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Control of sex-determination in poultry
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Executive Summary

The overall objective of this sub-project was to produce single sex lines of chickens, with animal welfare and economic benefits to the Australian and global poultry industry. The approach was to use advanced genetic technologies to modulate known and novel sex-determining genes in embryos, producing stable sex-reversed lines of birds.
Specifically, we had two major objectives:
Objective 1: Modulate male development via DMRT1
Objective 2: Modulate female development via R-SPO1 and FOXL2

The outcome of this research has two major impacts on industry in Australia and globally:

1. Feminisation of male commercial layers

   Impacts:

   - Positive impact on welfare issues relating to culling male chicks
   - Commercial impact delivered through improved production efficiency
   - Increased uptake of innovative *in ovo* vaccines
2. Masculinisation of females in broilers

   Impact:

   - Substantial commercial benefit and improved sustainability for industry

The research undertaken in the project was predominantly “blue sky” and built on preliminary work conducted in Poultry CRC I and previous collaborative research conducted between Craig Smith (MCRI) and Tim Doran (CSIRO) that confirmed to pivotal role of the *DMRT1* gene in males sex determination in avians. Modulation of the *DMRT1* gene was a major focus of this subproject and during the course of our research we have now developed a much broader and improved understanding of a larger set of genes that are involved in the complex trait of sex determination in the chicken. We now have an excellent opportunity to extend this knowledge, utilise a larger “tool box” of gene manipulation technologies and build on the exceptional performance of the science team and focus on key recommendations to deliver impact to the Poultry Industry.

Modulation of male development

Our research efforts focussed on the development and use of RCAS viral vectors for RNAi knockdown or over-expression of the male specific *DMRT1* gene. The outcome of this work was a better understanding that whilst RCAS is an excellent research tool for working with embryos, it is not a viable delivery vector for producing hatched sex-reversed birds. RCAS *DMRT1* knockdown in hatched chicks was not robust, and we therefore transitioned to Tol2 delivery and analysis of phenotype in G(1) progeny. Tol2 delivery results in sustained

transgene expression. G(1) birds showed some signs of sex reversal, but again this was not robust. We developed strategies for improving *DMRT1* knockdown via “mirizing” the *DMRT1* specific shRNA343. We have developed Tol2 constructs for delivery of miR343 and recommend a combinatorial strategy for delivery as knockdown alone is not strong enough for sex reversal.

A major outcome of this project was the discovery that over-expression of Aromatase via RCAS delivery induces complete male to female sex reversal in embryos. We subsequently shifted our focus towards the modulation of Aromatase together with knock-down of *DMRT1* with an improved shRNA (miR343). We developed Tol2 constructs for delivery of Aromatase over-expression and *DMRT1* knock-down to provide a dual or “combinatorial” approach to achieving female development. G(1) chicks with these combinatorial transgenes are already being generated and screened. We are yet to identify a positive G(1) chick and therefore recommend that the screening continues to evaluate this approach to develop male to female sex reversed birds.

DMRT1 over-expression via RCAS delivery was lethal to injected embryos and obviously not viable. To overcome this, we have pursued the identification, characterisation and validation of gonad-specific promoters or enhancers directing expression only in the gonads. We recommend that this work continues. As an alternative approach we have developed *in ovo* electroporation of RCAS as an improved method to examine sex determination genes. Using this technique, RCAS is confined to the gonads of embryos and does not lead to global expression and lethality. There is now a strong potential to hatch chicks and we recommend further work using this approach for *DMRT1* over-expression.

Towards the end of the project, we also pursued the over-expression of the *AMH* gene via the RCAS vector. The preliminary data indicates that this gene is an excellent candidate for further analysis as we have seen robust masculinisation of female embryos. We are currently extending analysis to hatched birds and will assess the potency of *AMH* in causing female-to-male sex reversal. This work will form the basis of a new CRC PRP.

Modulation of female development

Our research efforts focussed on over-expression and knock-down of the female specific genes *RSPO-1* and *FOXL2* and the identification of novel female sex determining genes (W linked genes) using RNA sequencing.

Over-expression of *RSPO-1* via electroporation resulted in a likely upregulation of Aromatase, but this was not robust. Over-expression of *FOXL2* resulted in the repression of male specific genes, however we did not observe upregulation of female genes and the

effect was not sufficient for modulation of phenotype. RNAi knock-down of both genes *in vivo* was also not effective in modulating sex phenotype to feminize males. This clearly demonstrates that modulation of *FOXL2* or *RSPO-1* alone will not be sufficient to strongly and consistently reverse sex. We recommend a combinatorial approach of double over-expression or knock-down of both genes simultaneously.

There is an ongoing need to identify, characterise and validate novel W linked genes that are potential regulators of *FOXL2*, Aromatase, *SOX9* or *DMRT1*. We have used RNA sequencing and this has been extremely successful in finding novel sex gene candidates. Analysis of our top 10 candidate genes is underway and we predict that a number of these genes will provide new targets for modulating sex. We have developed *in ovo* electroporation of RCAS as an improved method to examine the effect of these new candidate genes in the gonads of injected embryos. This will provide us with the increased potential to hatch chicks for further analysis.

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Introduction

The over-arching aim of the Poultry CRC for Sex Determination was to produce single sex lines of chickens. The project involved the use of molecular methods to modulate genes that control gonad development (ovary versus testis formation) at embryonic stages and hence female versus male development. The generation of single sex populations is highly desirable to the poultry industry, with two clear benefits:

(1) an economic advantage in producing all female hatchlings for the laying industry and all males for the broiler (meat) industry. All female or all male populations would greatly increase the efficiency of egg and meat production, respectively. In the broiler industry, males grow more rapidly and produce more meat than females.

(2) Animal welfare. Half of all hatchlings are currently culled because they are of the unwanted sex. Production of monosex populations would obviate the need for post-hatch culling, addressing and major animal welfare issue, and saving costs associated with the culling procedure.

To modulate chicken sex ratios, our team focussed on altering sexual development in chicken embryos, with the aim of yielding all male or all female hatchlings. To this end, the project involved cutting edge methods of gene manipulation and analysis. The team represented a collaborative association between CSIRO AAHL in Geelong (lead by Tim Doran), the Murdoch Childrens Research Institute (MCRI) in Melbourne (lead by Craig Smith) and commercial partners (the EW Group). Previous collaboration between CSIRO AAHL and MCRI demonstrated our ability to use RNA interference technology to knock down a key male-related gene, *DMRT1,* to induce feminised gonads in embryos (Smith *et al*., Nature, 2009). The *DMRT1* gene is located on the chicken Z sex chromosome and the MCRI group previously proposed it to be the master sex-determining switch in chicken (Smith *et al.,* Nature, 1999). By combining expertise in avian embryology and sex determination (the MCRI group) with expertise in RNA interference to modulate gene expression (CSIRO AAHL group), we demonstrated that DMRT1 was required for proper testis development and hence male sex determination (Smith *et al*., 2009). This widely publicised finding formed the basis of the current CRC for the sex modulation. Modulation of the DMRT1 gene formed the centrepiece of the CRC sub-project, with the aim of knocking down and over-expressing the gene in early embryos, and assessing sex modulation in hatched and sexually mature sex-reversed birds. Two other genes were also examined during the life of the CRC sub-project, the female associated genes, R-Spondin1 and FOXL2. These two genes are implicated in ovary formation and hence female development

in chicken and other species (Chue and Smith, 2012). We rationalised that modulation of one or both of these genes could also affect sexual development in chicken embryos.

Chicken sex determination

Sex is determined genetically in chickens and others birds by the inheritance of sex chromosomes. These chromosomes differ from those in mammals. In chicken, the male has two Z sex chromosomes while the female has Z and W sex chromosomes. Sex determination involves the expression of genes in embryonic gonads to direct testis or ovary development. The gonads then release hormones that masculinise or feminise embryos. Several lines of evidence now suggest that the Z-linked *DMRT1* gene regulates avian sex determination via a gene dosage mechanism (two doses in males, ZZ, one in females, ZW) (Lambeth et al., 2014). This gene is active in embryonic gonads, as outlined below. Despite extensive analysis by our team and others, no convincing master female gene has been identified on the W sex chromosome, which is unique to females (Smith and Sinclair, 2003; Ayers et al., 2013). The current thinking is that *DMRT1* triggers gonadal development into a testis in ZZ embryos, but lower expression in ZW embryos allows ovary development.

The DMRT1 gene and male development

DMRT1 (*Doublesex* and *Mab-3*-Related Transcription factor-1) encodes a zinc finger-like transcription factor related to sexual regulators in the fly, *Drosophila*, and the worm, *C. elegans*, (Raymond *et al*., 1998). *DMRT1* is only expressed in the gonads, where it is consistently linked to testis development (Raymond *et al*., 1999). We previously showed that *DMRT1* is male up-regulated in the embryonic gonads of chicken and other animal embryos (Smith *et al., Nature,* 1999). Chicken *DMRT1* is located on the Z sex chromosome, such that males (ZZ) have two doses, while females (ZW) have one (Nanda *et al*., 1999). Birds do not have a chromosome-wide dosage compensation mechanism, as seen in mammals, and *DMRT1* is more highly expressed in embryonic male chicken gonads compared to females, prior to and during gonadal sex differentiation (Smith *et al.,* 1999, 2003). We hypothesized that a higher dose of *DMRT1* in male chicken embryos (ZZ) may be testis determining. By exploiting our joint expertise, we were able to use RNA interference to reduce DMRT1 gene expression male embryos (ZZ), resulting in female-type ovaries in embryos (Smith et al., 2009). The further aim as part of the CRC was to take such embryos to hatching and sexual maturity, and to develop methodologies of producing all female hatchlings via stable knockdown of DMRT1.

The R-Spondin1 (R-SPO1) and FOXL2 genes and female development.

While our previous findings implicated sex-linked *DMRT1* in testis (male) development in the chicken, several lines of evidence also suggest that the un-related genes, *R-Spondin1* and *FOXL2*, play key roles in ovary (female) development. We therefore included genetic analysis of these two putative female determinants in the sub-project. R-Spondin1 was first identified as a gene required for female sex determination in humans, as it was found to be mutated in cases of XX male development (Parma et al., 2006). Similarly, in mouse models, genetic deletion of R-Spo1 caused masculinisation of the gonads. We showed that chicken R-Spo1 has a female-specific expression profile in developing gonads (Smith et al., 2008). This gene was therefore also targeted as part of the project, based on the hypothesis that knockdown of the gene in females would masculinse genetically female (ZW) embryos and hatchlings, while over-expression in genetic males (ZZ) would potentially feminise individuals.

The other high profile candidate ovary gene that we focussed upon was *FOXL2*. This gene encodes a fork-head transcription factor, shown to be required for normal ovary development in mammals. Loss-of-function mutations in FoxL2 disrupt ovary development in mouse embryos, and cause a condition called BPES in humans, associated with ovarian failure. Remarkably, recent data show that genetic ablation of the *FOXL2* gene in the adult mouse causes the mature ovary to convert into a testis (Uhlenhaut et al., 2009). *FOXL2* is conserved amongst animals, and we have shown that it is activated female-specifically in the embryonic chicken gonad, where it likely controls oestrogen synthesis (Hudson *et al*., 2007). Although it is not sex-linked, we hypothesised that *FOXL2* plays a crucial early role in female (ovary) determination in the chicken. In this CRC sub-project, we aimed to manipulate *FOXL2* expression in the embryonic gonads. We over-expressed the gene in males, and knocked it down in females.

During the three-year life of this CRC sub-project, two other lines of research emerged that were not part of the initial proposal, which focussed purely on *DMRT1*, *R-SPO1* and *FOXL2*. As we gained new knowledge, developed new technologies and as new ideas emerged, we carried out a gene expression screen to identify novel genes that might regulate gonad development (RNA-seq analysis). This yielded several new candidate sex-determining genes that are currently being analysed (see below). Secondly, we developed the hypothesis that a well known gene called *AMH* (encoding Anti-Müllerian Hormone) could have a more critical role in male development that previously appreciated. We over-expressed this gene in female embryos and found that it strongly masculinised the gonads

(Chapter 4). These two additional lines of research (RNA-seq for novel genes) and the role of AMH, now form the basis of a new CRC research project being proposed in 2014.

Objectives

The overall objective of the sub-project was to produce single sex lines of chickens, with economic and animal welfare benefits to the Australian and international poultry industry. The approach was to use advanced genetic technologies to modulate known and novel sex-determining genes in embryos, producing stable sex-reversed lines of birds.

Specifically, four research objectives were proposed:

Objective 1: Modulation of DMRT1:

(a) Produce all female hatchlings by sex-reversing male embryos (genetically ZZ) into females, by knocking down (repressing) the male-determining gene, *DMRT1*.

(b) Over-expressing *DMRT1* in female (ZW) embryos to induce male development, using gonad-specific promoters.

Objective 2: Modulation of R-SPO1 and FOXL2

(a) Over-expression and RNAi knockdown of *R-SPO1* modulate female development

(b) Over-expression and RNAi knockdown *of* FOXL2 to modulate female development

Objective 3: Explore and define commercially acceptable and efficient means of delivering sex-modifying RNAi molecules into chicken embryos

(a) Non-vector delivery of *DMRT1* or *FOXL2* RNAi molecules (short hairpin RNAs) into embryos.

Methodology

The project involved the use of advanced genetic technologies to modulate sex-determining genes in chicken embryos, hence yielding sex-reversed birds. This centred around the use of RNA interference technology (RNAi) to knock down specific genes, such as *DMRT1*, the development of tools to deliver the knockdown, such as viral and non-viral vectors, the transient delivery of short interfering RNAs (siRNAs), and the use of gonad-specific promoters to drive gene over-expression only in the gonads. Ultimately, non-transgenic methods of modulating sex were favoured, such as siRNA delivery. This was pursued as part of objective three above. Transgenic approached were also pursued to provide proof of principle that modulating specific genes could induce stable sex reversal.

For modulation of *DMRT1, R-SPO1* and *FOXL2*, the RCAS avian-specific viral vector was initially used. Genes or shRNAs plus GFP reporters were cloned into this vial vector. High titre virus was produced in vitro and injected into day 0 blastoderms. Effects upon embryonic and hatchling sex were then assayed, using gene expression studies and analysis of gonadal morphology. To produce stable transgenic lines over-expressing genes or knockdown constructs, the TOL2 transposable element was used via Direct Injection method described below:

Tol2 plasmids

The Tol2 plasmid system used was as described by Balciunas et al. (2006) and was kindly provided by Professor Stephen C. Ekker from the Mayo Clinic Cancer Center, Minnesota, USA. In this two-plasmid system, one plasmid contained the terminal Tol2 sequences flanking the shRNA under the control of a PolIII promoter or the over-expression transcript under the control of the CAGGS promoter. The other plasmid contained the transposase sequence under the control of the CMV IE promoter (designated pTrans). In this system, the Tol2 sequences and enclosed DNA will be incorporated into the target genomic DNA while the pTrans will not be incorporated.

Formulation of Lipofectime2000 CD complex for microinjection

The Lipofectamine2000 CD complex was prepared according to the manufacturer's instructions. Briefly, 0.6 μg of pMiniTol and 1.2 μg of pTrans were mixed with 45 μL of OptiPRO (Invitrogen) and incubated at room temperature for 5 minutes. At the same time, 3 μL of Lipofectamine2000 (Invitrogen) was added to 45 μL of OptiPRO and incubated for 5 minutes. The two solutions were then mixed together and allowed to complex for 20 minutes

before being injected. The mixture was stable for several hours at room temperature prior to injection.

Microinjection and detection of EGFP PGCs in injected embryos

A window was cut in the pointed end of a recipient egg to allow access to the stage 14 (HH) embryo. Using a micropipette, 1-2 μL of transfection complex was injected into the dorsal aorta using a pulled glass micropipette. The opening in the egg was sealed with parafilm and the egg was then incubated normally. To assess the success of the technique embryos were analysed at ED 7 and 14 or allowed to hatch. Gonads from ED 7 and 14 embryos were dissected away from the kidney and viewed under a fluorescence microscope for the expression of EGFP.

qPCR of semen from G0 roosters

Hatched chicks were grown to sexual maturity and quantitative real time PCR (qPCR) was used to detect the presence of Tol2 transgene in the semen. Semen samples were collected and DNA was extracted from 20 μl of semen diluted in 180 μl of PBS using the Qiagen DNeasy Blood and Tissue Kit following the manufactures instructions. The semen genomic DNA was then diluted 1/100 in ddH_20 for use in the PCR reaction. qPCR was carried out on a Mastercycler® ep realplex (Eppendorf Hamburg, Germany) following the manufactures instructions. In short 20 μl reactions were set up containing 10 μl of Taqman 2x Universal master mix (Applied Biosystems), 1 μl 20X FAM labeled Assay Mix (Applied Biosystems) and 9 μl of diluted DNA. Each sample was set up in duplicate with specific primers and probe for Tol2 (Fwd primer 5' CAGTCAAAAAGTACTTATTTTTTGGAGATCACT 3'; Rev primer 5' GGGCATCAGCGCAATTCAATT 3'; detection probe 5' ATAGCAAGGGAAAATAG 3') and a genomic control region from the chicken genome which acts as a template control (Fwd primer 5' GATGGGAAAACCCTGAACCTC 3'; Rev primer 5' CAACCTGCTAGAGAAGATGAGAAGAG 3'; detection probe 5' CTGCACTGAATGGAC 3').The PCR cycle parameters were an initial denaturing step at 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each rooster was tested at least twice and was classified positive if a C_T value of less than 36 was obtained for Tol2. A C_T of less than 32 for the control genomic region was used to indicate there was sufficient DNA in the sample tested.

Generation and analysis of G1 transgenic chicks

Roosters identified as positive from the qPCR screen were mated with wild-type females and offspring were visually screened with GFsP-5 (long wavelength blue) goggles (BLS LTD, Hungary) for the expression of EGFP.

The chicks shown to express EGFP where further analyzed by Southern blot hybridization analysis. Ten micrograms of genomic DNA isolated from blood samples was digested overnight with *Bam*H1 and resolved by gel electrophoresis on a 1% agarose TAE gel. The gel was then depurinated with 0.25 M HCl for 20 minutes and denatured twice with 0.5 M NaOH, 1.5 M NaCl for 25 minutes prior to being neutralised twice with 0.5 M Tris, 1.5 M NaCl (pH 7.0) for 25 minutes. The gel was treated in 10x SSC for 30 minutes and then transferred overnight to a nylon membrane (Hybond N) using a turboblotter (Whatman). The membrane was prehybridised for 4 hours at 68°C in 6x SSC, 5x Denhardt's and 0.5% SDS and hybridised overnight at 68°C with a ^{32}P labeled random primed probe made from a fragment of the EGFP sequence within pMiniTol-EGFP (Promega Prime-a-Gene Labeling System). The blot was washed for 20 minutes in 2x SSC, 0.1% SDS followed by two 20 minute washes in 0.1x SSC, 0.1% SDS at 68°C and then the membrane was autoradiographed at -80°C with an intensifier screen.

Chapter 1: Knockdown and over-expression of DMRT1

Summary

The objective of this part of the project was to modulate sex through manipulation of *DMRT1* gene expression. Two approaches were used to firstly knockdown DMRT1; delivery via the RCASBP.A avian viral vector (Replication Competent Avian Sarcoma virus, Bryan Polymerase, subgroup B) and via TOL2 transposon. The RCAS viral vector integrates into the host genome, spreading vertically to all daughter cells, but also horizontally to neighbouring cells (i.e., it is replication competent). The RCAS vector was initially used successfully to deliver DMRT1 shRNAs and modified miRNAs into chicken embryos, inducing male to female development in day 10.5 embryos (Smith et al., 2009). However, the virus proves quite toxic to embryos and very few reached the hatching stage. Those that did survive had low levels of GFP reporter expression. After one year, it was concluded that RCAS was not a suitable vector for expression of knockdown constructs in hatchlings or mature birds. The second approach used the TOL2 vector to deliver the same shRNAs proven to knock down DMRT1 in early embryos in the initial studies. This approach successfully produced transgenic birds expressing GFP reporter but clear male-to-female sex reversal could not be detected. The likely reason for this result was insufficient expression of the *DMRT1* RNAi molecules. This data will be expanded below:

RCAS delivery of *DMRT1* shRNA for male to female sex reversal

Determine chicken line for RCAS injection

The first part of this objective was to determine the susceptibility of the four commercial bird lines (broiler: B and D; layer: 66 and 76) to different subtypes of RCAS virus. RCAS infection requires the specific interaction between the envelope glycoprotein on the surface of the virus and the cognate receptor on the surface of the cell. The RCAS (ASLV) family of viruses has five primary envelope types: A, B, C, D, and E. From previous work we already knew that all four lines were not susceptible to the RCAS subtypes A, B and C. We therefore obtained the D and E subtypes and infected embryos from all four lines. Unfortunately embryos (20 from each of the 4 lines) were also not susceptible to both D and E subtypes. Embryos from the SPF line that we routinely use for RCAS infection work were included as controls and were susceptible to virus. As a back-up to embryos, infection of CEF cultures for each line was also conducted and again all were not susceptible to the D and E type

viruses. This result determined that all the planned RCAS vector work in this project would be conducted with the SPF line of chickens that we routinely use for RCAS infections.

We established a flock of the German SPF line at the Werribee Animal Health Facility to provide a consistent supply of eggs for all RCAS delivery work required in the project. We established a flock of 100 hens and 20 roosters.

Produce RCAS stocks for injections

Based on previous experience with the SPF line, we chose to use RCAS subtype A. We already had an existing DMRT1 shRNA knockdown construct (DMRT343 shRNA) which contained the chicken U6-4 promoter for shRNA expression. This construct has previously been shown to be effective in silencing DMRT1 expression in chicken embryos up to 18 days of incubation. Our goal now was to hatch chicks that were infected with these vectors. We know from previous work that hatchability of RCAS infected embryos is low. Therefore, we also re-engineered the U6-4 construct by replacing this promoter with the chicken H1 promoter. The U6 promoter is a strong promoter and in some applications the expression of shRNA from this promoter can be toxic to cells – including very early stage embryos where over expression of shRNAs competes with miRNA processing which is critical for cell differentiation. The H1 promoter is weaker than U6 and in some applications is preferable for shRNA expression. Therefore we developed two RCAS constructs for DMRT1 silencing, one for high expression of DMRT1 shRNA and another for low expression of DMRT1 shRNA. Both RCAS constructs also contain the GFP gene which allowed us to use GFP expression and fluorescence as a marker for spread of RCAS virus in injected embryos and chicks. High titre virus stocks for both RCAS vectors was made and used in injection experiments.

