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BRIEF COMMUNICATION

A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells

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Abstract Traditional methods of avian transgenesis involve complex manipulations involving either retroviral infection of blastoderms or the ex vivo manipulation of primordial germ cells (PGCs) followed by injection of the cells back into a recipient embryo. Unlike in mammalian systems, avian embryonic PGCs undergo a migration through the vasculature on their path to the gonad where they become the sperm or ova producing cells. In a development which simplifies the procedure of creating transgenic chickens we have shown that PGCs are directly transfectable in vivo using commonly available transfection reagents. We used Lipofectamine 2000 complexed with Tol2 transposon and transposase plasmids to stably transform PGCs in vivo generating transgenic offspring that express a reporter gene carried in the transposon. The process has been shown to be highly effective and as robust as the other methods used to create germ-line transgenic chickens while substantially reducing time, infrastructure and reagents required. The method described here defines a simple direct approach for transgenic chicken production, allowing researchers without extensive PGC culturing facilities or skills with retroviruses to produce transgenic chickens for wide-ranging applications in research, biotechnology and agriculture.

Keywords Avian · Transgenisis · Primordial germ cells

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Introduction

The production of transgenic chickens has increasing applications in biotechnology providing excellent model organisms for developmental biology research (Smith and Sinclair 2001; Modziac and Petitte 2004; Rashidi and Sottile 2009; Vergara and Canto-Soler 2012) and bioreactors for pharmaceutical proteins (Lillico et al. 2005; Ivarie 2006). Perhaps the application with the greatest global impact will be the increased security of chicken meat and egg production by generating chickens that are resistant to disease and/or have improved production traits (Clark and Whitelaw 2003). Research into the development of improved genetic engineering technologies facilitating the production of transgenic chickens is of crucial importance in light of the need to feed a growing population and maintaining global food

security. The use of transgenic agricultural crops has shown the value of transgenesis to improve production of agriculture commodities (Mittler and Blumwald 2010). A trait of major interest to the poultry industry is disease resistance and research using transgenic technology to develop avian influenza-resistant chickens is already gaining momentum (Lyall et al. 2011) and may expand as a novel control strategy to protect against other industry threatening diseases such as Marek's disease and Newcastle disease. Of equal importance is the protection of humans from potentially devastating zoonotic diseases such as avian influenza-controlling this virus in poultry will reduce the risk of the next flu pandemic.

Previously published methods of germ line transgenesis in avian species have been based on two approaches. The first approach uses recombinant lentivirus carrying a transgene that is injected into the blastoderm (stage X) (McGrew et al. 2004) or early stage chick embryo [stage 13–14 Hamburger and Hamilton (HH)] (Sun et al. 2012) and transducing the primordial germ cells (PGCs) to produce a germline transgenic chick. For commercial applications of avian transgenic technology, particularly for the food production sector, a non-viral method is essential for biosafety reasons. The second approach depends on a complex process involving the isolation, in vitro culture, modification by transfection and re-injection of PGCs to produce germ line transgenic chickens (Van de Lavoir et al. 2006). The establishment of a long-term culture system for stable PGC lines is both technically demanding and resource intensive.

The latest advances in chicken transgenesis have utilized "cut and paste" transposons in cultured PGCs (Macdonald et al. 2012; Park and Han 2012). These are a particular class of mobile genetic elements that transpose genetic information by a "cut and paste" process from one genomic location to another within the host genome (Ivics et al. 2009). Whilst this approach may help minimise epigenetic silencing (Macdonald et al. 2012), the establishment of cultured PGCs remains problematic. It requires access to specific and in some countries restricted biological components (including cross-species growth factors) as well as capability to inactivate (e.g. irradiation or mitomycin C) the required feeder cells to help maintain long-term PGC cultures. The transposon plasmids must be transfected into the PGCs, however, these cells are difficult to transform with high efficiency in vitro. Even with the use of the most effective electroporation equipment, only a small percentage of PGCs are transformed. In order to overcome this inefficiency, a reporter gene is often incorporated into the transposon to facilitate selection and enrichment of transfected PGCs. This process of amplifying pure cultures of modified PGCs greatly increases the chance of obtaining a germ-line transgenic chicken. However, the incorporation of a reporter gene into the chicken genome and expression of a non-native protein will not be acceptable for commercial production of poultry and would complicate regulatory issues around chicken meat and egg consumption. Avoiding reporter genes in the generation of transfected PGCs is a further complication for production of transgenic chickens.

