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Single sex selection for the egg laying industry to improve animal welfare
Sub-Project No. 1.5.5

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Executive Summary

The ability to detect and remove male chicks pre-hatch would be a big step forward to the egg laying and related industries. Currently culling male chicks post-hatch creates a major ethical dilemma for some countries. As a result the poultry industry has invested in developing solutions for this issue. In some European countries the need is urgent following the introduction of legislation to ban the culling practise. Growing male layer chicks is not a sustainable option for farmers. A sex selection application effectively negates the need to cull or grow out male chickens and would contribute to a more sustainable industry with a view to future food security. We have demonstrated that a Z-linked selectable marker can successfully be employed *in ovo* to identify male embryos and enable their removal from the production system at the earliest age.

We have used transposon technology to create chickens with the Z-linked marker gene. For translation to commercial practice it would be preferable to use the latest techniques of precision genome engineering to place the marker gene in the most suitable location on the Z chromosome. We have identified a number of suitable Z locations that could be used for development of this application. We have also clearly demonstrated that it is possible to detect expression of the Z-linked marker gene at any time from point of lay to hatch.

The outcomes of this project are really promising, however translation into industry practise requires industry to overcome their concerns about adoption. This is based on consumer perception in relation to GM technology. The marker gene is GM, but the biology of sex chromosome segregation means that the laying hen and the eggs produced are not GM. We have clear advice from gene technology regulators from a number of international jurisdictions that the hen and her eggs would not be considered GM and would not require regulation or labelling of product.

Contents

Introduction

The ability to detect and remove male chicks pre-hatch would be a big step forward to the egg laying and related industries. Currently culling male chicks post-hatch creates a major ethical dilemma for some countries. As a result the poultry industry has invested in developing solutions for this issue. In some European countries the need is urgent following the introduction of legislation to ban the culling practise. Growing male layer chicks is not a sustainable option for farmers. A sex selection application effectively negates the need to cull or grow out male chickens and would contribute to a more sustainable industry with a view to future food security.

Sex determination in chickens is defined by the presence of two sex chromosomes, Z and W. The males carry two copies of the Z chromosome (ZZ) and the females carry one Z and one W chromosome (ZW). This is in contrast to mammalian sex which is defined by XY for the male and XX for the female. Of key relevance to this project is the fact that the single copy of the Z chromosome carried by the female bird is always passed on to the male offspring only and it is to ALL of the male offspring. The biological process that controls this is meiosis.

ZZ♂ x ZW♀ → ZZ♂ + ZW♀

The innovation within this project is the incorporation of a selectable marker on the female parent derived Z chromosome that appears only in male offspring. The female offspring can only derive their Z chromosome from a non-modified breeding rooster. This can then be used to remove all males from the egg-layer production system at the earliest stage i.e. prior to incubation. Therefore all offspring that go into the production system will be female and completely GM free.

The benefits of this innovation include;

1) removal of males before incubation relieving the welfare issues associated with culling male chicks post hatch;
2) removal of the need for manual sexing of the hatched chicks;
3) improvement of efficiency and economics of in ovo vaccination technologies for the egg-layer industry;
4) A high value, consumer acceptable lead in to the application of precision genome engineering technology to poultry science and industry outcomes. For industry this

technology can have future impact on the development of disease resistant poultry, enhanced production traits, food-safety such as Salmonella/Campylobacter free products and allergen free eggs. For poultry science this will revolutionise our understanding of poultry biology, such as host pathogen interactions, developmental embryology and nutritional science, through the development of knock-out gene chickens.

The selection marker is a fluorescent protein (FP) that is inserted into the Z chromosome of female breeder birds using genetic engineering techniques that we have developed in our laboratory (Tyack et al, 2013). This modified chromosome (ZFP) is passed on only to male offspring and they all become ZZ^{FP}. All the female offspring by contrast only receive the W chromosome from the female parent and it is therefore impossible for them to carry the ZFP GM marker. They are not genetically modified and therefore cannot express the fluorescent marker.

The presence of the Z^{FP} marker in the male embryos would be detected by laser illumination and detection of the emitted wavelength of the FP. Industry currently uses white light illumination to “candle” eggs to determine if they are embryonated and productive. It should be possible to automate an equivalent test station that detects the FP in a similar manner and selectively removes the fluorescent (male) eggs.

