of cDNA sample and made up to final volume with Nuclease free water (Promega). PCR cycling was performed at 95^{o}C for 1 min, followed by 40 cycles of 95^{o}C for 15 sec; 61^{o}C for 30 sec and; 68^{o}C for 30 sec. Ct values were obtained at a standard threshold value of 0.2 for all reactions. This threshold value corresponded to the midway point of the logarithmic phase of all amplification plots. Ct values were exported to Microsoft Excel for analysing relative gene expression using the comparative Ct method. Relative levels of *DMRT1* mRNA were compared with the chicken house keeping 18S rRNA species across all cDNA samples (Figure 2.26). Quantitative RT-PCR analysis confirmed that *DMRT1* mRNA expression was specifically reduced in all pooled groups of male embryos when compare to control Group 1. Almost 40% of *DMRT1* gene expression knockdown was observed for Group 3 male embryos treated with the *DMRT1*-343-siRNA. It is interesting to note that Group 3 was also the group that resulted in the greatest degree of observed feminisation of male gonads at the macroscopic level.

2.3 References

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2.4 Tables

Table 2.1 Synthesized oligonucleotides used in this study

Name	Sequence	Location/Feature
TD135	5’- CGAAGAACCGAGCGCTGC -3’	cU6-1
TD139	5’- TATGGAACGCTTCACGAA -3’	Human U6 snRNA
TD152	5’- AGTGGAACGGAGCCTGGAGA -3’	cU6-2
TD174	5’-CGCCAAATCCATCGCTGCTC -3’	cU6-2
TD176	5’- CAGACAGACGTCAGGCTTTC -3’	cU6-3
TD72	5’- TTTTAGTATATGTGCTGCCG -3’	Human U6 snRNA
TD175	5’- GAATTGTGGGACGGCGGAAG -3’	cU6-4
TD148	5’- CTCGAGTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAA GATGAACTTCAGGGTCAGCGAATATCTCTACCTCCTAGG -3’	cU6-1-shEGFP
TD143	5’- CTCGAGTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAA GATGAACTTCAGGGTCAGCGAATACCGCTTCCTCCTGAG -3’	cU6-1v-shEGFP
TD196	5’- CTCGAGTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAA GATGAACTTCAGGGTCAGCGACTAAGAGCATCGAGACTG -3’	cU6-3-shEGFP
TD195	5’- CTCGAGTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAA GATGAACTTCAGGGTCAGCAAACCCCAGTGTCTCTCGGA -3’	cU6-4-shEGFP
TD149	5’- CTCGAGTTCCAAAAAAATAAGTCGCAGCAGTACAATCTCTTGAA TTGTACTGCTGCGACTTATGAATACCGCTTCCTCCTGAG -3’	cU6-1-shNS
M13	5’- GTTTTCCCAGTCACGAC -3’	Universal Forward primer
TD134	5’- CTCGAGTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAA GATGAACTTCAGGGTCAGCCAAACAAGGCTTTTCTCCAA -3’	mU6/shEGFP
LL91	5’-rGrAUrGrArArCUUrCrArGrGrGUrCrArGrC-3’	EGFP probe

‘r’ prefix to G, A and C represents ribonucleotide, and U indicates ribo-uridine.

Table 2.2 Forward shRNA oligonuleotide sequences.

Primer Name	Primer sequence
NP-OL	GGATCTTATTTCTTCGGAGTTCAAGAGACTCCGAAGAAATAAGATCC**TTTTTTGGAA**GGATCC
NP-mir17	GGATCTTATTTCTTCGGAGGATATATAGACTCCGAAGAAATAAGATCC**TTTTTTGGAA**GGATCC
NP-ggamir30a	GGATCTTATTTCTTCGGAGCTGTGAAGCAGCAGATGGGGCTCCGAAGAAATAAGATCC**TTTTTTGGAA**GGATCC
NP-hspmir30a	GGATCTTATTTCTTCGGAGCTGTGAAGCCACAGATGGGCTCCGAAGAAATAAGATCC**TTTTTTGGAA**GGATCC
CAVVP2/3-1-OL	*ATTC*GGAATTACAGTCACTCTATTTCAAGAGAATAGAGTGACTGTAATTCC**TTTTTTGGAA**
CAVVP2/3-1-mir17	*ATTC*GGAATTACAGTCACTCTATGATATATAGAATAGAGTGACTGTAATTCC**TTTTTTGGAA**
CAVVP2/3-1-ggamir30a	*ATTC*GGAATTACAGTCACTCTATCTGTGAAGCAGCAGATGGGGATAGAGTGACTGTAATTCC**TTTTTTGGAA**
CAVVP2/3-1-hspmir30a	*ATTC*GGAATTACAGTCACTCTACTGTGAAGCCACAGATGGGATAGAGTGACTGTAATTCC**TTTTTTGGAA**
CAVVP2/3-3-OL	GAAGGTGTATAAGACTGTATTCAAGAGATACAGTCTTATACACCTTC**TTTTTTGGAA**
CAVVP2/3-3-mir17	GAAGGTGTATAAGACTGTAGATATATAGATACAGTCTTATACACCTTC**TTTTTTGGAA**
CAVVP2/3-3-ggamir30a	GAAGGTGTATAAGACTGTACTGTGAAGCAGCAGATGGGGTACAGTCTTATACACCTTC**TTTTTTGGAA**
CAVVP2/3-3-hspmir30a	GAAGGTGTATAAGACTGTACTGTGAAGCCACAGATGGGTACAGTCTTATACACCTTC**TTTTTTGGAA**
shGFP	GGTGATGCTACATACGGAATTCAAGAGATTCCGTATGTAGCATCACC**TTTTTTGGAA**

[a] Letters in bold indicate PolIII promoter termination signal

Table 2.3 Oligonucleotides used to produce Chicken U6 promoter vectors

Primer Name	Primer sequence
ChU6#4F	TTTGCATGCGTACCTCCTTCTCGCAG
ChU6#4R	TTT**GTCGAC**AT**AAGCTT**AT**GTTTAAAC**CCCAGTGTCTCTCG
ChU6#1F	CATGCATGCAAACGCTAAGCAGGCACCTAAAG
ChU6#1R	TTT**CATATG**AT**ACTAGT**AT**ACCTGCGCAT**GAATATCTCTACCTCCTAGGCGG
ChU6#MP1F	TTTGCATGCGTACCTCCTTCTCGCAG
ChU6#MP1R	TTT**GTCGAC**AT**AAGCTT**AT**GTTTAAAC**CCCAGTGTCTCTCG
ChU6#MP2F	TATGTCGACAAACTCCAGGAGGTGCATGTTTG
ChU6#MP2R	TTT**CTCGAG**AT**GAATTC**AT***CCATGG*GACTAAGAGCATCGAGAC
ChU6#MP3F	CATCTCGAGAAACGCTAAGCAGGCACCTAAAG
ChU6#MP3R	TTT**CATATG**AT**ACTAGT**AT**ACCTGCGCAT**GAATATCTCTACCTCCTAGGCGG

[a] Letters in bold indicate restriction sites for ligation of shRNA annealed oligonucleotides
[b] Letters underlined indicate restriction sites for cloning promoter vectors
[c] Letters in italics indicate plasmid requires nuclease treatment after digestion

Table 2.4 Oligonucleotides used to produce shRNA expression vectors

Primer Name	Primer sequence
VP2/3-1F	*ATTC*GGAATTACAGTCACTCTATTTCAAGAGAATAGAGTGACTGTAATTCC**TTTTTTGGAA**
VP2/3-1R	*TA***TTCCAAAAAA**GGAATTACAGTCACTCTATTCTCTTGAAATAGAGTGACTGTAATTCC
VP2/3-2F	CAACTGCGGACAATTCAGATTCAAGAGATCTGAATTGTCCGCAGTTG**TTTTTTGGAA**
VP2/3-2R	*TCGA***TTCCAAAAAA**CAACTGCGGACAATTCAGATCTCTTGAATCTGAATTGTCCGCAGTTG
VP2/3-3F	GAAGGTGTATAAGACTGTATTCAAGAGATACAGTCTTATACACCTTC**TTTTTTGGAA**
VP2/3-3R	*TCGA***TTCCAAAAAA**GAAGGTGTATAAGACTGTATCTCTTGAATACAGTCTTATACACCTTC
VP1/2-1F	CAAGCGACTTCGACGAAGATTCAAGAGATCTTCGTCGAAGTCGCTTG**TTTTTTGGAA**
VP1/2-1R	*TCGA***TTCCAAAAAA**CAAGCGACTTCGACGAAGATCTCTTGAATCTTCGTCGAAGTCGCTTG
VP1-1F	*ATTC*GAAGGACTCATTCTACCTATTCAAGAGATAGGTAGAATGAGTCCTTC**TTTTTTGGAA**
VP1-1R	*TA***TTCCAAAAAA**GAAGGACTCATTCTACCTATCTCTTGAATAGGTAGAATGAGTCCTTC
VP1-2F	CATCAATGAACCTGACATATTCAAGAGATATGTCAGGTTCATTGATG**TTTTTTGGAA**
VP1-2R	*TCGA***TTCCAAAAAA**CATCAATGAACCTGACATATCTCTTGAATATGTCAGGTTCATTGATG
NS-F	
NS-R	

[a] Letters in bold indicate PollIII promoter termination signal
[b] Letters in italics indicate retriction site overhangs for ligation
[c] Letters underlined indicate loop sequence

Table 2.5 Primer List

Primer Name	Sequence (5'-3')
DMRT1-F	GAATTCATGCCCGGTGACTCC
DMRT1-R	AAGCTTCTACTCGCCCTCGAG
DMRT1-208T	GACTGCCAGTGCAAGAAGTTTCAAGAGAACTTCTTGCACTGGCAGTCTTTTTGGAAGGATCC
DMRT1-208B	TCGAGGATCCTTCCAAAAAGACTGCCAGTGCAAGAAGTTCTCTTGAAACTTCTTGCACTGGCAGTC
DMRT1-343T	GAGCCAGTTGTCAAGAAGATTCAAGAGATCTTCTTGACAACTGGCTCTTTTTGGAAGGATCC
DMRT1-343B	TCGAGGATCCTTCCAAAAAGAGCCAGTTGTCAAGAAGATCTCTTGAATCTTCTTGACAACTGGCTC
DMRT1-568T	CTGTATCCTTACTATAACATTCAAGAGATGTTATAGTAAGGATACAGTTTTTGGAAGGATCC
DMRT1-568B	TCGAGGATCCTTCCAAAAACTGTATCCTTACTATAACATCTCTTGAATGTTATAGTAAGGATACAG
DMRT1-694T	CTCCCAGCAACATACATGTTTCAAGAGAACATGTATGTTGCTGGGAGTTTTTGGAAGGATCC
DMRT1-694B	TCGAGGATCCTTCCAAAAACTCCCAGCAACATACATGTTCTCTTGAAACATGTATGTTGCTGGGAG
DMRT1 RNA probe	UrCU UrCU UrGrA rCrArA rCUrG rGrCU rC
pSlax-linkerT	GATCCGATATCGCGGCCGCAGATCTCTCGAGCTGCA
pSlax-linkerB	GCTCGAGAGATCTGCGGCCGCGATATCG
pEGFP-linkerT	GGCCGCAGATCTGAATTCCTGCAGGATATCAAGCTTGGATCCT
pEGFP-linkerB	CTAGAGGATCCAAGCTTGATATCCTGCAGGAATTCAGATCTGC
qDMRT1-F	TCAAGCCAGTCAGGAAAACAGT
qDMRT1-R	TCATGGCATGGCGGTTCT
qDMRT1-probe	FAM-CCATCCCTTTCATCTGCC-NFQ