Day 0 embryos infected with virus expressing DMRT1 shRNA

We developed and implemented an injection schedule for RCAS infection of day 0 chick embryos with both DMRT1 silencing constructs (RCAS(A)H1-DMRT343 and RCAS(A)U6-DMRT343). We completed a total of 30 weeks of injections (R1 – R40) and hatched and reared 48 confirmed males and 51 confirmed females. Of the 48 males, 27 were injected with the RCAS(A)H1-DMRT343 construct and 21 males with the RCAS(A)U6-DMRT343 construct. Of the 51 females, 28 were injected with the RCAS(A)H1-DMRT343 construct and 23 with the RCAS(A)U6-DMRT343 construct. 22 birds were culled due to RCAS related health issues – 15 were confirmed males and 7 were confirmed females. Of the 15 males, 8 were injected with the RCAS(A)H1-DMRT343 construct and 7 males with the RCAS(A)U6-DMRT343 construct. Of the 7 females, 5 were injected with the RCAS(A)H1-DMRT343

construct and 2 with the RCAS(A)U6-DMRT343 construct. We noted the increased number of male to female deaths and are uncertain of the significance (if any) of this observation. Overall, results indicate that there is no difference in the survival of embryos that receive a construct with either a strong U6 promoter or weaker H1 promoter. RCAS is a replication competent ALV that has been attenuated by deletion of the src oncogene. Although the src oncogene is deleted, RCAS is still capable of inducing tumours if it can integrate near an oncogene such as c-myc. We are aware of other research groups that have seen tumours in RCAS infected birds that have been kept for long periods (around 3-4 months or more). RCAS integration into an important locus is purely a random event, however it can happen and lead to an undesirable outcome.

Analysis of the hatched male birds was the clear focus of this milestone. Each bird was bled at 4 week intervals - the first blood sample taken at 4 weeks of age was used for the sex PCR test and also FACS analysis to measure EGFP expression in blood cells as an indicator of how successful RCAS infection is in each bird. Every 4 weeks we weighed each bird and recorded comb height, colour and appearance. All of this information was used to monitor any feminisation of these male birds and to help us select birds for analysis of gonad morphology and sexual phenotype in DMRT1 knockdown birds.

We also analysed RCAS injected embryos pre-hatch, to confirm previous *in ovo* results and to also confirm that our RCAS constructs were working (Injections R41 – R51 – see Table 1). We analysed day 13 and day 19 embryos and observed gonad feminisation in a number of male embryos at both these time points. We observed gonad feminisation in 6 males out of ~40 injected embryos at day 13 (Figure 1.1). The sex PCR was repeated to confirm genotype (1 band =male, 2 band = female). We did not see any feminised males using the RCAS H1 shDMRT1 construct (R42, R43, R45 and R47), so focused on the U6 construct for injections to analyse day 19 embryos. Of the 21 embryos analysed at day 19, 3 genotype males (10, 16 and 17) had female gonad development (Figure 1.2). Repeat PCR on the three embryos confirmed male PCR results. Based on these *in ovo* results we also decided to look post hatch at young chicks (R49, R50 and R51). Only 3 injected embryos survived to hatch and were analysed at day 1. Two of the chicks were male but did not have obvious feminisation of gonads.

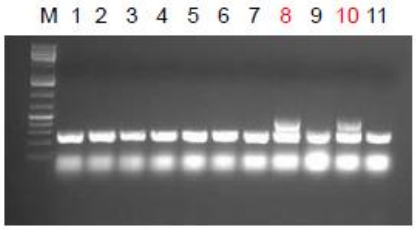
Gonadal sex and sexual phenotype will then be assessed at hatching and adult stages

All hatched birds from RCAS injections R1 to R40 were analysed for gonad morphology and sexual phenotype. We did not observed any modulation of secondary sexual phenotype in male birds that had reached adult stages. One male from U6 construct injection R36 (0056 - 6 weeks old) had a modulated left gonad (Figure 1.3). The right gonad was clearly a non-regressed testis, but the left gonad had a somewhat ovary type appearance. Our initial thoughts were that it could be a malformed testis or perhaps a tumour, but this was the very first time in all the birds that we culled that we had observed any male gonad with an abnormal appearance. Figure 1.3 includes gonad images of aged matched males (0072 and 0064), plus a close up of bird 0056 gonads. We took samples of both the right and left gonad for histology analysis and sections for expression of the male specific marker Sox9 and female specific marker aromatase. We also stained control sample gonad sections from age matched male and female chickens from (A)U6-DMRT343 construct injections (Figure 1.4 and 1.5). As expected the female control was positive for aromatase and negative for Sox9 and conversely the male was positive for Sox9 and negative for aromatase. The results for 0056 were extremely interesting (Figure 1.6), with strong aromatase expression detected in the left gonad and very low expression in the right gonad, suggesting some degree of feminisation. Both the left and right gonads were positive for the male specific marker Sox9 indicating that the feminisation is only partial, as was obvious from visual inspection of the gonads.

All culled birds (male and female) were analysed for EGFP expression using a UV light source and EGFP goggles. All of the birds had high levels of EGFP in some tissue, particularly in muscle and skin, indicating that the RCAS infections were successful. Not all of the birds, however, had high levels of EFGP throughout all expected tissue samples (liver was always negative for EGFP). Only a minority of the birds had detectable EGFP expression in their gonads and this did include bird 0056. Our extensive trial of the RCAS vector for shDMRT1 delivery suggests that a large viral load in hatched chicks is probably required for sufficient and perhaps sustained delivery of shRNA to gonads. We think it is likely that many embryos with high viral titres do not make it through to hatch.

Figure 1.1. RCAS injected embryos pre-hatch (day 13)

M – 1kb marker
1 – R41-14 (M)
2 – R41-21 (M)
3 – R41-24 (M)
4 – R41-36 (M)
5 – R41-41 (M)
6 – R41-42 (M)
7 – R41-47(M -control)
8 – R41-48 (F-control)
9 – R42-2 (M)
10 – F control DNA
11 – M control DNA



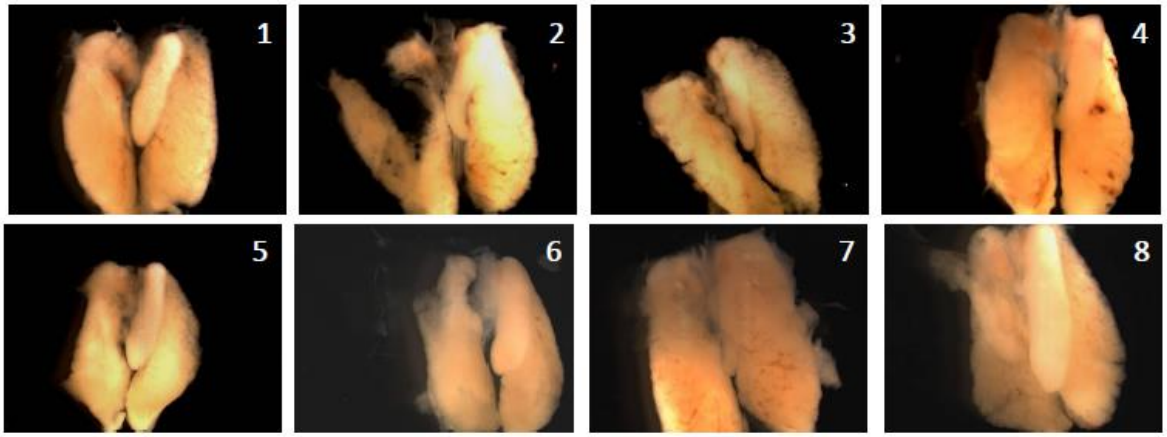


Figure 1.2. RCAS injected embryos pre-hatch (day 19)

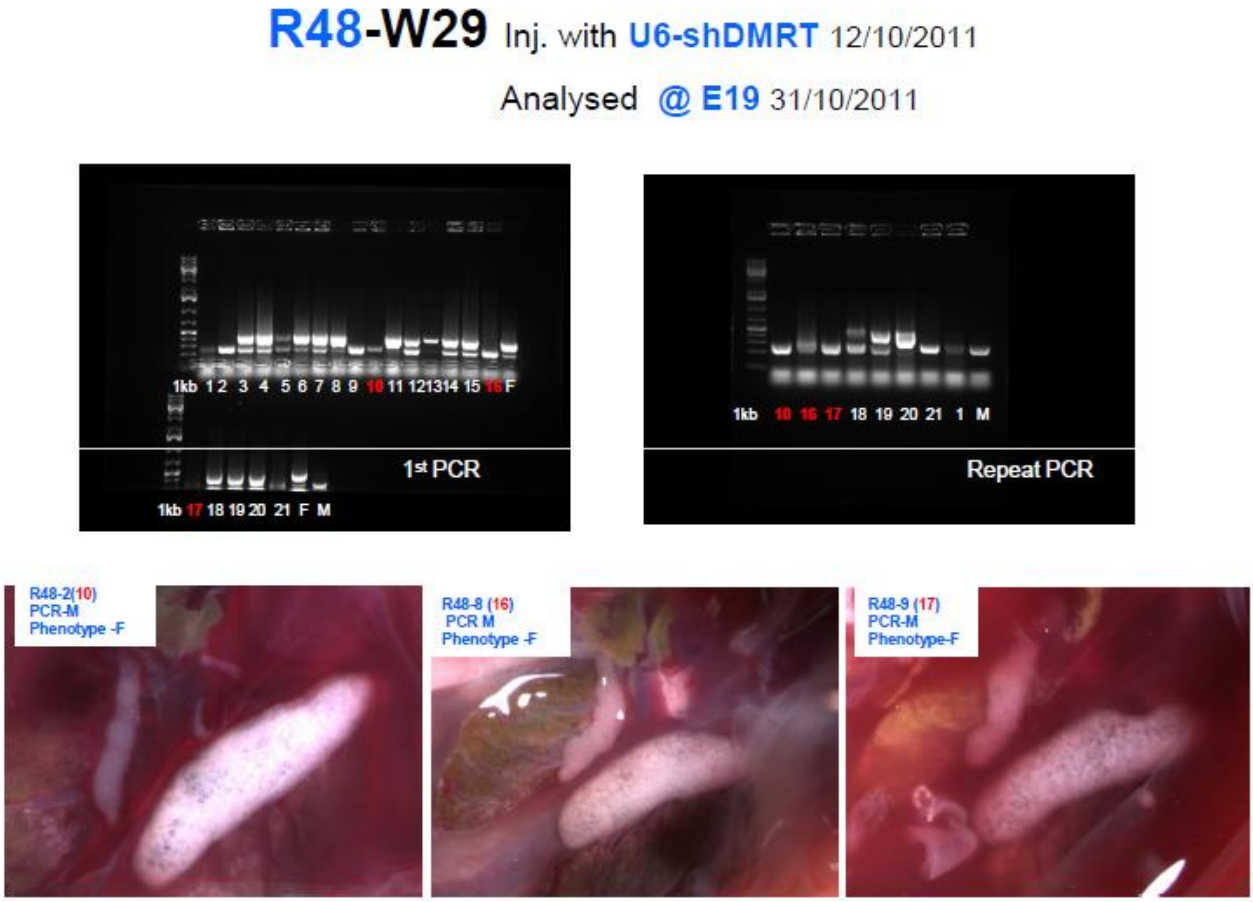


Figure 1.3. Gonad analysis of confirmed male bird 0056 and age matched males (0064 and 0072)

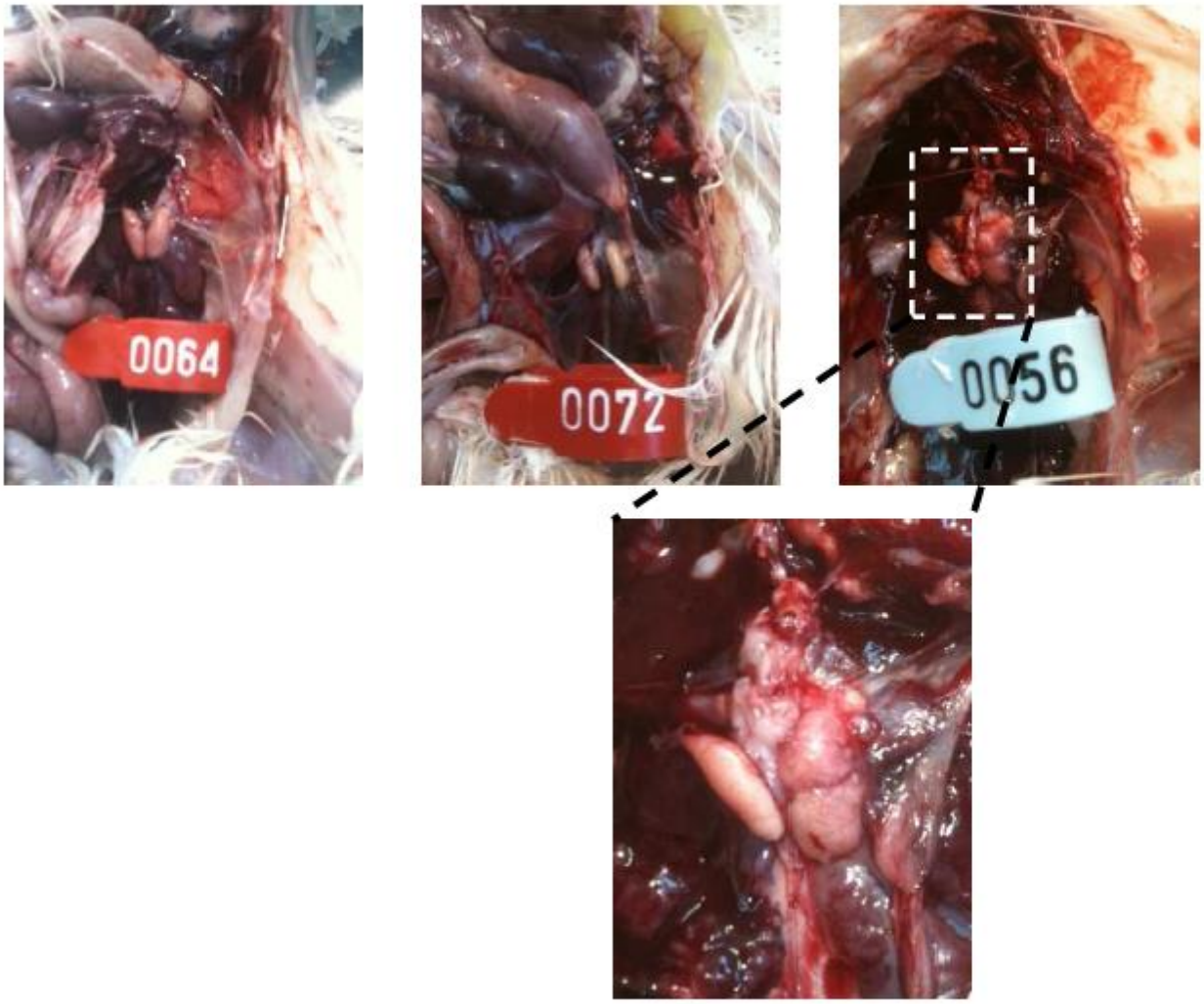


Figure 1.4. Aromatase and Sox9 expression on gonad sections from age matched female chicken from (A)U6-DMRT343 construct injections.

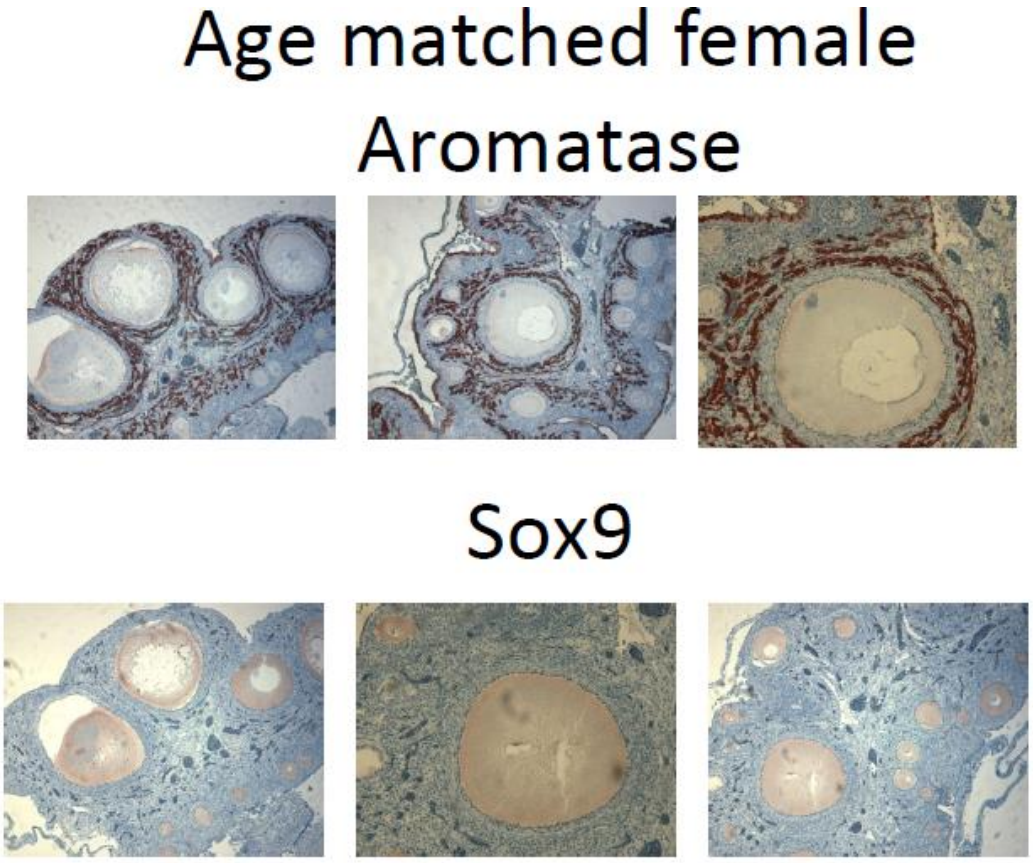
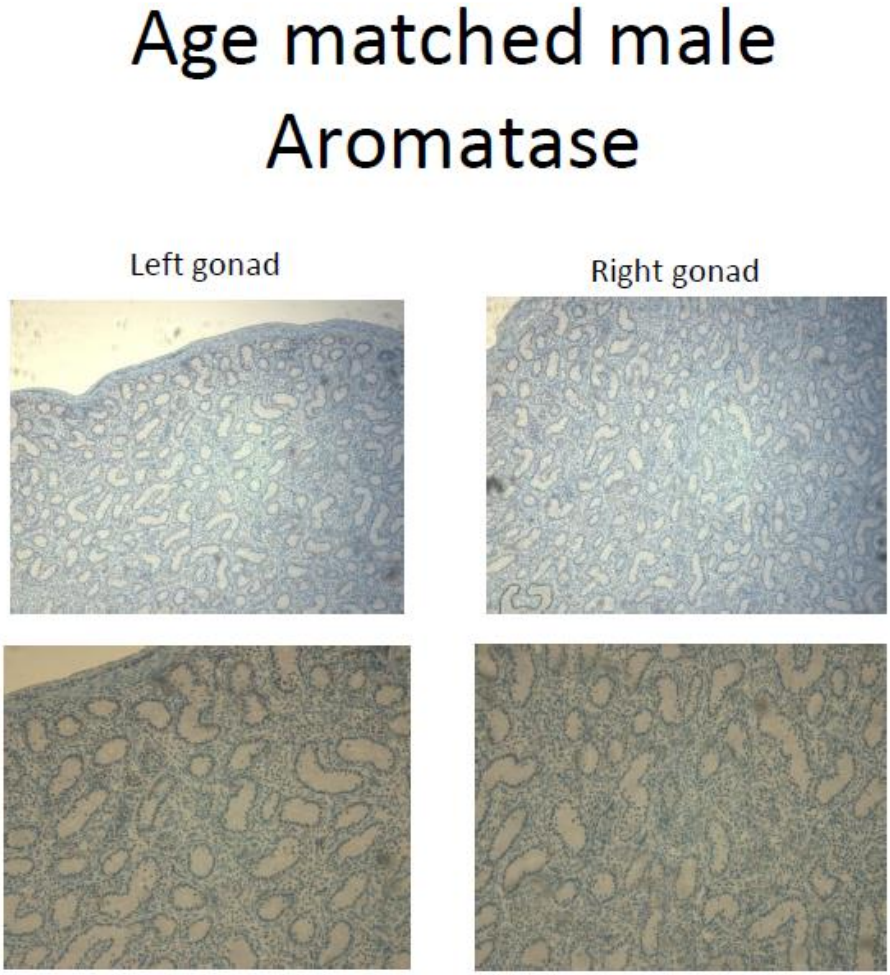
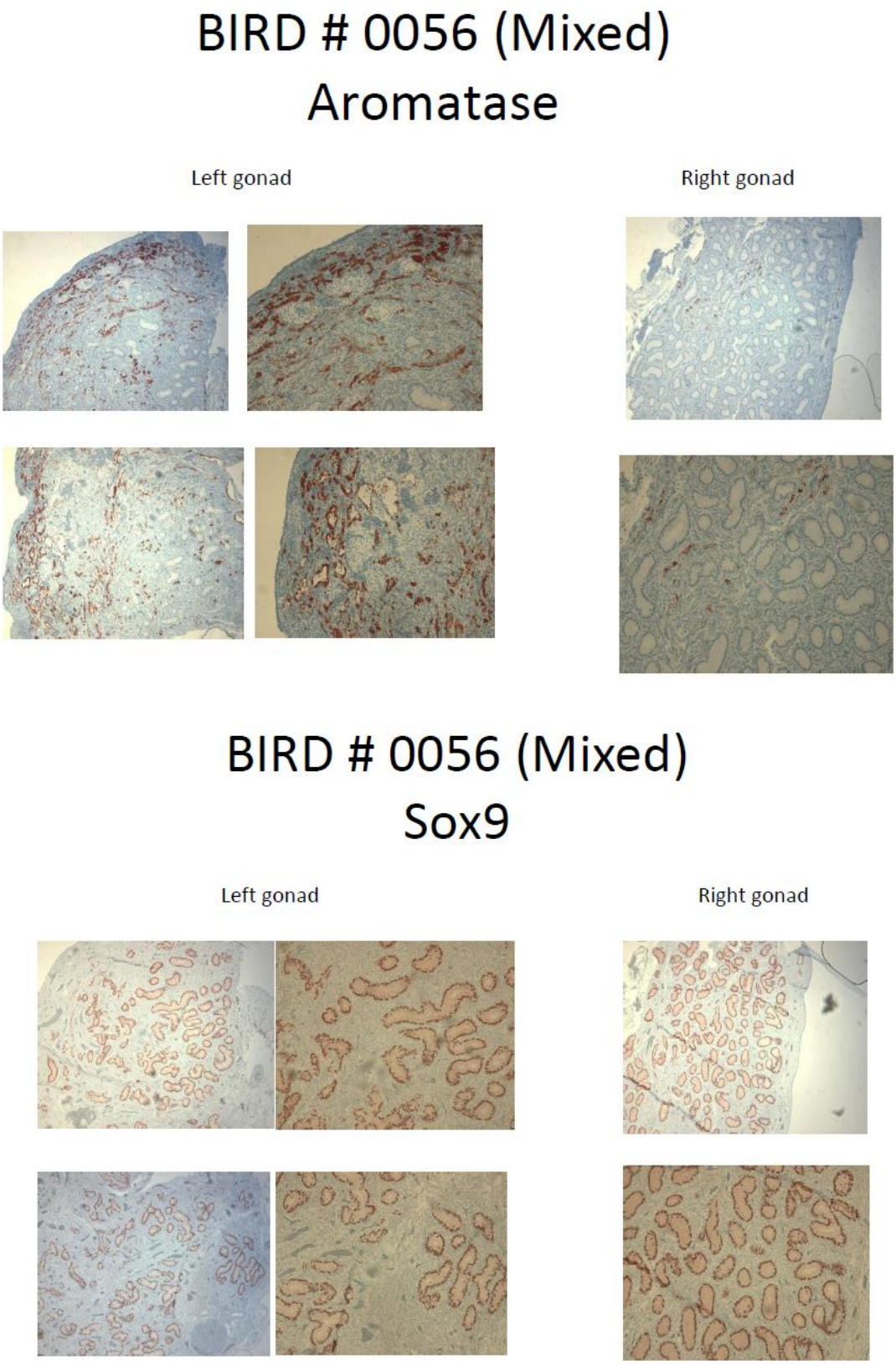


Figure 1.5. Aromatase and Sox9 expression on gonad sections from age matched male chicken from (A)U6-DMRT343 construct injections.