We sought to develop a more direct method for the in vivo transfection of PGCs with miniTol2 transposon plasmids to generate stable germ-line transgenic male chickens capable of passing the transgene onto the next generation. We have shown that this simplified technique can be as effective at generating transgenic chickens as the established but technically demanding methods involving the use of retroviruses or cultured PGCs. This method provides a new opportunity for researchers without extensive PGC culturing facilities or skills with retroviruses to undertake functional genomic studies within the chicken, and also help to drive the development of new applications for avian transgenesis in biotechnology and agriculture.

Materials and methods

miniTol2 plasmids

The miniTol2 plasmid system used in this study 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 pCAGGS promoter driving the enhanced green fluorescence protein (EGFP) open reading frame with a SV40 polyA sequence (designated pMiniTol-EGFP). 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 Lipofectime 2000 CD complex for microinjection

The Lipofectamine 2000 CD complex was prepared according to the manufacturer's instructions. Briefly, 0.6 μg of pMiniTol-EGFP and 1.2 μg of pTrans were mixed with 45 μl of OptiPRO (Invitrogen) and incubated at room temperature for 5 min. At the same time, 3 μl of Lipofectamine 2000 (Invitrogen) was added to 45 μl of OptiPRO and incubated for 5 min. The two solutions were then mixed together and allowed to complex for 20 min 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. Staining for chicken-Vasa homologue was also carried out to identify PGCs. Dissected gonads were dissociated using trypsin and cytospotted (Shandon) onto microscope slides prior to being fixed with 4 % paraformaldehyde. The cells where then permeabilised with 0.1 % Triton-X-100 in PBSA for 5 min, and the slides were washed three times in PBSA prior to being blocked with 5 % BSA in PBSA. The slides were then incubated with the primary antibody, Rabbit anti-Chicken VASA homolog (1:1,000 in 1 % BSA/PBSA) for 45 min at room temperature and washed three times in 1 % BSA/PBSA. Slides where then incubated with Goat anti-Rabbit IgG (H + L) Rhodamine Red (Invitrogen Molecular Probes) (1:100 in 1 % BSA/PBSA) for 45 min at room temperature and then washed three times in PBSA. The slides were then DAPI stained (5 μg/ml) and mounted in DAKO mounting medium.

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 miniTol-EGFP 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 2× Universal master mix (Applied Biosystems), 1 μl 20× 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 minTol2 (Fwd primer 5′ CAGTCAAAAAGTACTTATTTTTTGGA GATCACT 3′; Rev primer 5′ GGGCATCAGCGCAA TTCAATT 3′; detection probe 5′ ATAGCAAGGGA AAATAG 3′) and a genomic control region from the chicken genome which acts as a template control (Fwd primer 5′ GATGGGAAAACCCTGAACCTC 3′; Rev primer 5′ CAACCTGCTAGAGAAGATGAGAA GAG 3′; detection probe 5′ CTGCACTGAATGGAC 3′).The PCR cycle parameters were an initial denaturing step at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Each rooster was tested at least twice and was classified positive if a C_T value of <36 was obtained for minTol2. A C_T of <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 min and denatured twice with 0.5 M NaOH, 1.5 M NaCl for 25 min prior to being neutralised twice with 0.5 M Tris, 1.5 M NaCl (pH 7.0) for 25 min. The gel was treated in 10× SSC for 30 min and then transferred overnight to a nylon membrane (Hybond N) using a turboblotter (Whatman). The membrane was prehybridised for 4 h at 68 °C in 6× SSC, 5× 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 min in 2× SSC, 0.1 % SDS followed by two 20 min washes in 0.1× SSC, 0.1 % SDS at 68 °C and then the membrane was autoradiographed at −80 °C with an intensifier screen.