Objectives

The overall objective of this project was to evaluate a novel approach to single sex selection for the egg laying industry. The approach involved the generation of a Z-linked-fluorescent protein (FP) in a female lineage. When this hen is crossed with a wild-type male it will result in the fluorescent gene marker only going to the male offspring. All female offspring will be free of any genetic modification (see Figure 1 and 2 below). All that is required to remove "male eggs" prior to incubation is an automated illumination and detection systems to "see" the fluorescent male embryo through the shell, much like "candling" for viability.

The output of this project will be the demonstration that a Z-linked selectable marker can successfully be employed in ovo to identify male embryos and enable their removal from the production system at the earliest age. This will provide a valuable tool to the layer industry to improve animal welfare perceptions associated with male chick culling, to reduce cost of manual sexing and to enable more cost effective adoption of in ovo vaccination technologies for disease control. If successful it is very likely that this technology could be fast-tracked to commercial implementation. It is also a technology that could be transferred to production facilities worldwide.

It is important to note that although this approach uses transgenic (GM) technologies to address a major industry issue, the end product that enters the food chain is **not** a GMO.

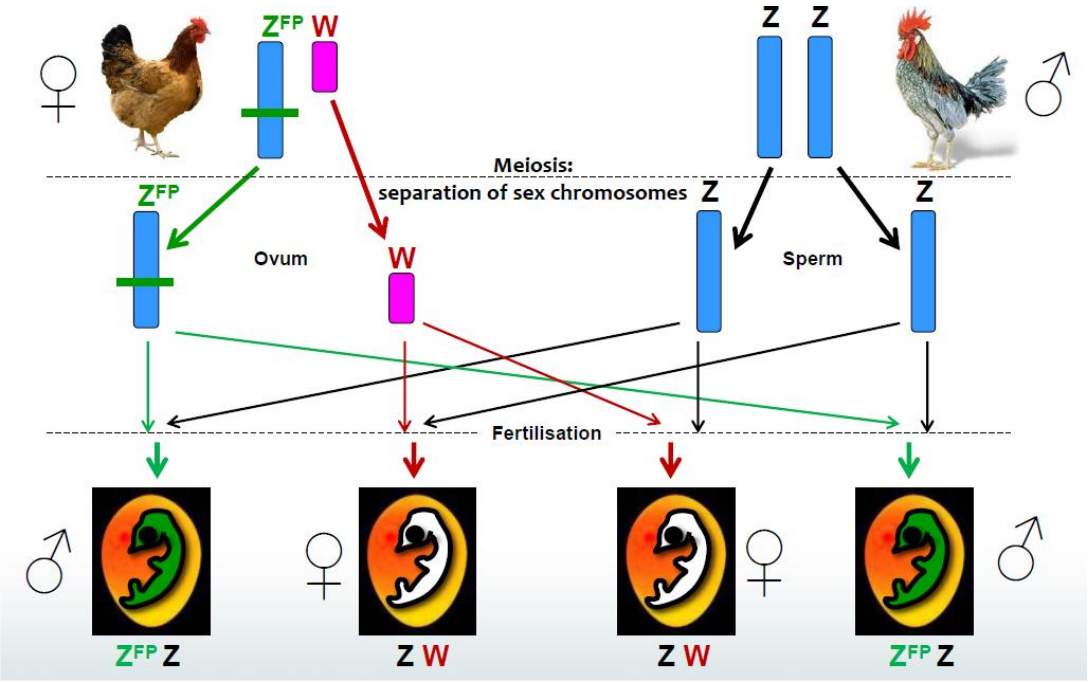


Figure 1. Sex selection concept.