Note: This Sox9 Antibody was used at 1:2000. at this dilution it does not stain strongly. Both 1:1500 and 1:1000 were also done. However, the cover slips which are glued down were lifted and therefore could not get a clear image of them.

Figure 1.6. Aromatase and Sox9 expression on gonad sections from bird 0056.



Tol2 delivery of *DMRT1* shRNA for male to female sex reversal

We decided to adopt the direct injection method (Tol2 transfection) as an alternate approach to RCAS delivery of DMRT1-343 shRNA. The strategy was to assess the knockdown effect of DMRT1 expression in G1 male offspring from selected directly injected males. We used line 66 birds for this work. Our target number of directly injected males was 40 and from these we selected 10 for breeding, on the basis of qPCR analysis of semen samples. We constructed Tol2-EGFP vectors that contain H1 promoter (pMAT-DMRT343-H1) or attenuated U6 promoters (pMAT-DMRT343-U6-107 & pMAT-DMRT343-U6-018) expressing shDMRT343. We conducted “glonad” assays to confirm that each of the Tol2 expression vectors were tolerated in directly injected embryos. We also confirmed expression of shDMRT343 expression via a DMRT1-EGFP fusion reporter assay in DF1 cells. We next developed a direct injection schedule to generate the target of 40 males. Approximately half of the selected 40 males were injected with the H1 construct, a quarter received the U6-107 construct and the remaining quarter received the U6-018 construct. Expression analysis was conducted on culled female samples to confirm that the shDMRT1 molecule was expressed from each construct.

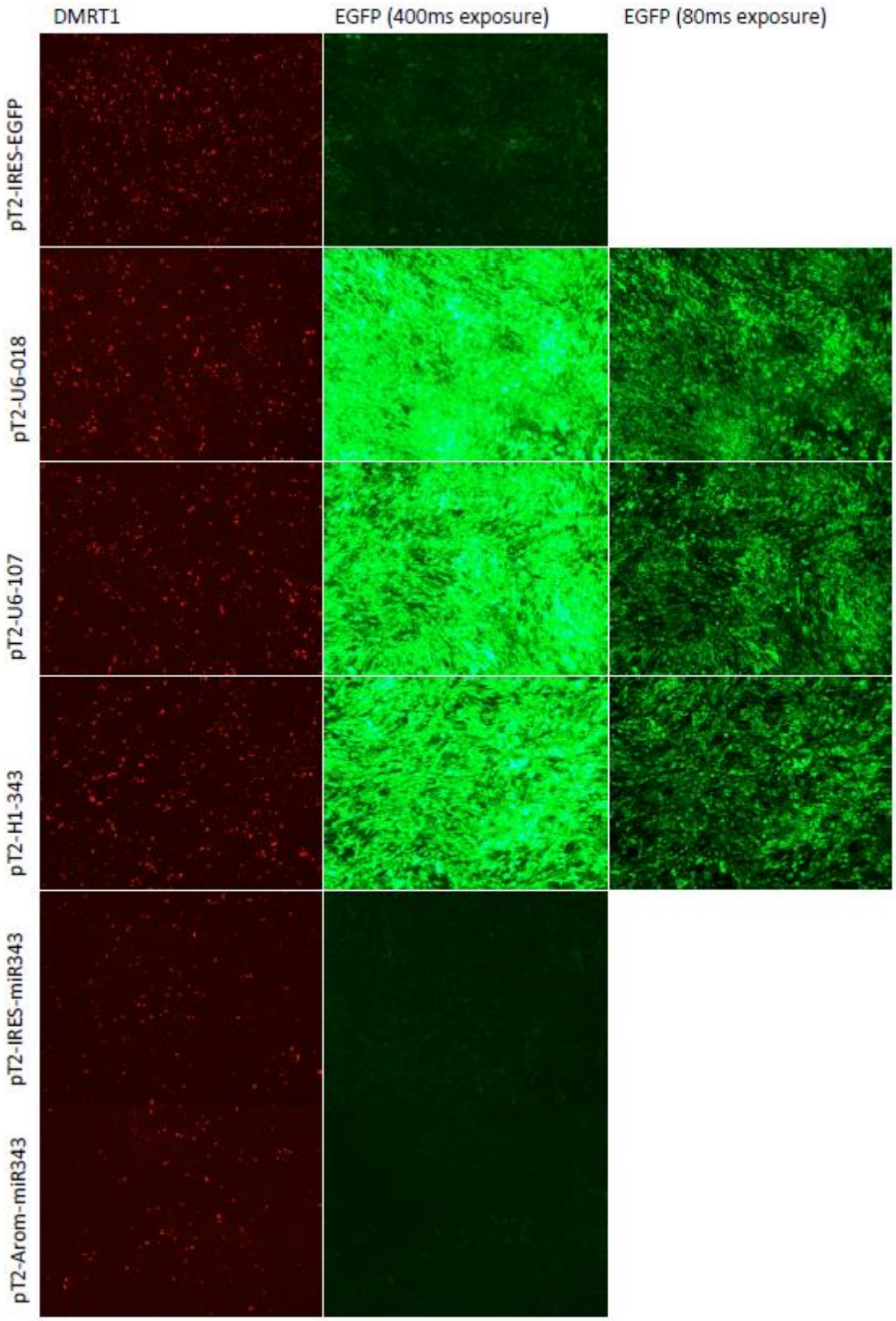
The Tol2 direct injection process generated 40 males that were raised to sexual maturity. We used three Tol 2 – EGFP constructs; one containing the H1 promoter (pMAT-DMRT343-H1) and two containing attenuated U6 promoters (pMAT-DMRT343-U6-107 & pMAT-DMRT343-U6-018) expressing shDMRT343. 26 of the selected 40 males were injected with the H1 construct, 8 received the U6-107 construct and the remaining 6 received the U6-018 construct. Semen samples from all the males were collected and analysed by qPCR to determine the percentage of transgenic sperm in their semen. Three roosters (22, 39 and 44) were chosen for breeding with the pMAT-DMRT343-H1construct and one rooster (46) with the pMAT-DMRT343-U6-107 construct. A breeding program was implemented with the 4 roosters. Transgenic G1 offspring were simply detected by EGFP expression and we generated a total of 103 positive chicks for assessment of knockdown of DMRT1 expression in the males. We produced 44 Tg +ve males from rooster 44 and 17 Tg +ve males from rooster 39. We did not detected Tg G1 progeny from roosters 22 and 46. We selectively culled male chicks at various stages of sexual development for analysis of gonad development. Selection was based on relative expression levels of the DMRT1 shRNA from blood samples. Chicks with the highest expression are maintained for sexual maturity whilst those with low levels were culled at earlier stages of development for analysis of gonad development and characterization of female specific markers using immune-histochemistry

on gonad tissue sections. We also analyzed young chicks that died early after hatch and it is these samples for which we have the most complete set of data.

We processed gonad samples from two early dead chicks 116 and 126. 116 was PCR sexed as a male and 126 as a female. Gonad tissues from both chicks were of decent quality and gave us a transgenic male and female sample to compare with existing non-transgenic controls. Histology analysis revealed clear DMRT1 knockdown and also a clear reduction in SOX9 expression in the transgenic male. The knockdown was modest and cords were observed in the gonads (fibronectin staining), however they appeared less organized than the control male sample. Also of interest was that the germ cells (CVH staining) were clearly less organised in the Tg +ve male compared to the Tg –ve control male (i.e. they did not line up around the cords as neatly) and were aggregating towards the edge suggesting the beginnings of a cortex. We potentially observed a small amount of aromatase up regulation, and perhaps there were a few SCP3 positive germ cells (meiotic). So at this stage, our assessment is that there is clearly an effect of the development of the gonads of the Tg+ve male chicks, but the level of DMRT1 silencing in these particular chicks might be too low to see the level of effect we were aiming for.

We have used TOL2 transposon to deliver GFP-H1 driven sh343 for DMRT1 knockdown in transgenic (Tg) birds. This vector yielded G0 birds with GFP +ve semen, and fully Tg G1 birds. However, gonadal phenotypes were only mildly sex-reversed (partially or not ovarian in ZZ birds). This could be due to the degree of integration of the transgene, or poor knockdown with the current construct. We detected reasonably normal DMRT1 protein expression in the G1 knockdown birds. We therefore developed and tested a variety of alternative TOL2-sh343 vectors for their ability to knockdown DMRT1 expression in vitro (in chicken DF1 cells, using immunofluorescence as the read out) (Figure 1.7). The DF1 cells were transfected with the various Tol2 constructs, or with new vectors (empty control = pT2-IRES-EGFP, miR-343 and EGFP = pT2-IRESmiR343, Aromatase and miR343 and EGFP = pT2-Arom-miR343), along with transposase. Cells were passaged for one week, FACS sorted, plated back down, transiently transfected with pCMV-DMRT1, then stained for DMRT1 protein expression. The best knockdown was obtained with the miRised-343 vectors (with or without an Aromatase open reading frame included). The original U6-sh343 did not show as good a knockdown. This suggests that the current method of Tg bird production may not knockdown as efficiently as the original RCAs delivered DMRTR1 sh343.

Figure 1.7 In vitro analysis of new Tol 2 constructs for knock down of DMRT1 and over expression of Aromatase.



We selected 3 G1 males and 9 G1 females with the highest levels of shRNA expression as determined by PAM-PCR and set up matings to generate G2 birds for analysis. These matings generated G2 chicks with multiple copies of the shRNA transgene and we used PAM-PCR and EGFP fluorescence to analyse expression of these chicks and determine if we were successful in improving levels of RNAi activity for increased silencing of DMRT1. Unfortunately we did not observe any noticeable increase in expression levels in the G2 chicks analysed and subsequently culled the chicks from these hatches. We also conducted a time course analysis to study gonad morphology and histological analysis of the urogenital system in male embryos and chicks. Samples were taken from five males at E17, D2, D8 and D15 for analysis.

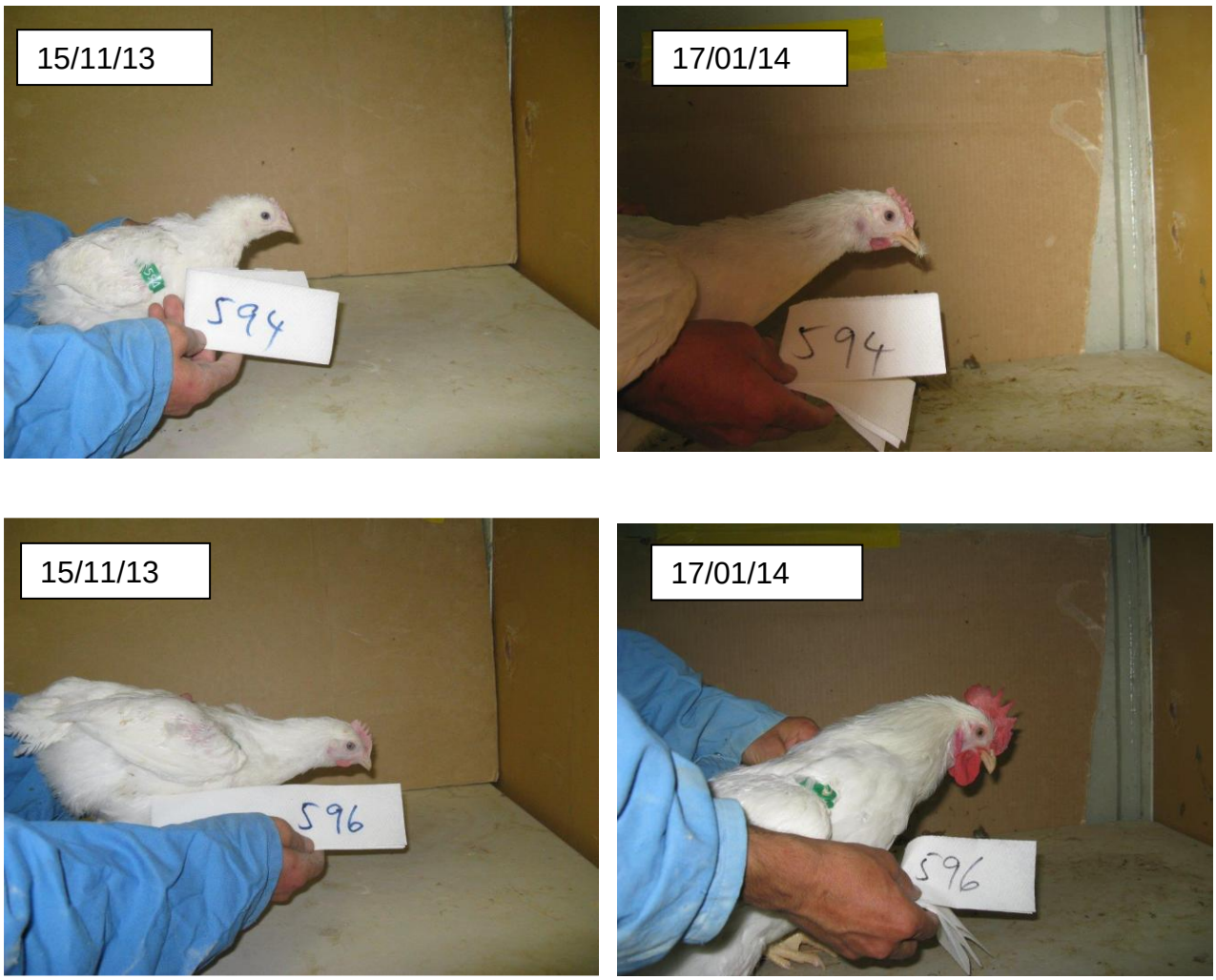
Tol2 delivery of Aromatase for male to female sex reversal

During this project we published a study showing that over-expression of Aromatase alone (from the RCAS viral vector) is sufficient to induce complete male-to-female sex reversal in chicken embryos, consistent with previous data. Based on this result we developed a number of new constructs. Two of the constructs were designed and developed for over expression of Aromatase (pT2-IRES-Arom and pT2-CMV-Arom). The third construct was designed and developed for improved silencing of DMRT1 using a newly developed "mirised" DMRT1 sh343 (pT2-IRES-mIR343). The fourth construct was pT2-IRES-Arom-mir343. This construct not only expresses Aromatase but also expresses the "mirised" DMRT1 sh343. We believe that improved knock down of DMRT1 in conjunction with over expression of Aromatase will have a synergistic effect on the feminization of male embryos. We also placed selected G1 females with Tol 2 shDMRT1 inserts in combinatorial matings with G(0) males generated using the aromatase over expression constructs.

Direct injection of the 4 new constructs was completed as planned. We generated 6 G(0) males for pT2-IRES-Arom, 13 G(0) males for pT2-CMV-Arom, 11 G(0) males for pT2-IRES-mIR343 and 17 G(0) males for pT2-IRES-Arom-mir343. Once all G(0) males for each construct reached sexual maturity we started testing semen samples using the Tol2 qPCR test. All selected males were measured for secondary sexual phenotype at 4 week intervals leading to sexual maturity (weight, comb size and colour). One of the pT2-Arom-mir343 G0 males (594) had some female characteristics suggesting that this bird is reasonably chimeric post direct injection with the Tol2 construct – this is possible and also very encouraging that we may observe a profoundly modulated phenotype in a fully transgenic G(1) male bird (Figure 1.8). We have focussed on a single mating with our best candidate G(0) rooster (Rooster 528 - %Tg 0.342) and placed this bird with 3 of the DMRT1 G(1) females described

above. The Tol2- Aromatase constructs do not express EGFP so we cannot use fluorescence as a screen for G1 chicks that have both the Tol2-Aromatase and Tol2-shDMRT1 constructs. We developed a specific endpoint PCR to detect the unique Tol2-Aromatase sequence from gDNA isolated from blood samples of two week old chicks. At hatch, we separate the EGFP +ve and EGFP –ve chicks and take blood samples from all chicks and determine sex via our PCR test. PCR +ve/EGFP +ve chicks will have both Tol2 constructs and PCR+ve/EGFP-ve chicks will only have the Tol2-Aromatase construct. We have so far screened ~150 G(1) chicks from this mating and not yet generated a Tg +ve G(1) chick. We will maintain the breeding until the end of the project to give us every chance of success.

Figure 1.8. G(0) Rooster 594 – chimeric expression of aromatase gene from Tol2 construct. Images taken on 15/11/2013 and again on 17/01/2014

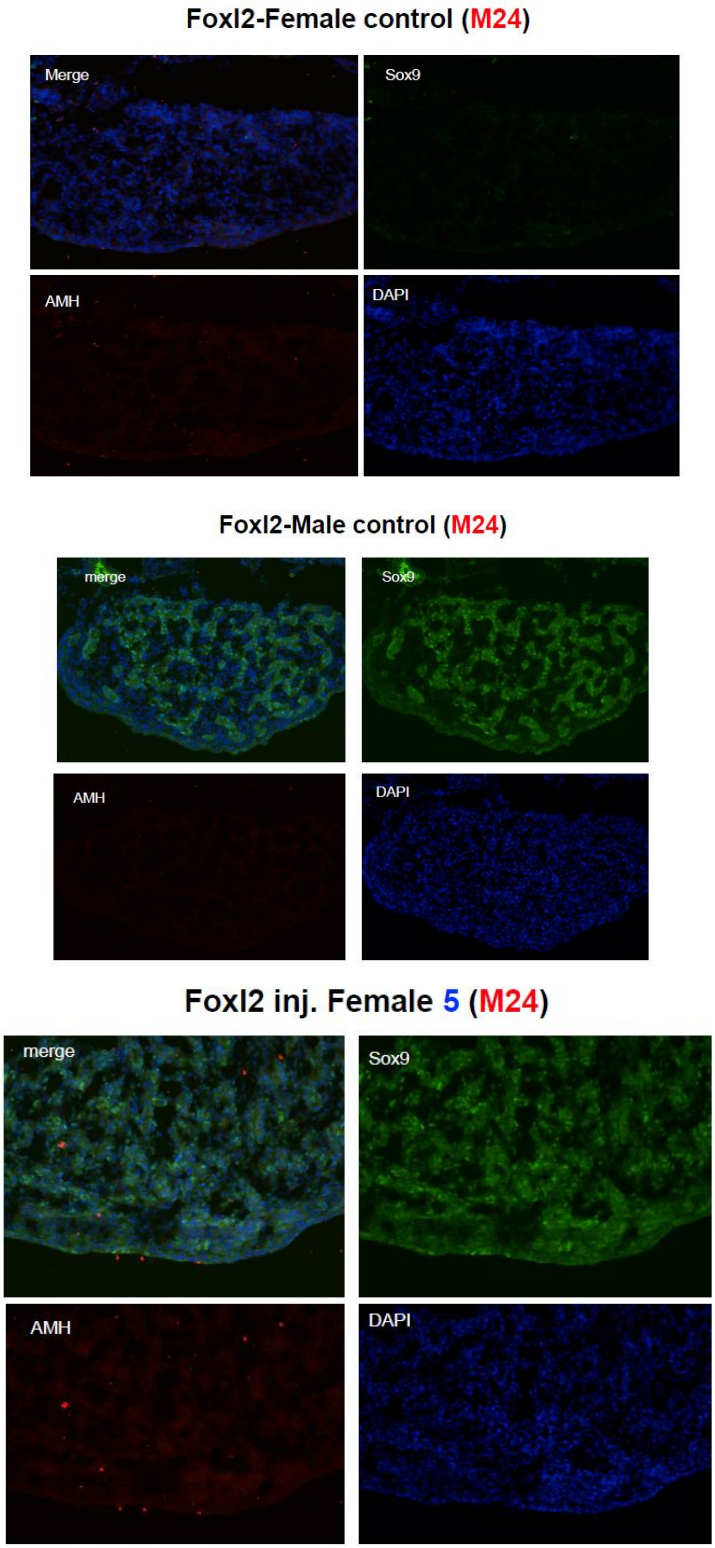


Direct injection of Aromatase and *FOXL2* siRNA for female to male sex reversal

During this project we also investigated the potential to directly deliver siRNAs to early stage embryos to modulate key sex determination genes. This was developed as an alternative to viral or transgene delivery. The target genes for this work were aromatase and FOXL2. We tested 3 siRNAs against an aromatase over-expression construct in DF1 cells. Two of the siRNAs gave potent silencing of aromatase as measured by antibody staining and we ordered a large scale synthesis of the best siRNA for in ovo work. We delivered the siRNA formulations via IV route to embryos, initially following our direct injection protocol and injecting day 2.5 embryos. We also investigated later time points (ie day 4 – 5) when we know we can measure aromatase mRNA and allow us to test silencing via qPCR. We also assessing phenotypic changes (including histopathology) to gonad development in injected embryos at staged intervals leading to hatch. In conjunction with this milestone, we also arranged large scale synthesis of a FOXL2 siRNA and assessed side by side with the aromatase silencing molecule.