'r' prefix to G, A and C represents ribonucleotide, and U indicates ribo-uridine

Table 2.6. Primer and Probe sequences

Sequence Name	Sequence 5' – 3'
DMRT1- 343-siRNA	GAGCCAGUUGUCAAGAAGAUU
DMRT1 TaqMan MGB probe	CCATCCCTTTCATCTGCC
DMRT1 Forward primer	TCAAGCCAGTCAGGAAAACAGT
DMRT1 Reverse primer	TCATGGCATGGCGGTTCT
18S rRNA TaqMan MGB probe	TGCTGGCACCAGACTTGCCCTC
18S rRNA Forward primer	CGGCTACCACATCCAAGGAA
18S rRNA Reverse primer	GCTGGAATTACCGCGGCT

Table 2.7. *DMRT1* embryo injection results

siRNA dose and injection route	No. embryos injected	No. viable at E10	% Male :Female Genotype (PCR sex test)	% Male:Female Macroscopic Gonad phenotype
Group 1 No injection control	48	48 (100%)	60:40	60:40
Group 2 I.V. – 100 pmol	51	39 (76%)	59:41	54:46
Group 3 I.V. – 200 pmol	53	50 (94%)	54:46	46:54
Group 4 Amnion – 100 pmol	81	33 (40%)	48:52	48:52
Group 5 Amnion – 200 pmol	53	40 (75%)	33:67	28:72

2.5 Figures

	OCT		PSE	TATA	
Consensus	ATTTGCAT		STSACCGTGWSTGTRAAR$_{(0-3)}$TG		
Mouse U6	-235 ATTTGCAT -228		-74 CTCACCcTaACTGTAAAGTa	----------TATAAATAT	-27
cU6-1	-207 ATTTGCAT -200		-65 CTtgCCcTaTCctTGAgGTTTc	----------TATAAATA	-24
cU6-1v	-207 ATTTGCAT -200		-65 CTtgCCcTaTCctTAAgGTTTc	----------TATAAATA	-24
cU6-3	-194 ATTTGggC -187		-66 CTCACCGctACTtaAAAATCATG	----------TTAAATA	-24
cU6-4	-232 ATTTGCAT -225		-66 GTCACtGTGTtctaAAAGAAcTTG	----------TTTAAATA	-24

Figure 2.1 Promoter element sequences of the mouse U6, cU6-1, cU6-1v, cU6-3 and cU6-4 snRNA promoters. The distal promoter region containing the OCT sequence and the proximal promoter region containing the PSE and TATA elements sequence are shown for each promoter. Matches to the consensus sequence delineated at the top of the OCT, PSE and TATA sequences are shown in upper case (PSE consensus is from Dahlberg & Lund 1988, and the OCT consensus is from Sturm *et al*. 1988). Each dash mark between the PSE and TATA represent one nucleotide.

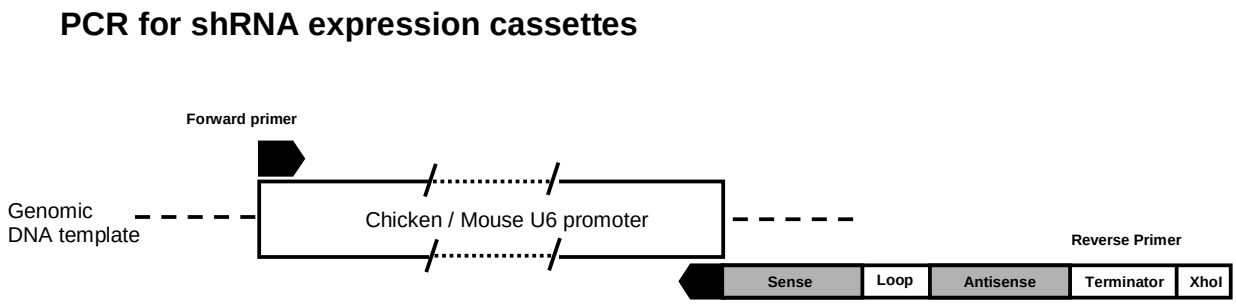


Figure 2.2 Schematic representation of the PCR strategy used to produce shRNA expression vectors. PCR used forward primers paired with reverse primers comprising all shRNA components. All final PCR products consisted of a chicken or mouse U6 promoter, shRNA sense, loop, shRNA antisense, termination sequence and *Xho*I site.

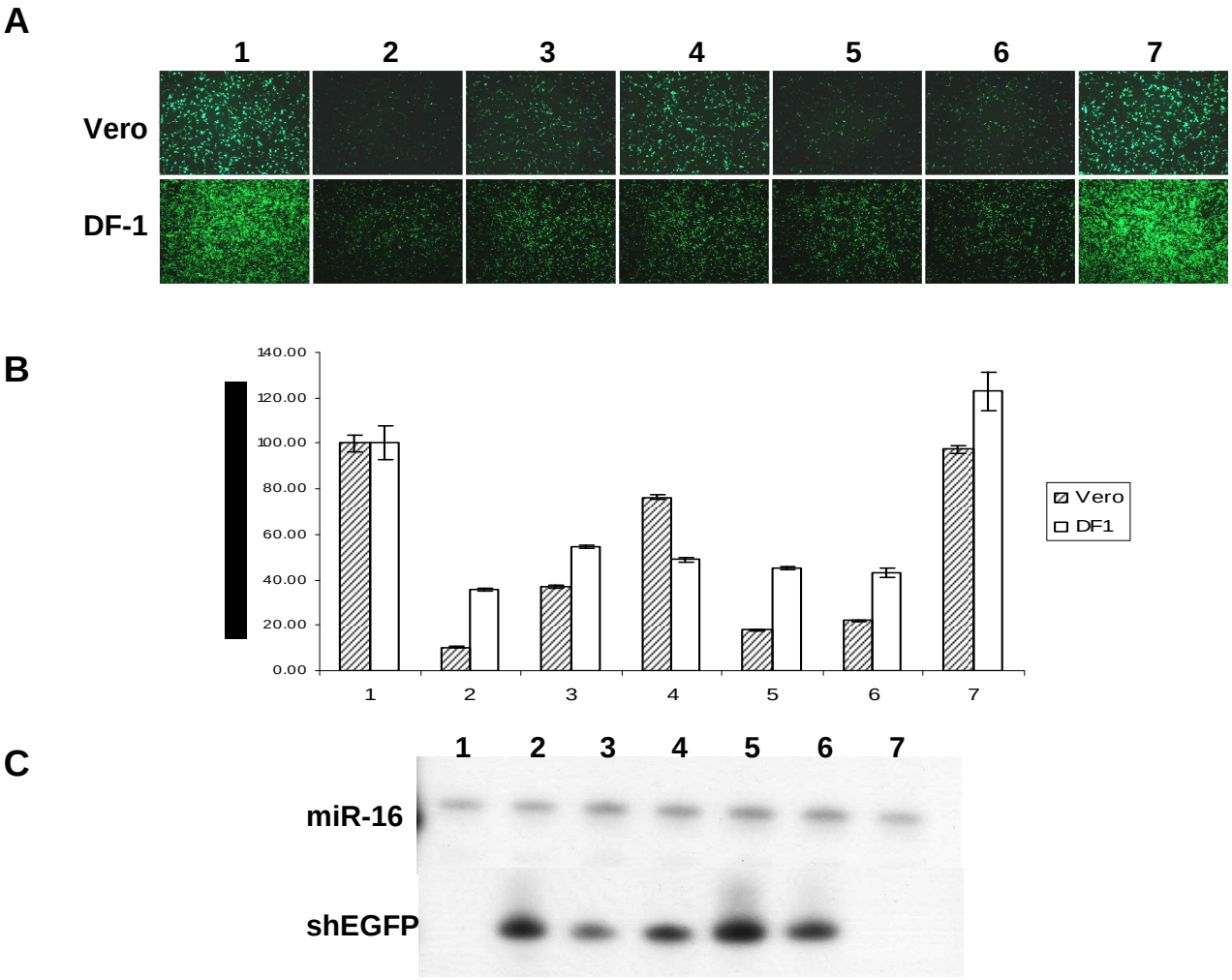
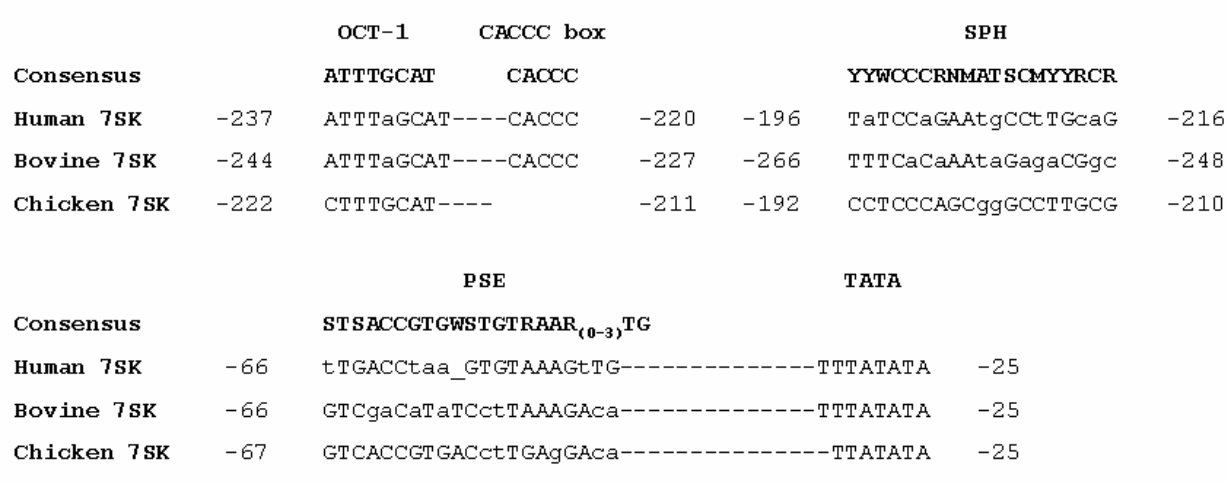


Figure 2.3 Inhibition of EGFP expression and detection of expressed shRNA from chicken and mouse U6 promoters. (1) cotransfection of pEGFP-N1 and pcU6-1v-irrshRNA; (2) cotransfection of pEGFP-N1 and pmU6-shEGFP; (3) cotransfection of pEGFP-N1 and pcU6-1v-shRNA; (4) cotransfection of pEGFP-N1 and pcU6-1-shRNA; (5) cotransfection of pEGFP-N1 and pcU6-3-shRNA; (6) cotransfection of pEGFP-N1 and pcU6-4-shRNA; (7) transfection of pEGFP-N1. (A): Fluorescence images of Vero and DF-1 cells transfected with the EGFP/shRNA vectors (Magnification 50x). (B): Mean fluorescence intensity for each transfection condition expressed relative to pcU6-1v-irrshRNA. Error bars indicate standard error calculated on each individual experiment completed in triplicate. (C): Detection of expressed shRNAs targeting EGFP (shEGFP) from Vero cell extracts.