Results

Characterisation of gonads from direct injected embryos

The miniTol transposon system used in this study is made up of two plasmids. The first plasmid contains the EGFP transgene under the control of the CAGGS promoter and is flanked by the Tol2 ITRs (pMiniTol-EGFP). The second plasmid (pTrans) encodes the Tol2 transposase under the control of the cytomegalovirus immediate-early promoter for *in trans* expression of the transposase and subsequent transposition of miniTol-EGFP from plasmid to chromosome in transfected PGCs. In this system, the miniTol-EGFP sequence will be incorporated into the target genomic DNA while the pTrans sequence will not be incorporated. pMiniTol-EGFP and pTrans were combined and formulated with Lipofectamine 2000 to produce the Lipofectamine 2000–miniTol complex. Here we describe a transposon plasmid:transposase plasmid ratio of 1:2 and a Lipofectamine 2000:plasmid ratio of 3 µl:1.8 µg. This could be further optimized for future experiments in particular using different plasmids.

This Lipofectamine 2000–miniTol complex was intravenously injected into 20 embryos at stage 14 HH and examined in two groups of 10 at embryo day (ED) 7 and at ED 14. Nine embryos were alive at ED 7 and 6 were alive at ED 14. Dissected gonads and whole embryos were analyzed under a fluorescence microscope and EGFP was observed extensively throughout the gonads of all embryos (Fig. 1a). Chicken-Vasa homologue antibody staining of gonadal cells confirmed that all of the EGFP positive cells were indeed PGCs (Fig. 1b), confirming that we had successfully transfected PGCs in vivo with the miniTol-EGFP plasmid. Fluorescence microscopy also identified EGFP expression in other cells throughout the embryo body, especially in the heart and brain, indicating that not just PGCs but other cell types were transfected as a result of the direct injection of the Lipofectamine 2000–miniTol complex into the bloodstream of stage 14 HH embryos.

Generation and characterization of transgenic chickens

We next intravenously injected 50 embryos at stage 14 HH with the Lipofectamine 2000–miniTol complex. In order to confirm successful transfection, ten embryos were analyzed at ED 14 and we observed EGFP expression in the gonads of all 10. Of the remaining 40 embryos, 16 survived to hatch of which 5 were female and 11 were male. The male chicks were grown to sexual maturity and their semen was then collected and tested using qPCR for the presence of the miniTol-EGFP transgene. From the qPCR results the 3 males with the highest levels of miniTol DNA in the semen (roosters 8, 9 and 11) were selected as founder roosters to breed for G1 germline transgenic offspring (Table 1). The selected roosters were each mated with 7 hens of the same line and a total of 419 G1 chicks were hatched and screened for visual whole-body EGFP expression using GFsP-5 goggles (BLS LTD, Hungary). A total of 5 out of the 419 chicks were positive for EGFP expression confirming stable integration of miniTol-EGFP into transfected PGCs of the founder roosters and germ line transmission of the transgene to the G1 offspring. Roosters 9 and 11 had germ line transmission of approximately 1.5 %. Of the 95 G1 chicks screened from rooster 8, none were found to be transgenic. Hatch data, efficiency of germ line transmission and transgenic chick production is shown in Table 1. Transgenic chicks could be easily identified with the GFsP-5 goggles because of high level external EGFP expression in tissues such as the beak, eyes, and skin on the legs and feet of positive chicks (Fig. 2a). Strong EGFP expression was also

