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In Vivo Genetic Ablation by Cre-Mediated Expression of Diphtheria Toxin Fragment A

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Summary

We generated a *ROSA26-eGFP-DTA* mouse line by introducing an *eGFP-DTA* (enhanced green fluorescent protein - diphtheria toxin fragment A) cassette into the *ROSA26* locus by homologous recombination in ES cells. This mouse expresses *eGFP* ubiquitously, but *DTA* expression is prevented by the presence of *eGFP*, a *Neo* cassette, and a strong transcriptional stop sequence. Mice carrying this construct are normal and fertile, indicating the absence of *DTA* expression. However, upon Cre-mediated excision of the floxed region *DTA* expression is activated, resulting in the specific ablation of Cre-expressing cells. As an example of this approach, we ablated *Nkx2.5* and *Wnt1*-expressing cells by using the *Nkx2.5-Cre* and *Wnt1-Cre* mouse lines, respectively. We observed loss of the precise tissues in which *Nkx2.5* and *Wnt1* are expressed. Apart from being a general GFP reporter, the *ROSA26-GFP-DTA* mouse line should provide a useful resource for genetic ablation of specific groups of cells.

Keywords

Cre; *loxP*; diphtheria toxin; *eGFP*; *Nkx2.5*; *Wnt1*; genetic ablation; heart; midbrain

The role of individual cells in complex tissues or within the whole organism can be examined by specific deletion of appropriate lineages. For example, during embryogenesis specific groups of cells (signalling centres) control the fate of neighbouring cells by emanating diffusible molecules (Meinhardt, 1983; Martinez *et al.*, 1991; Shimamura and Rubenstein, 1997; Placzek and Briscoe, 2005). Mechanical ablation of restricted embryonic regions has been useful in identifying these signalling centres and their role during development (Placzek *et al.*, 1995; Shimamura and Rubenstein, 1997; Thomas and Beddington, 1996). However, these experiments require very precise surgery on the developing embryo, which can often be a difficult task. In adulthood the loss of small numbers of strategically placed cells may result in conditions such as Parkinson's disease (Moore, 2005), Type I diabetes (Butler *et al.*, 2003), and Hirschsprung's disease (Amiel and Lyonnet, 2001). Thus, a versatile system to specifically ablate cells of any lineage during embryogenesis or in adulthood would be of substantial benefit for studies of development, as well as to model human diseases of various aetiologies.

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With the aim of developing such a system, we generated a *ROSA26-eGFP-DTA* mouse line, which combines the use of enhanced green fluorescent protein (GFP), diphtheria toxin A subunit (DTA), and Cre recombinase. eGFP is a mutated version of GFP that displays enhanced fluorescence in mammalian cells in vivo and in vitro (Srinivas *et al.*, 2001). Diphtheria toxin is secreted by pathogenic strains of *Corynebacterium diphtheriae* and is composed of two subunits, A and B. Subunit B is responsible for the internalisation of the toxin upon binding to its receptor. Once inside the cell, subunit A catalyses the inactivation of elongation factor 2, resulting in termination of protein synthesis and apoptosis of the target cell (Maxwell *et al.*, 1986; Palmiter *et al.*, 1987; Breitman *et al.*, 1990; Harrison *et al.*, 1991; Collier, 2001). The phage-derived *Cre/loxP* system has been successfully used in vitro and in vivo to conditionally inactivate numerous genes (Nagy, 2000). Cre recombinase recognises 34 basepair *loxP* DNA fragments and mediates excision of the DNA flanked by two *loxP* sites if they are placed in the same orientation.

We generated the *ROSA26-eGFP-DTA* mouse line by insertion of a conditionally expressed *DTA* construct into the *ROSA26* locus by homologous recombination in ES cells (Fig. 1a). In this construct, *eGFP* is upstream of *DTA* and is constitutively expressed under control of the *ROSA26* promoter. In contrast, *DTA* transcription is prevented by the presence of a floxed DNA region containing *eGFP*, a *Neo* cassette, and a triple *SV40* polyadenylation signal (tpA). This strategy allows the conditional expression of *DTA* upon Cre-mediated excision of the floxed region, which leads to specific death of Cre-expressing cells. *ROSA26^{eGFP-DTA/+}* and *ROSA26^{eGFP-DTA/eGFP-DTA}* mice were present in the expected proportions among offspring of heterozygous intercrosses (Table 1). Hence, it is unlikely that *DTA* expression is leaky from the *ROSA26* locus, as no toxic effect was detectable in these animals. When observed under ultraviolet light, ES cell clones, embryos, and mice carrying the *ROSA26-eGFP-DTA* allele showed ubiquitous green fluorescence (Figs. 1g, 2d, 3b).