		OCT-1	CACCC box			SPH	
Consensus		**ATTTGCAT**	**CACCC**			**YYWCCCRNMATSCMYYRCR**	
Human 7SK	-237	ATTTaGCAT----CACCC		-220	-196	TaTCCaGAAtgCCtTGcaG	-216
Bovine 7SK	-244	ATTTaGCAT----CACCC		-227	-266	TTTCaCaAAtaGagaCGgc	-248
Chicken 7SK	-222	CTTTGCAT----		-211	-192	CCTCCCAGCggGCCTTGCG	-210

		PSE	TATA	
Consensus		**STSACCGTGWSTGTRAAR$_{(0-3)}$TG**		
Human 7SK	-66	tTGACCtaa_GTGTAAAGtTG----------TTTATATA		-25
Bovine 7SK	-66	GTCgaCaTaTCctTAAAGAca----------TTTATATA		-25
Chicken 7SK	-67	GTCACCGTGACctTGAgGAca----------TTATATA		-25

Figure 2.4 Promoter element sequence alignment of chicken, bovine and human 7SK promoters.

The enhancer (DSE) of the chicken 7SK promoter contains OCT-1 and SPH motifs but no CACCC box. The basal promoter region features a PSE and TATA box with homology to consensus. Matches to the defined consensus sequences indicated at the top of the OCT-1 [21], SPH [12], PSE [20] and TATA sequences are shown in upper case. Nucleotide positions indicate the location (5' → 3') of each element in the promoter relative to the transcription start site (+1). Each dash mark between the OCT-1 and CACCC box and PSE and TATA box represents one nucleotide. The underscore in the human 7SK PSE indicates a shift in the sequence by one nucleotide for alignment. Nucleotide abbreviations in consensus sequences are according to the International Union of Biochemistry convention for GENBANK.

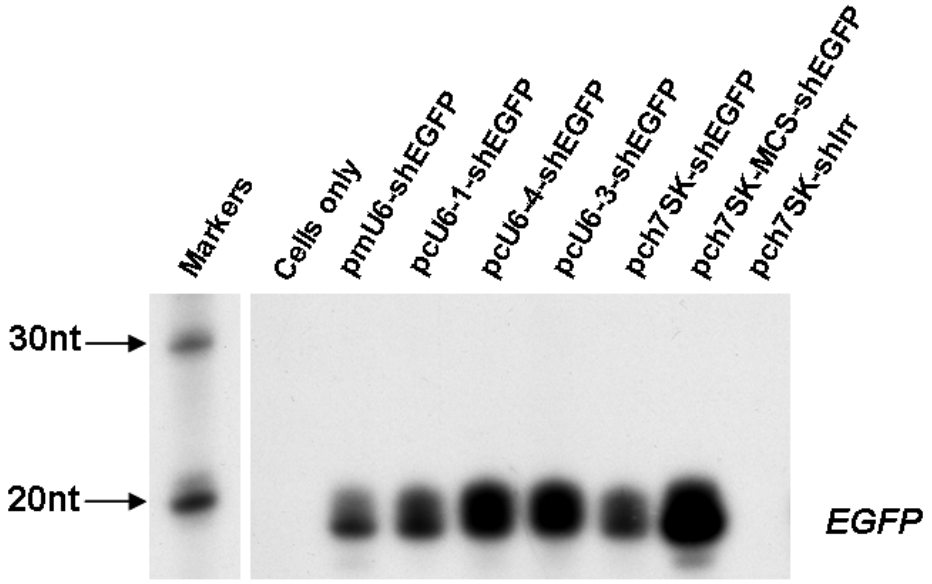


Figure 2.5 Detection of shEGFP expression from ch7SK-shEGFP expression constructs in DF-1 cells.

DF-1 cells were transfected with shEGFP expression constructs as indicated above each lane. RNA samples were probed in solution with ^{32}P-labelled shEGFP-specific LL91 RNA probe [14] and treated with RNAse A/T1. Protected shEGFP fragments were distinguished by comparison to RNA size markers (Decade™, Ambion).

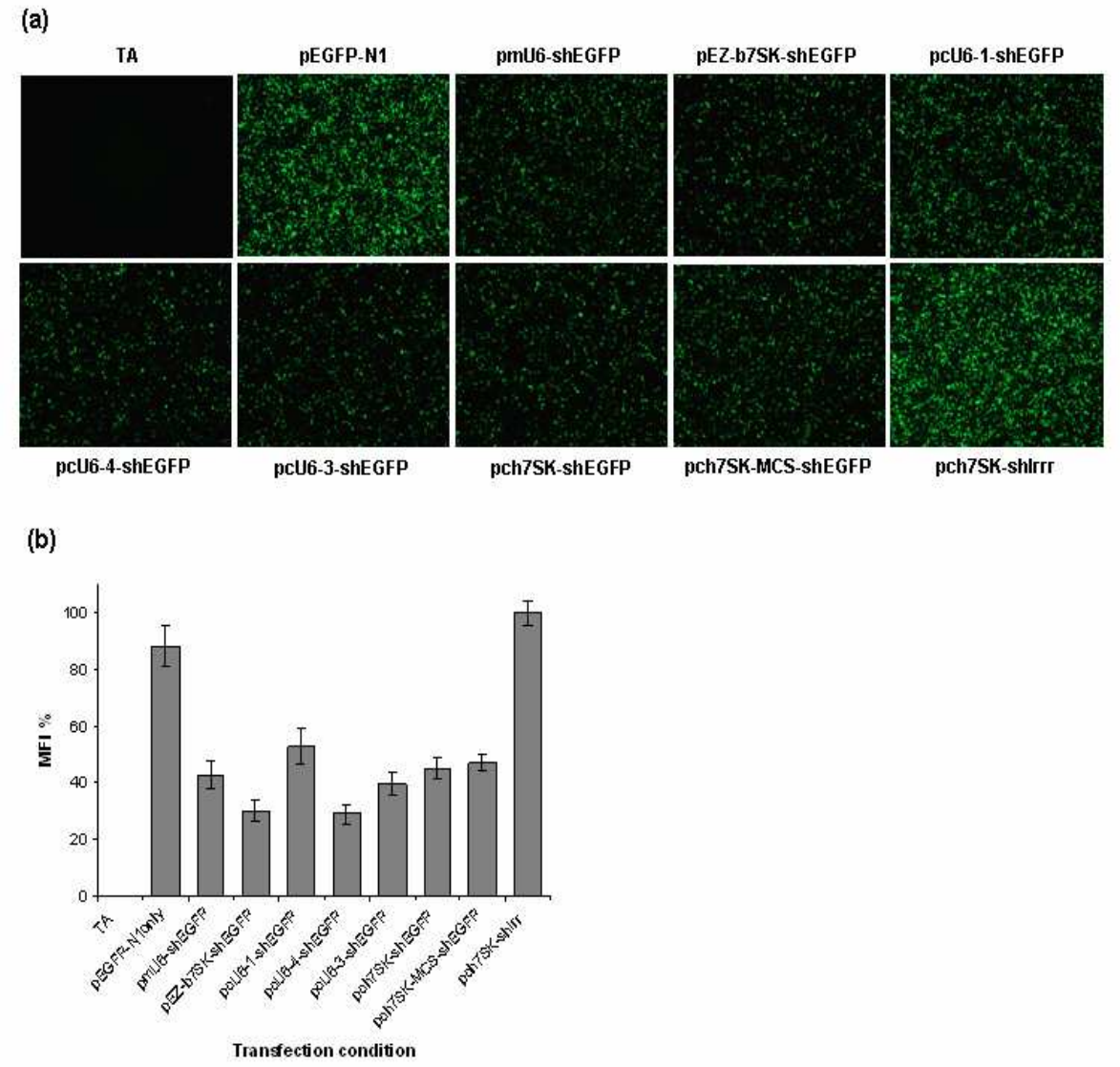


Figure 2.6 EGFP knockdown conferred by chicken 7SK and U6 promoters in DF-1 cells.

(a) Fluorescence microscopy images of DF-1 cells transfected with pEGFP-N1 only, or co-transfected with pEGFP-N1 and various shEGFP expression plasmids as indicated for each image. TA is transfection reagent-only control (no-plasmid DNA). Images presented are representative of results from three independent experiments at 60 hours post-transfection (Magnification 50x). (b) Flow cytometry results for EGFP knockdown assays in co-transfected DF-1 cells. shEGFP expression constructs co-transfected with pEGFP-N1 are indicated on the x axis. EGFP knockdown was measured as % mean fluorescence intensity (MFI %), normalised to the average MFI of the negative control pch7SK-shIrr cells (100%). Error bars represent standard error of the mean (SEM) calculated from three independent experiments. Where no bars are visible the MFI and or SEM is less than 1%.

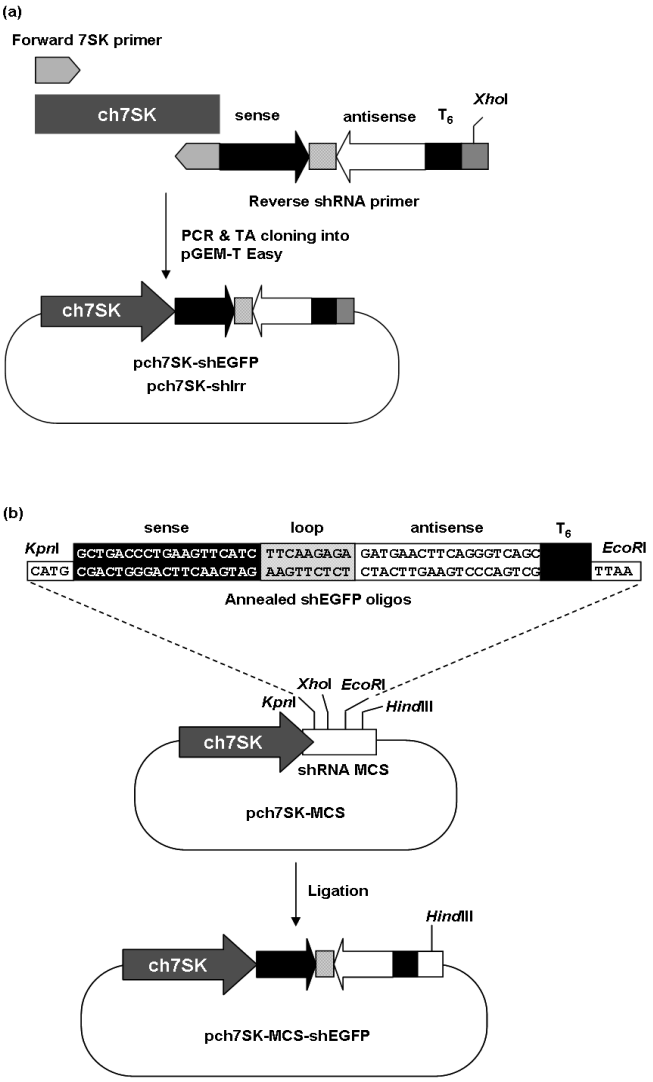


Figure 2.7 Construction of ch7SK-shEGFP expression vectors.

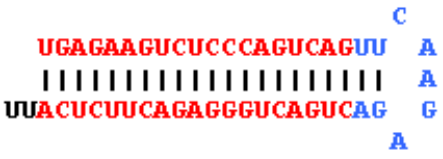
(a) pch7SK-shEGFP and pch7SK-shIrr vectors were engineered using one-step PCR. Expression cassettes were amplified from cloned ch7SK promoter template using a forward primer (right-pointing grey arrow), to the cloned ch7SK promoter sequence (blue) and reverse primers (left-pointing grey arrow) overlapping the last 20bp of the promoter. The reverse primers also encoded the sense (black), loop [5] (light grey) and antisense (white) shRNA sequences, pol III terminator (Black, T_6) and *Xho*I recognition sequence. (b) Construction of the pch7SK-MCS-shEGFP vector used an annealed oligonucleotide (oligo) cloning approach [8]. Complementary DNA oligos featuring the sense (black), loop [5] (grey) antisense (white), pol III terminator (T_6), and *Kpn*I and *Eco*RI overhangs were annealed and ligated *Kpn*I/*Eco*RI into the 3' multi-cloning site (MCS) of the pch7SK-MCS vector which contained a 315bp synthesised copy of the ch7SK promoter sequence.

