Chapter 3
Applications of *Tol2* Transposon-Mediated Gene Transfer for Stable Integration and Conditional Expression of Electroporated Genes in Chicken Embryos

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1 Introduction

Because of the high accessibility to developing embryos, avian embryos (chicken and quail) have long been used as a good model animal to study embryogenesis in vertebrates, especially amniotes (reviewed in Wolpert, 2004). The techniques used for "classical" avian embryology included tissue transplantations, tissue ablations, and cell-labeling by vital dye. At the end of the last century, the in ovo electroporation technique was developed by Nakamura and his colleagues, and this modern method opened a way to study the roles of developmental genes directly in living embryos (Funahashi et al., 1999) reviewed in (Nakamura et al., 2004; Yasuda et al., 2000; Yasugi and Nakamura, 2000). This powerful technique allows us to introduce genes (DNA, RNA, morpholino) into embryos in a tissue-specific way by targeting a restricted area of embryonic tissues. Thus, the electroporation technique using chickens has provided numerous novel insights into the understanding of early development in vertebrates, making the chicken a unique model animal.

One of few shortfalls of the original technique has been that expression of electroporated genes does not persist for a long period of time probably because the introduced plasmids, which are not integrated into the genome, degrade or become diluted as embryonic cells undergo massive proliferation. Although a spontaneous genomic integration of electroporated genes could occur, this incidence must be extremely low. Since in most cases the electroporation is performed at embryonic day 1~2 (E1~E2), the "short life" of introduced genes hampers the analysis of the effects by introduced genes at late stages, i.e., from E5 onward, when a variety of organogenesis proceeds. At these late stages, the electroporation is difficult to perform because the embryo is much less accessible.

To overcome this problem and obtain long-lasting expression of electroporated genes, we have recently developed a method of transposon-mediated gene transfer (Sato et al., 2007). Transposons are genetic elements that move from one locus in

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the genome to another. *Tol2* is an autonomous type of transposon, originally found in medaka fish (Koga et al., 1996), and encodes a transposase and transposable elements. Kawakami and his colleagues developed a method of Tol2-mediated gene transfer that enables efficient transgenesis in zebrafish (Kawakami et al., 1998). Since then, the Tol2-mediated transgenesis has also been used in other vertebrate species including frogs, mice, and human (Balciunas et al., 2006; Kawakami, 2004; Kawakami, 2005; Kawakami, 2007; Kawakami and Noda, 2004; Kawakami et al., 1998).

In this review, we first describe the method of the Tol2-mediated stable integration of electroporated genes with early somites as an example. Most of the explanations are with the constitutively active promoter, CAGGS (combination of CMV-IE enhancer and chicken β-actin promoter) (Momose et al., 1999; Niwa et al., 1991). Subsequently, we will show that the expression of stably integrated foreign genes can also be controlled in a stage-specific manner. This is enabled by the tetracycline-induced conditional expression system (tet-on), the method we recently optimized for chickens (Watanabe et al., 2007).

2 Procedure

2.1 Tol2-Meditated Gene Transposition in Chicken Embryo

A background of the Tol2 gene and the Tol2-mediated transgenesis in zebrafish embryo are described in (Kawakami, 2005; Kawakami, 2007). Briefly, when a DNA plasmid that contains a transposon construct carrying a gene expression cassette is introduced into vertebrate cells with the transposase activity, the transposon construct is excised from the plasmid, and the cassette is subsequently integrated into the host genome (Kawakami, 2005) (see also Fig. 3.1a). We here explain the Tol2-meditated stable transgenesis with an example of EGFP and DsRed2 (Clontech).

2.1.1 Plasmids

Two kinds of plasmid vectors are required for the stable integration: pCAGGS-T2TP (T2TP: Tol2 transposase) and pT2K-CAGGS-EGFP (pT2K: a plasmid of transposon construct carrying transposable elements). pCAGGS-DsRed2, which does not contain Tol2-construct, is used as a reference. The above-mentioned three kinds of plasmids, 5 μg/μl for each, are mixed to prepare a DNA cocktail with the following ratio, pCAGGS-T2TP : pT2K-CAGGS-EGFP : pCAGGS-DsRed2 = 1 : 2 : 0.5. The cocktail is then added with Fast green (2% final) prior to the electroporation. Devices and settings for in ovo electroporation should be optimized for a targeting tissue as described elsewhere (Fukuda et al., 2000; Nakamura et al., 2000; Nakamura et al., 2004; Yasuda et al., 2000; Ohata and Takahashi, Chapter 5 in this volume).