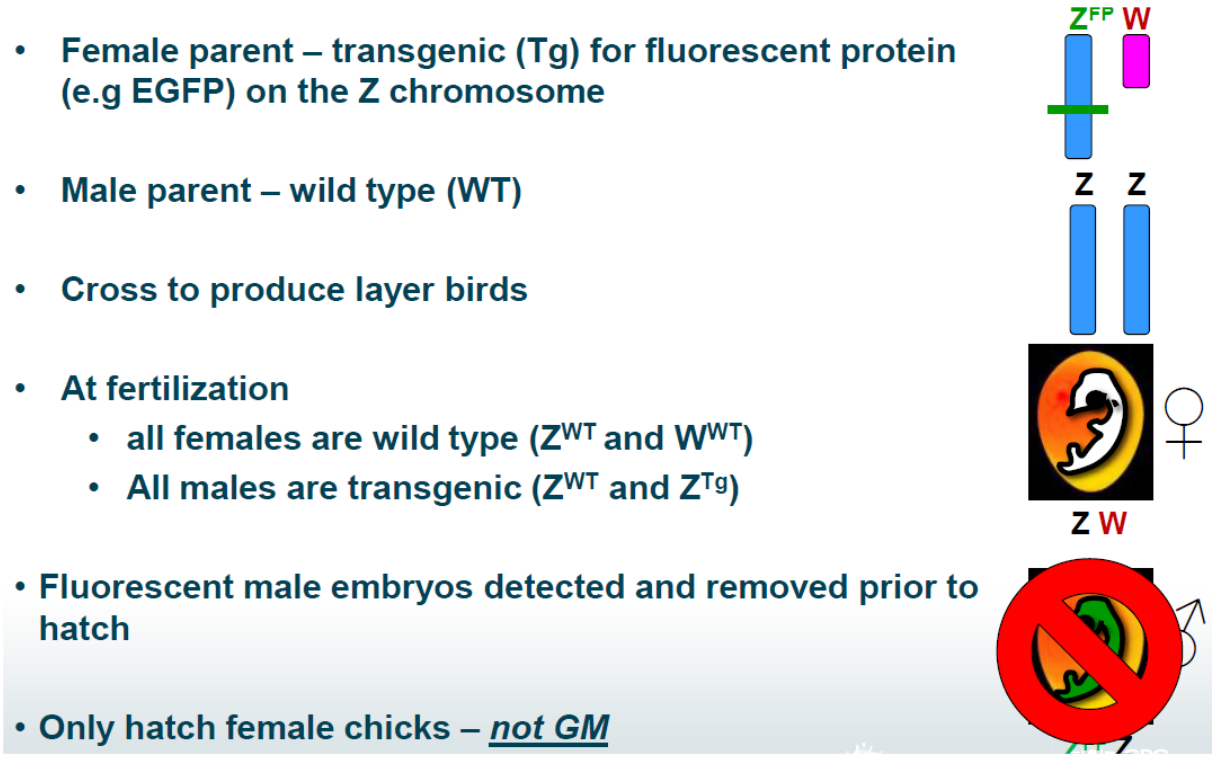


Figure 2. Sex selection overview.

Methodology

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 EGFP Fluorescent Protein gene 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 manufacturer's

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.

Insertion site analysis for Z-linked location of Tol2-EGFP construct

For insertion site analysis, gDNA from chicken primordial germ cells (PGCs) (transfected with a Tol2-EGFP plasmid) was digested with AccI, BglII, EcoRV, HindIII, XbaI or XhoI, which are each present within the Tol2-EGFP sequence, re-ligated overnight at 4°C, then used for

template in PCR with primer sets that anneal to either the Tol2 left hand end (LHE), or Tol2 right hand end (RHE). Amplified products were then separated by gel electrophoresis on a 2% TAE/agarose gel, and various bands were excised, gel purified and sequenced using internal primers. Resultant fragments sequences were then BLAT searched (http://genome.ucsc.edu/cgi-bin/hgBlat?hgsid=460376161_4142cBAsUbLfaBr1YFTCha5r7DEw&command=start) and identified regions were used to design specific primers for each chromosomal location for further validation by PCR with specific primer sequences for either the Tol2 LHE or RHE.

Chapter 1. Generation of birds that carry Z chromosome integrations

We have successfully used Tol2 transposon technology in combination with direct transfection of embryonic primordial germ cells (PGCs) to specifically and stably integrate the EGFP gene into the Z chromosome of the chicken. The technology takes advantage of the fact that avian embryonic PGCs migrate through the vasculature on their path to the gonad where they become the sperm or ova producing cells. We have developed a method to stably transform PGCs in vivo and successfully generate germ-line transgenic chickens using the miniTol transposon system – a two component plasmid system (pMiniTol-EGFP - CAGGS promoter and pTrans - Tol2 transposase, CMV promoter).