		OCT-1			SPH	
Consensus		**ATTTGCAT**			**YYWCCCRNMATSCMYYRCR**	
Chicken U4B	-218	cTTTGCAT	-211	-206	CTTCCCAGCATGCCTCGCG	-216
Chicken 7SK	-222	cTTTGCAT	-215	-192	CCTCCCAGCggGCCTTGCG	-210

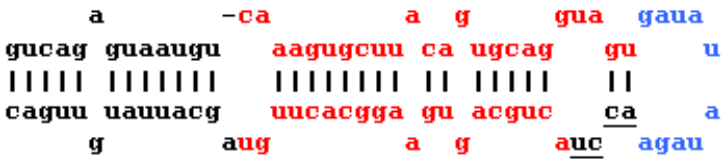
Figure 2.8 Alignment of the enhancer regions of the chicken 7SK and U4B promoters.

Nucleotide positions of the OCT-1 and SPH element for the ch7SK and cU4B [27] promoters are given relative to the transcription start site (+1). The underscore indicates a shift in nucleotides for sequence alignment. Conserved nucleotide sequences are defined in upper-case.

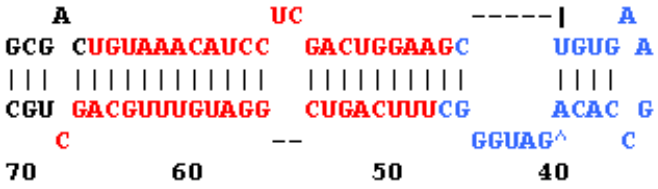
psilencer loop sequence –
Brümmelkamp *and al.* • Science 296, 550-553 ΔG -38.9



Gallus gallus mir-17 ΔG -33.0



Homo sapien mir-30a ΔG -37.3



Gallus gallus mir-30a ΔG -36.0

Figure 2.9. Schematic representation of influenza H1 NP targeting shRNAs with different microRNA loops A) Native pre-miRNAs. The red letters are the siRNA sequences, black letters indicate extra miRA stem sequences, blue letters indicate the loop sequence used and underlined letters indicate loop bases not present in the shRNA constructs. B) NP targeting shRNAs with microRNA loops predicted through mFOLD. The red letters are the siRNA sequences and the blue letters indicate the loop sequences used.

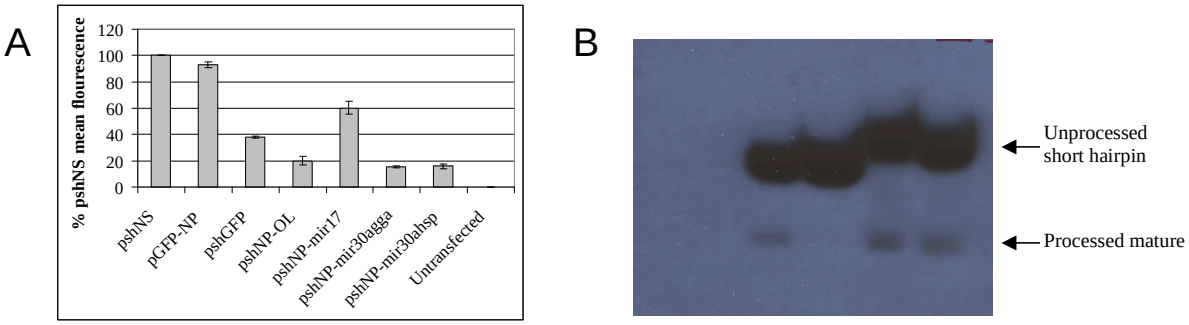


Figure 2.10a. Silencing of EGFP-NP fusion mRNA by shRNAs in DF1 cells**.** DF1 cells were co-transfected with 1 μg of the relevant vectors for 72 h. Column 1; pshNS control, column 2; pEGFP-NP alone, column 3; pshGFP control, column 4; pshNP-OL, column 5; shNP-mir17, column 6; shNP-mir30agga, column 7; shNP-mir30ahsp, column 8; untransfected. Columns 1 and 3-7 were co-transfected with 1μg of pEGFP-NP. Cells were then assayed by flow cytometry and analysed in Microsoft Excel. Values are shown as percentages of the negative control shRNA (shNS), as the mean of three separate experiments in duplicate ± standard deviation.

Figure 2.10b. Verification of loop expression by Northern Blot**.** Northern Blot of NP targeted shRNA molecules. Lane 1; uninfected cells: Lane 2; pshNS control: Lane 3; pshNP-OL: Lane 4; pshNP-mir17: Lane 5; pshNP-mir30agga: Lane 6; pshNP-mir30ahsp.

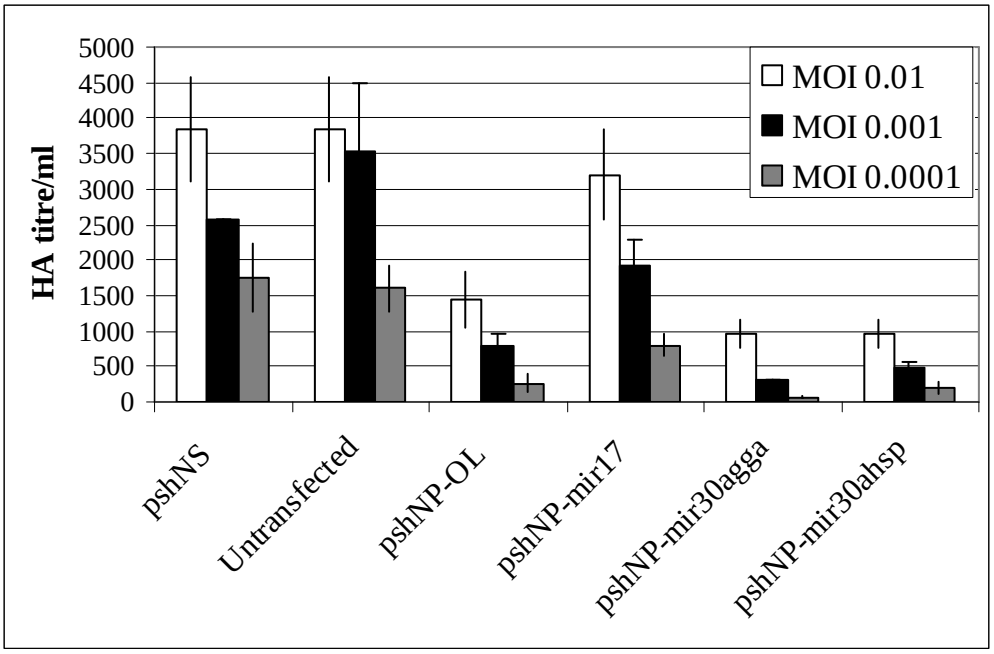


Figure 2.11. Silencing of influenza A PR8 by shRNAs in MDCK cells**.** MDCK were electroporated with 2.5 μg of DNA in nucleofector solution T with Amaxa program T20. Column 1; pshNS control, column 2; untransfected, column 3; pshNP-OL, column 4; shNP-mir17, column 5; shNP-mir30agga, column 6; shNP-mir30ahsp. Transfected cells were incubated for 24hrs then infected with influenza A PR8 virus for 48 h. Supernatants were assayed for influenza A virus by HA assay. Graph depicts two separate experiments in duplicate ± SEM.

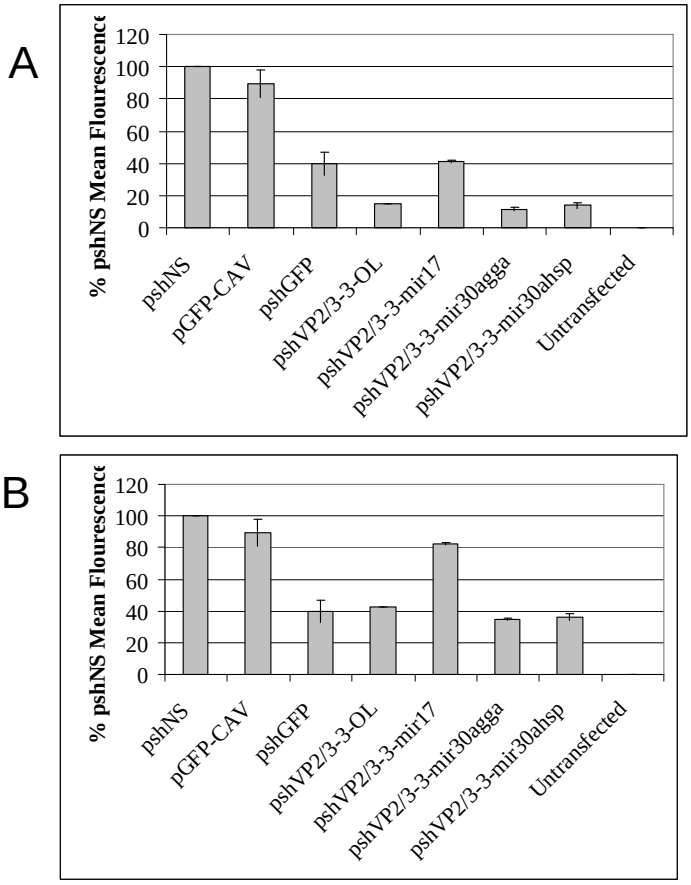


Figure 2.12. Silencing of targeted EGFP-CAV fusion mRNA by shRNAs in DF1 cells. **A.** DF1 cells were co-transfected with 1 μg of the relevant vectors for 72 h or 96 h. Column 1; pshNS control, column 2; pEGFP-CAV alone, column 3; pshGFP control, column 4; pshVP2/3-1-OL, column 5; pshVP2/3-1mir17, column 6; pshVP2/3-1mir30agga, column 7; pshVP2/3-1mir30ahsp, column 8; untransfected. Columns 1 and 3-7 were co- transfected with 1 μg of pEGFP-NP. **B.** Column 1; pshNS control, column 2; pEGFP-NP alone: column 3; pshGFP control, column 4; pshVP2/3-3-OL, column 5; pshVP2/3-3mir17, column 6; pshVP2/3-3mir30agga, column 7; pshVP2/3-3mir30ahsp, column 8; untransfected. Columns 1 and 3-7 were co- transfected with 1 μg of pEGFP-NP. Cells were then assayed by flow cytometry and analysed in Microsoft Excel. Values are shown as percentages of the non-silencing control shRNA (shNS), as the mean of three separate experiments in duplicate ± standard deviation.