We initially focused on IV delivery using lipofectamine2000 following our direct injection protocol in E2.5 embryos. We analysed gonad morphology post-injection in E10 embryos and stained gonad sections for the male specific gene *SOX9*. To date our most encouraging results were obtained with the *FOXL2* siRNA where we observed signs of female-to-male sex reversal via activation of the male *SOX9* gene in E10 female gonads (Figure 1.9). We delivered 200 pmol of siRNA per embryo and survivability was high.

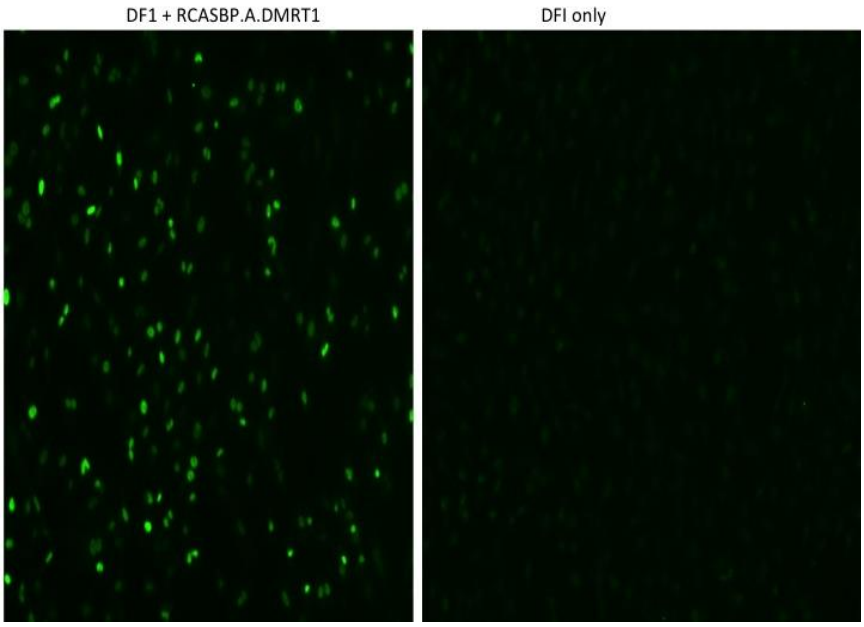
Figure 1.9. Activation on *SOX9* in female E10 embryo directly injected with *FOXL2* siRNA



Over-expression of DMRT1 to induce female-to-male sex reversal

To over-express DMRT1 in chicken embryos, RCAS viral vector was initially used. The DMRT1 ORF was cloned into RCASBP.A. vector and high titre virus produced in DF1 chicken fibroblastic cells. An in-house antibody was raised that would detect DMRT1 protein. This antibody detected robust over-expression of DMRT1 from RCASBP virus in vitro (Fig. 1.10 below).

Figure 1.10: Detection of DMRT1 protein in DF1 cells 5 days after transfection with RCASBP.A.DMRT1 DNA. Immunostaining for DMRT1 using in-house antibody.



Injection of high titre virus into day 0 blastoderm consistently caused embryo lethality by E4.5. This is not surprising, since this approach would deliver the transcription factor throughout most cells. Most recently, we have used gonad-specific electroporation at E2.5,

in which DNA is injected into the left body cavity and taken up by cells across via an electrical current. This resulted in areas of DMRT1 over-expression throughout the gonad and activation of male markers, SOX9 and AMH, in female gonads. (Figure below) (Lambeth et al., 2014), The data further support our proposal that DMRT1 is the master male determinant in chickens.

Fig 1.11. Over-expression of DMRT1 in embryonic chicken gonads. In control females, DMRT1 protein is lowly expressed. In control males, it is highly expressed in organising seminiferous cords (green). In female + RCASBP.DMRT1, DMRT1 expression is elevated in scattered cells throughout the gonad (right panel).

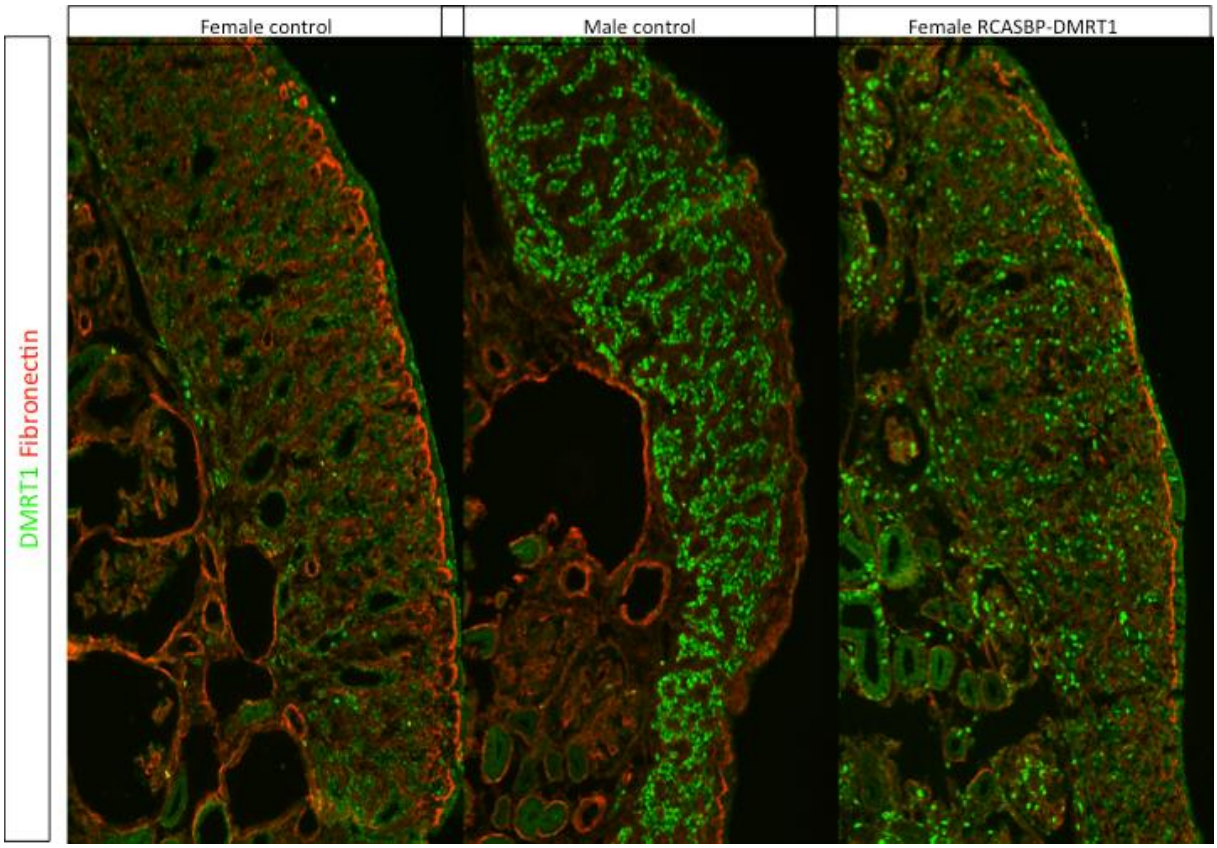
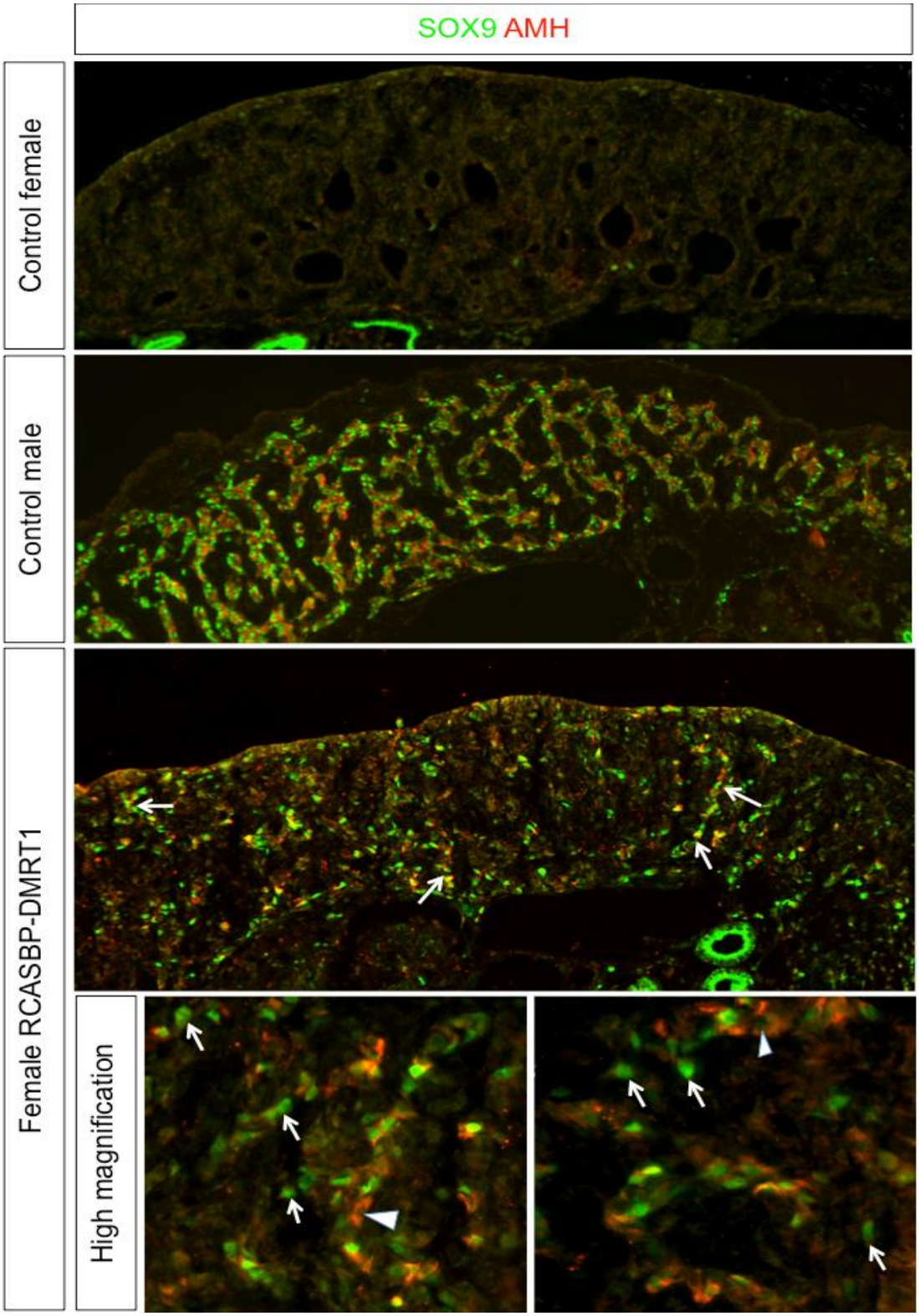


Figure 1.12. Activation of the male pathway genes, SOX9 and AMH, following over-expression of DMRT1.

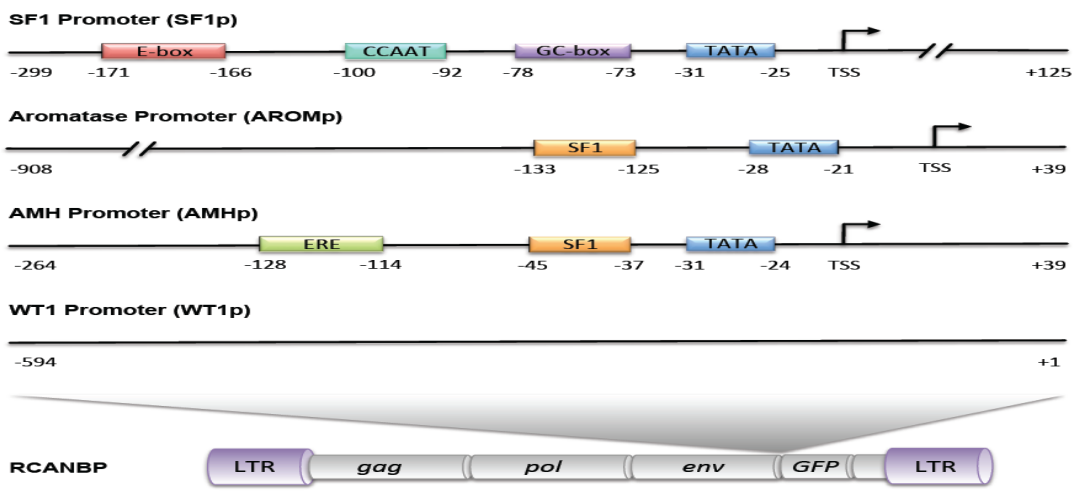


Over-expression of DMRT1 using tissue-specific (gonad-specific) promoters

The data shown above confirm that over-expression of DMRT1 in the gonads can direct differentiation along the male pathway, and hence cause female-to-male sex reversal. To overcome the problem of embryo lethality in transgenic birds over-expressing DMRT1, a gonad-specific mechanism of expression is required. Years 2 and 3 of the CRC were spent attempting to identify a suitable promoter or enhancer that would drive *DMRT1* expression only or predominantly in the developing gonads, and hence direct male development. Such a promoter could then be used to produce transgenic birds (via TOL2, since RCAS vector proved toxic to hatchlings as described above).

A number of candidate promoters and/ or enhancers were tested for the ability to drive gene expression (GFP reporter or DMRT1) in embryonic chicken gonads. Putative regulators of genes expressed in the goands were tested, including *AMH, SFI* and *WT1* promoters, and also the mouse *SF1* enhancer elements. The latter has been shown to drive strong GFP expression in the gonads. The data are summarised on figure 1.13 below. For these experiments, the RCANBP viral vector was used to test promoter/enhancer activity. RCANBP lacks a splice acceptor that is present in RCASBP, so that internal genes are no transcribed. It allows the insertion of regulatory sequences that drive expression of a chosen transgene independent of transcriptional activity of the virus itself. Various gonadal regulatory regions driving GFP were cloned into RCANBP, as shown below:

Figure 1.13. Construct design for gonad-specific expression.

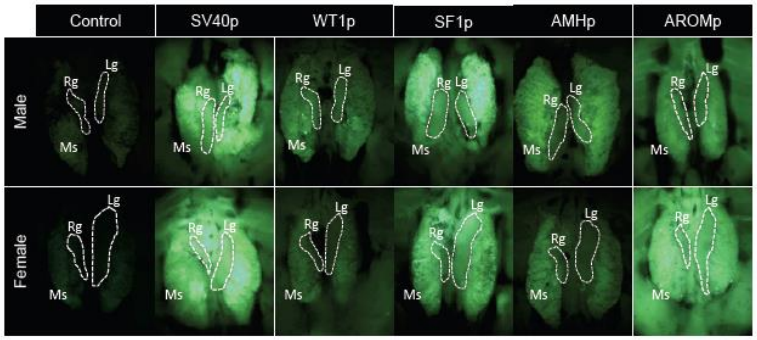


High titre viruses were produced and used to infect day 0 blastoderms. Initially, GFP reporter expression was then assessed in embryos harvested at day 10.5. At least 80 embryos were

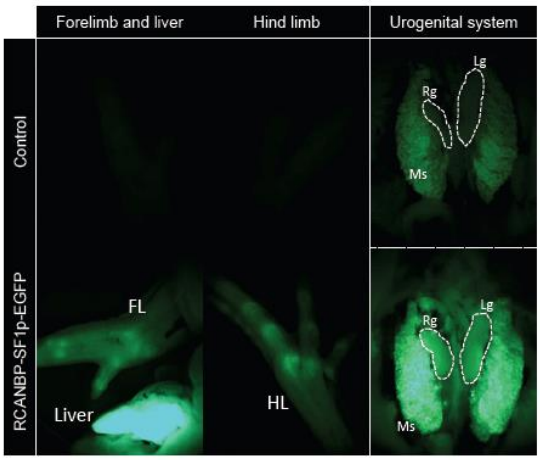
used for each construct. Figure 1.v below shows the results of these experiments. In negative controls (not injected) no GFP was detected. Positive controls included RCANBP virus carrying CMV promoter/enhancer. This construct caused widespread GFP expression in the embryo in a non-tissue specific way, as expected. The WT and AMH promoter yielded little if any reporter expression in the gonads. Aromatase promoter produced strong GFP expression in the gonads, but also strong expression throughout the embryo. Meanwhile, the SF1 promoter yielded good GFP expression in the gonads with a low level of expression elsewhere in the embryo (Figure 1.14 below).

Figure 1.14.A. GFP reporter expression in the urogenital system of E10.5 embryos following injection of RCANBP-promoters into day 0 blastoderms. The highest level of expression, with minimal expression elsewhere in the embryo, was observed when using the SF1 promoter construct. B. Low level expression of RCASNBP.B.SF1-GFP outside the UGS.

A

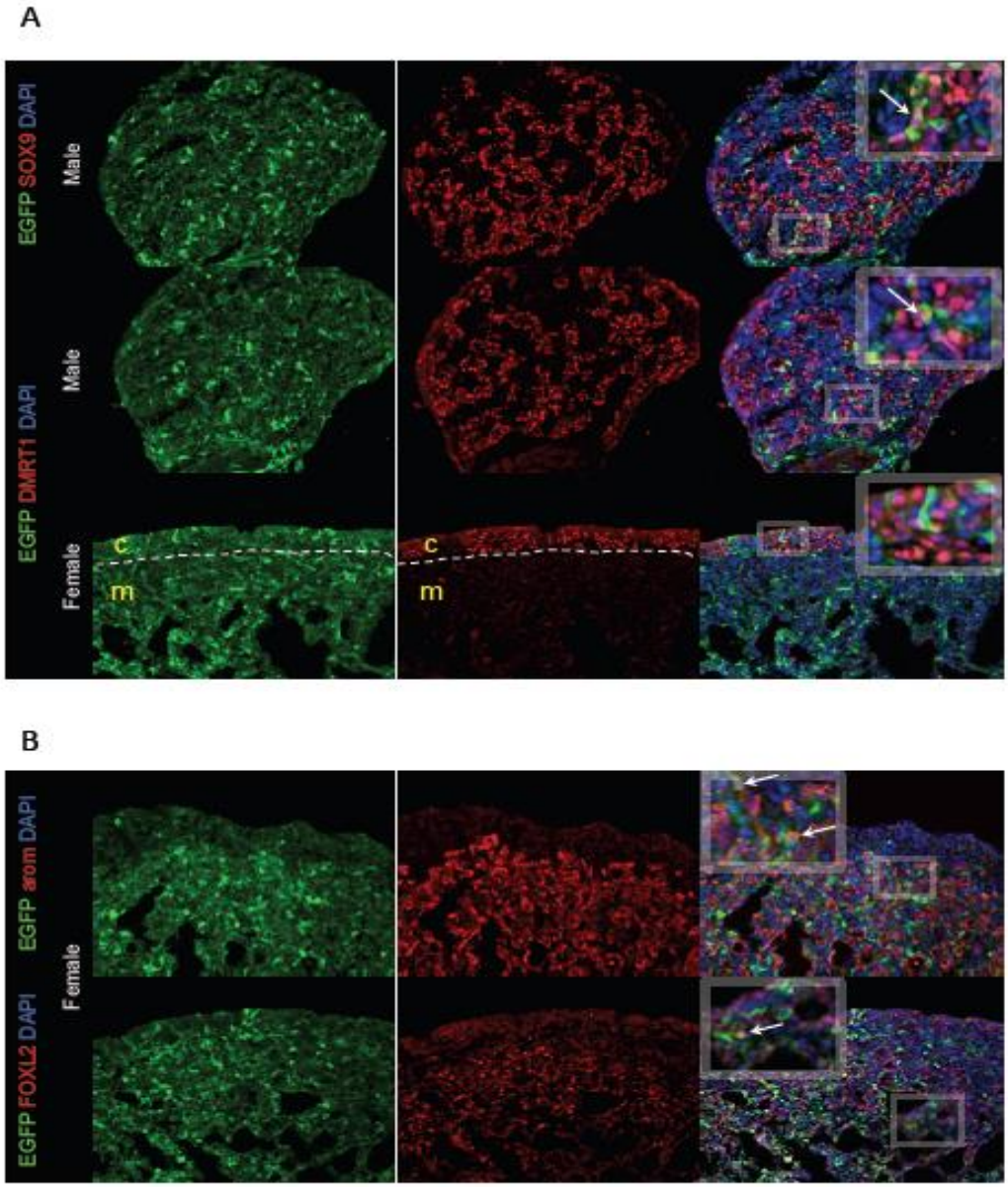


B



In tissue sections, GFP expression was detectable in the gonads, where is co-localised with markers, such as DMRT1, SOX9, Aromatase and FOXL2, as shown below in figure 1. 15. This was expected, since SF1 is expressed in the somatic cells of both sexes.

Figure 1.15. Expression of GFP from RCAN.SF1 promoter in E10.5 gonads (green), compared to male and female sex markers (red). Boxed inserts show high pwer view and co-localisation of the GFP and markers.