We have generated over 100 germline transgenic G1 chickens using the Tol 2 transposon. We have analysed transgene copy number using Southern blot and the number of Tol2 insertions can vary from 1 to 7 copies (Figure 3). The majority of G1 chicks (63%) have just a single transgene insertion. We have also characterised the region of the genome that the insertions have occurred (Figure 4) – 49.4% were in introns, 1.3% were in exons (these are regions that we would actively avoid), 24.6% were in repeat regions, 3.9% were in UTRs (untranslated regions) and 20.8% are in unknown regions, ie not characterised in the current version of the chicken genome. Chromosomal distribution of the inserts is as follows: 12.3% are located in the Z chromosome (which is our target chromosome for this project); Chromosome 3 is 18.5%; Chromosome 1 is 13.8%; and Chromosome 5 is also 12.3% (Figure 5). All of these chromosomes are the biggest and therefore statistically expected to have the most number of insertions. From our overall Z Chromosome data we have been able to identify 8 locations on the Z Chromosome that are suitable for a transgene insertion. They are in locations that do not impact on the viability of the chicken and have no detrimental impact on Z gene expression and regulation. Three of these locations are suitable to be developed as target sites for the Precision Genome Engineering of the RFP gene in female breeding birds.

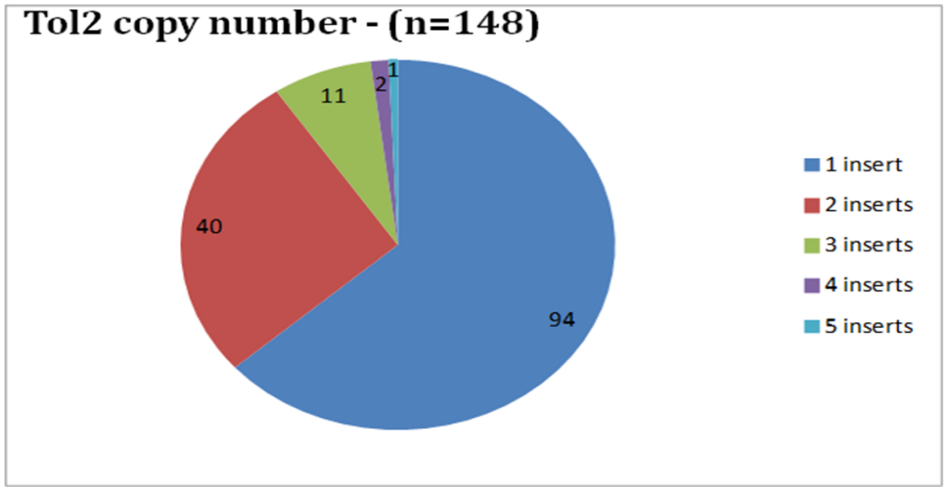


Figure 3. Tol2 copy number in G1 transgenic chickens.

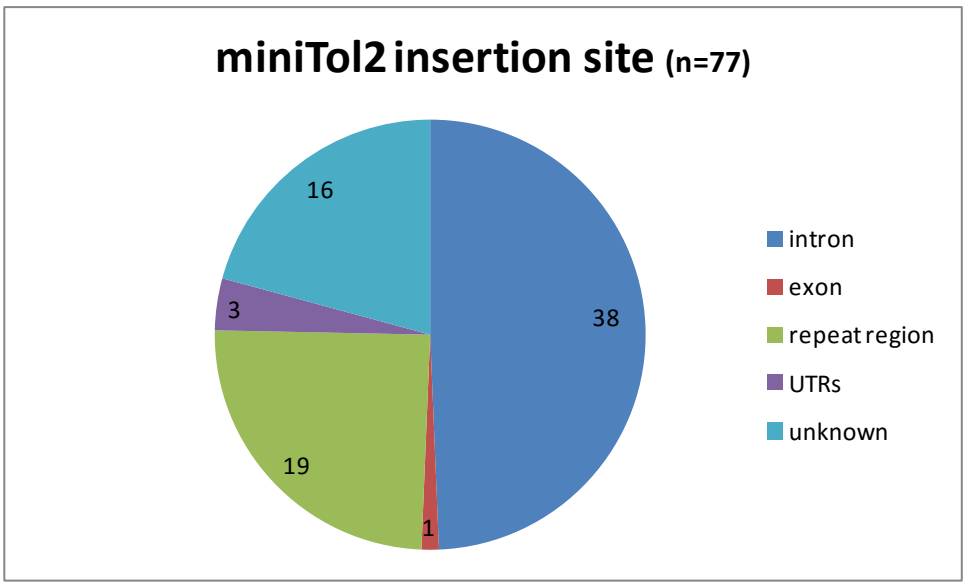


Figure 4. Characterisation of Tol2 insertion sites.