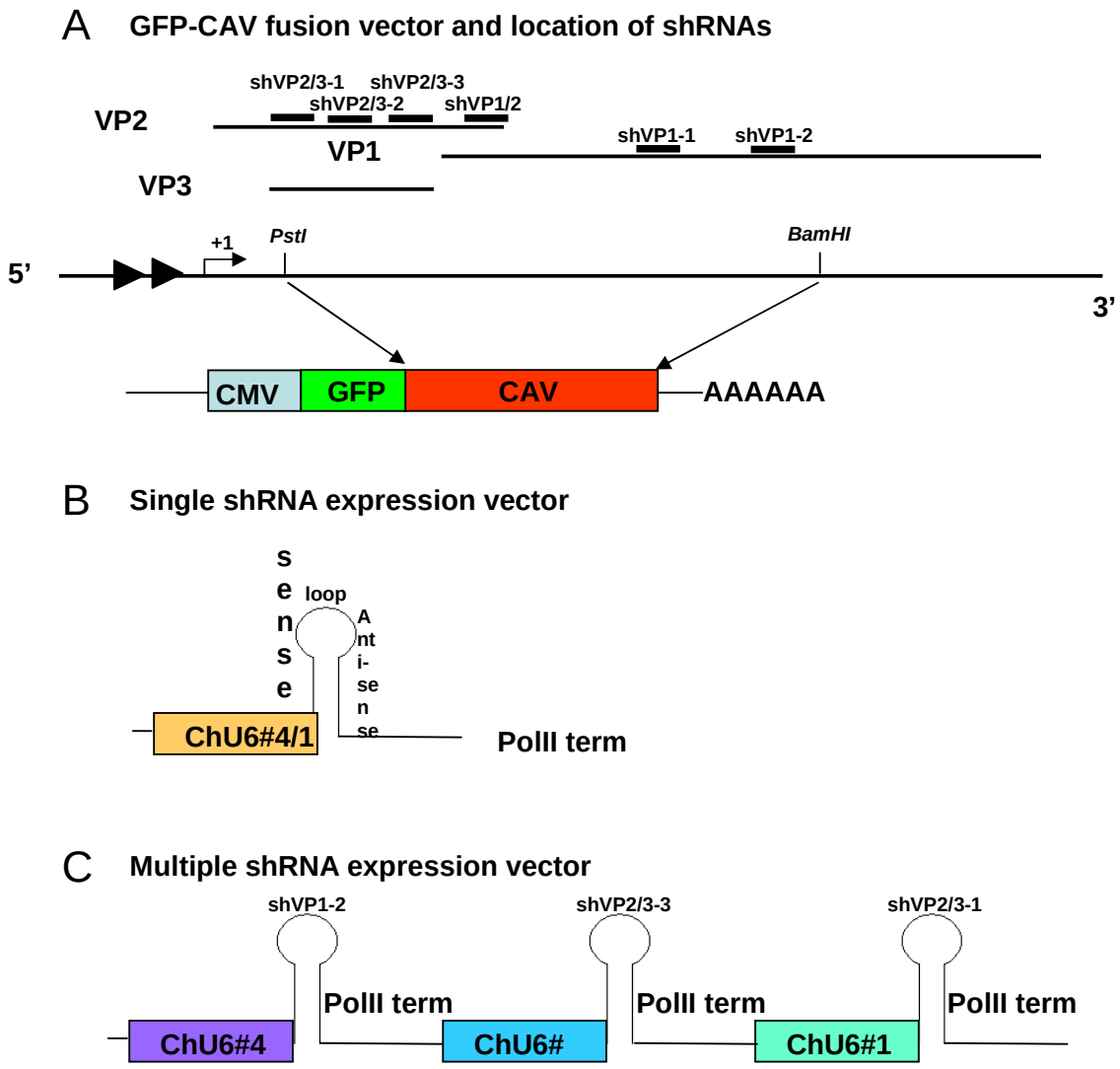


Figure 2.13 Schematic representation of vectors. (A) Schematic representation of the linearised CAV genome. Sequences encoding open reading frames for the three known CAV proteins are indicated. The transcriptional start site, is indicated by an arrow and +1. The location of the promoter/enhancer repeat region is shown by two arrow heads. Location of the CAV targeting shRNAs are indicated by short lines. Not to scale. The region of the CAV genome included in the fusion plasmid is shown. (B) Schematic representation of the shRNA expression vectors. One vector contains either the ChU6#4 or ChU6#1 promoter. (C) Schematic representation of pCAV-MW vector.

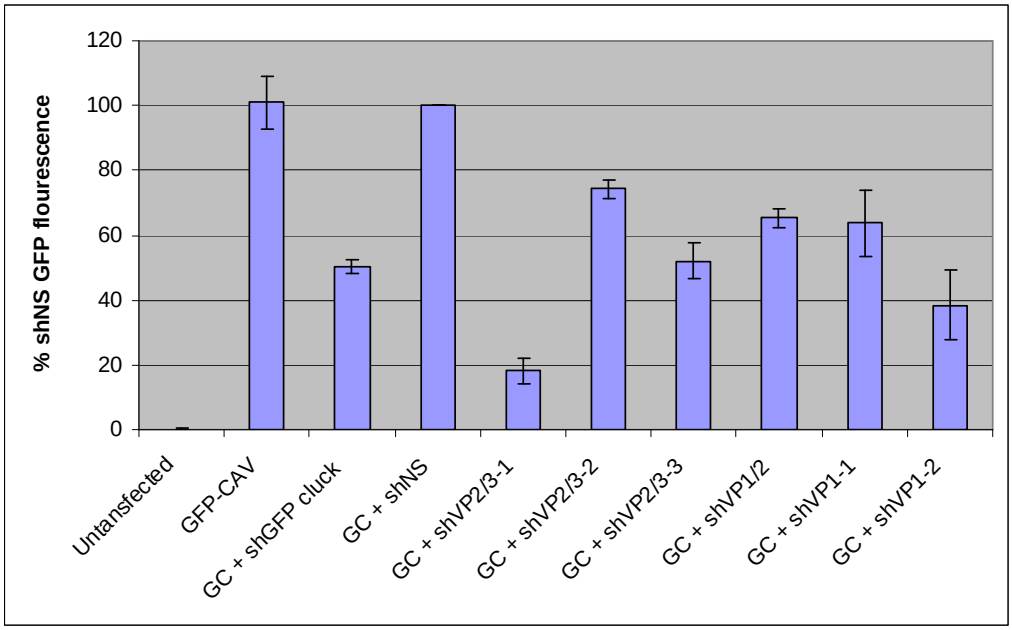


Figure 2.14. shRNAs targeting the CAV genome silence the GFP-CAV fusion mRNA in DF1 cells. DF1 cells were co-transfected with 1µg of pEGFP-CAV and 1µg of the relevant shRNA vector for 4hrs. Except for the untransfected which had no DNA and the GFP-CAV alone which had 1µg of the GFP-CAV vector. Media containing the transfection agent was replaced with normal growth media and the cells incubated for a further 68hrs. Cells were then assayed by flow cytometry and analysed in microsoft excel. shRNAs appear in order from 5' end of the CAV mRNA. Values are shown as percentages of the negative control shRNA (shNS), as the mean of three replicates ± standard deviation.

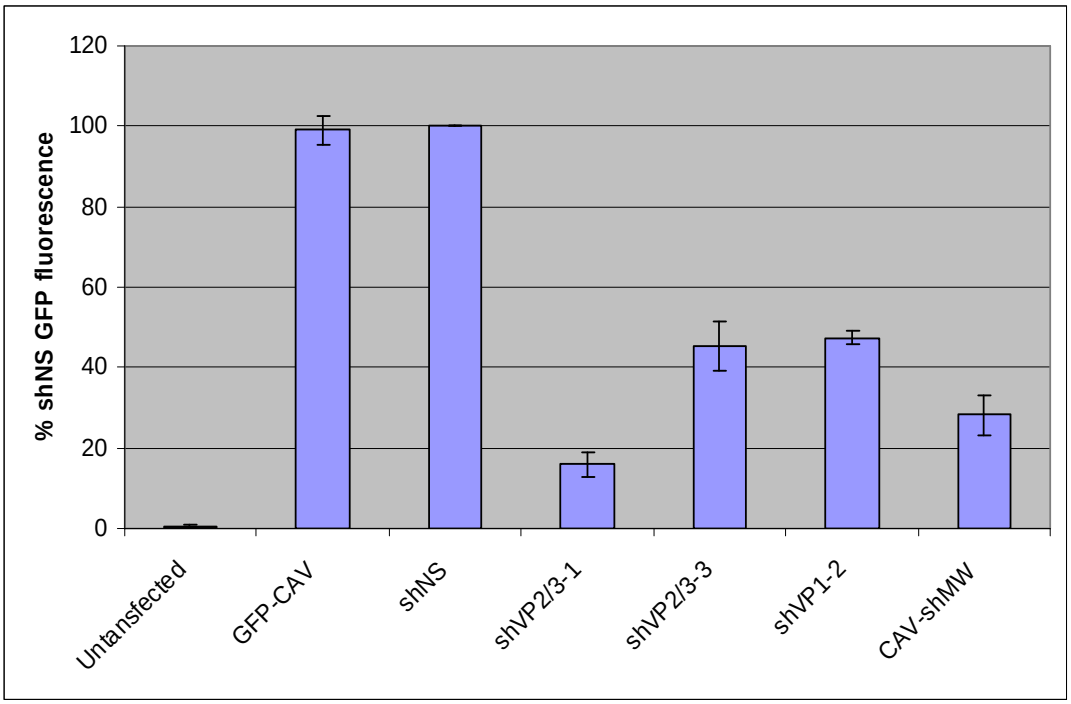
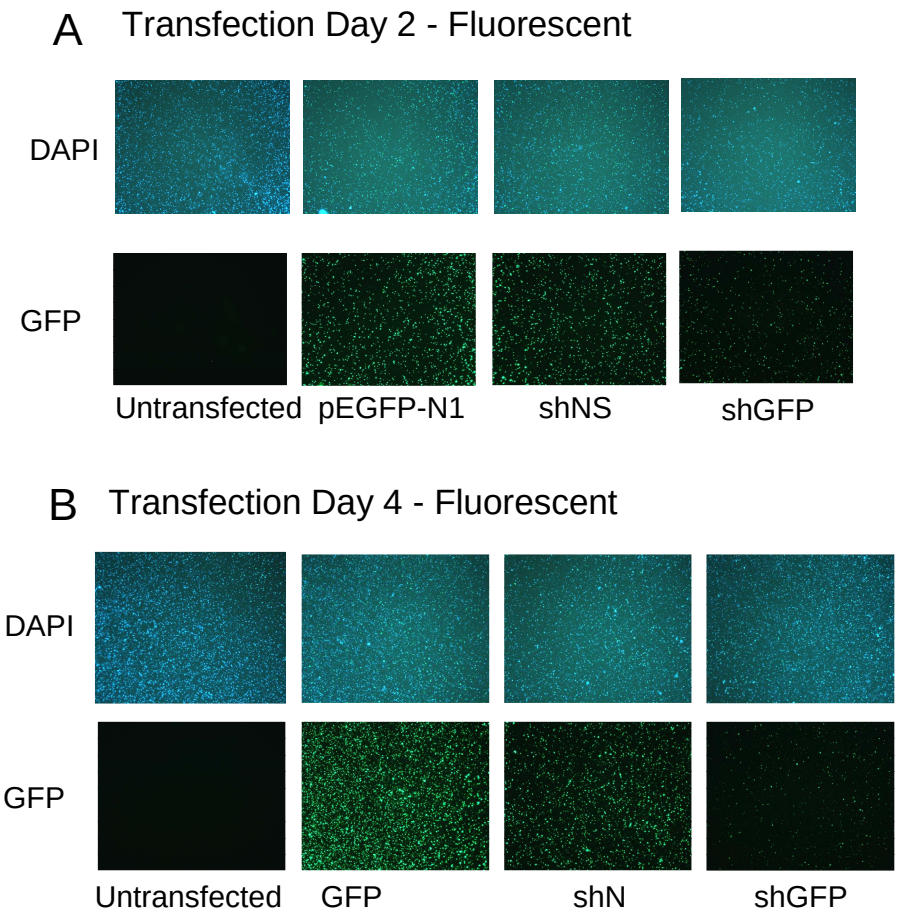


Figure 2.15. Expression of multiple shRNAs targeting the CAV genome from a single plasmid silence the GFP-CAV fusion mRNA in DF1 cells. DF1 cells were co-transfected with 1μg of pEGFP-CAV and 1μg of the relevant shRNA vector for 4hrs.Except for the untransfected which had no DNA and the GFP-CAV alone which had 1μg of the GFP-CAV vector. Media containing the transfection agent was replaced with normal growth media and the cells incubated for a further 68hrs or 92hrs. Cells were then assayed by flow cytometry and analysed in microsoft excel. The individual shRNA run are the ones present in the MW. Values are shown as percentages of the negative control shRNA (shNS), as the mean of three replicates ± standard deviation.