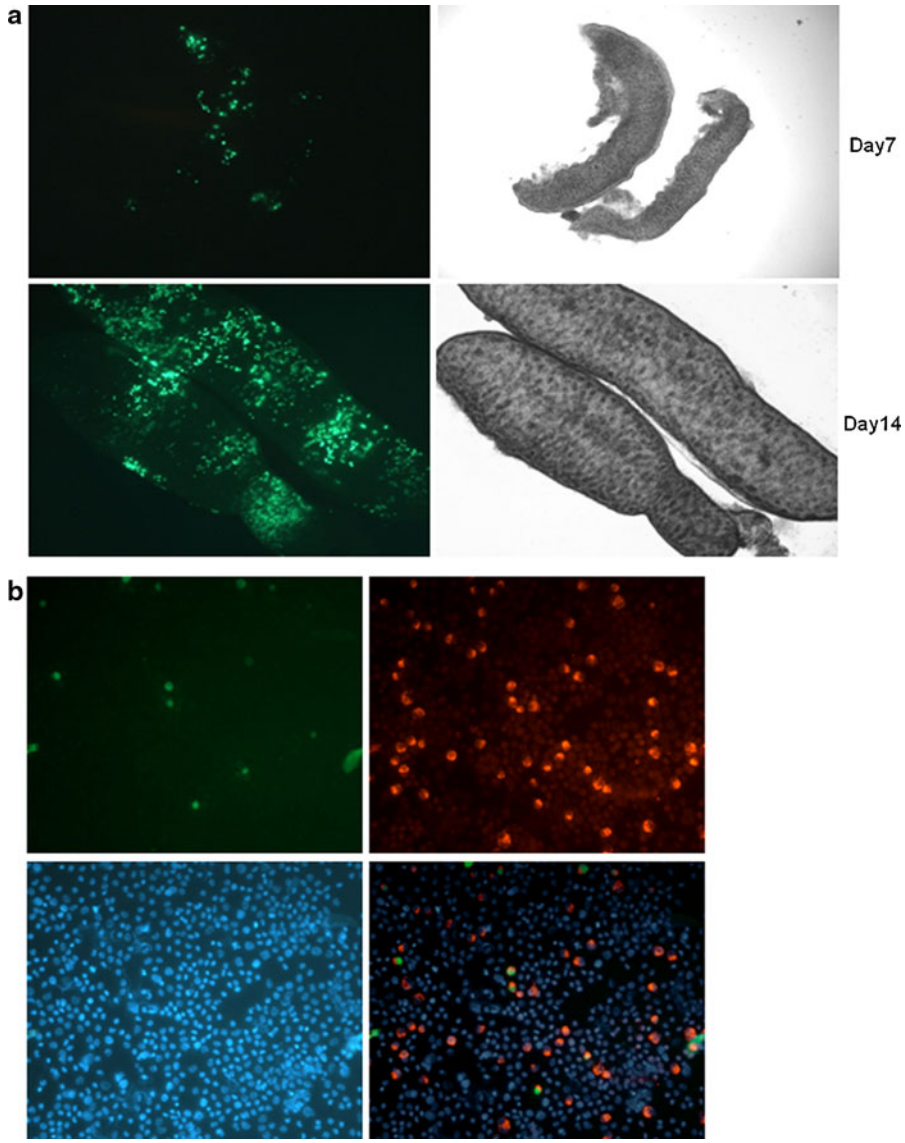

Fig. 1 Analysis of gonads from direct injected embryos **a** EGFP and corresponding bright field images of representative gonads from ED 7 and ED 14 embryos directly injected with the Lipofectamine 2000–miniTol complex at ED 2.5. Magnification ×50. **b** Composite image of dissociated gonads from a representative ED 14 showing PGC's stained by chicken-Vasa homologue (*red*), some expressing EGFP (*green*) and all gonadal cell nuclei stained by DAPI (*blue*). Magnification ×50. (Color figure online)

observed throughout all internal tissues examined including brain, heart and kidney (Fig. 2b–d).

Southern blot analysis of genomic DNA from the 5 positive G1 chicks revealed that a single transposition event had occurred in 4 of the 5 chicks and a double transposition event had occurred in 1 chick (Fig. 2e). Based on restriction fragment size each transposition appears to have targeted a unique genomic location within transfected PGCs.

Discussion

The results presented in this paper demonstrate a simpler, more direct method for the in vivo transfection of PGCs with miniTol2 transposon plasmids to generate stable germ-line transgenic male chickens capable of passing the transgene onto the next generation. Our method is a significant improvement on the previously published method which demonstrated that transfection

Table 1 Germline transmission and transgenic chick production

G0 rooster no.	Relative levels of miniTol in semen[a]	Offspring hatched	Transgenic offspring	Percentage transgenesis
1	0			
2	0.100			
3	0			
4	0.053			
5	0			
6	0			
7	0			
8	0.109	95	0	0
9	0.145	131	2	1.53
10	0			
11	0.221	193	3	1.55