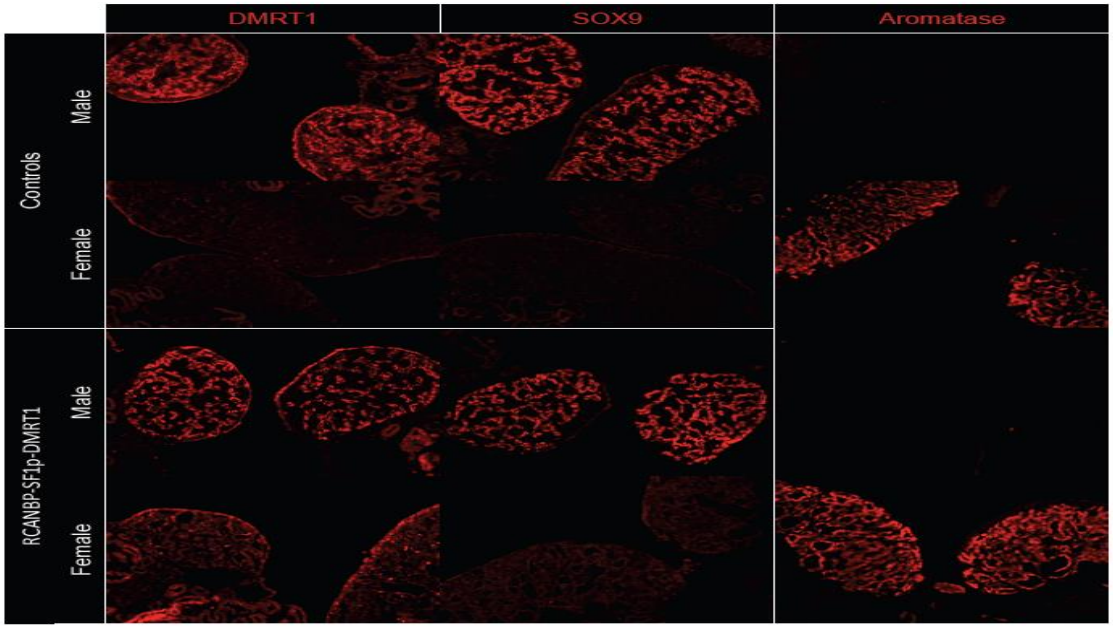


The RCAN.SF1 promoter was then used to express DMRT1 in a gonad-specific manner. High titre virus (RCANBP.A.SF1promoter-DMRT1) was used to infect day o blastoderms and

the embryogenesis allowed to proceed to day 10.5. However, the gonads (of both sexes) appeared normal. Female gonads appeared to be normal ovaries. Immunofluorescence showed elevated DMRT1 in female gonads – delivered in a tissue specific manner by RCANBP – but normal female markers were still expressed (e.g., Aromatase). This is shown below in figure 1.16. We conclude from these experiments that while *SF1* promoter is a potentially useful promoter in which to drive gene expression in the the embryonic gonads, it does not deliver sufficient DMRT1 over-expression to masculinise female gonads.

Most recently, we have carried out ChIP-seq studies to identify novel promoter or enhancer elements that could be used to drive gene expression in the gonads. ChIP-seq stands for Chromatin immunoprecipitation followed by deep sequencing. By this method, gonadal DNA is sheared and then immunoprecipitated with antibodies that recognise active chromatin, and hence promoter or enhancers that are active (e.g, histone H3K4- trimethylated). Such histone ChIP captures genes “in the act” of becoming active. The precipitated DNA is then subjected to deep sequencing and the sequence reads mapped back to the chicken genome, revealing peaks where active chromatic is found. Preliminary data, using K3K4-tri methylation, are very promising. We have identified large numbers of novel regulatory regions (a first for gonadal studies) and this will form the basis of a new CRC proposal.

Fig 1.16. Modest over-expression of DMRT in female gonads suing RCANBP.SF1.promoter. The gonads are not masculinised, as SOX9 is not expressed



Publications that derived from the work in Chapter 1

Lambeth, L, Cummins, D, Doran, T, Sinclair, AH and **Smith, CA** (2013). Ectopic expression of a single gene, aromatase, is sufficient for gonadal sex reversal in male chicken embryos. ***PLoS One*** (DOI: 10.1371/journal.pone.0068362).

Cummins, David; Tyack, Scott; Doran, Tim. Characterisation and comparison of the chicken H1 RNA polymerase III for short hairpin RNA expression. Biochemical and Biophysical Research Communications. 2011; 416(1-2):194-198.

Chapter 2: Modulation of *R-SPO1* and *FOXL2* genes

Summary

This chapter summarises the research conducted at MCRI on the modulation of *R-SPO1* and *FOXL2* in chicken embryos (Objective 2). Knockdown and over-expression of *R-SPO1* had no consistent effect upon the phenotype of embryonic gonads. In the case of over-expression, this was likely due to toxicity of the gene within cells. In the case of knockdown, the reasons for a lack of phenotype remain unknown, but may be related to insufficient expression of the RNAi molecules (miRNAs). Alternatively, *R-SPO1* may not be required for ovarian development in chicken. In contrast, modulation of *FOXL2* produced gonadal phenotypes. Over-expression of *FOXL2* in male embryonic gonads blocked the male pathway, while knock down allowed activation of the male pathway in females. *FOXL2* is therefore a candidate gene for further analysis.

Over-expression and RNAi knockdown of *R-SPO1* to modulate female development

For the gene knockdown studies, short hairpin RNAs (shRNAs) were cloned into the RCAS viral vector carrying GFP reporter, and tested for their ability to knock down R-SPO1 or FOXL2 in vitro (in the DF1 fibroblastic cell line).

R-SPO1. Firstly, an in-house antibody was raised chicken R-SPO1. Using immunofluorescence, this antibody recognised R-SPO1 protein over-expressed *in vitro* (figure 2.1) and in gonads in vivo (Figure 2.2). R-SPO1 is a signalling molecule and is expressed in the cell and at the cell membrane. For R-SPO1 knockdown, Several shRNAs were identified that showed robust knockdown (at least 75% by immunostaining). This is shown in figure 2.3 and 2.4. Short hairpin RNAs that showed robust knockdown were converted into an shRNA or miRNA format and cloned into RCAS viral vector for propogation of high titre viral stocks. The active RCAS virus was then used to infect either early blastoderms (day 0) or for gonadal electroporation (EP) whereby the viral DNA is transferred into gonadal cells via an electric current after local injection. Treatment of embryos or gonads with the RCAS virus expressing R-SPO1 knockdown constructs failed to affect endogenous R-SPO1 expression or alter gonadal phenotype, as shown in figure 2.5. The reason for this is unclear, since the siRNA (and its miRNA derivative in RCAS) can robustly knock down R-SPO1 in vitro. We have not at this stage assessed the expression level of the mature miRNA, but it may be suboptimal.

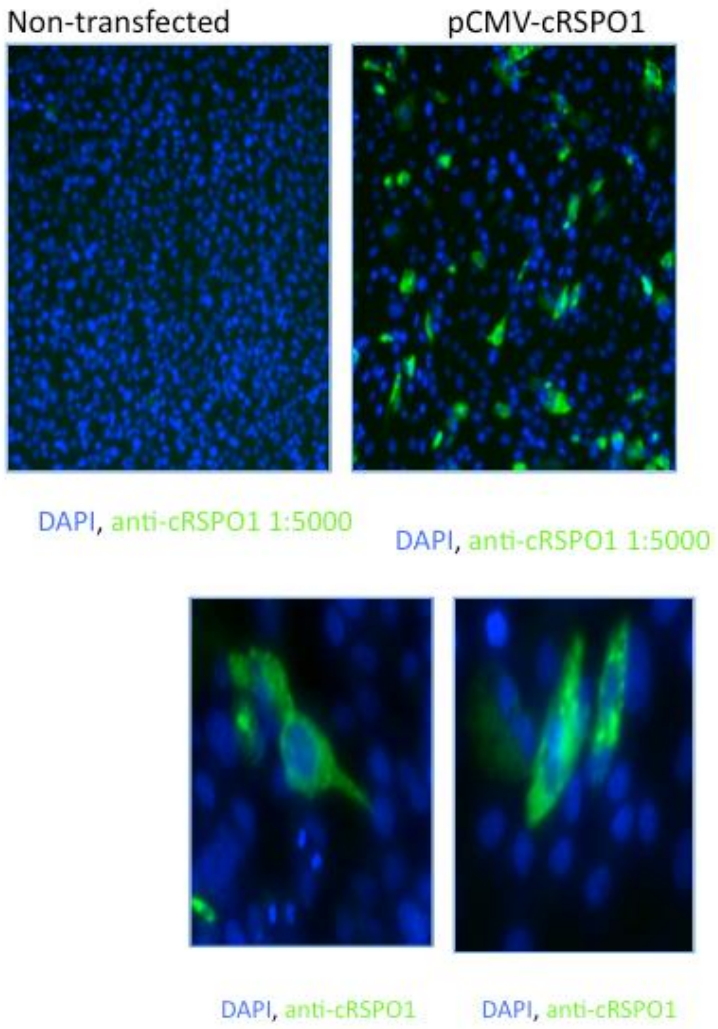


Figure 2.1: Validation of R-SPO1 antibody in vitro. The in-house anti-chicken R-SPO1 antibody raied In rabbit recognises the over-expressed protein in DF1 cells transfected with pCMV plasmid Expressing the open reading frame but not in untransfacted DF1 cells. C and D show high power views.

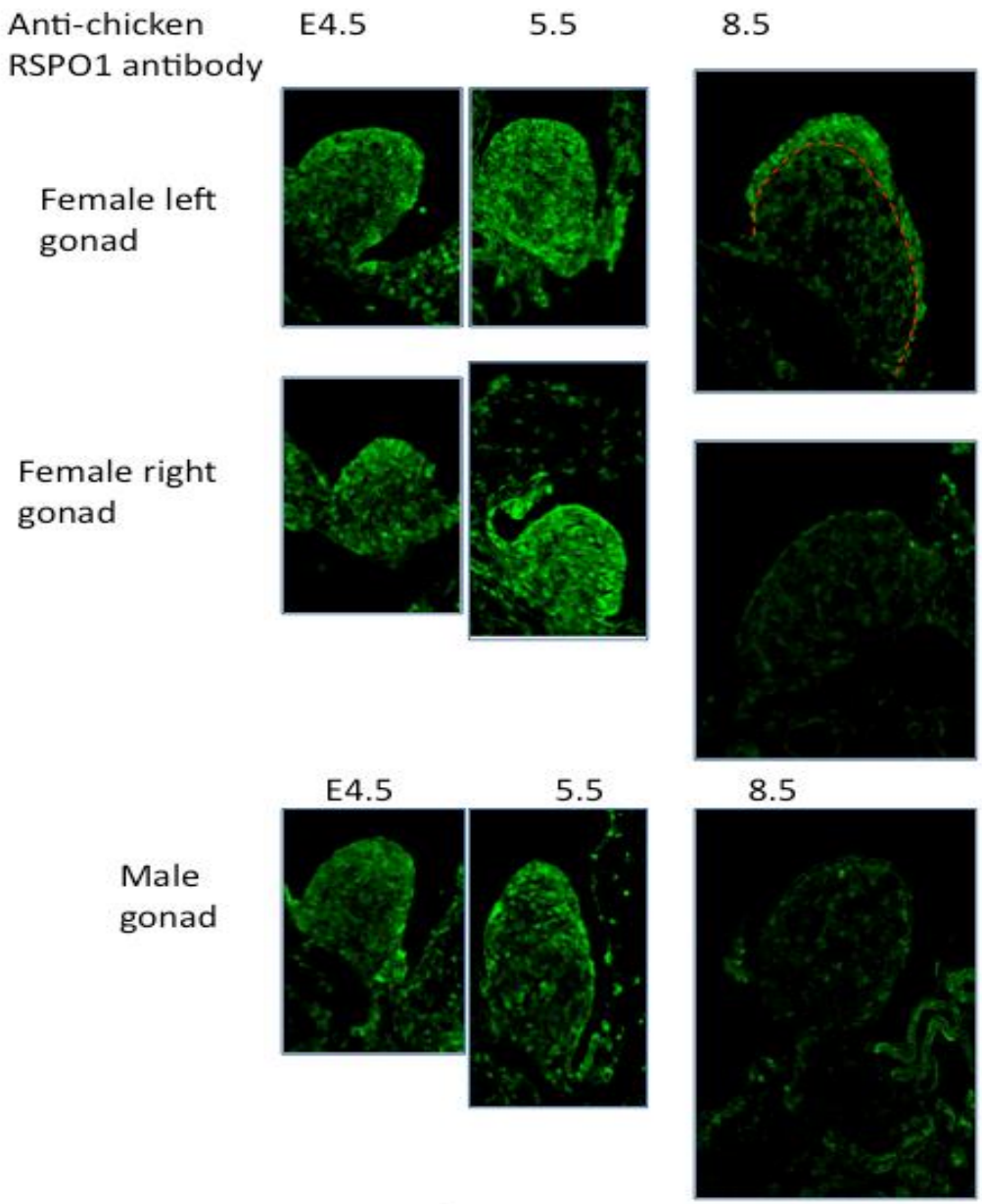


Fig 2.2 Expression of R-SPO1 protein in embryonic day (E) 4.5, 5,5 and 8.5 gonads, using in house antibody (green). The protein is more highly expressed in female gonads and becomes concentrated in outer cortex (outlined in E8.5 above).

Fig 2.3: R-SPO1 Knockdown strategy

ATGCAGCTTGGACTGTTTGTGGTGGTGGTTTTTCTAAGCTCGATGGATCTAACAGGCGGCAGCAAAGTGGTGAAGGGC
AAGAGGCAAAGGCGAATTAGCACTGAGCTGAGCCAGGGCTGTGCCAGGGGCTGCGACCTGTGCTCTGAGTTCAACGGG
TGCCTGAGATGTTCCCCCAAGCTCTTCATCCTTCTGGAGAGGAACGATATCCGGCAAATTGGGATCTGCCTCCCATCC
TGTCCACTGGGATACTTTGGCCTTCGCAATACAGACATGAACAAGTGCATCAAATGCAAAATCGAGAACTGTGAGTCC
TGCTTCAGCCGAAACTTTTGCACAAAATGTAAGGAAGGTTTGTATTTGCACAAAGGGAGATGTTACGTCACGTGCCCC
GAAGGCTACTCTGCTGCCAATGGCACCATGGAGTGCAGCAGTCCTGCGCAATGTGAAATGAGTGAGTGGGGGCCCTGG
GGGCCCTGCTCCAAGAACAGGAAGCTGTGTGGCTTCAAGAAGGGGAACGAGGACCGAACGCGGCGGATCCTGCAGGCT
CCCTCTGGGGACGTGTCCCTGTGCCCCGCCACCACGGAGGTGCGCAGATGCACTGTGCAGAAGAGCCAATGCCCCGAA
GGGAAAAGGAAGAAAAAGGACGAGCAAGGAAAGCAAGATAATACAAACGGGAACAGAAATCGGAAAGACACCAAAGAT
GCAAAGTCTGGCACCAAGAAGAGGAAGAGCAAACAGAGGGGGGCTGTGGCCCCCACCACATCCGCCAGCCCTGCCCAA
TAG

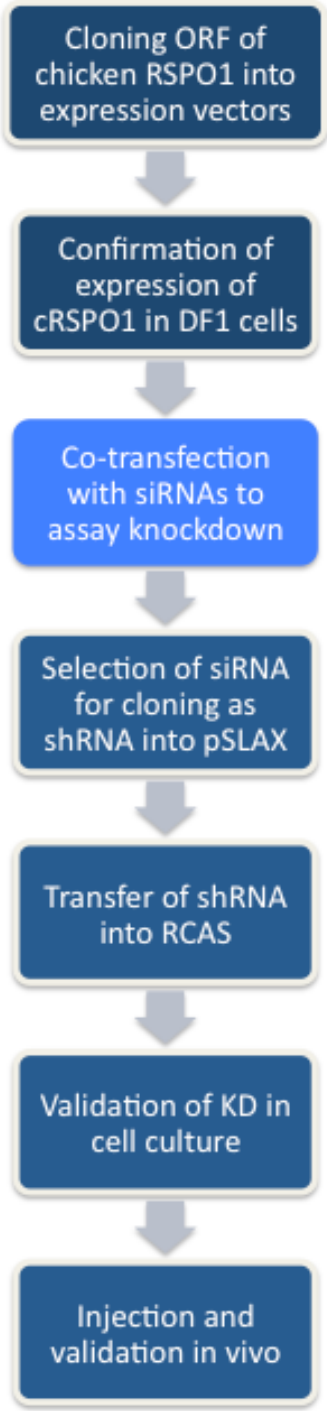


Figure 2.3
R-SPO1 knockdown strategy.
Five short interfering RNAs (siRNAs) were designed against different regions of chicken R-SPO1 cDNA. They were then individually tested in vitro for their ability to quench R-SPO1 protein Expression ,assessed by immunofluorescence. Those showing robust knock down were converted into short hairpin RNAs (siRNAS) cloned into RCAS viral vector and used to infect gonads.

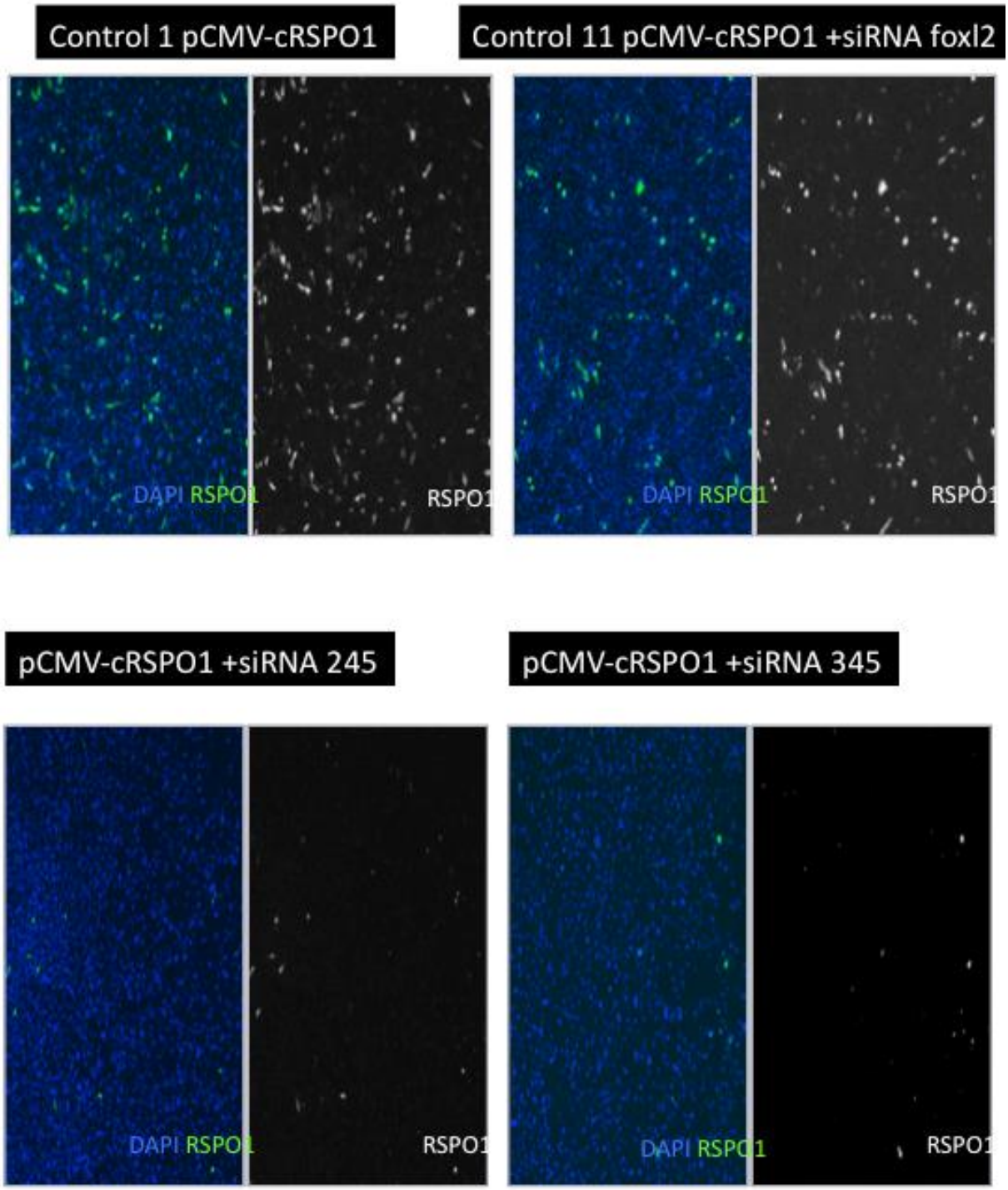


Fig 2.4. Knockdown of R-SPO1 expression DF1 cells after treatment with siRNAs. Controls in upper panels show R-SPO1 expression In absence of silencing siRNAs, or in presence of irrelevant siRNA. Bottom panels show robust knock down with siRNa245 or siRNA345

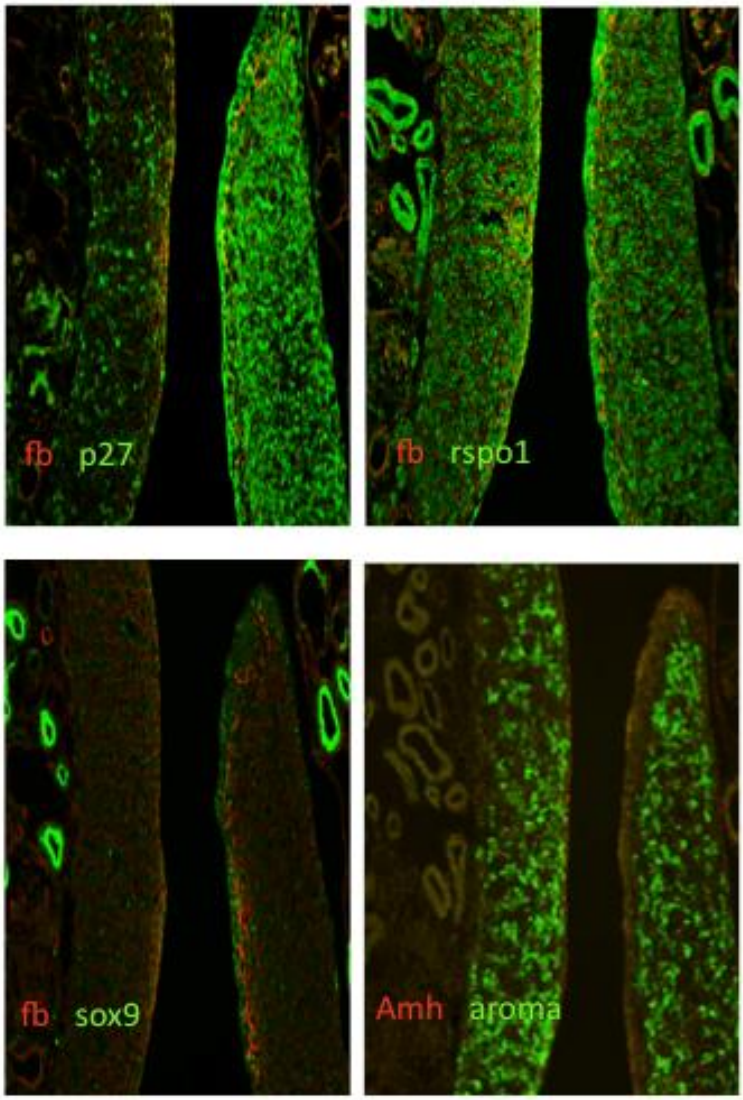


Fig 2.5. No effect of treating gonads with RCAS virus expressing R-SPO1 knockdown constructs (RCAS. Anti-R-SPO1345.) Day 9.5 embryonic gonads, after electroporating virus. Upper left panel shows expression of P27, a marker for the RCAS virus. It shows robust delivery to the left gonad. However, R-SPOI is expressed normally in the left gonad (upper right panel). The male marker, SOX9, is not activated and the female marker, Aromatase, is not disturbed.