A
Transfection Day 2 - Fluorescent
DAPI
GFP
Untransfected
pEGFP-N1
shNS
shGFP
B
Transfection Day 4 - Fluorescent
DAPI
GFP
Untransfected
GFP
shN
shGFP

C

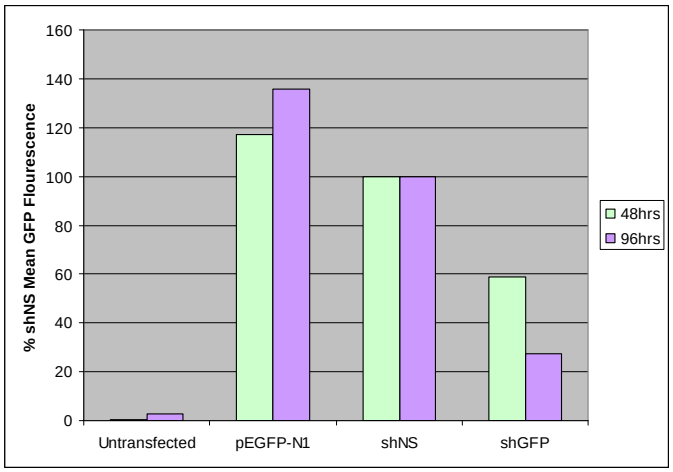


Figure 2.16. MSB-1 cells have the RNA silencing pathway. MDCC-MSB1 cells were electroporated with 10µg pEGFP-N1 alone or with 5µg pEGFP-N1 and 5µg of the relevant shRNA and incubated for 48hrs. (A) An aliquot of cells were taken, spun and resuspended in PBS with DAPI, then allowed to settle onto a microscope slide, images were taken by a fluorescent microscope. The remainder was incubated for a further 24hrs (B) An aliquot of cells were taken, spun and resuspended in PBS with DAPI, then allowed to settle onto a microscope slide, images were taken by a fluorescent microscope. (C) 1x106 cells were removed, washed twice with FACS wash and then analysed for GFP by flow cytometry. Mean fluorescent intensity were obtained and analysed by microsoft excel. Values are shown as percentages of the negative control shRNA (shNS).

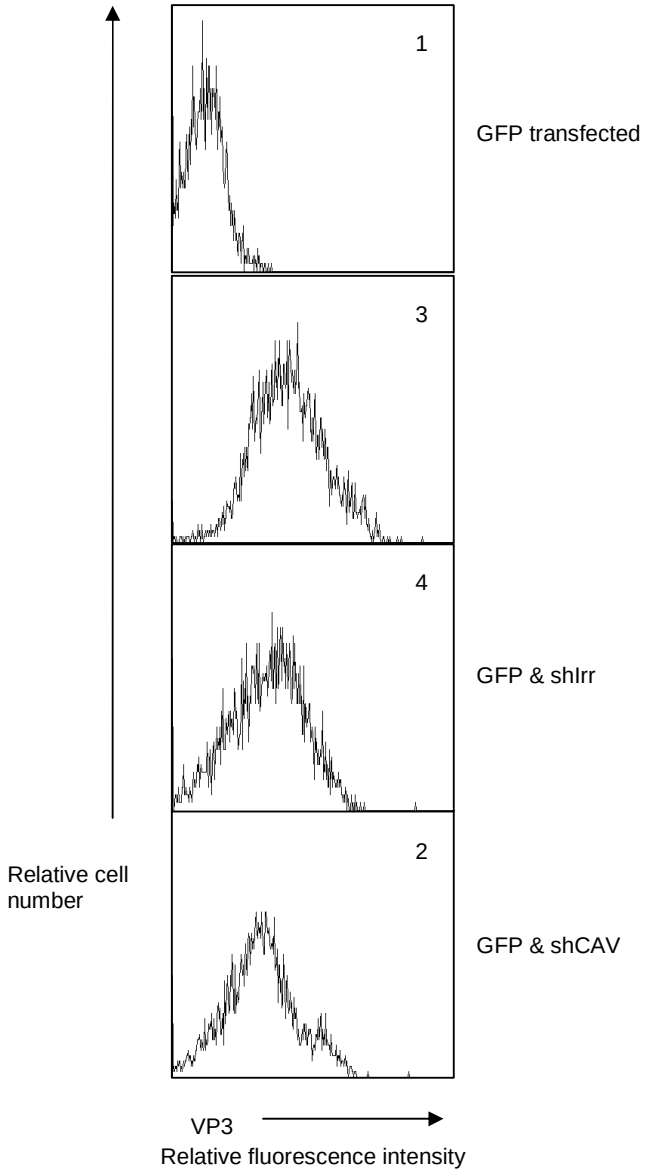


Figure 2.17. An example of GFP detection and staining for CAV VP3 by FACS. MDCC-MSB1 cells were electroporated with 10μg of each of the relevant vectors and incubated for 4hrs. 1x106 cells were then infected for 1hr with CAV269/7 at an MOI of 2. Cells were added to growth media and incubated for 72 or 96hrs. 1x106 cells were then fixed, stained for CAV VP3 using anti-VP3 and anti-mouse APC and detected by flow cytometry. Cell quest was used to analyse the results. Cells in the lower left quarter of the dot plot are negative for both GFP and virus. The upper left quarter contains CAV infected cells, the lower right quarter contains just GFP transfected cells and the upper right contains CAV VP3 positive and GFP positive cells. Cells in the right half of the dot plot were gated and the

APC histogram analysed for mean fluorescence. A decrease in mean fluorescence indicates less virus present.

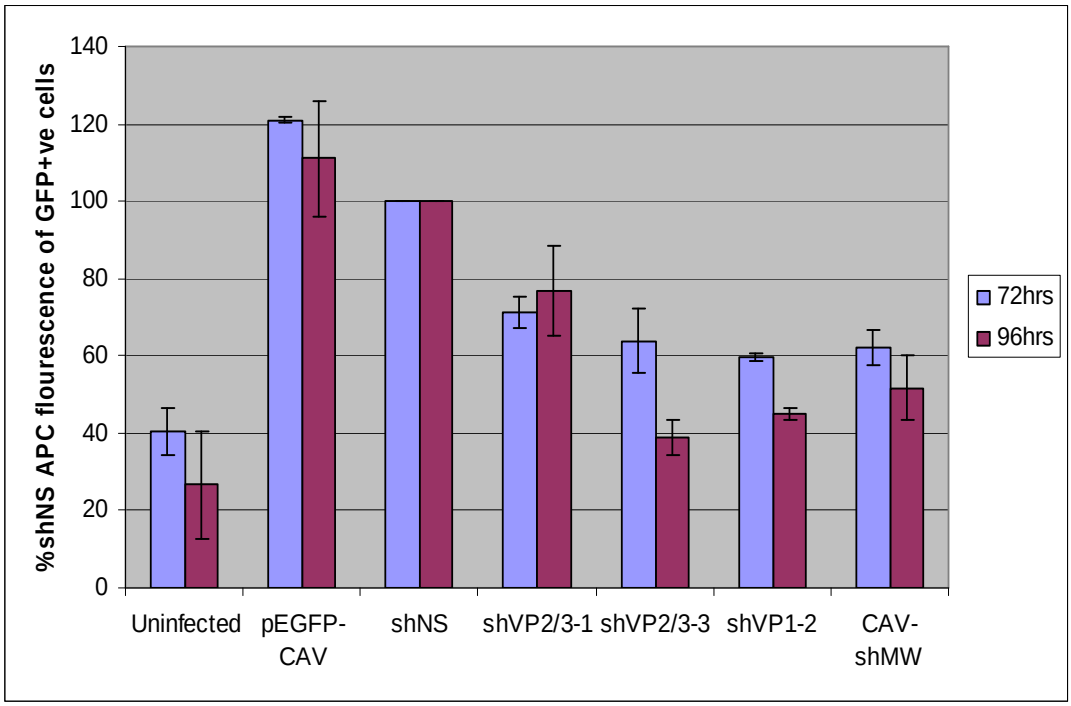
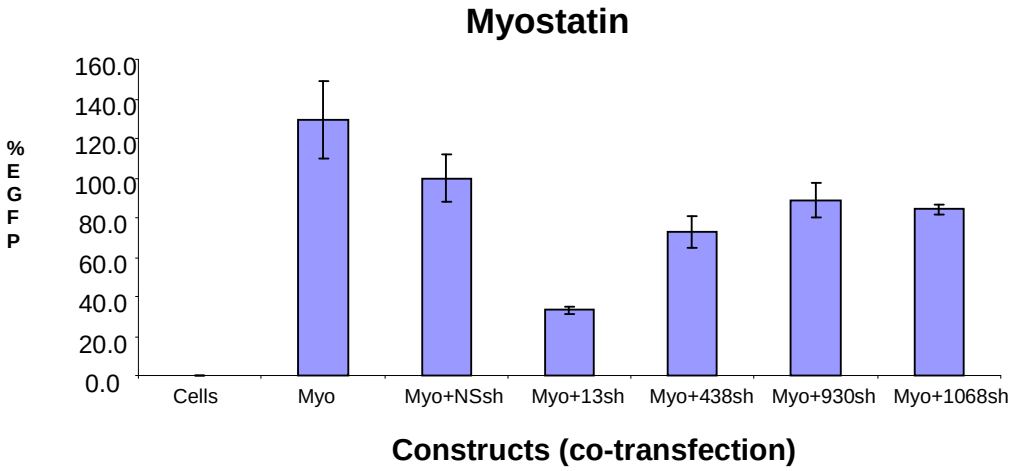


Figure 2.18. shRNAs targeting the CAV genome can inhibit expression of CAV VP3. MDCC-MSB1 cells were electroporated with 10µg of each of the relevant vectors and incubated for 4hrs. 1x106 cells were then infected for 1hr with CAV269/7 at an MOI of 2. Cells were added to growth media and incubated for 72 or 96hrs. 1x106 cells were then fixed, stained for CAV VP3 using anti-VP3 and anti-mouse APC and detected by flow cytometry. Cell quest was used to obtain the results. The mean APC fluorescent intensity of GFP positive cells was then analysed in microsoft excel. Values are shown as percentages of the negative control shRNA (shNS), as the mean of three replicates ± standard error of the mean.



