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Short communication

Expression of green fluorescent protein in the chicken using in vivo transfection of the piggyBac transposon

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ABSTRACT

The chicken is a well-established model system for studying developmental biology and is recognized as one of the top food production animals in the world. For this reason the chicken is an excellent candidate for transgenic applications, as the technology can be applied to both areas of research. Transgenic technology has not been broadly utilized in the chicken model, however, primarily due to difficulties in targeting germ cells and establishing germ line transmission. Transgenic technologies using non-replicating viral particles have been used in the chick, but are unsuitable for many applications because of size and sequence restraints and low efficiency. To create a more versatile method to target chick germ line stem cells, we utilized the transposable element system *piggyBac* paired with an in vivo transfection reagent, JetPEI. *piggyBac* has been previously shown to be highly active in mammalian cells and will transpose into the chicken genome. Here, we show that JetPEI can transfect multiple chick cell types, most notably germline stem cells. We also show that pairing these two reagents is a viable and reproducible method for long-term expression of a transgene in the chicken. Stable expression of the green fluorescent protein (GFP) transgene was seen in multiple tissue types including heart, brain, liver, intestine, kidney and gonad. Combining an in vivo transfection strategy with the PB system provides a simple and flexible method for efficiently producing stable chimeric birds and could be used for production of germ line transgenics.

The chick embryo has been a leading model system in developmental biology research because of the availability of embryos, short incubation period and ease of experimental embryology (Stern, 2005). Over the past two decades, however, developmental research in the chick has diminished due to transgenic technological advances in other systems that have proven very useful in advancing biomedical research. Indeed, transgenic chicken technology has only recently been revived from interest in the chicken as a bioreactor for producing therapeutic proteins or as a mechanism to develop disease resistance (Lyall et al., 2011). Of current transgenic methods, viral injection has shown the highest efficiency (McGrew et al., 2004), but viral integration systems have sequence and size restraints (Scott et al., 2010) and transgene expression from viral promoters may be silenced in the germ line. For these reasons, transposons have become and alternative to viruses for integration and expression of transgenes.

PiggyBac (PB) is an extremely efficient transposon system which has been shown to be active in mammalian cells (Ding et al., 2005; Wilson et al., 2007) and more active than other transposon systems in many cell types (Wu et al., 2006). PB uses a precise "cut and paste" mechanism to excise and integrate its transposon and cargo into novel locations in the host genome (Fraser et al., 1996). Previous reports show PB integration into the chick embryo and stable expression of green fluorescent protein in vivo (Lu et al., 2009) as well as efficient genomic integration in PGC's in culture (Macdonald et al., 2012; Park and Han, 2012). Combining transposons with in vivo transfection has been shown effective for chimera production and germline transgenesis by direct injection into the vasculature of developing chick embryos (Tyack et al., 2013). JetPEI (Polyplus Transfection, Illkirk, France) is a cationic polymer that bundles and delivers DNA through electrostatic interactions with DNA and cell membranes, and has been shown highly versatile and efficient at cellular transfection (Akinc et al., 2005; Boussif et al., 1995). It does not induce an inflammatory response (Bonnet et al., 2008) and has been used to deliver DNA and RNA in vivo to multiple tissue types (Hassani et al., 2007; Liao and Yau, 2007; Vernejoul et al., 2002; Wiseman et al., 2003). In this work,

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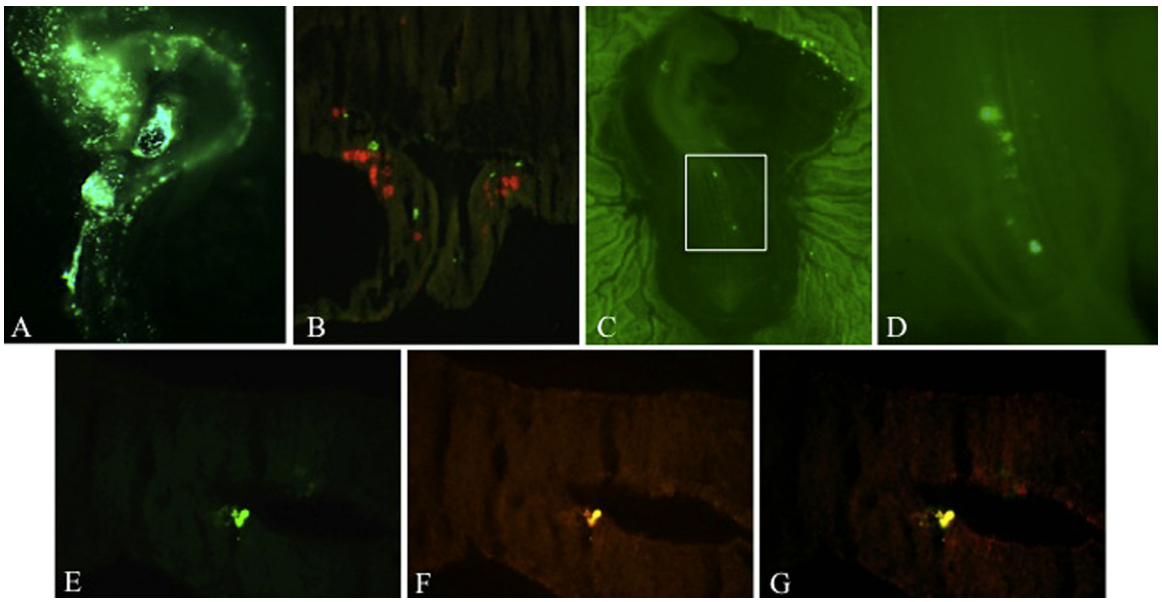

