

Corrigendum

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The e-press version of the article that was published on the 26th January contains the incorrect URL www.geneworks.com on p. 940. The correct URL is www.geneworks.net

Both the published print and online versions of this article are correct.

The authors apologise to readers for this mistake

Ubiquitous GFP expression in transgenic chickens using a lentiviral vector

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Summary

We report the first ubiquitous green fluorescent protein expression in chicks using a lentiviral vector approach, with eGFP under the control of the phosphoglycerol kinase promoter. Several demonstrations of germline transmission in chicks have been reported previously, using markers that produce tissue-specific, but not ubiquitous, expression. Using embryos sired by a heterozygous male, we demonstrate germline transmission in the embryonic tissue that expresses eGFP uniformly, and that can be used in tissue transplants and processed by *in situ* hybridization and immunocytochemistry. Transgenic tissue is identifiable by both fluorescence microscopy and immunolabeling, resulting in a permanent marker identifying transgenic

cells following processing of the tissue. Stable integration of the transgene has allowed breeding of homozygous males and females that will be used to produce transgenic embryos in 100% of eggs laid upon reaching sexual maturity. These results demonstrate that a transgenic approach in the chick model system is viable and useful even though a relatively long generation time is required. The transgenic chick model will benefit studies on embryonic development, as well as providing the pharmaceutical industry with an economical bioreactor.

Key words: GFP, Transgenic, Lentiviral, Chick, Development

Introduction

Production of transgenic chicks has been technically challenging, in part, owing to the nature of the reproductive system of the hen and the processing of the egg as it passes down the oviduct, the need for the shell in development, and the difficulty in isolating germ/ES cells. Additional significant challenges include the requirement for laboratory and animal housing facilities in close proximity to each other, and the relatively long generation time required to produce birds of reproductive age. A number of approaches have been pursued with varying degrees of success: manipulation of oocytes/spermatozoa or newly laid eggs; production of chimeras using primordial germ cells or chick ES cell equivalents; and gene transfer using viral vectors (Mozdziak and Petitte, 2004; Petitte et al., 2004; Sang, 2004). Oncogenic viruses have shown promise (Ishii et al., 2004), including avian leucosis virus (ALV) and reticuloendotheliosis virus (REV) (Salter et al., 1986; Salter et al., 1987; Bosselman et al., 1989a; Bosselman et al., 1989b), and Moloney murine leukemia virus (MoMLV) (Mizuarai et al., 2001). A newly reported MoMLV-transduced chick, utilizing the vesicular stomatitis virus G glycoprotein (VSV-G) vector pseudotyping system, expresses GFP under control of the rous sarcoma virus (RSV) promoter

(Kwon et al., 2004). G0 birds produced using this system are chimeric, but G1 analysis has yet to be reported.

Recently, lentiviral vectors have been favored, based on their ability to transduce dividing and non-dividing cells (Naldini et al., 1996; Pfeifer et al., 2002), a relatively large transgene capacity of 8 kb, and the apparent resistance of transduced cells to gene silencing, which is a problem with oncogenic viral vectors. The promoter/enhancer elements driving gene expression are of some importance, depending on whether ubiquitous or tissue-specific expression is desired. The cytomegalovirus immediate-early gene promoter/enhancer (CMV) is a highly efficient promoter in many vertebrates, but in chick it seems to be less efficient than β -actin (Colas and Schoenwolf, 2003; Krull, 2004). McGrew et al. (McGrew et al., 2004) have reported a CMV-driven GFP transgene in chick that shows conserved tissue expression in the germline, but not ubiquitous expression. A newly reported CAGGS enhancer/promoter containing the β -actin promoter successfully drives ubiquitous GFP expression in mice, but awaits full analysis in G1 birds (Sang, 2004).

We describe results from embryos expressing GFP ubiquitously under the control of the phosphoglycerol kinase (PGK) promoter, obtained from eggs produced by a lentiviral-

generated rooster (G2) mated to wild-type hens. The approach used to generate transgenic chicks provides a model for the production of a new generation of avian transgenics for investigating the gene and tissue interactions important in embryonic development. Other applications include expressing pharmaceutical products in egg albumen (Harvey et al., 2002a; Harvey et al., 2002b), and imparting disease resistance to poultry flocks through genetic manipulation.