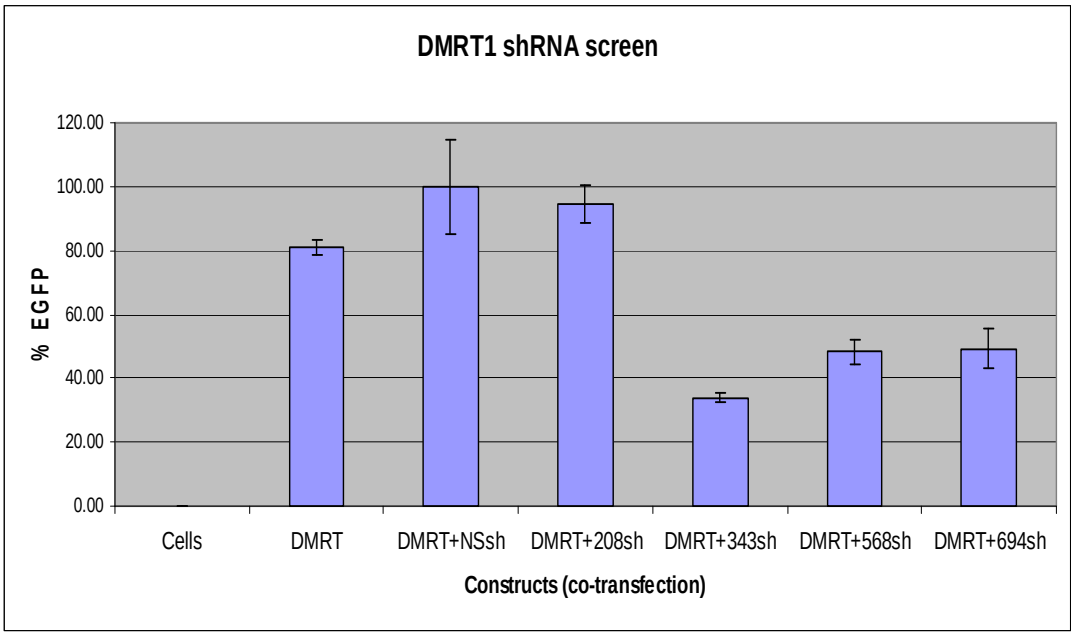


Figure 2.19. RNAi silencing of chicken DMRT1 and Myostatin in Embryonic Fibroblast cells.
Flow cytometry results for gene knockdown in DF1 cells is shown. DMRT1-shRNA expression constructs co-transfected with pEGFP-DMRT1 is indicated on the x-axis. DMRT1 knockdown was measured as percent mean fluorescence intensity (% MFI), normalised to the average MFI of the non-silencing control (NSsh) construct (100%). Error bars represent the standard error of the mean calculated from three independent experiments.

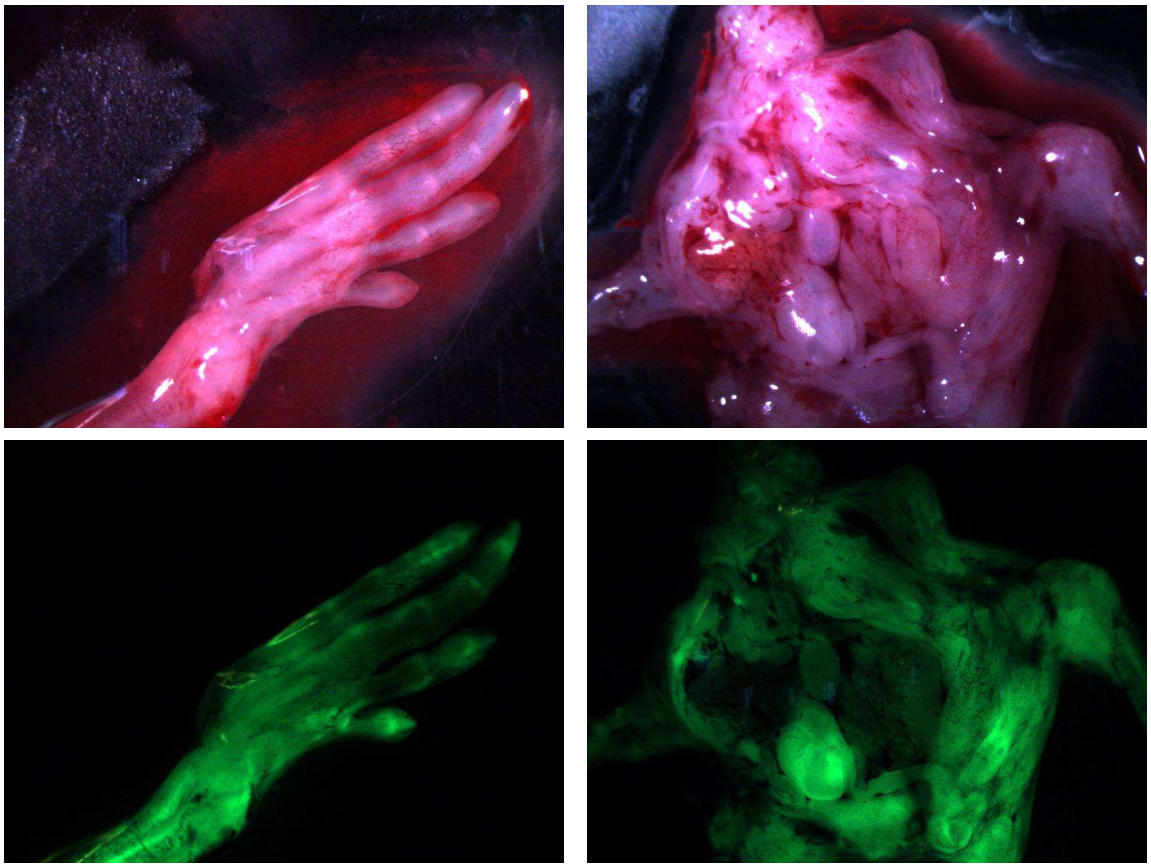


Figure 2.20. *In ovo* RCAS delivery of EGFP to chicken embryos. Day 4 embryos were injected with virus and incubated until Day 10. Embryos were then removed from the shell and examined for expression of EGFP with fluorescence microscopy.

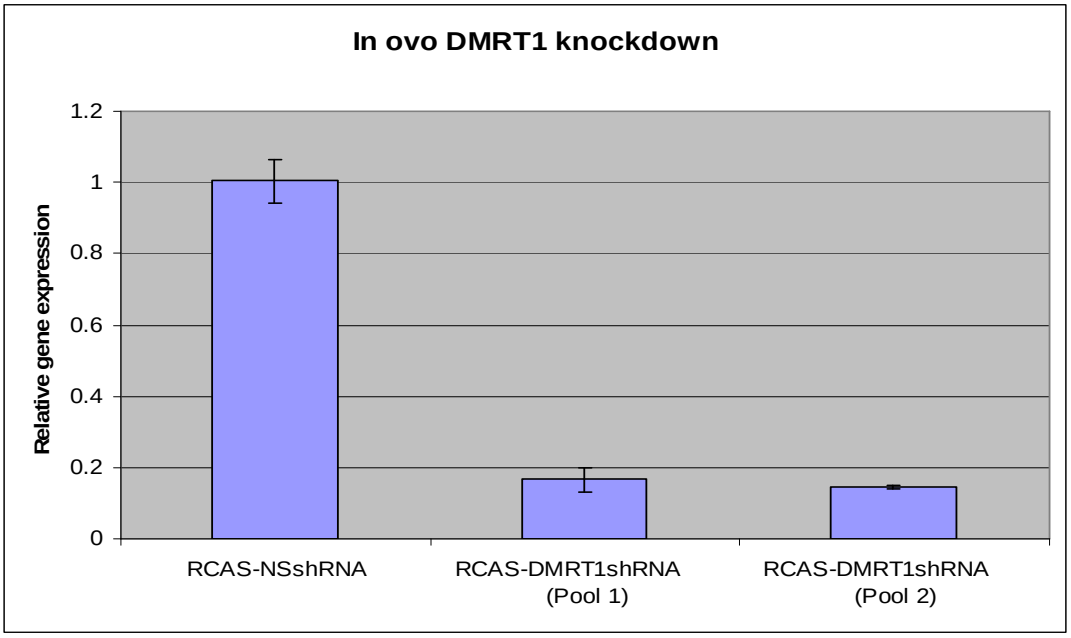


Figure 2.21 In ovo DMRT1 knockdown using RCAS-DMRT1shRNA

Knockdown of DMRT1 mRNA was achieved by *in ovo* administration of RCAS-DMRT1shRNA intra venously at embryonic day 4 (E4). Genetic male gonads were isolated at E10 and pooled into two groups (pool 1 and pool 2) as indicated. Gene expression of DMRT1 in the gonads of RCAS-DMRT1shRNA treated embryos was quantified relative to that of male gonads isolated from RCAS-NSshRNA (non-silencing control) infected chick embryos.

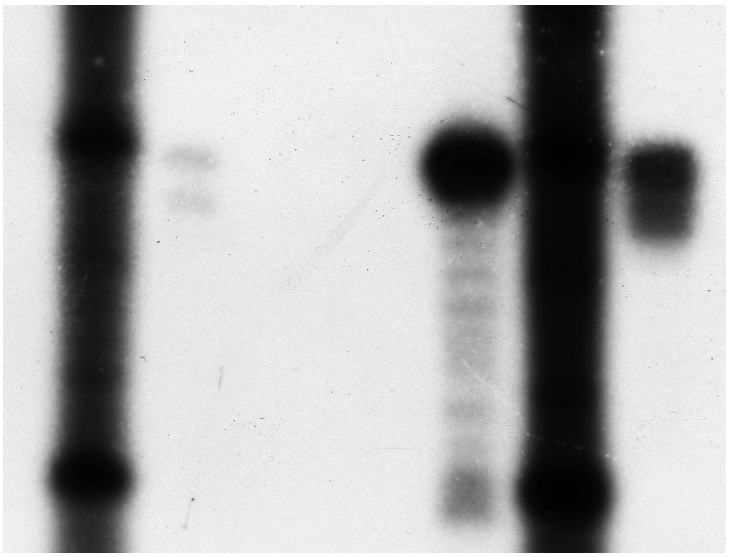


Figure 2.22 Detection of RCAS expressed DMRT1 shRNAs

An RNase protection assay was performed to detect DMRT1 shRNA expression on small RNAs isolated from (1) Genetic male gonads taken from RCAS-DMRT1shRNA infected chicks at embryonic day 10 (E10), (2) Genetic male gonads taken from uninfected chicks at E10, and (5) RCAS-DMRT1shRNA infected DF1 cell culture. Size standards are indicated (M). +/- RNAse controls are shown in lanes 3 and 4 respectively.