To prove the principle that specific cell ablations can be achieved during mouse development, we crossed *ROSA26-eGFP-DTA* and *Nkx2.5-Cre* mice. *Nkx2.5-Cre* is a knockin of *Cre* into the *Nkx2.5* locus (Moses *et al.*, 2001). When crossed with the general *ROSA26lacZ* reporter mouse, *lacZ* expression is weak in the heart crescent at headfold stages, but is robust in the developing heart tube at early somite stages (Moses *et al.*, 2001). In accordance with this expression pattern, we found that *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* compound heterozygous embryos showed a fully penetrant phenotype characterised by the absence of the heart at the 12-14 somite stage (n = 11) (Fig. 2a-f). All other embryonic regions were normal by morphological, histological, and in situ hybridisation analyses. This phenotype was not observed in littermate embryos with any other genotype: *ROSA26^{eGFP-DTA/+}* and *Nkx2.5^{Cre/+}* single mutants as well as wildtype embryos were all entirely normal. To further confirm the absence of the heart, we analysed the expression of specific heart markers such as *Nkx2.5* and alpha-cardiac actin (Sassoon *et al.*, 1988; Lints *et al.*, 1993; Lemonnier and Buckingham, 2004). Neither of these markers was expressed in *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* compound heterozygous embryos (n = 9) (Fig. 2g,h and data not shown). *Cre* expression was restricted to the heart in *Nkx2.5^{Cre/+}* embryos, but was absent from *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* embryos (n = 5) (Fig. 2i,j). At 9.5 days postcoitum (dpc), *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* compound heterozygous embryos were very small, morphologically abnormal, and in some cases partially resorbed (data not shown), suggesting that a functional cardiovascular system is required for survival between 8.5-9.5 dpc during mouse embryogenesis (Copp, 1995).

We also crossed the *ROSA26^{eGFP-DTA/+}* mice with *Wnt1-Cre* transgenics (Jiang *et al.*, 2000). *Wnt1-Cre* is a transgenic line that drives *Cre* expression in the prospective midbrain at presomitic stages. At later stages, *Cre* expression is restricted to the dorsal aspects of these brain regions and throughout the dorsal hindbrain and spinal cord (Echelard *et al.*, 1994;

Rowitch *et al.*, 1998; Jiang *et al.*, 2000). At 9.5 and 10.5 dpc, *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* compound embryos showed abnormal morphology of the brain region (n = 10) (Fig. 3a-d). The brain rostral to the otic vesicle was significantly smaller and the cranial neural tube was open dorsally (exencephaly). Forebrain tissue was distinguishable and optic cups developed bilaterally (Fig. 3C,D). The isthmus, a constriction of the neural tube at the boundary between the midbrain and hindbrain (MHB), developed normally in *Wnt1-Cre, ROSA26^{eGFP-DTA/+}*, and wildtype embryos, whereas it was absent in *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* mutants (Fig. 3a and data not shown). No other regions of the embryos showed gross abnormalities at these stages. The brain phenotype observed in *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* is similar to that described in *Wnt1* null mutant embryos (McMahon and Bradley, 1990; Thomas and Capecchi, 1990).

To characterise this phenotype further we used in situ hybridisation with neural markers specific for restricted regions of the neural tube (Fig. 4). The most anterior *Wnt1* expression domain, which encompasses the midbrain and posterior forebrain, was much reduced in *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* compound embryos at 8.5 dpc (n = 6) (Fig. 4a,b, bracketed region). Weak *Wnt1* expression was detected at the MHB and dorsal hindbrain in the compound mutant at this stage (Fig. 4a,b, arrowhead and black arrow, respectively). However, normal *Wnt1* expression was detected in the dorsal neural tube at spinal cord levels (Fig. 4a,b, red arrow). *Cre* expression, which has been previously shown to recapitulate *Wnt1* expression (Echelard *et al.*, 1994; Rowitch *et al.*, 1998; Jiang *et al.*, 2000), was also absent from the midbrain and posterior forebrain of *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* compound embryos at 9.0 dpc, except for a small patch of cells ventrally within the MHB (n = 4) (Fig. 4c,d).