Materials and methods

Viral stocks and injection

A replication-defective HIV-1 lentiviral vector, with packaging vectors deleted, containing eGFP under the control of the PGK promoter, was obtained from Tranzyme LLC (RTP, NC). Published guidelines for biosafety level 2 were followed for injecting the virus. Unincubated fertilized eggs were obtained from Charles River Laboratories SPAFAS Avian Products and Services (Roanoke, IL), and were accompanied by a Quality Control Sheet that listed the test dates and methods used to screen for 28 avian pathogens within the flock. The eggs were maintained on their sides at room temperature upon arrival and until microinjection, which happened over the course of two days. When eggs were kept on their sides, embryos floated to the uppermost point of the shell. Thus by windowing this area of shell, embryos were exposed directly beneath the window for subsequent injection.

The upper surface of the egg was cleaned with a solution of 70% ethanol in water (v/v) and then blotted dry with a laboratory tissue. A small (0.3-cm diameter) hole was abraded into the shell using a hand-held rotary tool fitted with an abrasive stone bit. A small hole was then cut through the shell membrane with a #11 blade disposable scalpel. A glass capillary was attached to a microinjection apparatus that was attached to a micromanipulator. A solution of lentivirus, at a titer of 10^5 to 10^7 infectious particles/ml, was drawn into the glass capillary that had been pulled to a tip thickness of a few hundred microns using a Sutter model P-30 pipette puller. The glass capillary was then positioned into the subgerminal space of the exposed embryo and 2–5 μ l of the suspension was delivered.

The space beneath the window in the egg was then filled with PBS containing penicillin (100 I.U./ml) and streptomycin (100 mg/ml) (Media Tech Cellgro), and the window was then covered with a donor shell membrane harvested from eggs grown under sterile conditions and maintained in the PBS/antibiotic solution described above. After drying in a Class II biological safety cabinet, the membranes were covered with Opsite[®] surgical tape and placed in a Natureform model NMC-4000 Incubator until hatching. Two days before hatching the eggs were moved to a Natureform model NMC-4000 Hatcher. Eggs were handled in accordance with standard safety procedures for modified organisms.

Analysis of transgenic birds

To identify individuals containing the transgene, DNA was extracted from the blood of hatchlings and from the semen of mature roosters, using a PUREGENE DNA purification kit (Gentra Systems, Minneapolis, MN), and then analyzed by PCR. PCR amplifications were performed in a volume of 40 μ l, using PCR supermix (Invitrogen), 2 μ l of genomic DNA template, and 0.2 μ M of each primer. Primers were as follows:

semen, 5'-ACTCACAGTCTGGGGCATCAAG-3' and 5'-CCACC-TTCTTCTTCTAATCCTTCG-3'; and

blood, 5'-GGACAGCAGAGATCCAGTT-3' and 5'-CGGTGG-TGCAGATGAACTT-3'.

PCR conditions for both semen and blood were: 94°C for 2 minutes; then 94°C for 45 seconds, 63°C for 10 seconds, cooling at 0.1°C/sec to 62°C, 62°C for 1 minute and 72°C for 1 minute, for 34 cycles; followed by 72°C for 10 minutes and then storage at 4°C.