Fig. 1. The piggyBac transposon will express GFP in chick embryos after transfection with JetPEI. Five micrograms of piggyBac transposon 5′-CAG-GFP-3′ was diluted into JetPEI and 5% glucose in a volume of 10 μl according to manufacturer's protocol (http://www.polyplus-transfection.com/2009/08/dna-sirna-delivery-in vivo-jetpei/), using a nitrogen/phosphate (N/P) ratio of 8 to calculate amount of JetPEI needed. One to two microliters was injected into the vitelline artery and embryos were incubated for 24 h post-injection (Van Raay et al., 2008). Fluorescent images show widespread GFP expression (A) at 24 h, though not in the germ cells (B) when injected at this time point. When injected at Stage X of development (Andacht et al., 2004), GFP is seen in cells locating to the developing germinal ridge (C and D), including germ cells. Green cells are expressing GFP from plasmid 5′-CAG-GFP-3′. Red cells are positive by immunohistochemistry (Lassiter et al., 2010) for SSEA-1/EMA-1 germ cell specific antibodies from the Developmental Studies Hybridoma Bank.

we evaluated using JetPEI to transfect chick embryo cells in vivo with PB plasmids to express GFP throughout development of the chick. We found that utilizing JetPEI in combination with PB is an effective method for creating chimeric chicks that express GFP in the embryo through adulthood.

We first modified previously described *piggyBac* plasmids (Cadinanos and Bradley, 2007) for use in this study. The PB transposase gene replaced the GFP gene in plasmid pCAG-GFP (Addgene) by restriction digest cloning, giving plasmid PBase. The CAG-GFP fragment of pCAG-GFP was cloned into the PB transposon, replacing the PURO gene, by restriction digest cloning, giving plasmid 5′-CAG-GFP-3′ that constitutively expresses GFP from the CAG promoter. All plasmids were verified by sequencing. We next injected a solution of JetPEI and 5′-CAG-GFP-3′ into the vitelline artery of ten Stage 23 (HH) chick embryos to assess the ability of JetPEI to transfect chick embryo cells. After 24 h, strong GFP expression was seen over the entirety of the chick embryo, verifying that JetPEI will transfect developing chick tissues (Fig. 1A). To examine the ability of JetPEI to transfect germ cells, gonadal ridge tissue was sectioned and stained with germ cell specific antibodies for detailed analysis. No co-labeling of germ cell markers with cells expressing GFP was observed (Fig. 1B). This observation is not surprising since germ cells at this developmental stage have already left the vasculature, making them unlikely targets of a vasculature injection, and populated the poorly vascularized germinal ridge. All injected embryos showed similar expression to images shown. To determine if JetPEI could transfect non-dividing PGC's prior to mobilization to the gonadal ridge, we injected 20 freshly laid Stage X embryos with the same solution of JetPEI/5′-CAG-GFP-3′ and incubated for 72 h. Little overall fluorescence was seen from lack of integration of the PB transposon and dilution of the GFP signal from cell division. GFP expression was seen, however, corresponding to the developing gonadal ridge (Fig. 1C and D). Immunohistochemistry identified a positively staining germ cell expressing GFP (Fig. 1E–G), demonstrating that JetPEI can transfect slowly dividing germ cells when injected at an early stage.

We then sought to achieve long-term GFP expression through integration of the PB transposon into the chick genome. The PBase helper plasmid was mixed in solution with 5′-CAG-GFP-3′ and JetPEI, and injected into Stage X embryos. Eighty embryos were injected and incubated until hatch, and analyzed at one day, two weeks, seven weeks and 25 weeks post-hatch to determine expression stability of the GFP transgene. Forty-two of the 80 embryos injected hatched (52.5%) and all chicks sampled showed localized GFP expression, regardless of age or sex. Tissues from all three germ layers expressed GFP, with intestine and heart being the most common. Fig. 2 shows representative samples of GFP expression in varying tissue types including intestine, gizzard, heart, brain, pelvic bone and testes from 7-week-old chickens. Other tissues expressing GFP included liver, breast muscle, eye, and gut connective tissue (data not shown). Immunohistochemistry of GFP positive testes shows EMA-1/SSEA-1 positive germ cells expressing GFP (Fig. 2G–I) in tissue surrounding the semeniferous tubules. Co-expression of GFP in germ cell antibody positive cells (Fig. 2I) indicates that the GFP transgene was stably integrated into those cells. Three male chicks were kept until sexual maturity and their sperm was analyzed for the GFP transgene by PCR. Positive reactions were consistently seen in DNA isolated from 1 rooster's sperm, which was verified as GFP by sequencing. This rooster was bred multiple times (~150 offspring), but no positive transgenic chicks were detected by visual GFP expression. This rooster was subsequently euthanized for welfare reasons unrelated to our experiments so no further chicks could be analyzed. Positive PCR reactions were not seen using sperm DNA of the other 2 roosters. These birds were euthanized and their testes submitted for immunohistochemistry, with only a single EMA-1/SSEA-1 positive germ cell expressing GFP identified (Fig. 3).

Transgenic technology in the chicken has progressed but has not reached the efficiency or reproducibility of the mouse model. Improvements in technology would be highly beneficial in studying both short term (1st generation) and long-term (subsequent generations) biological processes. Here we show that combining the *piggyBac* transposon with JetPEI transfection reagent is a