[a] The relative levels of integrated miniTol DNA in semen were calculated by comparing the mean Ct values from genomic and miniTol qPCR from two semen samples

of PGCs can be achieved by injecting plasmid DNA-Liposome complexes into the bloodstream of stage 14 HH embryos (Watanabe et al. 1994). The previous study confirmed that although it was possible to introduce exogenous DNA into gonadal germ cells by transfecting circulating PGCs in vivo, it was a very inefficient and unstable process. Furthermore, they were unable to demonstrate that this approach was able to generate transgenic birds. We have now adopted the latest developments in Lipofection technology and significantly advanced this approach to stably transform PGCs in vivo and successfully and efficiently generate transgenic offspring expressing the EGFP gene carried in a transposon. Compared with circular or linear plasmid DNA previously used (Watanabe et al. 1994), transposable elements such as piggyBac and Tol2 have now been validated as efficient vectors for the genetic manipulation of PGCs and the chicken genome (Macdonald et al. 2012; Park and Han 2012; Sato et al. 2007).

Combining our direct injection method with precision genome manipulation involving zinc finger nucleases (ZFNs) (Kim et al. 1996) and TALENs (Bedell et al. 2012) will allow breakthroughs in functional genomic studies in the chicken, providing the opportunity to set and test hypotheses in phenotype development and disease states that until now have been very difficult to establish. Both ZFN and TALEN systems are based on plasmid delivery to the cell and are therefore suitable to the direct injection method for generating specific events in the genome of avian species. The ability to precisely study host gene function, for example in host-pathogen interactions, will transform our ability to understand and control important infections in chickens such as zoonotic highly pathogenic avian influenza virus (H5N1) or food bourne pathogens such as Salmonella and Campylobacter. Conversely, transposon mediated insertion of RNA interference (RNAi) transgenes into the chicken genome that express short hairpin RNAs or microRNAs can be used to stably knockdown the expression of targeted genes. This will not only greatly benefit functional genomic studies within the chicken, but offer entirely new ways of controlling the replication of intracellular pathogens.

While we have shown our transgenesis technique to be successful in chickens, the transfer of the technology to other avian species is expected to be equally successful. In zebra finches, a model avian species used in scientific research, there is the potential to make specific modifications to the genome and provide new possibilities for research with translational outcomes for human neuroscience. This direct injection method provides the ability to produce "knock-out" birds with greater simplicity and presents new models for a range of diseases and allows us to ask new fundamental biological questions that have up to now relied on often inappropriate mouse models. Furthermore, for ducks, turkeys and other avian species of agricultural importance there is the potential for improvement in production traits and disease resistance.

Transgenesis has proven to be an invaluable tool in animal biology and is predicted to follow plant science to produce animals specifically bred for food production that are resistant to industry-threatening diseases. It was almost a decade ago when Clark and Whitelaw (2003) proposed in their review "A future for transgenic livestock" that the advent of the then new method of RNAi for modifying genomes will underpin a resurgence of research using transgenic livestock. They suggested this may be an important alternative to traditional breeding and could lead to the generation of farm animals that are more resistant to infectious disease such as the possibility of genetically engineering poultry to make them resistant to avian influenza. This example and others has now become a real possibility using the combination of improved methods