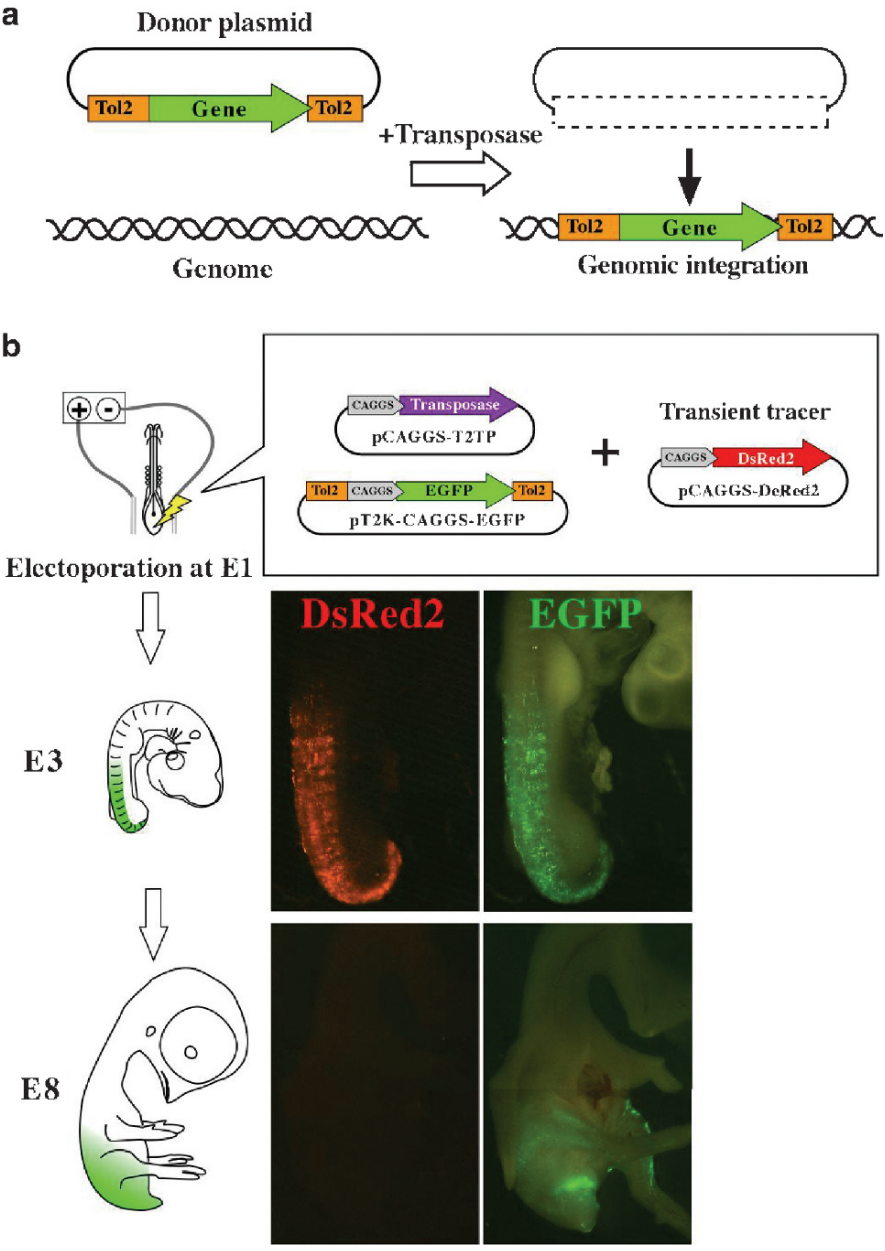

Fig. 3.1 Integration of foreign DNA into the chromosome by Tol2-mediated transposition in chicken embryos. (**a**) When Tol2 transposase is present, a gene cassette (*green*) flanked by the Tol2 transposable elements (*orange*) is excised from a donor plasmid, and becomes integrated into the genome of a host cell. (**b**) Persistent expression of electroporated genes in developing embryos. pCAGGS-T2TP and pT2K-CAGGS-EGFP are co-electroporated along with a transient tracer pCAGGS-DsRed2 into the prospective somitic cells of E1 (HH 8) embryo. Although both DsRed2 and EGFP signals are seen until E3 embryo, DeRed2 signal disappears afterward whereas EGFP signal persists until late stages (*See Color Plates*)