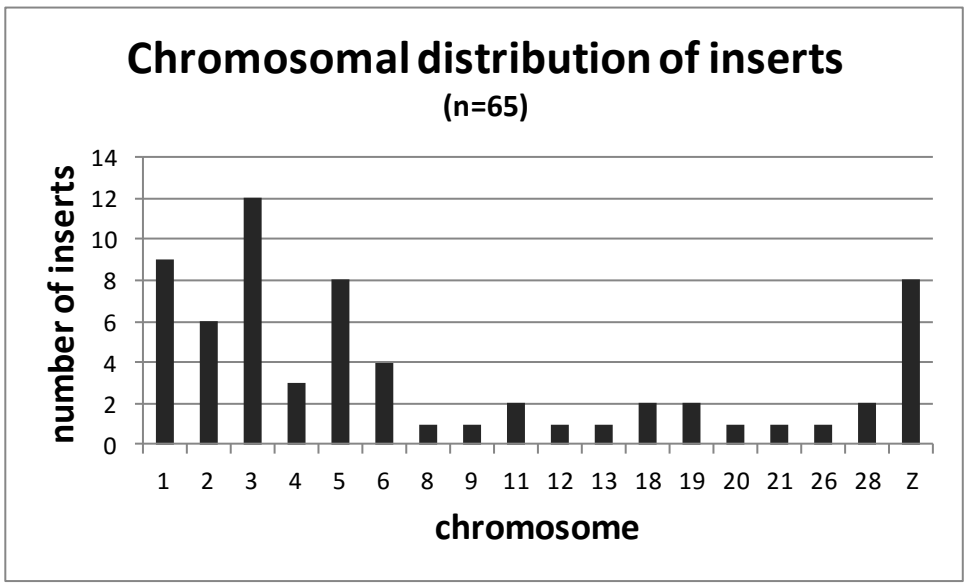
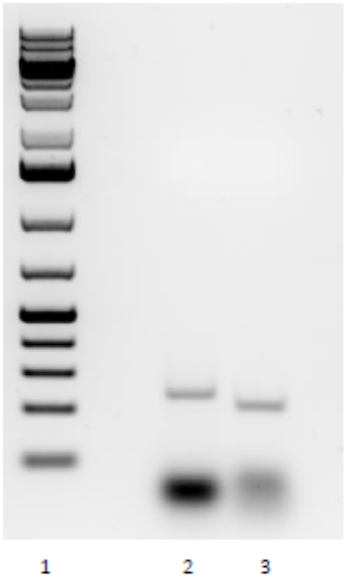


Figure 5. Chromosomal distribution of Tol2 integration.

Using the Tol2 system we have successfully developed a line of chickens in which the hens carry the FP (Tol2 EGFP construct) on the Z chromosome. Segregation analysis confirms that only male offspring inherit this marker when hens are mated with wild type roosters. Specific primer sets were designed to genotype male and female chicks and to also validate the Z chromosomal location (Figure 6). The primer set for the Z chromosome insertion confirmed location of the Tol2- EGFP cassette into an intron of chicken Talin1 (chTLN1).



Gel analysis PCR gDNA PGC-Tol2-EGFP with Tol2 specific and chromosomal specific primer sets.

Lane 1 Generuler 1kb plus DNA ladder
Lane 2 Tol2-RHE-F/TD792 (chrZ-R)
Lane 3 Tol2-F8/TD792 (chrZ-R)

Figure 6. Specific PCR to Z-link insertion of Tol2-EGFP transgene.

To complete this major project objective and finalise the project work we have isolated, cultured and stored PGCs from this line of birds so that they are available in the future for further development of the application and continued analysis and assessment.

Chapter 2. *In ovo* detection of the Z-linked FP

Prior to beginning this project we had generated 5 G1 transgenic chickens with Tol2- EGFP and subsequently used G2 progeny from one of these birds that contains a single insert for the specific objective of testing in ovo detection of EGFP (Figure 7).