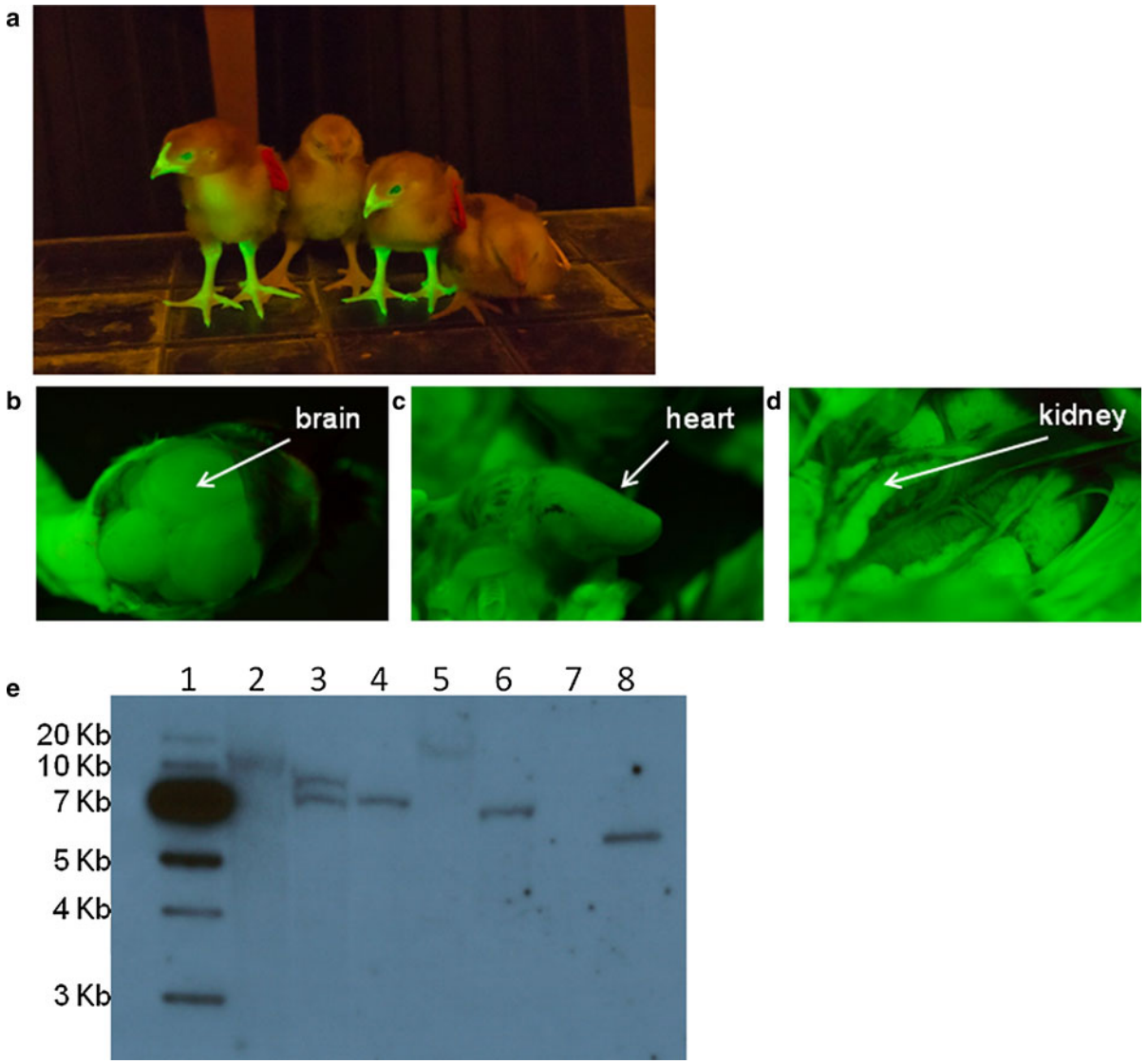

Fig. 2 Analysis of G1 offspring **a** G1 offspring from positive G0 roosters injected with Lipofectamine 2000–miniTol. G1 offspring were visualised for EGFP expression, image shows two positive G1 offspring with two non transgenic siblings. **b** EGFP expression from the brain, **c** heart and **d** kidney of a positive G1 chick. **e** Southern blot. Genomic DNA was analysed to determine the copy number of miniTol transposon by digestion with *Bam*HI to generate junction fragments and hybridised with a probe to the EGFP sequence. *Lane 1* 1 Kb DNA markers (GeneRuler), *Lane 2* G1-1, *Lane 3* G1-2, *Lane 4* G1-3, *Lane 5* G1-4, *Lane 6* G1-5, *Lane 7* non transgenic, *Lane 8* positive plasmid control (EGFP). G1-1 and G1-3 are offspring from G0 rooster 9 and G1-2, G1-4 and G1-5 are offspring from G0 rooster 11

for genetic modification of chickens and the emergence of RNAi as an antiviral strategy. The ability to produce virus resistant livestock will increase the welfare status of production animals, contribute to increasing the quality and safety of food production particularly in intensively reared animals such as poultry and serve to enhance future food security worldwide. Perhaps more importantly, developing animals that are resistant to zoonotic viruses with pandemic potential such as H5N1 and H1N1 influenza is a key strategy for reducing the risk of pandemic emergence in humans (Lyall et al. 2011). Our development of a direct and readily adoptable method for avian transgenesis is an important step forward in realizing these applications

for chickens and other bird species used in research, biotechnology and agriculture.

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Conflict of interest None.

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