Expression analysis

EC culture (Chapman et al., 2001), grafting (Garcia-Martinez et al., 1993), and in situ hybridization (ISH) (Chapman et al., 2001; Chapman et al., 2002) were carried out as described previously. Immunocytochemistry (ICC) was performed on whole-mount embryos or paraffin sections (12 μ m) using an anti-GFP antibody from Molecular Probes, at 1:400 dilution, with a secondary goat anti-rabbit Alexa Fluor[®] 488-conjugated antibody (Molecular Probes), or with a HRP-conjugated goat anti-rabbit secondary antibody for DAB labeling, at 1:200 dilution, according to our standard protocol (Lopez-Sanchez et al., 2004). Briefly, for whole-mount immunocytochemistry, after fixation in 4% paraformaldehyde (PFA) overnight, embryos were washed in PBS, then washed for 4×30 minutes in PBT (PBS, 0.1% Tween-20 and 0.2% BSA), and then 5% goat serum/PBT before addition of the primary antibody and incubation overnight at 4°C. After washing for 4×30 minutes in PBT, and once in 5% goat serum/PBT, the secondary antibody was added and embryos incubated overnight at 4°C. For fluorescence visualization, embryos were rinsed several times with PBS and imaged using a Nikon SMC1500 microscope, with a GFP filter and a QImaging RTV5.0 Megapixel camera. A standard exposure time of 20 seconds was used for fluorescence imaging to enable a comparison of the fluorescent signal strength between embryos. For developing the DAB stain, embryos were rinsed for 3×5 minutes in PBT, 4×30 minutes in PBT, and for 10 minutes in 0.3 mg/ml DAB in PBT and then developed using 0.03% hydrogen peroxide in DAB/PBT. Once the desired staining was reached the reaction was stopped by several rinses in PBT and embryos were fixed in 4% PFA/PBT before imaging and paraffin sectioning. For immunohistochemistry on sections, slides were deparaffinized for 2×5 minutes in Xylene, 2×5 minutes in 100% EtOH, 5 minutes in 95% EtOH, and 5 minutes in 3% H₂O₂/MeOH, then rinsed three times in distilled water, followed by three times in PBS. Sections were then incubated at 37°C for 1 hour in 5% goat serum/PBT (PBS, 0.1% Triton and 0.2% BSA), and then 2 hours in PBT with primary antibody diluted 1:400. After rinsing three times in PBT, the sections were incubated with a fluorescent secondary antibody, diluted 1:200, for 1 hour at 37°C, before washing for 3×5 minutes in PBS and mounting in Slowfade (Molecular Probes) for visualization and imaging.

Results

An HIV-1 derived lentiviral vector (Tranzyme LLC; RTP, North Carolina) was used because of its ability to integrate into the genome of dividing and non-dividing cells, significantly increasing the host cell range. Enhanced green fluorescent protein (eGFP) was inserted into the replication-deficient vector under the control of a phosphoglycerol kinase promoter (PGK). Retroviral suspension (2–5 μ l) was microinjected into the subgerminal space of each windowed egg containing a stage X embryo (Eyal-Giladi and Kochav, 1976). Following injection, eggs were resealed with Opsite[™] surgical tape and incubated to hatching. Of the 473 microinjected eggs, 19 G0 chicks hatched (4%) and were grown to maturity. Of these 19, semen was collected from six mature roosters and analysed for the presence of the transgene by PCR (Fig. 1A). Although three of the roosters were positive, only one rooster (rooster 6) passed the transgene onto its offspring when mated to wild-type hens. Blood samples from 637 offspring from this rooster were analyzed by PCR, revealing 4 positives (0.63%). Positives were further identified by the presence of fluorescent lymphocytes. Of the resulting G1 birds produced, two roosters and a hen survived to adulthood, and the roosters have been crossbred to wild-type hens. The semen of one G1 rooster was

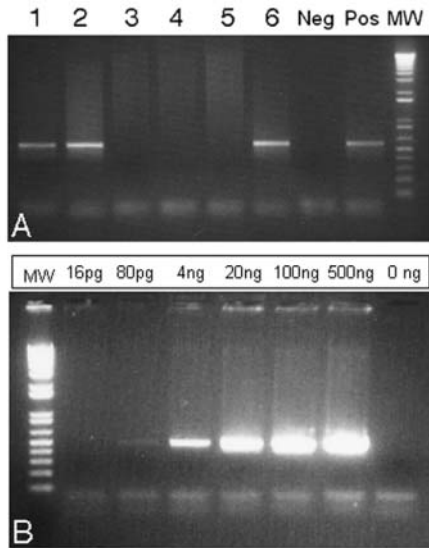


Fig. 1. PCR transgene analysis. (A) Semen from six chimeric G0 roosters was analyzed by PCR for the presence of the transgene. Three roosters were positive for the transgene in the germline, but only rooster 6 was able to pass the transgene onto his offspring. (B) Two mature offspring from rooster 6 had their semen tested for the presence of the transgene. Increasing template quantity from one of these G1 heterozygous roosters showed the expected quantity-dependent band increase. Neg, negative control; Pos, positive control; MW, molecular weight marker.