The extent of the ablation was analysed further by in situ hybridisation with *Otx3*, a neural marker expressed in midbrain and posterior forebrain territories with a sharp posterior limit at the MHB (Gogoi *et al.*, 2002; Ohtoshi *et al.*, 2002). No *Otx3* expression was detected in *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* compound embryos, suggesting ablation of these regions at 8.5 dpc (Fig. 4e,f). The presence of forebrain and hindbrain tissue was demonstrated by analysis of *Bf1*, *Fgf8*, and *Hoxb1* expression (Tao and Lai, 1992; Crossley and Martin, 1995; Gavalas *et al.*, 1998). Both forebrain markers, *Bf1* and *Fgf8*, were expressed in the developing telencephalon in *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* embryos in a similar pattern to that observed in control littermates (Fig. 4g-j). However, *Fgf8* expression in the MHB was not detectable, suggesting a requirement of *Wnt1* expressing cells for normal *Fgf8* expression (Matsunaga *et al.*, 2002). *Hoxb1* expression in rhombomere 4 was indistinguishable between mutants and control littermates (Fig. 4g,h).

We estimate a time lapse of less than 24 h between the onset of *Cre* expression and cell death in both *ROSA26^{eGFP-DTA/+}; Nkx2.5^{Cre/+}* and *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* embryos. Strong *lacZ* expression is first detected in *ROSA26-lacZ; Nkx2.5^{Cre/+}* embryos only at the 1-2 somite stage (Moses *et al.*, 2001). Since we first observed the absence of the heart in *ROSA26^{eGFP-DTA/+}; Nkx2.5^{Cre/+}* at the 8-10 somite stage (data not shown), this suggests a minimum time lapse of around 14-18 h to achieve heart ablation.

The detection of *Wnt1* and *Cre* expression in *ROSA26^{eGFP-DTA/+}; Wnt1-Cre* embryos (Fig. 4a-d) can be explained by taking into account the dynamic pattern of *Wnt1* expression during early development. *Wnt1* is first expressed in the prospective midbrain region of the presomitic embryo (Echelard *et al.*, 1994; Rowitch *et al.*, 1998). By the 5-6 somite stage embryo, *Wnt1* transcripts are still detected in the prospective midbrain, but have now also appeared in the dorsal aspects of the hindbrain. A few hours later (8-10 somites) *Wnt1* expression is, in addition, observable in the spinal cord (Echelard *et al.*, 1994; Rowitch *et al.*, 1998). Therefore, there is an anterior to posterior wave of *Wnt1* expression during these early stages of development. The *Wnt1-Cre* construct used to make the *Wnt1-Cre* mouse line has been shown to drive *Cre*

expression in the same manner as the endogenous *Wnt1* (Jiang *et al.*, 2000). Therefore, the prediction is that in *ROSA26^{eGFP-DTA/+};Wnt1-Cre* compound embryos, *DTA* expression, and therefore cell ablation, should occur first in the midbrain region, later in the dorsal hindbrain, and finally in the dorsal spinal cord. This fits well with our observations (Fig. 4a-d and data not shown). We estimate a minimum time lapse of around 16-20 h between the onset of *Cre* expression in the midbrain (presomitic stages) and cell ablation (8-10 somites), which is similar to that observed in *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* mutants.

Similar *DTA* mouse lines to the one described here have been generated recently (Brockschneider *et al.*, 2004; Matsumura *et al.*, 2004; Sato and Tanigawa, 2005). In comparison with the mouse line of Brockschneider *et al.* (2004), our mouse line offers the advantage of expressing *eGFP* rather than *lacZ* ubiquitously. Moreover, Cre-mediated recombination excises not only *eGFP* and the polyadenylation signals, but also the *Neo* cassette in our construct. In contrast, in the *DTA* mouse of Brockschneider *et al.* the excision leaves behind the entire *Neo* cassette, which might impair normal transcription of the *ROSA26* locus, reducing expression of *DTA*. This could explain the longer time lapse between the onset of *Cre* expression and cell death in their system (36-48 h).

The *DTA* mice of Matsumura *et al.* (2004) and Sato and Tanigawa (2005) are transgenic lines that were generated by microinjection into the pronuclei of fertilised eggs. As pronucleus microinjection normally leads to tandem incorporation of the transgene, Cre recombination can cause aberrant expression of *DTA*, depending on which *loxP* sites are utilised. This might explain the incomplete ablation of *Cre*-expressing cells in one of the *DTA* mouse lines. In contrast, the mouse described here was obtained by homologous recombination in ES cells, which enables specific recombination between the two unique *loxP* sites and, so, expression of *DTA* in all *Cre*-expressing cells. The time between initial *Cre* expression and cell death was not estimated in these two transgenic *DTA* mouse lines (Matsumura *et al.*, 2004; Sato and Tanigawa, 2005).

In conclusion, we have characterised a *ROSA26-eGFP-DTA* mouse line that can be used as a general GFP reporter and as a tool for genetic cell ablation experiments. We have proven that this system can be used successfully to ablate specific embryonic regions. We expect this system to be useful not only in developmental biology studies, but also in the field of stem cell research and human medicine, for example, to generate cell-depleted animals and model degenerative diseases caused by increased apoptosis in mice.