Figure 7. EGFP +ve chicks compared with EGFP –ve chicks.

We looked at fluorescence at Day 2.5, 10 and 18 of embryogenesis using a GFP detection light source placed and goggles with filter to detect fluorescence (Figure 8). This was successful and has opened the possibility of using fibre optics and microendoscopy to detect a fluorescent embryo. The fibre optics are hair width and can easily be incorporated into needles that are inserted into an egg to detect, mark and remove male embryos (Figure 9). We have also pursued detection of fluorescence through the egg shell. A publication by Shafey et al 2002 (Asian-Aust.J.Anim.Sci 2002, Vol15, No2:297-302) indicates that Red Fluorescent Protein (RFP) wavelengths have the greatest opportunity of penetrating the egg shell. Based on this we will pursue RFP in future work for the precision insertion into the Z Chromosome. We have established a connection with Dr David Wand (CSIRO Manufacturing) who has expertise in micro fibre optic detection technology. We are exploring ultra-fine detection devices that could be combined with existing egg injection platforms (Embrex) for rapid detection and removal of a fluorescent male embryo.

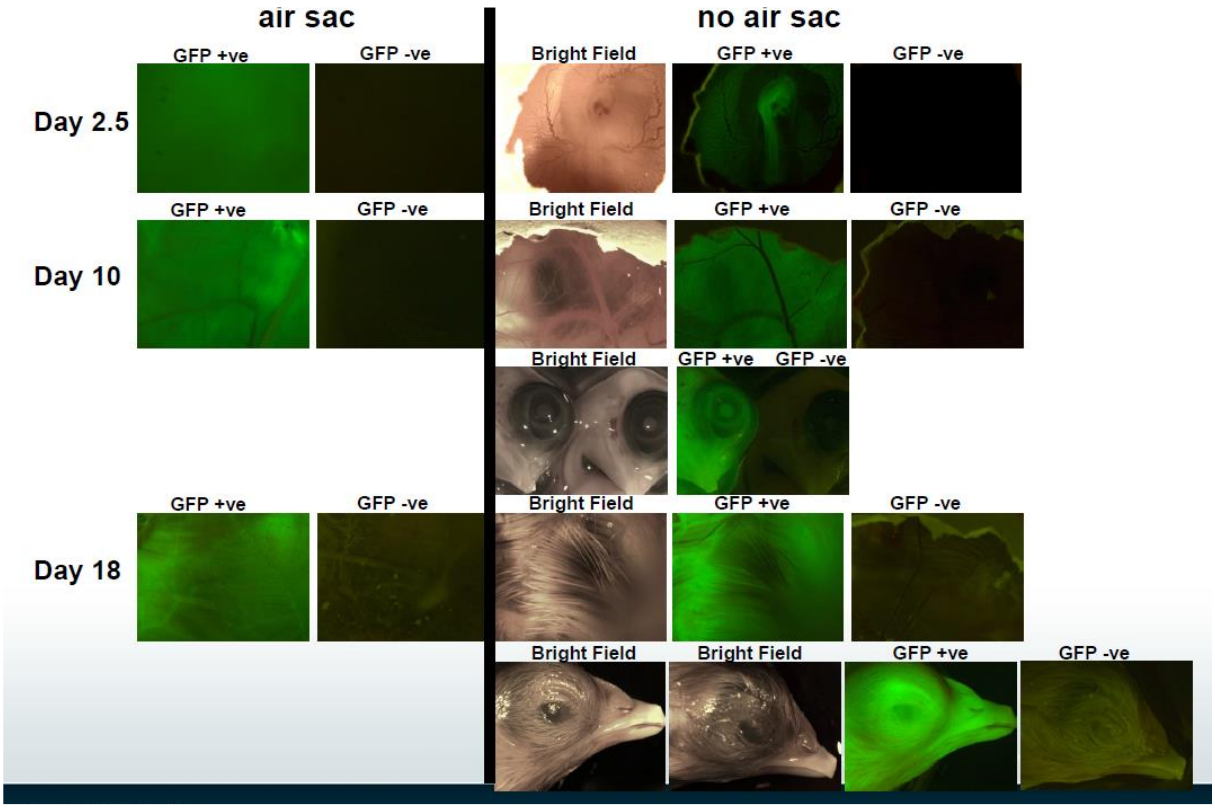


Figure 8. Detection of fluorescence in embryos with or without air sac.