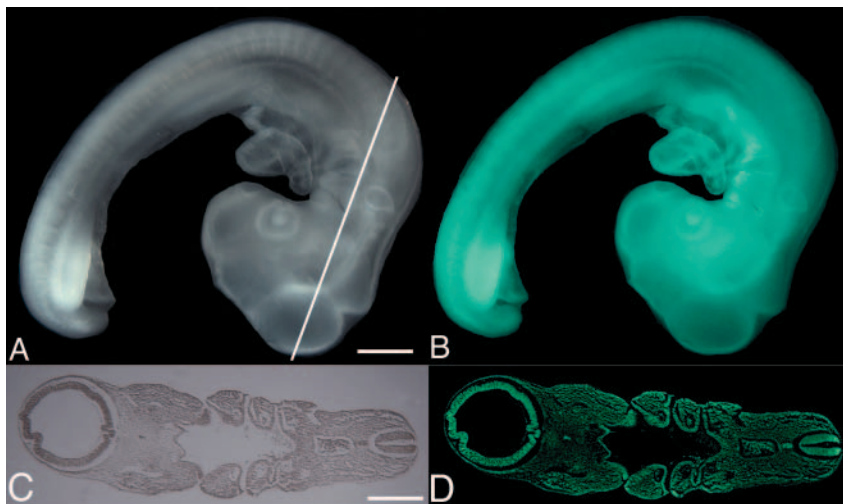


Fig. 2. GFP expression in whole-mount and sectioned transgenic embryos. (A-D) Examination by brightfield (A,C) or fluorescence (B,D) illumination of a live whole-mount 72-hour embryo (A,B) and a 72-hour embryo after sectioning and processing with anti-GFP antibody (C,D). GFP expression is ubiquitous (B,D). White line in A shows angle of the section. Scale bar: in A, 150 μ m for A,B; in C, 100 μ m for C,D.

analyzed by PCR and demonstrated template quantity dependence (Fig. 1B). Analysis of 170 eggs hatched from eggs sired by one of the heterozygous G1 roosters showed 68 G2 offspring positive for the transgene by PCR (40%). Mature offspring from heterozygote individuals have been crossbred to produce homozygous G3 individuals with two goals: (1) to eliminate the need for individual testing before experiments are performed (i.e. all G4 offspring will be positive/homozygote for the transgene); and (2) to obtain brighter fluorescence (owing to the double dose of GFP present in homozygotes).

Recent reports of GFP transgenic chicks have not demonstrated ubiquitous tissue expression (Harvey et al., 2002a; Harvey et al., 2002b; McGrew et al., 2004; Sang, 2004). In principle, the PGK promoter used in this study should result in ubiquitous expression of GFP from the onset of development. To test the timing of the onset, and the extent, of GFP expression, embryos were harvested from unincubated eggs and every two hours following incubation until HH stage 12 (48 hours) (Hamburger and Hamilton, 1951). In early embryos, expression was initially weak; development to at least HH stage 9 was required to produce a strong enough signal to be reliably detected using the fluorescence microscope (see Table 1). In total, GFP fluorescence could be detected only in 25% (12/48) of live embryos analyzed by fluorescence microscopy, whereas the use of an anti-GFP antibody (which enhances detection of the signal) revealed that 52% of the embryos produced GFP protein at these early stages. Expression was ubiquitous, and included the embryonic

and extraembryonic tissues. By HH stage 13, expression could be reliably detected in all transgenic embryos by GFP fluorescence. Thereafter, the strength of the fluorescent signal continued to increase until approximately HH stage 16/17 (Fig. 2A-D), after this stage the signal stabilized and remained at a constant level during the remainder of development; E18 was the latest developmental stage analyzed as whole embryos (E4-E18: 12/32 embryos were GFP positive). Organs, including heart, lung, kidneys, liver, pancreas, intestine, gizzard, eye and brain, were dissected from E14 embryos for more detailed analysis. Fresh, unfixed organs from GFP-positive embryos were individually analyzed and all were found to have uniform GFP expression in their organs by fluorescence microscopy (Fig. 3A,B,D,E,G,H,J,K), whereas no organs were GFP-positive from embryos that had no whole mount GFP-expression or from control non-transgenic embryos (data not shown). Following processing of GFP-positive organs by paraffin histology, 12- μ m sections were cut