2.1.2 Persistent Expression of pT2K-CAGGS-EGFP-Derived Signals Until Late Stages

To achieve a successful transgenesis in presomitic mesoderm (PSM)/somites, foreign genes are introduced by electroporation into their precursors at earlier stages, i.e., Hamburger and Hamilton stage 8 (HH8), when presumptive somites reside in the anterior end of the primitive streak. The details about the somitic electroporation are described by Ohata and Takahashi, Chapter 5 in this volume (see also Sato et al., 2002). Expression of a gene driven by the CAGGS-promoter, in this case DsRed2, starts to be detected approximately 3 h after the electroporation (Watanabe et al., 2007), and the signals usually disappear by the end of E4 (Sato et al., 2007). In contrast to pCAGGS-DsRed2, EGFP signals generated by pT2K-CAGGS-EGFP are retained for a prolonged period of time because of the Tol2-mediated genomic transposition (Fig. 3.1b) (Sato et al., 2007). The stable expression of EGFP indeed requires the activity of Tol2-transposase because electroporation with pT2K-CAGGS-EGFP without pCAGGS-T2TP did not yield the retained expression. Persistent expression of EGFP in somite-derived tissues was observed until E8, the latest stage examined in our hands. In addition, in vitro analyses using DF1 cells of a chicken fibroblast cell line showed that ~40% of transfected cells retained EGFP signals permanently when transfected with pT2K-CAGGS-EGFP and pCAGGS-T2TP (Sato et al., 2007; Y.T., unpublished data). Therefore, it is expected that the EGFP-positive cells found in the E8 embryo would last much longer until these cells die. For the electroporated embryos to develop in a good condition until late stages, it is recommended that antibiotics (penicillin/streptomycin) are added in HANKS solution to prevent bacterial contaminations.

2.2 Conditional Expression of Stably Integrated Transgenes

It is known that some of developmental genes are repeatedly used during early morphogenesis and organogenesis. Therefore, the technology would be useful that controls stage-specific expression of foreign genes introduced into embryos. The tet-on inducible expression system has widely been used, mainly in vitro, for conditional manipulation of foreign genes. We recently optimized the tet-on expression method for chicken embryology (Watanabe et al., 2007). We have therefore developed a vector set to combine the tet-on method with the Tol2-mediated stable integration so that we can control the onset of expression of electroporated genes at late stages (~E5 onward) (Sato et al., 2007).

2.2.1 Tet-on Inducible Expression

Materials required for the tet-on induction are: the tet-dependent transcriptional activator rtTA2^{S}-M2 (Urlinger et al., 2000), the tet-responsive element (TRE),

and the tetracycline-related drug, doxycycline (Dox, Clontech). rtTA2^S-M2 activates TRE to turn on the transcription of its driving gene only when rtTA2^S-M2 is bound with Dox. Thus, by timing the Dox administration into embryos, one can control the onset of expression of a TRE-driven gene (details described in Watanabe et al., 2007). rtTA2^S-M2 is now commercially available as Tet-on Advanced, Clontech.

2.2.2 Plasmid Preparation for Tol2-tet-on Expression System

Three kinds of plasmids are required. The first one is pT2K-CAGGS-rtTA2^S-M2. The second is pT2K-BI-TRE-EGFP. "BI-TRE" consists of bidirectionally-acting minimal promoters (BI) downstream of TRE. In this article, we explain our methods with a pT2K-BI-TRE-EGFP, which has a vacant slot for the opposite direction. This vector was modified from pBI-EGFP, Clontech. The third plasmid is pCAGGS-T2TP. pCAGGS-DsRed2 is also recommended to ensure a successful electroporation into the embryo because EGFP signal is silent until Dox administration (Fig. 3.2).

These plasmids, 5 μg/μl for each, are mixed to prepare a DNA cocktail with the following ratio, pCAGGS-T2TP : pT2K-CAGGS-rtTA2^S-M2 : pT2K-BI-TRE-EGFP : pCAGGS-DsRed2 = 2 : 1 : 2: 0.5. The cocktail is then added with Fast green (2% final) prior to the electroporation. A Dox solution is prepared at 0.1 mg/ml in HANKS.

2.2.3 Dox-Induced Expression of Stably Integrated Genes

The set of above-mentioned plasmids is electroporated into the presumptive somitic region of embryo at HH 8. Several hours after the electroporation, embryos with successful transgenesis are selected by the signals of DsRed2 (with EGFP signal being silent). To induce EGFP expression, 500 μl of Dox solution is administered into an egg shell using a syringe needle in between the embryo and yolk. The penetration of Dox into embryonic cells may vary from tissue to tissue. In the case of early somites (E2), TRE-driven EGFP starts to be expressed ~3 h post-Dox (Watanabe et al., 2007). For further developed tissues derived from somites of E5 embryos, one shot of Dox administration could start and maintain EGFP signals for a few days. However, from E10 onward, it is recommended that a Dox solution is administered every day with one shot per day because Dox appears to be quickly metabolized in developed embryos (Tanabe et al., 2006).

The Tol2-tet-on-inducible system has been successful for conditional expression of electroporated genes, including the dominant-negative form of N-cadherin in developing neural retina until E12 of chicken (Tanabe et al., 2006), and the constitutively active form of Notch in developing somites of chicken and quail until E6 (Sato et al., 2008).