R-SPO1 over-expression

For over-expression, the *R-SPO1* open reading frame (ORF) was cloned into either RCAS viral vector or TOL2 transposon. Over-expression of R-SPO1 from the RCAS vector was always very poor in DF1 cells, making purification of high titre virus extremely difficult. As shown in fig 2.6, the RCASBP virus spread normally in cells, but did not show immunoreactive R-SPO1 expression. This suggests that either the protein was not produced correctly, or that it was toxic to cells. The latter seems most likely.

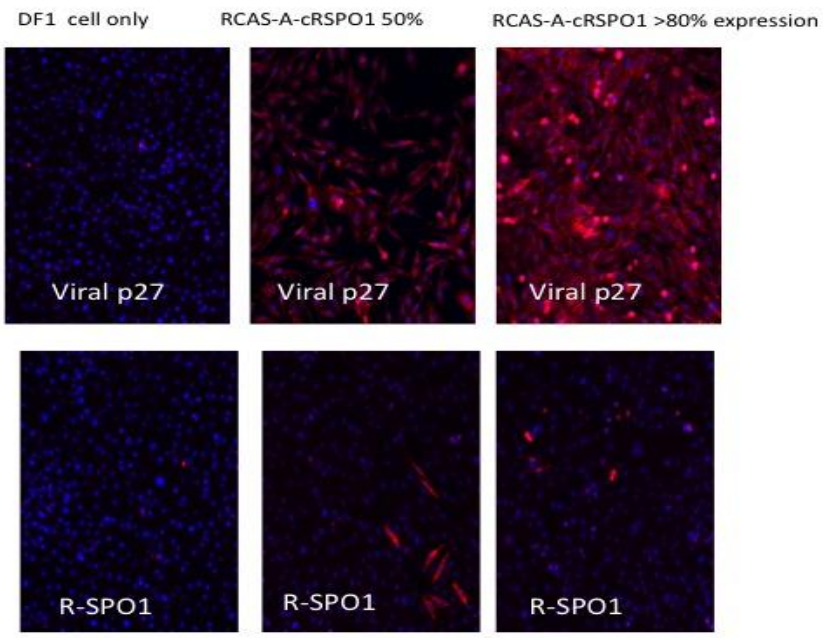


Figure 2.6. Expression of p27 viral marker (upp panels) versus expression of R-SPO1 transgene following infection of DF1 cells with RCASBP.A.R-SPO1. Cells were immunostaining at 50% infection and after over 80% infection. The data show robust expression of the virus Itself (p27), but very little R-SPO1 over-expression (red).

Since R-SPO1 could not be expressed in vitro from RCAS virus, an alternative strategy was tested. The ORF was cloned into the TOL2 vector (PT2) and then electroporated, together with transposase plasmid, into the left gonadal primordium at E2.5. The transposase facilitates integration of the TOL2 transgene into the genome. Gonadal electroporation (EP) was a novel technology developed by us during the life of the CRC project. Using

electroporation at an early time point (E2.5) we could achieve very strong and specific expression of a transgene into the targeted gonad (and Müllerian duct). This is shown in figure 2.7, in which TOL2. GFP has been electroporated into the left gonad, yielding robust GFP expression.

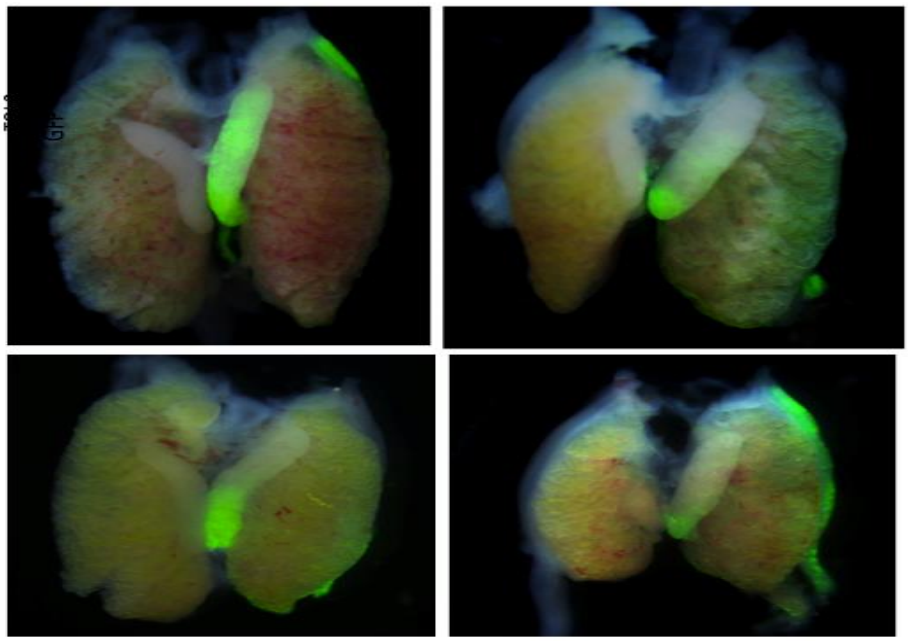


Figure 2.7. Gonadal electroporation of TOL2.GFP.
DNA was injected into the left body cavity, then subjected to electroporation at E2.5, and gonads examined several days later. Variable GFP expression is detected in the left targeted gonad, as shown in some representative specimens

The gonadal EP approach was used to over-express R-SPO1 only in the targeted gonad. This experiment was carried out on three separate occasions, involving some 50 embryos electroporated each time. With a typical survival rate of 40% to E9.5, at least 10 specimens of each sex were analysed in each of the three experiments. Results showed no obvious delivery of R-SPO1 into the gonads (Fig 2.8), where a feminising effect on male development was predicted. In one male gonad however, some ectopic expression of the key female gene, Aromatase, was detected (Fig 2.9). This could not be repeated and it is possible that over-expression of R-SPO1 is again lethal to the cells in which is expressed.

Conclusions. The over-expression and knock down of R-SPO1 failed to give clear results, and no gonadal phenotype was observed with either approach. This was partly due to the difficulty in expressing the gene, due to presumed toxicity in cells. R-SPO1 activates the potent Wnt signalling pathway, so it is not unreasonable to suspect that over-expression of such a potent growth factor as R-SPO1 could be detrimental to cells. However, while the

siRNA and miRNA constructs showed good knock down in vitro, it remains unclear why robust knock down could not be achieved in embryos. Expression of shRNAs from RCAS or TOL2 vectors have both previously been validated for DMRT1 knockdown, indicating that the strategy is technically sound.

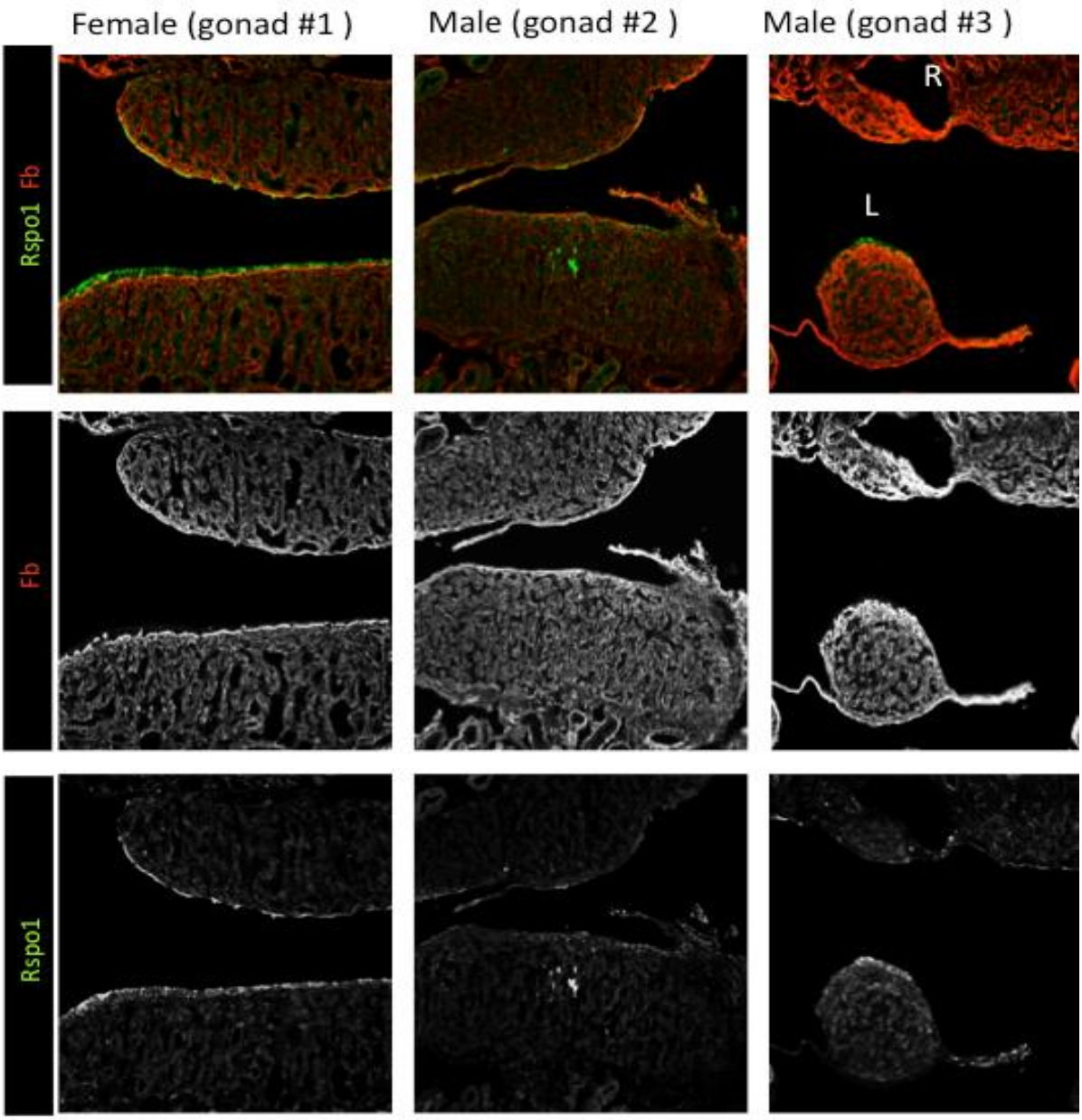


Fig 2.8 Expression analysis of R-SPO1 in E9.5 gonads following gonadal EP with Tol2.R-SPOI DNA. Very little over-expression of R-SPO1 is detectable (green). Fb = fibronection counterstain in red. The outer cortical expression in the female gonad (left panels) Represents endogenous R-SPO1 protein expression.

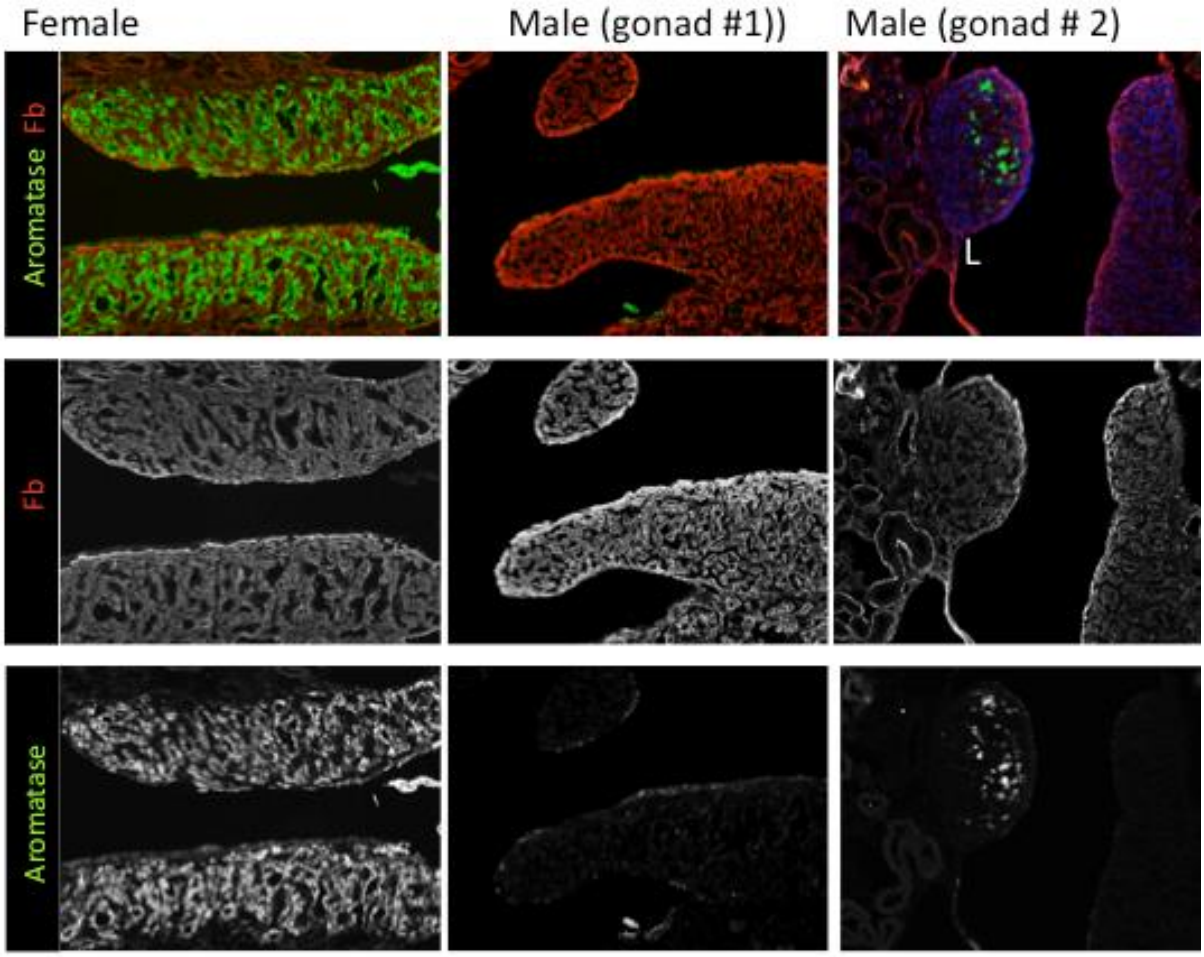


Figure 2.9 Ectopic expression of Aromtase in a male gonad over-expressing R-SPO1. (top right). Nevertheless, the gonad remained testicular in appearance (Fibronectin staining, middle panels). Bottom panel shows Aromatase expression in b/w for clarity.

Over-expression and RNAi knockdown *of* FOXL2 to modulate female development

In parallel with the R-SPO1 studies, the putative female gene, FOXL2, was also studied. As for R-SPO1, an in-house antibody was raised against chicken FOXL2 to facilitate the research. This antibody revealed female-specific expression of FOXL2 in embryonic gonads, just prior to aromatase expression, which it likely regulates (Fig 2.10).

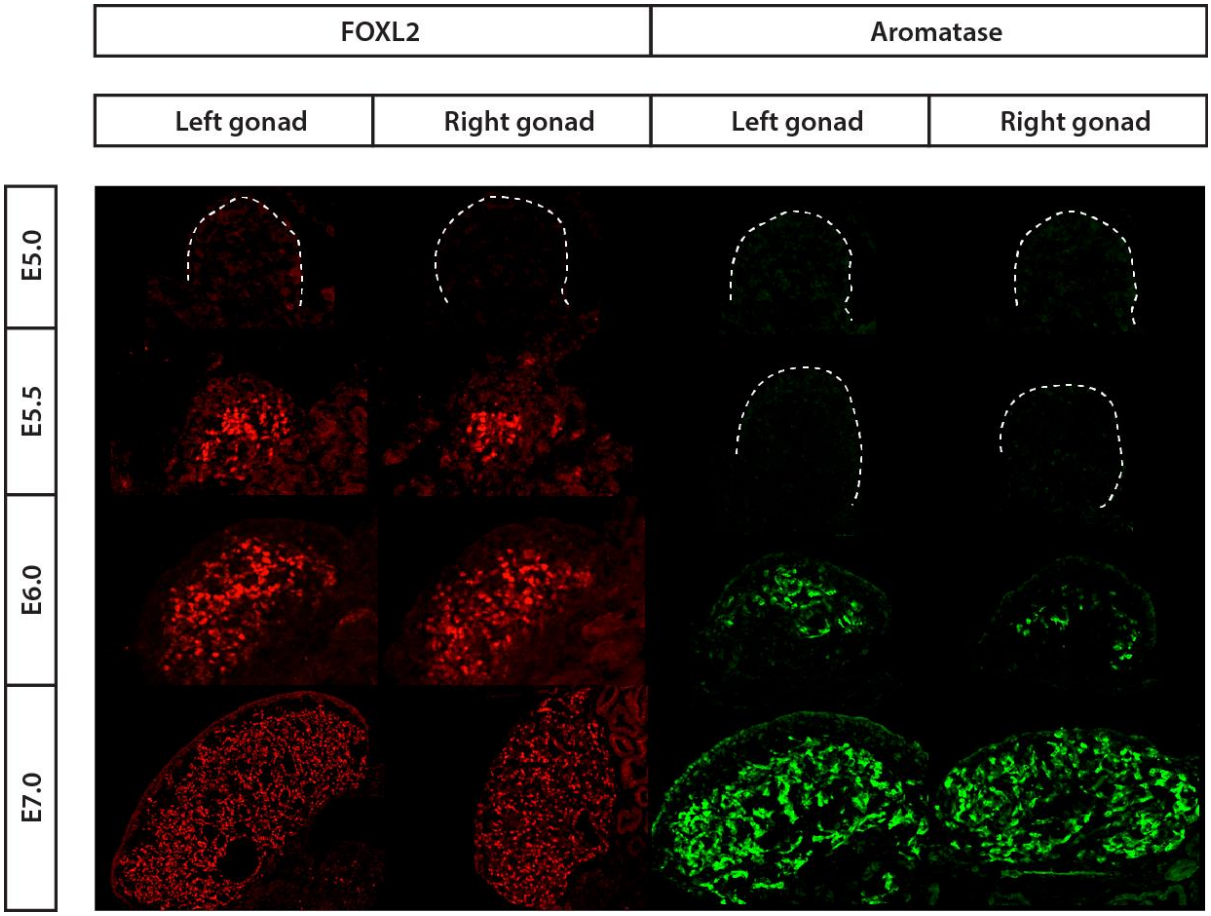


Fig. 2.10. cFOXL2 and aromatase expression during gonadal sex differentiation in female embryonic chicken gonads, determined by immunofluorescence. cFOXL2 (shown in red) expression is sexually dimorphic and is only observed in ZW females. cFOXL2 is first detected at E5.5 (HH stage 28) in the nuclei of medullary cord cells. Aromatase (shown in green) expression is also sexually dimorphic and is also observed in the medullary cord cells but at E6.0 (HH stage 29). Both genes are expressed in the left and right gonads of females. 10µm transverse sections were examined.

For over-expression, FOXL2 ORF was cloned into the RCASBP.A. viral vector, as for R-SPO1. When this viral vector was concentrate and injected into day 0

blastoderms, its caused embryo lethality prior to gonadal sex differentiation. The DNA was therefore electroporated into the coelom of day 2.5 embryos, and gonads examined several days later. This resulted in disrupted testis development in male (ZZ) embryos. The expression of both male markers, SOX9 and AMH, was suppressed in those parts of the gonad mis-expressing FOXL2. Interestingly, the key aromatase protein (which synthesises the oestrogen needed for ovary development)

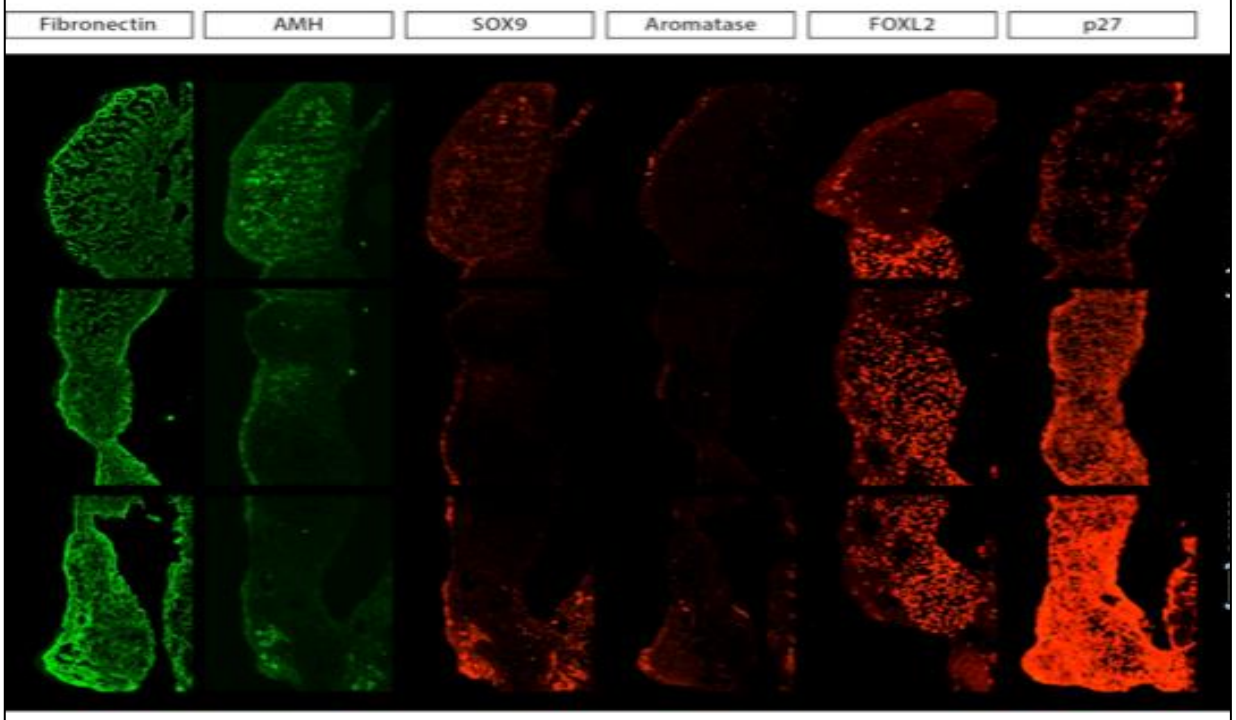


Figure 2.11 Mis-expression of FOXL2 blocks the male developmental pathway . E7.5 male (ZZ) gonads do not normally express FOXL2. Mis-expression is demonstrated by expression throughout the gonads (red; second panel from the right), as well as the RCAS viral vector (p27; right panel). In the gonad shown in this figure, AMH (green) and SOX 9 expression (red) are lost in those parts of the gonad mis-expressing FOXL2. However, Aromatase is not ectopically activated.

was not induced in male gonads mis-expressing FOXL2 (Fig. 2.11). The failure to detect aromatase activation may be due to a factor blocking its expression in male

gonads (such as DMRT1) or due to the requirement of female factors in addition to FOXL2.