Table 1. Numbers of living embryos with detectable GFP fluorescence and the same embryos with enhanced fluorescence following processing using a rabbit anti-GFP antibody and secondary anti-rabbit Alexa Fluor 488 antibody

	No fluorescence	GFP fluorescence	No fluorescence	GFP fluorescence	No fluorescence	GFP fluorescence	Total fluorescence
Stage	1-4	1-4	5-8	5-8	9-12	9-12	
Live embryo (%)	15/18 (83.3%)	3/18 (16.7%)	16/21 (76.2%)	5/21 (23.8%)	5/9 (55.5%)	4/9 (44.5%)	12/48 (25%)
Alexa Fluor 488 (%)	9/18 (50%)	9/18 (50%)	9/21 (42.9%)	12/21 (57.1%)	5/9 (55.5%)	4/9 (44.5%)	25/48 (52.1%)

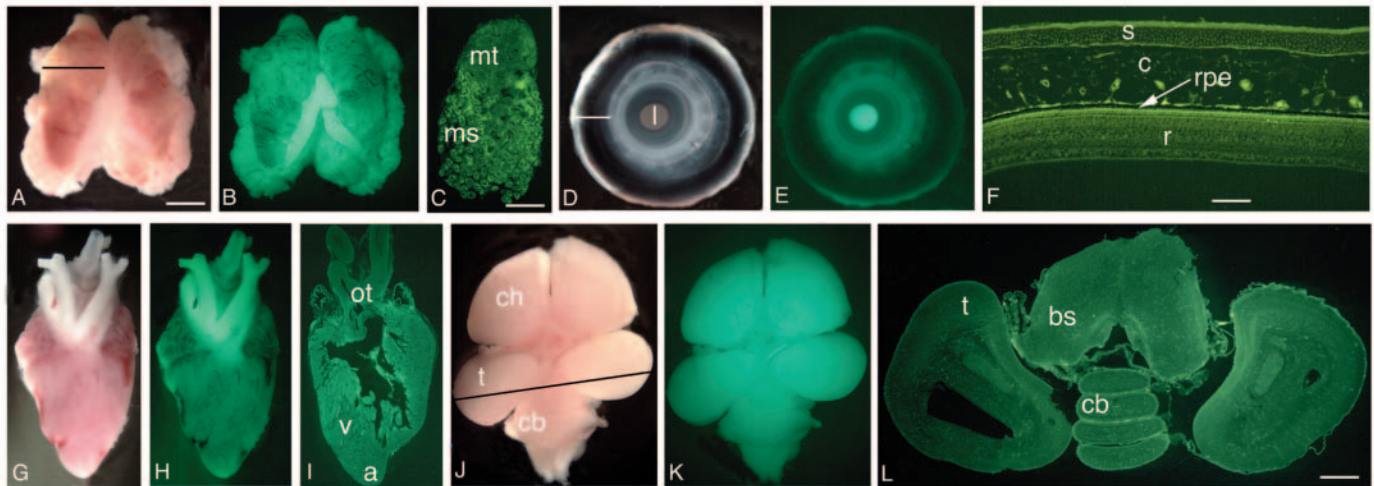


Fig. 3. E14 chick organs express GFP uniformly. Fresh organs from E14 embryos were dissected out and checked for GFP using fluorescence microscopy. Kidneys (A-C), eye (D-F), heart (G-I) and brain (J-L) are all positive for GFP expression. Lung, liver, pancreas, intestine and gizzard also uniformly express GFP (not shown). (C) Transverse section of the left kidney shown in A. (F) Magnified image of the right anterior portion of the transverse section of the eye in D. The heart in G was sectioned through the plane of the paper. L is an oblique section through the caudal part of the brain in J, including the tectum, cerebellum and brain stem. Lines in A, D and J indicate level of sections in C, F and L, respectively. a, apex; bs, brain stem; c, choroid; cb, cerebellum; ch, cerebral hemisphere; l, lens; ms, mesonephros; mt, metanephros; ot, outflow tract; r, retina; rpe, retinal pigment epithelium; s, sclera; t, tectum; v, ventricle. Scale bars: in A, 150 μm for A, B, D, E, G-K; in C, 50 μm ; in F, 40 μm ; in L, 35 μm .