MATERIALS AND METHODS

Generation of the Targeting Construct and the *ROSA-eGFP-DTA* Mouse Line

The components of the *ROSA26* targeting vectors were a gift from P. Soriano and S. Srinivas (Soriano, 1999; Srinivas *et al.*, 2001). To avoid possible interference with translation of *DTA* due to the presence of an ATG within the *loxP* sites, we generated a *pBigT-invloxP* by inverting the orientation of both *loxP* sites. Therefore, the first ATG in our construct after *loxP* recombination is the *DTA* initiation codon. We inserted *eGFP* (ClonTech, Palo Alto, CA) downstream of the splice acceptor and the 5' *loxP* of the *pBigT-invloxP*, followed by a *PGK Neo* cassette, a triple *SV40* polyadenylation signal, and *DTA*. *DTA* was obtained from I. Maxwell (Maxwell *et al.*, 1986; Harrison *et al.*, 1991), but we corrected its C-terminus by polymerase chain reaction (PCR) to generate the same sequence of *DTA* as described originally (Maxwell *et al.*, 1987). The *PacI-AscI* DNA fragment of *pBigT-invloxP* was cloned into the *ROSA26PA* to generate the final targeting vector (Fig. 1a).

CCE ES cells (129/SvEv) were electroporated with the linearised construct depicted in Figure 1 and a total of 300 clones were picked, expanded, and frozen following standard protocols

(Martinez-Barbera *et al.*, 2000). Homologous recombination in the *ROSA26* locus was identified by Southern blot using an external probe. This probe is a 140-bp DNA fragment that was excised from the *pROSA26-5'* plasmid by *EcoRI/HindIII* digestion (Soriano, 1999; Srinivas *et al.*, 2001). A total of six clones showed the correct band pattern with the external probe (Fig. 1b) and a *Neo* probe (data not shown). Three clones were used for blastocyst injection (C57BL6/J background) and all three gave germline transmission. *ROSA26^{eGFP-DTA/+}* heterozygous mice were intercrossed to generate *ROSA26^{eGFP-DTA/eGFP-DTA}* homozygous mice. Mice of these genotypes were viable in both C57BL6/J and CD1 backgrounds.

Genotyping of Mice and Embryos

Genotyping was carried out by Southern blot and PCR as described (Soriano, 1999; Srinivas *et al.*, 2001). Primers were R26R1 (5'-aaagtcgctctgagttgtat-3'), R26R2 (5'-gcgaagagttgtcctcaacc-3'), and R26R3 (5'-ggagcgggagaaatggatatg-3'). Embryos were genotyped after in situ hybridisation as described (Martinez-Barbera *et al.*, 2000). Detailed protocols are available upon request.

In Situ Hybridisation and Histology

In situ hybridisation was performed as described (Martinez-Barbera *et al.*, 2000) using probes to α -cardiac actin, *Cre*, *Wnt1*, *Otx3*, *Bfl1*, *Nkx2.5*, *Hoxb1*, and *Fgf8*. For histology, embryos were fixed in 4% paraformaldehyde, dehydrated in ethanol, embedded in wax, and sectioned at 8 μ m as described (Martinez-Barbera *et al.*, 2000).

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FIG. 1.

Targeting of the *ROSA26* locus. **a:** From top to bottom, diagrams of: *pBigT-invloxP* plasmid; *pROSA26PA* plasmid containing genomic *ROSA26* sequences and *PGK-DTA* for negative selection in ES cells; wildtype *ROSA26* locus; targeted *ROSA26* locus before and after Cre-mediated excision of the *loxP*-flanked DNA (*eGFP*, *PGK-Neo* cassette and a triple *SV40* polyadenylation signal). **b,c:** Examples of mice genotyped by Southern blot (**b**) and PCR (**c**). For Southern blot, genomic DNA was digested with *EcoRV* and hybridized with the probe indicated in **a**. PCR genotyping was performed as described (see Materials and Methods) using the indicated primers (arrows in **a**). **d,e:** Brightfield photographs of wildtype (**d**) and targeted (**e**) ES cell colonies. **f,g:** Fluorescence photographs of the colonies in **d** and **e**. Note that the targeted ES cell colony expresses eGFP. Scale bars = 100 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