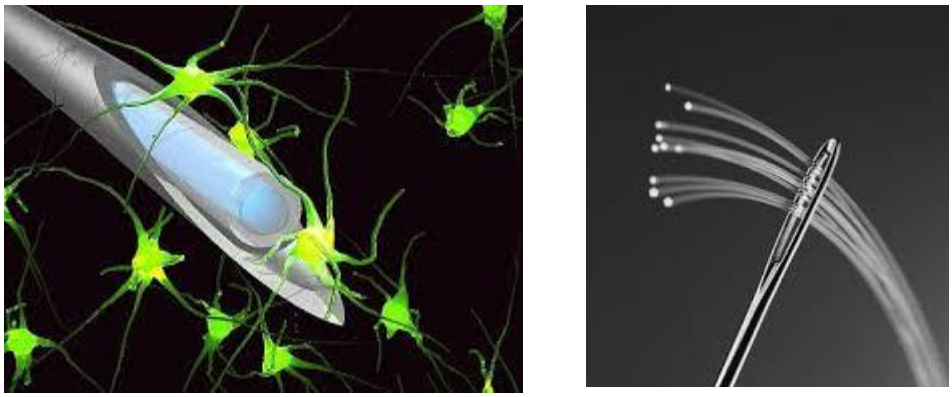


Figure 9. Schematic of fibre optics for incorporation into needles.

Discussion of Results

We have successfully achieved the objectives of this project. Using the Tol2 transposon system in combination with our technology to directly transform avian germ cells *in ovo*, we have created a breeding hen with a specific insertion of a FP gene on the Z chromosome. The FP gene is inserted within an intron of the Z-linked Talin1 gene (chTLN1). As expected, meiotic segregation of this marked Z chromosome to male offspring was confirmed. From our overall Z Chromosome data we have been able to identify 8 locations on the Z Chromosome that are suitable for a FP transgene insertion. They are in locations that do not impact on the viability of the chicken and have no detrimental impact on Z gene expression and regulation. Three of these locations are suitable to be developed as target sites for the Precision Genome Engineering of the GFP or RFP gene in female breeding birds. Red Fluorescent Protein (RFP) wavelengths have the greatest opportunity of penetrating the egg shell and is best suited for the development of a detection system that does not require a needle to penetrate the egg shell.

We have cultured and stored down primordial germ cells (PGCs) from transgenic hens that harbour the Z-linked FP transgene. These cells can be used to re-establish a line of transgenic chickens for future breeding and for future development and refinement of an industry adoptable detection system.

We have explored a fibre optic approach to detect FP marked male embryos, however it may well be possible to develop a light transmission and detection through the egg-shell. “Candling” is a standard method used to determine fertility of set eggs. This relies on visible light transmission both into and out of the egg. A number of efficient fluorescent proteins have wavelengths in the red and near infrared spectrum. These have been tailored to the needs of researchers seeking to study gene expression in animals in vivo in deep tissues. A number of proteins have recently been produced that can be detected at 7 to 12 mm deep inside a mouse body, which is highly opaque to visible light but transmits red and infrared light. We conclude that it should be a straightforward exercise to select an appropriate fluorescent protein to facilitate *in ovo* detection through the shell.

Implications

We have demonstrated that a Z-linked selectable marker can successfully be employed *in ovo* to identify male embryos and enable their removal from the production system at the earliest age. An added benefit is the potential to direct the marked male eggs for other valuable applications such as human influenza vaccine production. This new technology can be automated and integrated into existing farming practises potentially making it easy for industry to adopt.

Implications for adoption of the technology exist. The process will involve the use of one parent (the female) which is a genetically modified organism (GMO). However due to the innovation of the approach the transgene is ONLY passed on to the male offspring, which are removed from the production facility at a very early stage *in ovo*. This “risk” presents two issues:

a) Adoption by industry: we have considered how this technology could be implemented in a commercial environment. We have had a long term working relationship with Aviagen (partner of the CRC) and have had discussion with them about this technology. They have indicated to us that it can be integrated into their breeding strategy at the grandparent level of their breeding program.

b) Consumer perceptions. The resulting female progeny of the GMO parent are completely free of the genetically modified material, by virtue of the sex determination process. The progeny are not GMO and neither is the material that they produce – the egg which makes its way to the consumer’s plate. However it is possible that some consumer groups/activists may object to any use of GM in the production process. This would be balanced against welfare / ethical and production benefits.