and subjected to immunohistochemistry using an anti-GFP antibody. Secondary detection of the antibody using an Alexa Fluor[®] 488-conjugated antibody and fluorescence microscopy revealed GFP in all cells of the tissue sections. Dense cell

clusters appeared brighter than tissue areas with a low cell density. Sections from control embryos had no fluorescence (data not shown). These results demonstrate that the PGK promoter is effective at producing ubiquitous expression in embryos from unincubated stages onward.

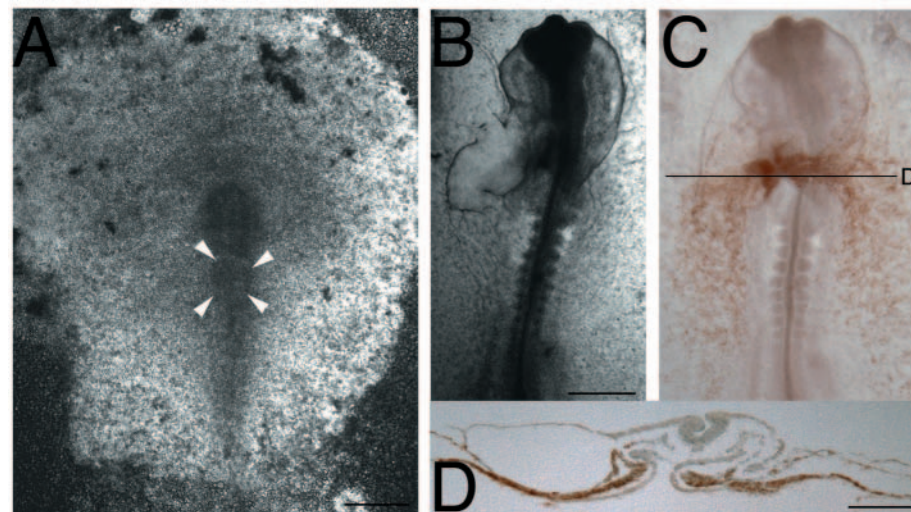


Fig. 4. Primitive streak graft using transgenic chick tissue. Wild-type chick host embryo at HH stage 3d grafted with a segment of the primitive streak obtained just caudal to Hensen's node from a transgenic chick donor embryo. (A) The location of the grafted tissue in the host (white arrowheads) is shown immediately after grafting. After 1 hour the graft had fully integrated. (B) The grafted embryo after 20 hours of incubation in EC culture. (C) Following immunocytochemistry with anti-GFP antibody and DAB, the grafted cells can be identified within the heart tube and as bilateral streams extending caudally into the area pellucida. The horizontal line indicates the level of the section shown in D. (D) Paraffin sections of the embryo (12 μm) show that the labeled cells (brown) contributed mainly to the heart mesoderm at the level (anterior intestinal portal) of the fusing lateral body folds. Scale bars: in A, D, 150 μm ; in B, 250 μm for B, C.

Fate mapping of embryos by transplanting cells carrying a stable tissue marker is an obvious application of this technology. By transplanting cells from chick donors to chick hosts potential confounding factors can be avoided, such as potential species differences that might exist, for example, with traditional quail/chick transplantation chimeras. Transplanted cells can potentially be followed in the live embryo by fluorescence, processed for molecular markers using RNA probes or antibodies, and also permanently marked for identification following sectioning. Our interest in early development lead to transplant experiments to determine the ability of GFP cells to integrate into non-transgenic embryos. Cells were followed by fluorescence microscopy and embryos were processed for in situ hybridization and immunocytochemistry.