Lack of cardiac tissue in *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* compound heterozygous embryos. **a:** 9.0 dpc *Nkx2.5^{Cre/+}* embryo. **b:** *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* compound mutant lacking the heart (arrow). **c,d:** Fluorescence photographs of the embryos shown in **a** and **b**. Only the compound heterozygote expresses eGFP. **e,f:** Haematoxylin-eosin staining of transverse sections at the level of the heart in *Nkx2.5^{Cre/+}* (**e**) and *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* compound heterozygote (**f**) at 9.0 dpc. The heart is absent from the compound heterozygous embryo, and the anterior cardinal veins (arrowheads) and dorsal aorta (arrows) appear dilated. Foregut, neural tube, and cranial mesenchyme all appear morphologically normal. **g-j:** Whole-mount in situ hybridisation with alpha-cardiac actin (*aCa*) (**g,h**) and *Cre* (**i,j**) riboprobes on *Nkx2.5^{Cre/+}* (**g,i**) and *ROSA26^{eGFP-DTA/+};Nkx2.5^{Cre/+}* compound heterozygotes (**h,j**) at 9.0 dpc. These cardiac markers are not detectable in compound heterozygotes. Scale bars = 200 μm (**a-d,g-j**); 100 μm . (**e,f**) a, atrial chamber; cv, common ventricle; f, foregut. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

FIG. 3.

Ablation of the midbrain region in *ROSA26^{eGFP-DTA/+};Wnt1-Cre* compound embryos. **a:** Left to right: *Wnt1-Cre*, *ROSA26^{eGFP-DTA/+}*, and *ROSA26^{eGFP-DTA/+};Wnt1-Cre* 10.5 dpc embryos. Only the *ROSA26^{eGFP-DTA/+};Wnt1-Cre* compound embryo shows defects in the anterior neural tube. Other regions of the embryo appear normal by gross morphology. **b:** Fluorescence photographs of embryos in (a). Only those containing the *ROSA26-eGFP-DTA* targeted allele express eGFP. **c,d:** Magnified views of the head of 9.0 dpc *ROSA26^{eGFP-DTA/+}* and *ROSA26^{eGFP-DTA/+};Wnt1-Cre* embryos. Optic (arrowhead) and otic (arrow) vesicles are well developed in the compound mutant, but there is a significant lack of brain tissue between these vesicles. Scale bars = 500 μ m (a,b); 200 μ m (c,d). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

FIG. 4.

Whole-mount in situ hybridisation analysis of *Wnt1-Cre* and *ROSA26^{eGFP-DTA/+};Wnt1-Cre* embryos. **a,b:** *Wnt1* expression rostral to midbrain-hindbrain boundary (MHB) (arrowheads) is absent in the *ROSA26^{eGFP-DTA/+};Wnt1-Cre* compound embryo (**b**) when compared with the control (**a**) at 8.5 dpc (i.e., region shown by bracket in **a** is absent in **b**). *Wnt1* expression in the dorsal aspects of the hindbrain is reduced in intensity (black arrows), but is normal in the spinal cord (red arrows). **c,d:** Expression of *Cre* at 9.0 dpc. The lack of midbrain tissue rostral to the MHB (arrowhead) is particularly evident at this stage, as shown by the significant reduction of *Cre* expression in the MHB (arrowhead) and rostral to it (bracket in **c**). **e,f:** *Otx3* is not detectable in the midbrain and posterior forebrain of *ROSA26^{eGFP-DTA/+};Wnt1-Cre* compound

embryos at 8.5 dpc (**f**). **g,h**: Double in situ hybridisation with *Bfl* (arrowhead) and *Hoxb1* (asterisk). Expression of these markers is detectable in *ROSA26^{eGFP-DTA/+};Wnt1-Cre* compound embryos (**h**), although quantity of brain tissue in between is severely reduced. **i,j**: *Fgf8* expression appears normal in the anterior neural ridge of the *ROSA26^{eGFP-DTA/+};Wnt1-Cre* compound embryo (arrow), but there is no MHB expression of *Fgf8* (black arrowheads). *Fgf8* expression in the tail bud (white arrowheads) and the branchial region (white arrows) is normal in compound heterozygous embryos. Scale bars = 200 μ m.

Table 1
Frequency of Genotypes Among Offspring From $ROSA^{eGFP-DTA/+}$ Heterozygous Intercrosses

Genetic background ^a	Total number of offspring	Number of offspring of each genotype ^b		
		+/+	+/-	-/-
C57BL/6J	44	12	25	9
CD1	43	7	20	16

^a $ROSA^{eGFP-DTA/+}$ heterozygotes were backcrossed twice with C57BL/6J or CD1 mice.

^b Distribution of offspring among genotypes does not differ from the expected 1 (+/+): 2 (+/-): 1 (-/-) ratio on either C57BL/6J or CD1 background $p > 0.05$ in each case).