These data suggest that over-expression of FOXL2 alone may be insufficient to induce ovarian development in male (ZZ) embryos.
Knockdown of *FOXL2* in female (ZW) embryos. For *FOXL2* knockdown, several siRNAs were tested and two were shown to induce robust knockdown (siRNA 523 and 689) and were reconfigured into miRNAs for expression from the RCAS viral vector. Figure 2.12 below shows knockdown of FOXL2 protein expression in DF1 Cells.

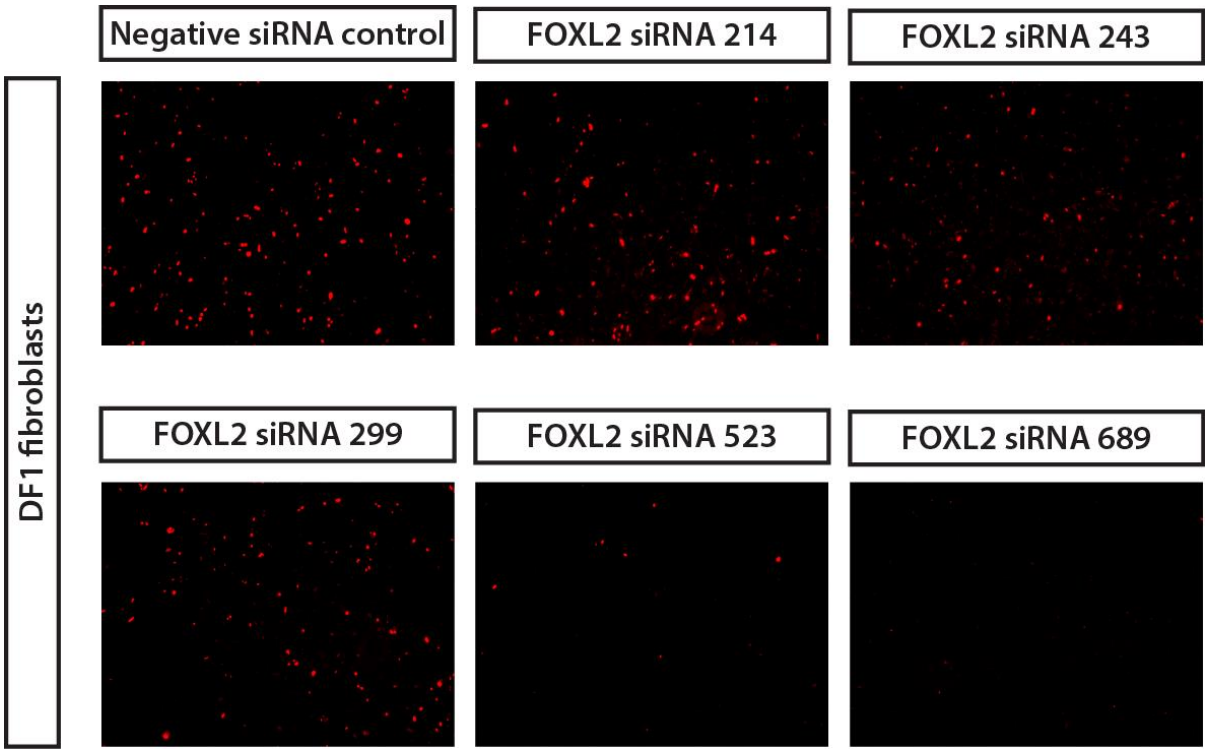


Fig. 2.12. RNAi-mediated knockdown of cFOXL2 in chicken DF1 fibroblast cells. Chicken DF1 fibroblast cells were transfected with a plasmid directing cFOXL2 expression and siRNA targeting cFOXL2. Several siRNA tested did not produce good knockdown of cFOXL2 and expression observed was similar to that in DF1 cells transfected with Qiagen All Stars negative control siRNA. siRNA 523 and 689 produced significant knockdown of cFOXL2.

RCAS virus expressing miRNAs was electroporated into E2.5 gonads and embryogenesis allowed to proceed to day 7.5. Knockdown of gonadal *FOXL2* was demonstrated by immunofluorescence. Knockdown caused ectopic activation of the male gene, SOX9, in female gonads, but failed to cause a loss of aromatase, suggesting that FOXL2 may not be required for Aromatase activation (Fig 2.13). The data suggested that FOXL2 could be used to manipulate gonadal development.

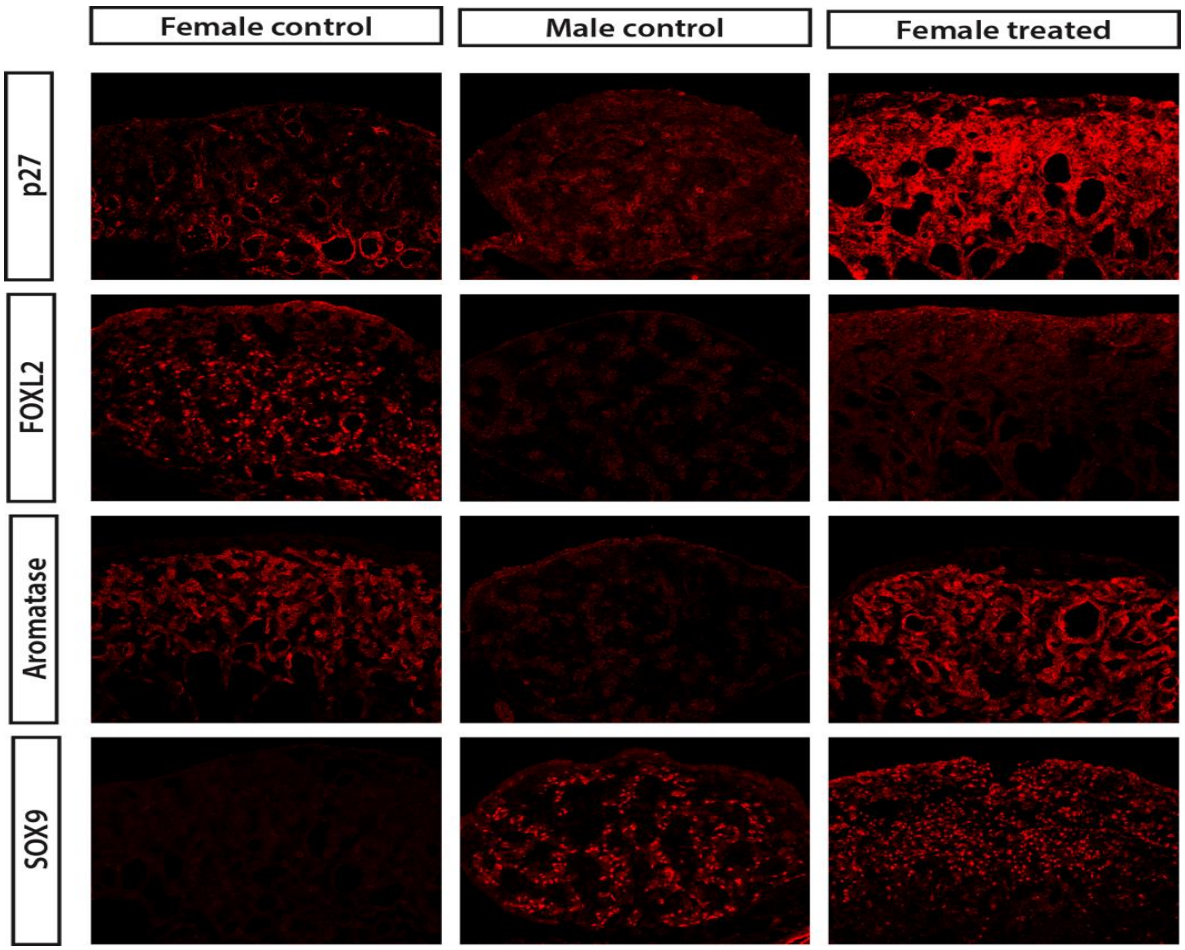


Fig 2.13. Immunofluorescence analysis of ZW female embryonic chicken gonads expressing shRNA knocking down cFOXL2. p27 staining is specific for the RCASBP(Y) virus and allows the extent of infection to be assessed. cFOXL2 expression is normally sexually dimorphic and exclusive to ZW females. No expression is observed in ZZ males. cFOXL2 was not detected in ZW female embryos expressing shRNA that knockdown cFOXL2. Aromatase expression is female-specific and is observed in treated ZW-females despite the absence of cFOXL2 expression. SOX9 is a male-specific marker of testis differentiation. Ectopic SOX9 expression is observed in ZW-females treated with cFOXL2 shRNA.

Publications that derived from the work in Chapter 2

Lambeth, LS and Smith, CA (2011) Disorders of sexual development in poultry. ***Sex Dev***, 2012;6 (1-3):96-103. Epub 2011 Nov 16.

Lambeth, L. and **Smith, CA**. (2103). Short hairpin RNA-mediated gene silencing. ***Methods Mol. Biol***., 942; 205-232.

Lambeth L, Raymond C, Roeszler KN, Kuroiwa A, Nakata T, Zarkower D and **Smith CA** (2014). Over-expression of DMRT1 induces the male pathway in embryonic chicken gonads. ***Dev. Biology****,* 398; 160-172.

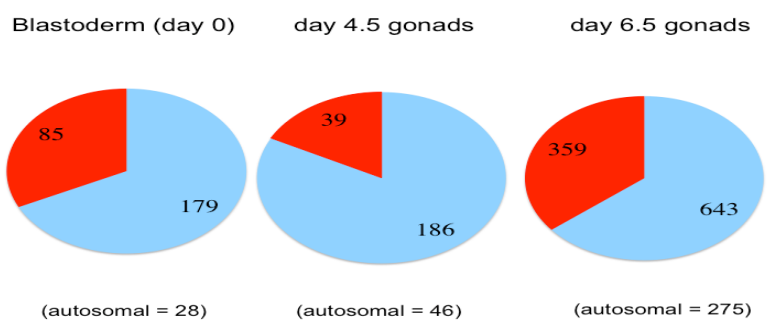
Chapter 3: Identification of novel chicken sex-determining genes

Summary

During the course of the sub-project, technical advances led to the opportunity to seek novel sex-determining genes in chicken. Although we have hypothesised that sex determination in poultry is driven by the Z-chromosome-linked *DMRT1* gene, the possibility still cannot be discounted that the female-specific W chromosome carries a master female (ovary) determinant. Expertise in bioinformatics at the MCRI was harnessed to carry out RNA-seq on embryonic tissues, whereby all of the gene transcripts in female and male embryos were identified and sequenced. This was carried out for day 0 blastoderms and day 4.5 (HH stage 25) gonads. The work formed the basis of a new research objective and milestone in year 2 (milestone 6: "Identification and characterisation of novel female sex determination genes on the W chromosome" and milestone 10: "Functional analysis of candidate W genes – HINTW and BTF3-W."). This research resulted in the full characterisation of the W transcriptome, fully annotating the 26 or so W-linked genes. No new candidate ovary determinants were identified, again placing the spotlight back on the Z-linked DMRT1 gene as being the master sex determinant in birds.

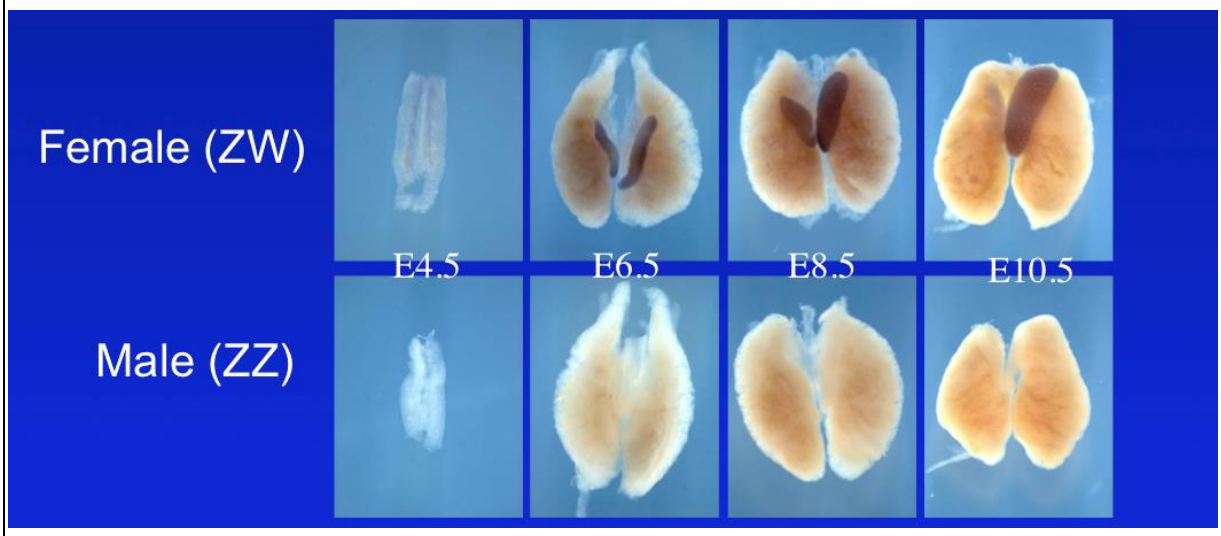
RNA was extracted from blastoderms or day 4.5 gonads, pooled by sex, DNAsed to remove genomic contamination, and sent to AGRF in Melbourne for deep RNA-sequencing. The results are summarised in figure 3.1 below.

Fig 3.1. Summary of RNA-seq data for the three tissues examined. Red = # genes female up regulated; blue = # genes male up-regulated



Most genes expressed in a sexually dimorphic fashion were male up-regulated (fig 3.1). This is partly explained by the lack of global Z chromosome dosage compensation in chicken and hence most Z-linked genes are more highly expressed in males. To validate the RNA-seq, the expression patterns of known sex genes were examined in the RNA-seq dataset. This validated the RNA-seq data, as it confirmed known expression patterns (e.g., FOXL2 up-regulated in females, DMRT1 expressed in both sexes). Genes specific to the W sex chromosome were those that mapped back to the W, or the unknown “random” chromosome in the latest build of the chicken genome. This yielded a list of some 26 genes that we were able to fully annotate on the W sex chromosome. This included some interesting novel W genes that were chosen for further analysis based on strong female-specific expression in the gonads. The gene *BTF3-W*, for example, was expressed in female gonads, and it encoded a transcription factor (see fig 3.2 below).

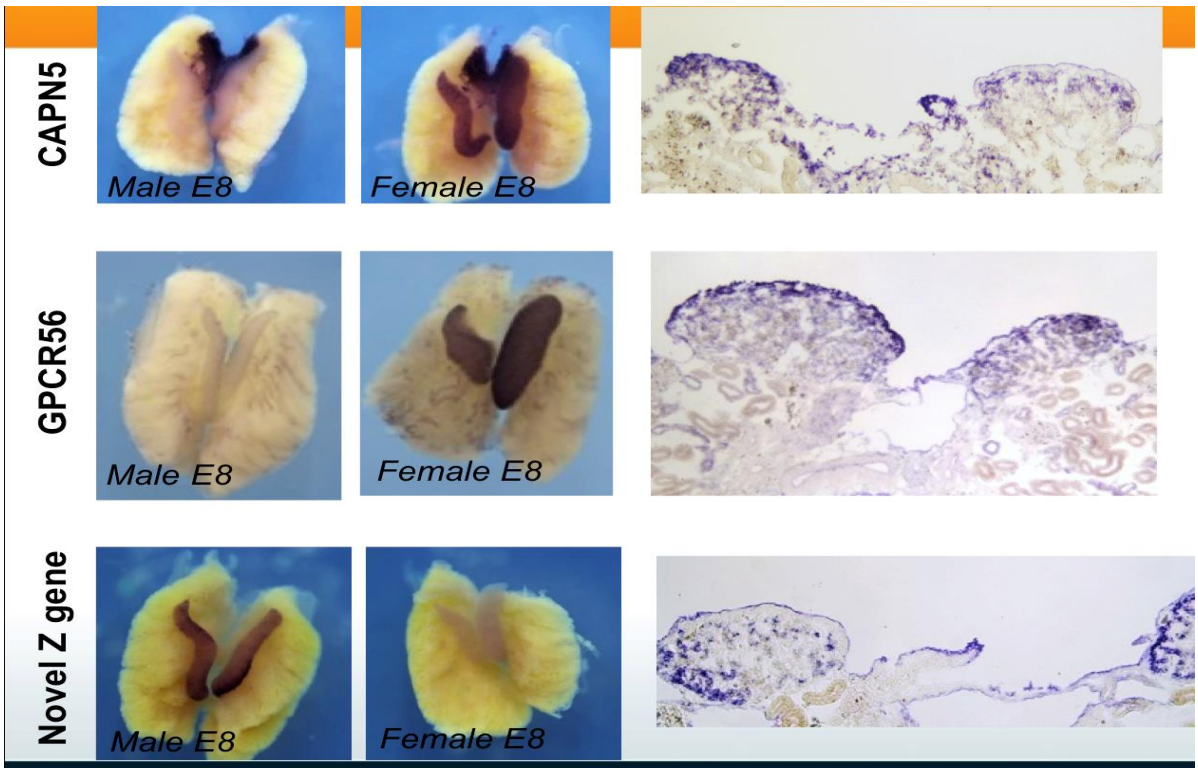
Fig 3.2. Expression profile of a novel W-linked gene, *BTF3-W*. This gene is expressed female-specifically, as determined by RNA-seq and confirmed by RNA whole mount in situ hybridisation. The purple staining shows expression in female gonads during
The time of gonadal sex differentiation



To test the possible function of *BTF3-W*, the ORF was cloned into both the RCAS viral vector and the TOL2 vectors. These vectors expressed the insert in DFI cells in vitro, as assessed by immunofluorescence or RT-PCR. This candidate female gene was electroporated into embryonic chicken gonads. Over-expression did not alter male (testis) development, but it did cause elevated cell proliferation in female gonads. No further work has been carried out on *BTF3-W*, but knockdown of the gene using viral or TOL2 vectors is planned. Meanwhile, the W-linked HINTW gene has long been a candidate ovary determinant, since it is highly expressed throughout female embryos, and it is diverged from its Z homologue, such that it has been proposed to operate as a dominant negative. However, our lab previously reported that mis-expression of HINTW in male embryos failed to disturb normal testis / male development (Smith et al., 2009).

More recently, we have examined other good candidate novel sex genes derived from the RNA-seq studies. Three genes of interest (two autosomal, one Z-linked) are *CAPN5, GPR56* and *Novel Z Protein* of unknown function (Fig 3.3). These genes show male or female-restricted expression and are the subject of on-going functional analysis at MCRI. If they prove important for gonadal sex differentiation, they will be tested in transgenic chickens (TOL2-mediated transgenics).

Fig 3.3. Novel sex genes derived from RNA-seq screen show confirmed sex-restricted expression in female or male gonads (purple staining in whole mounts, and in sectioned gonads.) Tissues were tested for expression at embryonic (E) 8.0.



Publications that derived from the work in chapter 3

Ayers, KL, Davidson, N, Demiyah D, Roeszler KN, Grützner, F, Sinclair , AH, Oshlack, A and **Smith, CA** (2013). RNA sequencing supports cell autonomous sex identity in chicken embryos and allows comprehensive annotation of W-chromosome genes. ***Genome Biol***. 2013 14 (3):R26.

Cutting AD, Ayers K, Davidson N, Oshlack A, Doran T, Sinclair AH, Tizard M and Smith CA (2014). Identification, expression, and regulation of Anti-Müllerian Hormone type-II receptor in the embryonic chicken gonad. ***Biology of Reproduction***, in press (accepted Feb 2104).

Ayers, K. L, Sinclair. AH and **Smith, CA** (2012). The molecular genetics of ovarian differentiation in the avian model. ***Sex Dev***. 2013;7:80-94.

Chapter 4: Anti-Müllerian Hormone and chicken sex determination

Summary

During the final 6 months of the CRC for Sex Determination, an analysis was carried out on the testis-derived hormone, AMH (Anti-Müllerian Hormone). The work was carried out at MCRI and involved CRC-funded doctoral student, Andrew Cutting, and postdoctoral researcher, Luke Lambeth, working with Craig Smith. AMH is a well characterised component of the testis pathway in mammals. It is a hormone secreted by Sertoli cells of the developing testis. It directs regression of the paired Müllerian ducts in males, which otherwise form most of the female reproductive tract (ref). In mammals, AMH is a “downstream” part of the male pathway, being activated by the SOX9 gene (and SF1 plus WT1). We and others have shown that AMH mRNA is also expressed in embryonic chicken gonads, but in both sexes, which is consistent with the fact that the right Müllerian duct regresses in females. However, mRNA expression is always higher in males, and we can only detect the protein in male gonads during embryonic life. It is unclear whether this is due to the lack of sensitivity of protein detection, or whether this is due to legitimate absence in females. Furthermore, AMH mRNA appears prior to SOX9 mRNA in chicken, suggesting that it is not regulated by SOX9, as in mammals. Most recently, a duplicated copy of AMH has been shown to be the master sex determinant in a fish species, which variants in the AMH type II receptor also have a key male-determining role in other fish species (reviewed in XX).