Transplant potential was tested using non-transgenic hosts and potential transgenic donors placed into EC Culture (Chapman et al., 2001) at HH stage 3. Hosts had a portion of the rostral primitive streak removed and replaced

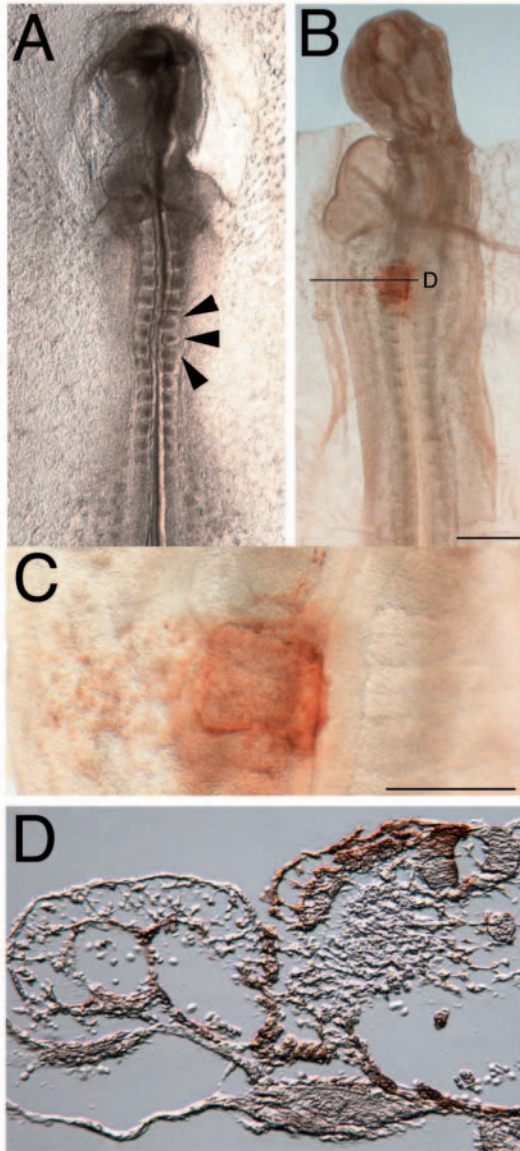


Fig. 5. Somite graft using transgenic chick tissue. (A) Three somites from a transgenic embryo (black arrowheads) were grafted into the left side of a 48-hour wild-type chick host embryo (ventral side up in EC culture). (B) After a 24-hour incubation, the integrated graft was observed on the left side of the whole-mount embryo (dorsal view) after processing for immunocytochemistry with an anti-GFP antibody and DAB staining. (C) Higher magnification view of the integrated graft in B. (D) Section at the level indicated by the horizontal line in B. Paraffin section (12 μm) at the level of the donor somitic tissue demonstrates that cells integrate into the host embryo in the neural tube, dermomyotome and heart. Scale bars: in B, 200 μm for A,B; in C, 200 μm for C,D.

with a homotopic and isochronic tissue graft from donor embryos (Fig. 4A). Only 50% of donor embryos were positive for GFP because a heterozygous male had sired the transgenic embryos. Donor tissue was not scored for GFP, as the embryos were insufficiently fluorescent for positive embryos to be reliably detected by fluorescence microscopy alone (i.e. in the absence of anti-GFP antibody). In 14 grafted embryos, 12 had good integration of the graft (Fig. 4B), and nine of these were GFP positive after ICC processing using either a fluorescent secondary antibody ($n=3/4$) or a DAB-labeled secondary antibody ($n=6/10$) to produce a permanent marker (Fig. 4C,D). Fluorescence or DAB was seen in 11/22 potential donor transgenic embryos used as positive controls, but not in embryos where the primary or secondary antibody had been omitted ($n=4$). Another experiment used somite transplants excised from GFP-positive 48-hour embryos. GFP-positive somite grafts were placed into six wild-type host embryos into the caudal segmental plate mesoderm. Following overnight incubation, the five embryos with intact grafts were processed for DAB labeling. All five of these embryos were positive for GFP (data not shown). In another experiment where grafts were taken from 48-hour embryos (Fig. 5A) without scoring for GFP and then placed homotopically and isochronically, three out of seven embryos were positive for GFP after processing by ICC (Fig. 5B-D).