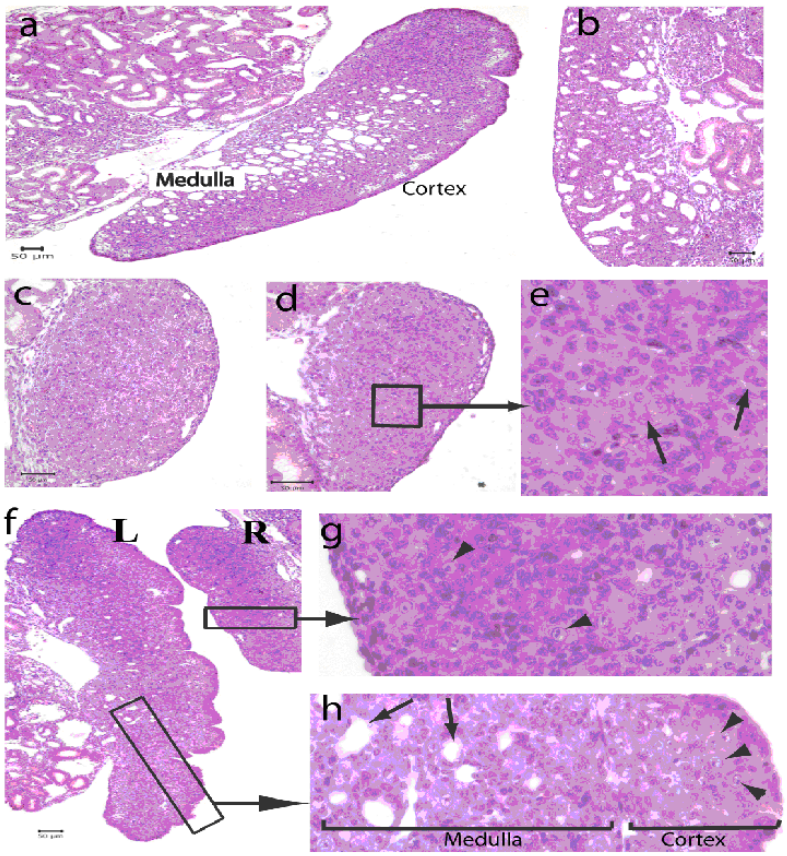


Figure 2.23 Feminisation of embryonic male (ZZ) chicken gonads following knockdown of *DMRT1*. Longitudinalhistological sections of gonads from day 10 chicken embryos treated at day 0 with scrambled control miRNA or DMRT1 miRNA563. This figure shows gonadal histology from embryos showing high GFP expression, and hence miRNA delivery. **a,** Left gonad from a control female (ZW) treated with scrambled miRNA, showing normal ovarian development (a thickened cortex and vacuolated medulla.) **b,** Smaller right gonad of a control female, showing a highly vacuolated medulla and no cortex. **c,** Right testis of a control male, showing seminiferous cords in the medulla and reduced surface epithelium. **d,** left testis of a control male. **e,** High magnification view of the boxed region in d, showing well defined seminiferous cords in control male testis (arrows). **f,** Feminised gonads of a male embryo (ZZ) treated with DMRT1 knockdown virus miRNA563, showing a large ovarian-shaped left (L) and smaller right (R) gonads. **g,** High magnification view of the right gonad, showing medullary germ cells but poorly organised cords (compare to e above). **H,** High

magnification view of the left gonad, showing a vacuolated medulla (arrows) and thickened female-like cortex containing numerous germ cells (arrowheads). No seminiferous cords are apparent.

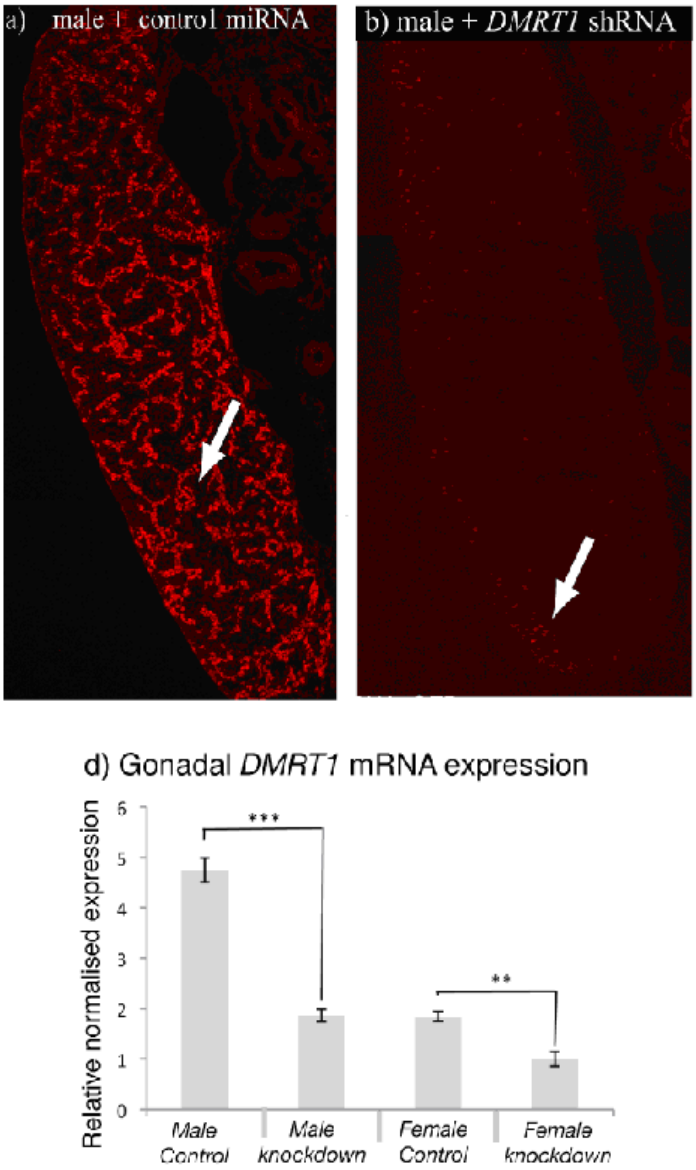


Figure 2.24 Feminisation of genetic male (ZZ) chicken embryos following knockdown of *DMRT1*. **a**, Normal expression of DMRT1 protein in a 10 day old genetic male gonad (ZZ) treated with non-silencing scrambled control sequence. DMRT1 is strongly expressed in the Sertoli and germ cells of the organising testis cords throughout the gonad (e.g., arrow). A longitudinal section is shown. **b**, Significant reduction of DMRT1 protein in the left gonad of a feminised male (ZZ), treated with *DMRT1* knockdown shRNA343. Some residual DMRT1 protein is present in germ cells (e.g, arrow). Longitudinal section. **d**, *DMRT1* mRNA expression in control and knockdown gonads (quantitative RT-PCR). Both male and female gonads treated with knockdown constructs (pooled miRNA563 and

shRNA343) show significantly reduced *DMRT1* expression (mean +/- SEM; *** p< 0.0001 ; ** p < 0.0025; n =3.

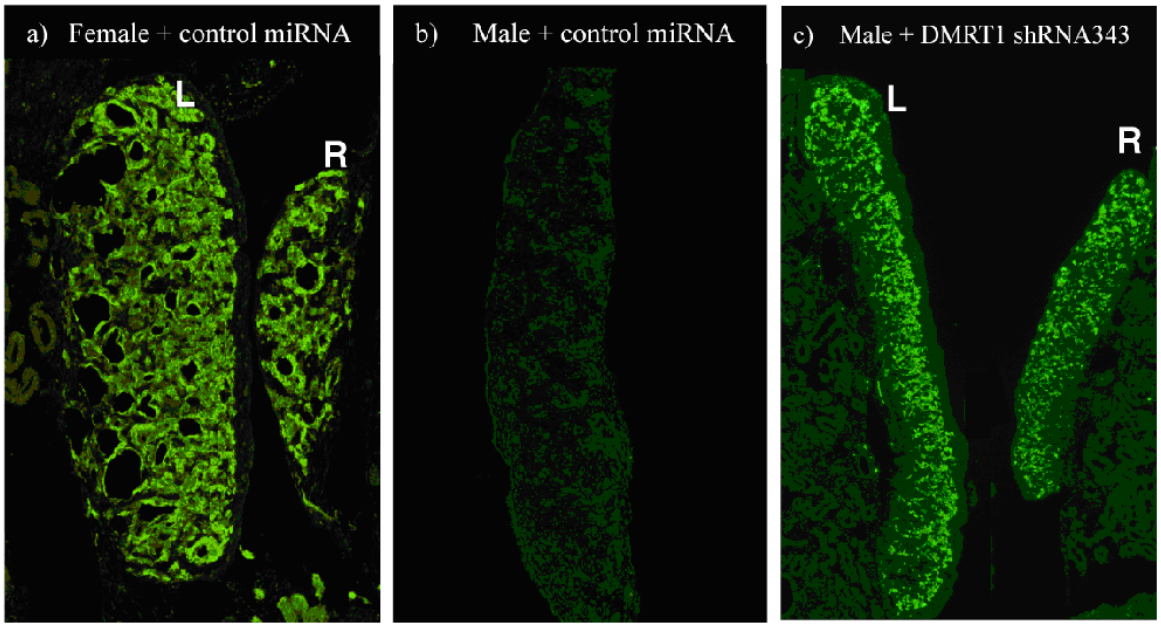


Figure 2.25 Ectopic expression of female markers in embryonic male gonads following DMRT1 knockdown. Immunofluorescence and quantitative RT-PCR. L = left gonad; R = right gonad. **a**, Strong bilateral expression of aromatase protein in control female gonads treated with miRNA. **b**, No aromatase expression in control male gonads treated with scrambled miRNA. **c**, Ectopic aromatase expression in both male gonads following treatment with DMRT1 shRNA343. Note the female-like size asymmetry between the left and right gonads.

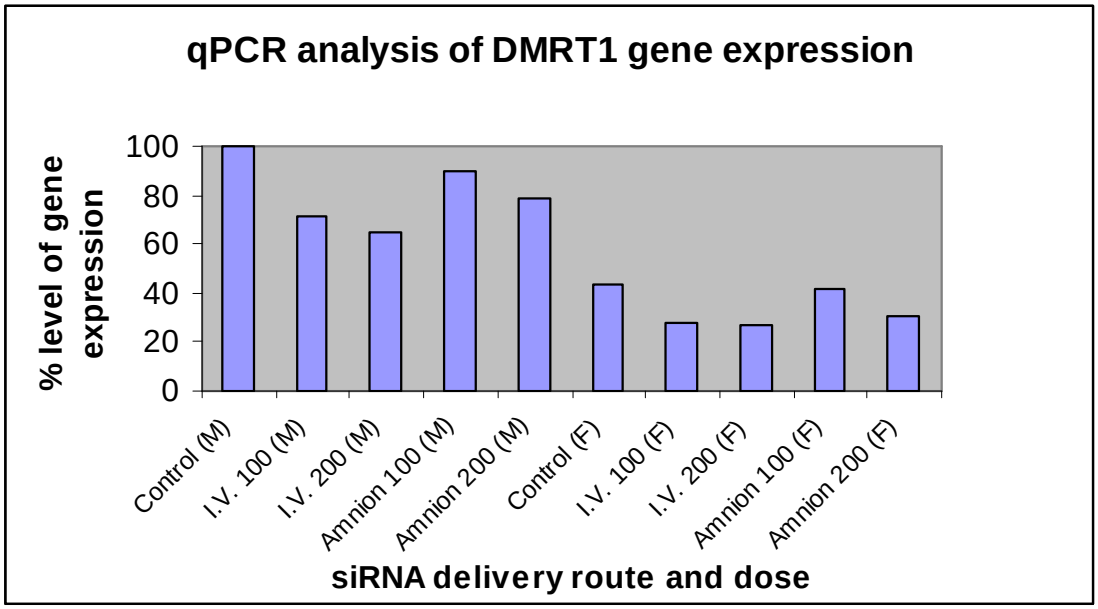


Figure 2.26 cDNA was then used to quantify relative *DMRT1* gene expression levels in the pooled male and female gonad samples from each treatment group. Quantitative RT-PCR analysis confirmed that *DMRT1* mRNA expression was specifically reduced in all pooled groups of male embryos when compare to control Group 1. Almost 40% of *DMRT1* gene expression knockdown was observed for Group 3 male embryos treated with the *DMRT1*-343-siRNA. It is interesting to note that Group 3 was also the group that resulted in the greatest degree of observed feminisation of male gonads at the macroscopic level.