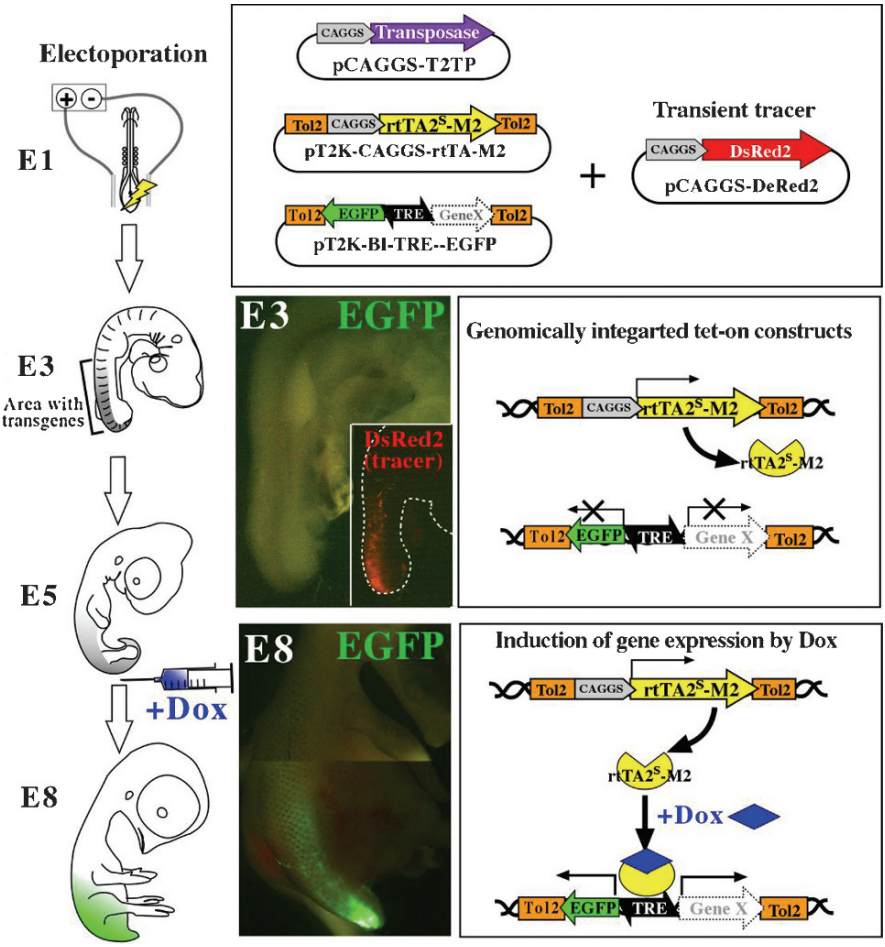

Fig. 3.2 Conditional expression of integrated genes using the tet-on inducible expression system. Three plasmids, pCAGGS-T2TP, pT2K-CAGGS-rtTA2^S-M2, and pT2K-BI-TRE-EGFP, are co-electroporated along with a transient tracer pCAGGS-DsRed2 into the presumptive somite. Even without Dox, early embryos with successful transgenesis can be seen by DsRed2 signals (inset in the image of E3). With a Dox injection at E5, these embryos start to express the genomically integrated EGFP gene in somite-derived tissues. See text for details (*See Color Plates*)

3 Comments

3.1 Future Perspectives

The gene integration method by the Tol2-transposition must be useful in a variety of experimental approaches. These include functional RNAi-knockdown of endogenous gene by short hair-pin type of RNA (shRNA). The shRNA-mediated

knockdown approaches have been attempted for many developmental genes, and some but not all of these are successful, i. e., *Pax2* (Watanabe et al., 2007) and *Ephrin B2* (Sato et al., 2008). Thus, if a shRNA-encoding cassette is manufactured for the use of Tol2-transposition system, a persistent knockdown of gene function could be possible. In addition, by combining with the tet-on inducible expression, conditional knockdown of developmental genes at later stages should be also available.

The stable integration of foreign genes must be also useful if combined with a tissue- or cell type-specific enhancer. Kondoh's group has recently described an efficient *in silico* screening of non-coding DNA elements that are conserved between amniotes. Many of these elements serve as tissue-specific enhancers (Uchikawa et al., 2004). It would be plausible if tissue- or cell type-specific labeling/gene manipulation could be performed for a prolonged period of time until late stages of embryogenesis. Such manipulations using chicken embryos should be much less time-consuming with low cost compared with manipulations in mice. Last, the stable integration method we have described here is expected to be useful for generating transgenic birds, a technology that has not been very successful until present.

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