Recommendations

We have clearly demonstrated that it is possible to create a breeding hen with a Z-linked marker gene that is stably inherited by male offspring only. We have used transposon technology to create this insertion. For translation to commercial practice it would be preferable to use the latest techniques of precision genome engineering to place the marker gene in the most suitable location on the Z chromosome. We have already identified a number of suitable Z locations that could be used for development of this application. We have also clearly demonstrated that it is possible to detect expression of the Z-linked marker gene at any time from point of lay to hatch. The approach that we employed would be consistent with future development of a fibre optic system incorporated into a needle that would be inserted into the egg, much as the Embrex *inovoject* system currently does. It is likely that use of the red fluorescent protein (RFP) as the marker could facilitate development of a non-invasive laser-based detection system. We recommend this as the next phase of this research in partnership with the breeding industry.

The outcomes of this project are really promising, however translation into industry practise requires industry to overcome their concerns about adoption. This is based on consumer perception in relation to GM technology. The marker gene is GM, but the biology of sex chromosome segregation means that the laying hen and the eggs produced are not GM. We have clear advice from gene technology regulators from a number of international jurisdictions that the hen and her eggs would not be considered GM and would not require regulation or labelling of product. It is our considered opinion that all major breeders would need to adopt this technology for it not to become a marketing tool for commercial opportunism. We recommend a high level discussion between the major breeders and their subsidiaries to undertake a risk/benefit analysis of the technology and to negotiate a consensus approach to adoption.

Acknowledgements

We would like to thank Malta Advanced Technologies for providing breeding flocks. We would like to thank the staff of the Small Animal Facility at AAHL and also staff of the Werribee Animal Health Facility. Mini Tol2 transposon plasmids were kindly provided by Professor Stephen C. Ekker, Mayo Clinic Cancer Center, Rochester, MN, USA.

Plain English Compendium Summary

Sub-Project Title:	Single sex selection for the egg laying industry to improve animal welfare
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Sub-Project Overview	The ability to detect and remove male chicks pre-hatch would be a big step forward to the egg laying and related industries. Currently culling male chicks post-hatch creates a major ethical dilemma for some countries. As a result the poultry industry has invested in developing solutions for this issue. In some European countries the need is urgent following the introduction of legislation to ban the culling practise. Growing male layer chicks is not a sustainable option for farmers. Sex selection effectively negates the need to cull or grow out male chickens and contributes to a more sustainable industry with a view to future food security.
Background	Thanks to recent advancements in gene technology, it is now possible for scientists to specifically place a biological marker on the sex determining chromosome of the chicken. This discovery provides a simple solution to meet a pressing need for the industry and a leading opportunity for the adoption of biotechnology in animal agriculture. Being male or female is determined by sex chromosomes, both in humans and in chickens. By harnessing technology to mark the sex determining chromosome in chickens, the males can be identified before hatching and removed during the incubation. The process uses a gene that marks only the chromosome that says “become male”, resulting in only the male chickens being marked and the females not. The unmarked females go on to lay eggs for our plate.
Research	The technology used for the sex selection process builds on ten years of experience with chicken genome engineering and gene editing. The skills for the job were developed in collaboration with industry and university partners. The process of marking the sex chromosome starts by carefully opening the shell of a fertilised egg to expose the embryo. A snippet of DNA that encodes for the marker gene, known as green fluorescent protein (GFP) is then microinjected into the bloodstream and taken up by germ cells that go on to become the ovum and the sperm of the adult chicken. Once in these cells the DNA homes in on the sex chromosome and uses a precise ‘cut and paste’ process to lock into this specific target. Male eggs that carry the marker gene make the GFP which can be seen and then detected through the shell using UV light.
Implications	We have demonstrated that a Z-linked selectable marker can successfully be employed in ovo to identify male embryos and enable their removal from the production system at the earliest age. An added benefit is the potential to direct the marked male eggs for other valuable applications such as human influenza vaccine production. This new technology can be automated and integrated into existing farming practises potentially making it easy for industry to adopt.
Publications	Manuscript in preparation.