Fate mapping relies on being able to process embryos after fixation and to molecularly identify grafted cells by ISH. Transgenic embryos were scored for GFP by fluorescence microscopy, fixed and processed by ISH for *Ganf* ($n=7$), *Sox2* ($n=12$), *Dkk1* ($n=7$) and *Chd* ($n=7$), and then subjected to anti-GFP ICC using Alexa Fluor[®] 488-tagged secondary antibodies (Fig. 6A-

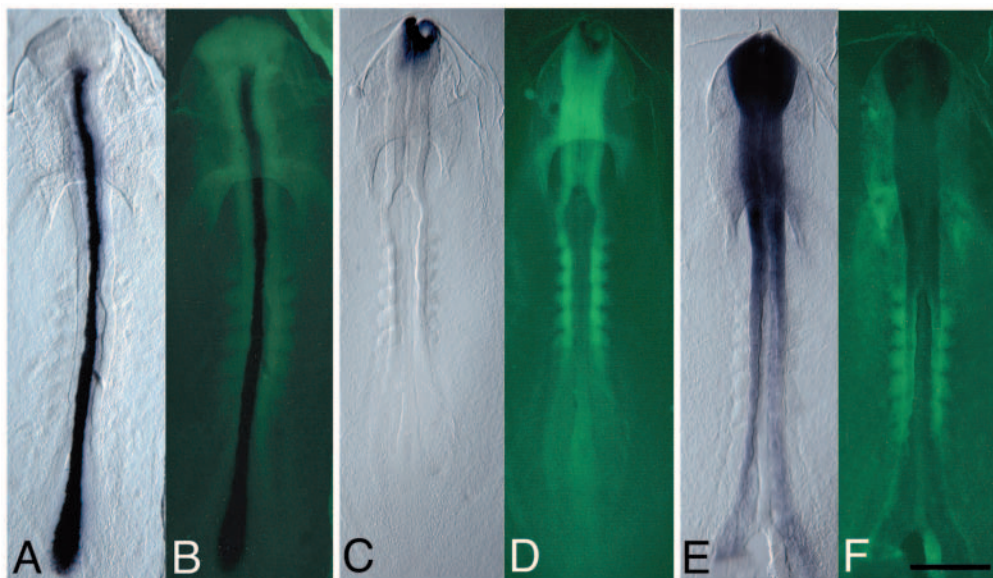


Fig. 6. In situ hybridization (ISH) and GFP expression in whole-mount embryos. *Chd* (A,B), *Ganf* (C,D) and *Sox2* (E,F) were used as ISH probes in whole-mount embryos. Anti-GFP antibody with an Alexa Fluor[®] 488-tagged secondary antibody was used to restore fluorescence following ISH (B,D,F). Scale bar: in F, 200 μm for A-F.

F). The anti-GFP antibody was required as fixation and ISH quench GFP fluorescence. In all cases, the fluorescence was detectable following ISH and ICC, demonstrating that tissue from transgenic embryos can be identified both by the GFP marker using ICC, and molecularly by ISH.

Discussion

We have produced GFP heterozygous transgenic chickens to the G2 generation, and G3 homozygote birds that are due to mature and begin egg production soon, thus demonstrating the feasibility of using lentiviral vectors to transduce exogenous genes into the chick genome. Transgenic chickens are an ideal bioreactor for the production of pharmaceutical products, and, together with, a variety of permanent cell markers, will be a useful additional tool to developmental biologists. The results of our culture, transplant, ISH and ICC experiments demonstrate that the PGK promoter allows for ubiquitous expression in embryonic tissue from unincubated stages up to E18. With the first draft of the chicken genome now available, the potential for using lentiviral vectors to produce chickens that can be used in analyzing gene function is timely. Other markers that may benefit cell-tracing experiments using transgenic chickens include, but are not limited to, β -gal, DSRed, YFP, RFP and other variants. Another useful addition would be the production of a Cre/Lox chick, where microinjection of Tamoxifen, for example, would activate the marker in a localized position. For example, in cells expressing Shh, injection of tamoxifen would turn the marker on, and even if the cell later turned off Shh expression the marker would remain, allowing identification of cells.

Once licensing agreements are finalized, eggs from transgenic birds will be available for distribution to interested parties (www.geneworks.net).

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