Methodology and Results

Studied conducted in the 1980s showed that genetically female chicken embryos developed functional testes rather than ovaries when a late stage embryonic testis was transplanted onto the (female) embryo at early stages. The robust masculinising effect was attributed to (but never proven to be) AMH. Given the early expression of the hormone, and high expression males, over-expression was attempted by cloning the AMH ORF into RCAS viral vector and then infecting day 0 blastoderms. A commercial antibody was used to assess AMH over-expression. Embryos examined at E11.5 showed remarkable masculinisation, with a complete loss of Müllerian ducts and masculinised gonads. These results are shown in figure 4.1 below. The urogenital systems of female embryos mis-expressing AMH lack Müllerian ducts. This served as a positive control for the experiment. The gonads of female embryos mis-expressing AMH did not develop as ovaries. Rather, they were small

masculinised gonads. This also applied to males over-expressing AMH (Fig. 4.1). Immunostaining of gonads showed that males over-expressing AMH had small DMRT1+

testes, while female gonads were grossly masculinised, lacking a typical cortex, lacking aromatase and with elevated male-tile DMRT. (fig 4.2).

Fig 4.1. Over-expression of AMH masculinises the chicken embryonic urogenital system. Gonads are small and testicular-like, while the Müllerian ducts are destroyed.

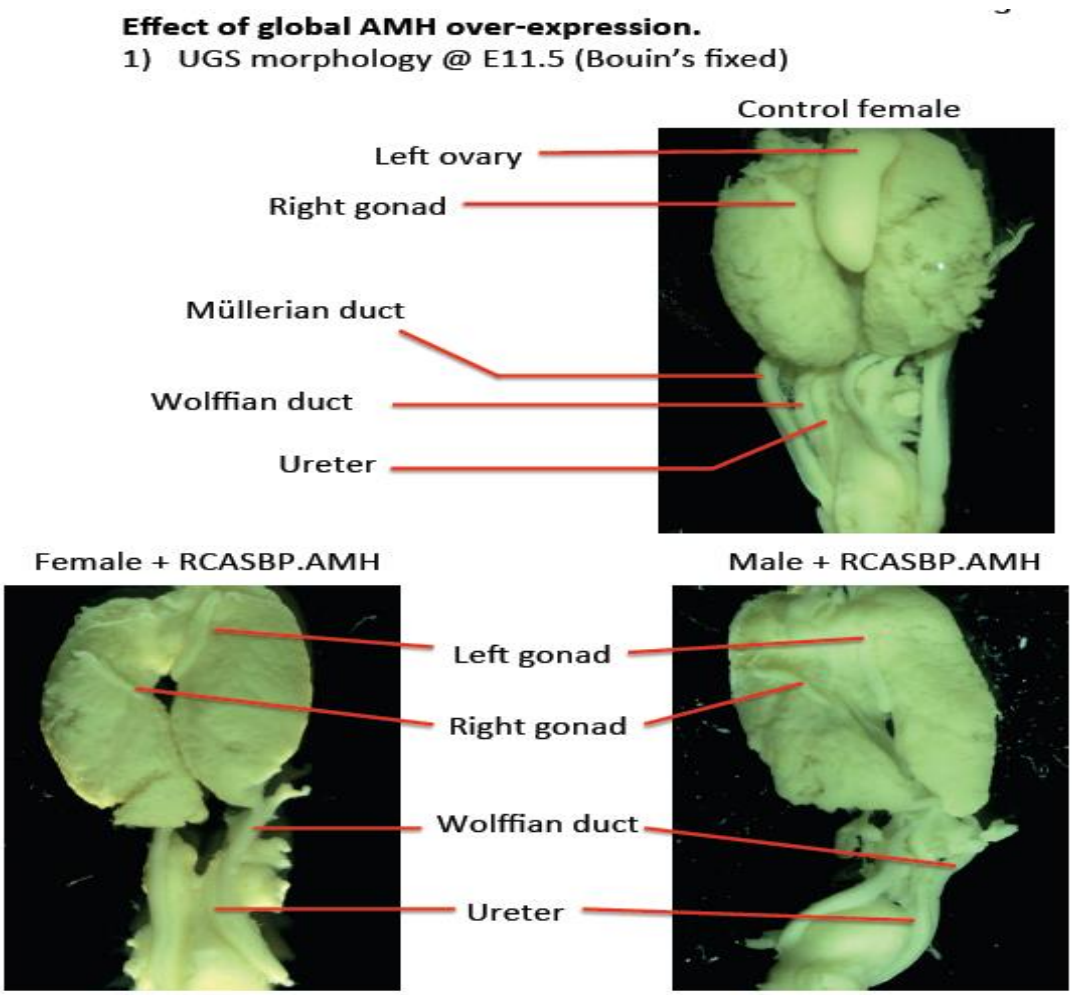
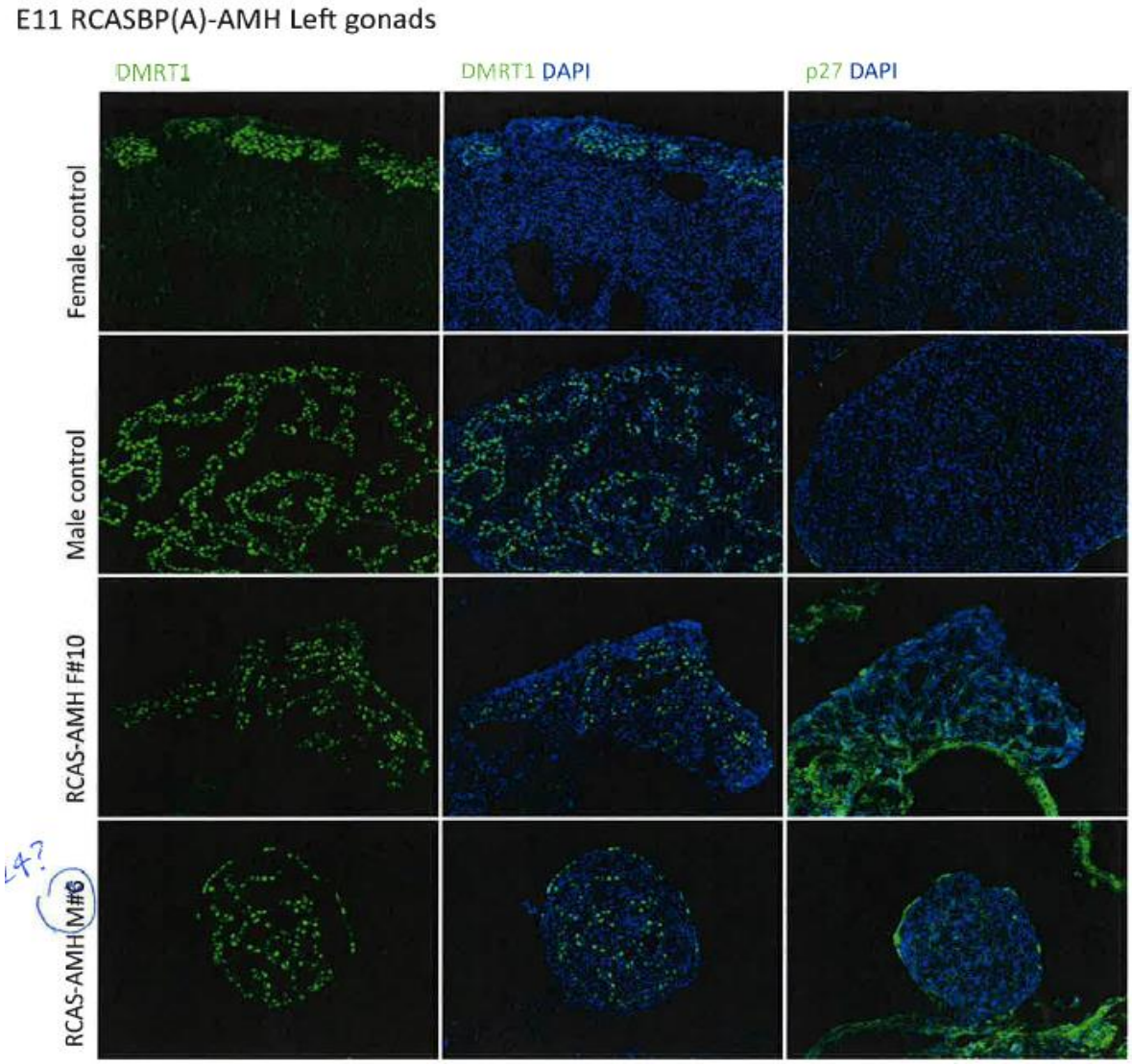


Figure 4.2. Immunostaining of E11.5 chicken embryonic gonads treated with RCAS virus over-expressing AMH, showing masculinisation of female gonads (no ovarian structure, and DMRT1 up-regulation.) P27 viral antigen expression shows modest amounts of virus in gonads.



Conclusion

This preliminary *AMH* data indicate that the gene is a very good candidate for further analysis. We are currently extending analysis to hatched birds, with the aim of examining gonadal structure and external sexual phenotype when AMH is over-expressed. The potency of AMH in causing female-to-male sex reversal that has been observed here will form the basis of a new CRC proposal.

Discussion of Results Compared to Objectives

This three year CRC sub-project did not yield single sex lines of birds, but made a substantial contribution towards this goal. The ultimate aim of achieving all male or all female birds was a very ambitious undertaking in the three-year time frame, but the data generated will form the groundwork for future attempts to manipulate poultry sex ratio by modifying gene expression in embryonic gonads.

The main objectives of this research were to stably knock down and over-express the testis-determining *DMRT1* gene, yielding feminised ZZ hatchlings and masculinised ZW embryos and hatchlings, and similarly modulate the female genes, *R-SPO1* and *FOXL2*. Two main obstacles prevented the production of sex-reversed birds: (1) achieving robust and lasting *DMRT1* knock down and (2) achieving gonad-specific over-expression of genes (as global over-expression is lethal).

The primary line of research was the production of birds showing robust knockdown of *DMRT1* and hence male-to-female sex reversal. Our previous data showed that delivery of *DMRT1* shRNAs via the RCAS viral vector at day 0 caused strong feminisation of embryos by day 10.5. However, taking such genetically modified birds to hatching and sexual maturity proved very difficult, due to toxicity of the viral vector in late stage embryos and hatchlings This was an unforeseen outcome, and led to the adoption of an alternative delivery vehicle: TOL2 transposon-mediated genomic integration. Our team at CSIRO AAHL developed a rapid and efficient method of transducing migrating PGCs with TOL2 vector carrying GFP reporter together with attenuated Pol III promoter driving short hairpin RNA against DMRT1. Using this strategy, manipulated embryos become chimeric for the transgene, with the aim of achieving an acceptable level of germline transmission to G1 birds that would be hypothesised to show the effects of DMRT1 knock down (feminisation). This approach was used successfully to generate G0 birds that had a high percentage of germ cells expressing the GFP reporter. These birds were taken to sexual maturity and were able to produce G1 fully transgenic offspring expressing GFP reporter. However, the level of DMRT1 shRNA production was variable and there was no consistent effect upon the morphology of the gonads (i.e., no ovarian development in GFP+ male G1 birds). The reasons for this are unclear, but may reflect negative regulatory control of exogenous shRNA production by cells during development. Another consideration that became apparent during the course of the project is that, given the normally high expression of DMRT1 in germ cells, knock down of the gene may produce sex-reversed gonads that might be sterile. Further analysis will focus not on DMRT1 knock down, but identifying suitable promoters that would drive DMRT1 0ver-

expression in the somatic cells of the gonads. This should induce female-to-male sex reversal.

The other main objectives of this sub-project were genetic manipulation of female genes, R-SPO1 and FOXL2. Unforeseen problems were encountered in our attempts to over-expression R-SPO1 in cells and embryos, and we suspect that over-expression is toxic. An alternative is the over-expression of the related and downstream effector of R-SPO1, WNT4. We are currently testing the ability of WNT4 to modify gonadal development. Previous studies in mammals and chicken suggest that WNT4 could be used to feminise embryos. In the case of *FOXL2*, this gene shows promise as a potential target for production of sex-reversed transgenic birds. Local over-expression of FOXL2 blocked the male pathway, but more work needs to be done, since FOXL2 over-expression alone was insufficient to activate the critical Aromatase gene.

Additional research that was developed during the course of the project focussed on the identification of novel sex genes, using state-of-the-art RNAS-seq technology. A number of new candidate sex genes were identified, including the W-linked *BTF3-W* gene. Future work will focus on functional analysis of these genes. At present, however, the RNA-seq did not yield any new and convincing candidate W (female) determining genes. Together with our recent finding that over-expression of *DMRT1* can induce the male pathway, the data again points towards *DMRT1* as being the master sex determinant in birds.

The data generated during this project will form the basis of a new CRC proposal, in which we will focus on over-expression of candidate sex genes, which is the area in which we achieved the most promising results. Specifically, over-expression of AMH has excellent potential as a mechanism of producing all male birds. The identification of a suitable gonad-specific promoter that would drive gene expression only or primarily in the gonads is another key aim. Again, AMH promoter would be an ideal candidate. The preliminary data using ChIP-seq is novel and provides a sound base for seeking suitable promoters.

Implications

The major objective of this subproject was to develop improved understanding of the genes responsible for sex determination in poultry, develop advanced gene-based technologies such as RNA interference (RNAi) and gene over-expression to modulate the transcription of these genes to impact on gonad development and finally to investigate commercially viable approaches to modulate key genes in early stage embryos. The outcome of this research has two major impacts on industry in Australia and globally:

(a) Feminisation of male commercial layers

Impacts:

- Positive impact on welfare issues relating to culling male chicks
- Commercial impact delivered through improved production efficiency
- Increased uptake of innovative *in ovo* vaccines

To achieve a significant impact for industry our research goal is to achieve stable and permanent sex reversal of males with a commercial objective to produce feminised males with 95% of female egg production.

(b) Masculinisation of females in broilers

Impact:

- Substantial commercial benefit and improved sustainability for industry

To achieve a significant impact for industry there is less need for stability as females do not require full sex reversal to grow like males. Commercial objective is to produce feminised females with 90% of male growth.

Recommendations

The overall objective of this sub-project was to produce single sex lines of chickens, with animal welfare and economic benefits to the Australian and global poultry industry. The approach was to use advanced genetic technologies to modulate known and novel sex-determining genes in embryos, producing stable sex-reversed lines of birds.
Specifically, we had two major objectives:
Objective 1: Modulate male development via DMRT1
Objective 2: Modulate female development via R-SPO1 and FOXL2

The research undertaken in the project was predominantly "blue sky" and built on preliminary work conducted in Poultry CRC I and previous collaborative research conducted between Craig Smith (MCRI) and Tim Doran (CSIRO) that confirmed to pivotal role of the *DMRT1* gene in males sex determination in avians. Modulation of the *DMRT1* gene was a major focus of this subproject and during the course of our research we have now developed a much broader and improved understanding of a larger set of genes that are involved in the complex trait of sex determination in the chicken. We now have an excellent opportunity to build on this knowledge, larger "tool box" of gene manipulation technologies and the exceptional performance of the science team and focus on key recommendations outlined below to deliver impact to the Poultry Industry.

Modulation of male development

Our research efforts focussed on the development and use of RCAS viral vectors for RNAi knockdown or over-expression of the male specific *DMRT1* gene. The outcome of this work was a better understanding that whilst RCAS is an excellent research tool for working with embryos, it is not a viable delivery vector for producing hatched sex-reversed birds. RCAS *DMRT1* knockdown in hatched chicks was not robust, and we therefore transitioned to Tol2 delivery and analysis of phenotype in G(1) progeny. Tol2 delivery results in sustained transgene expression. G(1) birds showed some signs of sex reversal, but again this was not robust. We developed strategies for improving *DMRT1* knockdown via "mirizing" the *DMRT1* specific shRNA343. We have developed Tol2 constructs for delivery of miR343 and recommend a combinatorial strategy for delivery as knockdown alone is not strong enough for sex reversal.

A major outcome of this project was the discovery that over-expression of Aromatase via RCAS delivery induces complete male to female sex reversal in embryos. We subsequently

shifted our focus towards the modulation of Aromatase together with knock-down of *DMRT1* with an improved shRNA (miR343). We developed Tol2 constructs for delivery of Aromatase over-expression and *DMRT1* knock-down to provide a dual or “combinatorial” approach to achieving female development. G(1) chicks with these combinatorial transgenes are already being generated and screened. We are yet to identify a positive G(1) chick and therefore recommend that the screening continues to evaluate this approach to develop male to female sex reversed birds.

DMRT1 over-expression via RCAS delivery was lethal to injected embryos and obviously not viable. To overcome this, we have pursued the identification, characterisation and validation of gonad-specific promoters or enhancers directing expression only in the gonads. We recommend that this work continues. As an alternative approach we have developed *in ovo* electroporation of RCAS as an improved method to examine sex determination genes. Using this technique, RCAS is confined to the gonads of embryos and does not lead to global expression and lethality. There is now a strong potential to hatch chicks and we recommend further work using this approach for *DMRT1* over-expression.

Towards the end of the project, we also pursued the over-expression of the *AMH* gene via the RCAS vector. The preliminary data indicates that this gene is an excellent candidate for further analysis as we have seen robust masculinisation of female embryos. We are currently extending analysis to hatched birds and will assess the potency of *AMH* in causing female-to-male sex reversal. This work will form the basis of a new CRC PRP.

Recommendations:

1. Continued development of Tol2 constructs for combinatorial approaches:
    a. Over-expression and knockdown of two genes simultaneously (i.e. Aromatase and *DMRT1).*
    b. Optimisation of shRNA constructs for RNAi knockdown of two genes in the same pathway (i.e. *DMRT1* and *AMH*).
2. Continue work to identify, characterise and validate gonad-specific promoters or enhancers directing expression of key genes such as *DMRT1* only in the gonads.
3. Analyse the effect of *AMH* over-expression in post-hatch birds. Develop new PRP for assessment of *AMH* protein delivery to female embryos to stimulate female-to-male sex reversal.

Modulation of female development

Our research efforts focussed on over-expression and knock-down of the female specific genes *RSPO-1* and *FOXL2* and the identification of novel female sex determining genes (W linked genes) using RNA sequencing.

Over-expression of *RSPO-1* via electroporation resulted in a likely upregulation of Aromatase, but this was not robust. Over-expression of *FOXL2* resulted in the repression of male specific genes, however we did not observe upregulation of female genes and the effect was not sufficient for modulation of phenotype. RNAi knock-down of both genes *in vivo* was also not effective in modulating sex phenotype to feminize males. This clearly demonstrates that modulation of *FOXL2* or *RSPO-1* alone will not be sufficient to strongly and consistently reverse sex. We recommend a combinatorial approach of double over-expression or knock-down of both genes simultaneously.

There is an ongoing need to identify, characterise and validate novel W linked genes that are potential regulators of *FOXL2*, Aromatase, *SOX9* or *DMRT1*. We have used RNA sequencing and this has been extremely successful in finding novel sex gene candidates. Analysis of our top 10 candidate genes is underway and we predict that a number of these genes will provide new targets for modulating sex. We have developed *in ovo* electroporation of RCAS as an improved method to examine the effect of these new candidate genes in the gonads of injected embryos. This will provide us with the increased potential to hatch chicks for further analysis.

Recommendations:

Modulation of female genes is promising but will require more advanced strategies. We recommend continued analysis of top W linked candidate genes using *in ovo* electroporation of RCAS vector. These genes will provide new targets for modulation of sex.

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Appendices

Publications derived from this CRC sub-project

1. Cummins, David; Tyack, Scott; Doran, Tim. Characterisation and comparison of the chicken H1 RNA polymerase III for short hairpin RNA expression. Biochemical and Biophysical Research Communications. 2011; 416(1-2):194-198.

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receptor in the embryonic chicken gonad. *Biology of Reproduction*, in press (accepted Feb 2104).

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14. Ayers, KL, Smith, CA and Lambeth, LS (2013). The molecular genetics of avian sex determination and its manipulation. Genesis, 51; 325-336.

IP Summary for project

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Granted Australia

Methods for modulating sex in avians. Doran T., Moore R., Lowenthal J.W., Smith C. And Sinclair A 2009
International Patent Application PCT/AU2009/001627 filed 16/12/2009
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International Patent Publication Number WO 2010/088742

Sex Determination Genes. Katie ayers, Nadia Davidson, Alicia Oshlack, Andrew Sinclair and

Application number: PCT/AU2013/000405 filed 19/04/2013

Status: Pending

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Plain English Compendium Summary

Sub-Project Title:	Modulation of sex determination in poultry
Poultry CRC Sub-Project No.:	1.3.1
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Sub-Project Overview	The aim of this project was to produce single sex lines of chickens. This used molecular methods to modulate genes that control gonad development (ovary versus testis formation) at embryonic stages and hence female versus male development.
Background	Sex is determined genetically in chickens and other birds by the inheritance of sex chromosomes. These chromosomes differ from those in mammals. In chicken, the male has two Z sex chromosomes while the female has Z and W sex chromosomes. Sex determination involves the expression of genes in embryonic gonads to direct testis or ovary development. Several lines of evidence now suggest that a gene on the Z chromosome called *DMRT1* regulates avian sex determination via a gene dosage mechanism (two doses in males, ZZ, one in females, ZW).
Research	The project involved cutting edge methods of gene manipulation and analysis. By combining expertise in avian embryology and sex determination with expertise in RNA interference which enables us to specifically switch off a gene, we demonstrated that DMRT1 was required for proper testis development and hence male sex determination. Four other genes were also examined, the female associated genes, R-Spondin1, FOXL2 and Aromatase and the male gene AMH. Modulation of one or a combination of these genes can also affect sexual development in chicken embryos.
Implications	The generation of single sex populations is highly desirable to the poultry industry, with two clear benefits: (1) an economic advantage in producing all female hatchlings for the laying industry and all males for the meat industry. All female or all male populations would greatly increase the efficiency of egg and meat production, respectively; (2) Animal welfare. Production of monosex populations would obviate the need for post-hatch culling of male chicks in the layer industry, addressing a major animal welfare issue, and saving costs associated with the culling procedure.
Publications	1. Cummins, David; Tyack, Scott; Doran, Tim. Characterisation and comparison of the chicken H1 RNA polymerase III for short hairpin RNA expression. Biochemical and Biophysical Research Communications. 2011; 416(1-2):194-198. 2. Cutting, AD, Bannister, SC, Doran, TJ, Sinclair, AH, Tizard, MV, Smith CA. (2011). The potential role of microRNAs in

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