Published in final edited form as:

Dev Neurobiol. 2009 May ; 69(6): 401–406. doi:10.1002/dneu.20714.

A tetracycline- inducible and skeletal muscle specific Cre recombinase transgenic mouse

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Abstract

We have generated a transgenic mouse that expresses Cre recombinase only in skeletal muscle and only following tetracycline-treatment. This spatiotemporal specificity is achieved using 2 transgenes. The first transgene uses the human skeletal actin (HSA) promoter to drive expression of the reverse tetracycline-controlled transactivator (rtTA). The second transgene uses a tetracycline responsive promoter to drive expression of Cre recombinase. We monitored transgene expression in these mice by crossing them with ROSA26 *loxP*-LacZ reporter mice, which express β -galactosidase when activated by Cre. We find that expression of this transgene is only detectable within skeletal muscle and that Cre expression in the absence of tetracycline is negligible. Cre is readily induced in this model with tetracycline analogs at a range of embryonic and postnatal ages and in a pattern consistent with other HSA transgenic mice. This mouse improves upon existing transgenic mice in which skeletal muscle Cre is expressed throughout development by allowing Cre expression to begin at later developmental stages. This temporal control of transgene expression has several applications, including overcoming embryonic or perinatal lethality due to transgene expression. This mouse is especially suited for studies of steroid hormone action, as it uses tetracycline, rather than tamoxifen to activate Cre expression. In summary, we find that this transgenic induction system is suitable for studies of gene function in the context of hormonal regulation of skeletal muscle and/or interactions between muscle and motoneurons in mice.

Keywords

skeletal muscle; Cre-*loxP*; Tet-On; transgenesis; neuromuscular systems

INTRODUCTION

Manipulating gene expression in skeletal muscle is of interest for a wide array of fundamental and applied research questions relating to gene function in neuromuscular development, health and disease. Embryonic or perinatal lethality resulting from manipulating gene expression can prove to be a significant hurdle to research. These inherent limitations of traditional transgenic or gene targeting approaches may be overcome by using constructs which provide spatial or temporal control over transgene expression. Notably, the Cre/*loxP* system is used in mice to allow for conditional activation or inactivation of mutations. Cre recombinase is a bacteriophage P1 enzyme that can catalyze efficient site-specific DNA recombination between two appropriately configured 34-

basepair *loxP* sites. Any genetic sequence of interest, if flanked by *loxP* sites, can be subsequently removed in the cells which express Cre recombinase (Kuhn and Torres, 2002). Inducible *loxP* mutants are becoming increasingly available as the popularity of this system grows.

Transgenic mice exist that express Cre specifically within cells of skeletal muscle fiber lineage (i.e. myocytes), either constitutively (Minou et al., 1999), or conditional upon tamoxifen administration (Schuler et al., 2005). The selective estrogen receptor modulator tamoxifen, although commonly used to induce genetic models, is a mixed agonist/antagonist of estrogen receptor with incompletely understood properties (e.g. see Katzenellenbogen and Katzenellenbogen, 2002). Tamoxifen treatment may therefore complicate studies that involve steroid hormones effects on neuromuscular systems, which increasingly use genetic models (for recent reviews of this area of research see Breedlove et al., 2002; Monks et al., 2008; Sengelaub and Forger, 2008). An alternative to activation of transgenes with tamoxifen is provided by the reverse tetracycline-controlled transactivator (rtTA) system. The rtTA transcription factor requires tetracycline, or its analog doxycycline (Dox), to bind tetracycline response elements (TRE) and thereby promote gene transcription. TRE can effectively drive gene expression in mammalian cells when used in conjunction with the minimal human cytomegalovirus (CMV) promoter (Gossen et al., 1995). This regulatory system is also known as the Tet-On system.

Recently, we have reported that overexpression of androgen receptor in myocytes results in male perinatal lethality (Monks et al., 2007; Monks et al., 2008), hampering studies of consequences of such overexpression in adulthood. Because we wish to study adult action of androgen receptor in this cell type, we have therefore developed a system that would allow us to bypass this perinatal lethality without directly manipulating steroid hormone receptors with tamoxifen. Here we describe this novel transgenic system that allows for tetracycline induction of Cre recombinase in myocytes. In principle, this system would allow for conditional activation or inactivation of any *loxP*-engineered gene or transgene within this cell type. Studies of gene function in myocytes help us understand not only muscle development, but also interactions between muscle and innervating motoneurons.

METHODS

Transgene construction and generation of Tg mice

The CMV promoter from a modified pTet-On plasmid (Le et al., 2008) was replaced with a 2.9 kb KpnI/NotI fragment of the HSA promoter to give the recombinant plasmid pHSA-rtTA (Fig 1). The construction of the TRE-Cre transgene has been previously described (Le et al., 2008). Plasmid sequences were removed from transgenes prior to microinjection as follows. A 5.1-kb fragment of the HSA-rtTA transgene was excised from the plasmid pHSA-rtTA with KpnI and BpuE1. The 3.5-kb TRE-Cre fragment was isolated from pTRE-Cre with HindIII and XhoI (Fig. 1). Following gel purification, both transgenes were co-injected into pronuclei of (C57BL/6 × C3H)F2 zygotes in the transgenic facility of the Van Andel Research Institute (Grand Rapids, MI). Zygotes were then implanted into pseudo-pregnant foster mothers, and resulting founder mice were genotyped using genomic DNA by polymerase chain reaction (PCR) with the HSA-rtTA specific primers a (5'-ACT GAG AGG TGG GAA GCT CA-3') and b (5'-GGC GAG TTT ACG GGT TGT TA-3') and the Cre-specific primers c (5'-AGG TGT AGA GAA GGC ACT TA -3') and d (5'-CTA ATC GCC ATC TTC CAG CA -3'). The sizes of the amplified products are 284 bp for HSA-rtTA and 412 bp for Cre respectively. HSA-rtTA/TRE-Cre double transgenic founders were used for further analyses. Founder lines were maintained by back-crossing transgenic mice to wild-type C57BL/6 mice.

Transgene induction in triple transgenics—The tetracycline analog doxycycline (Dox) was administered to induce transgene expression. Dox was dissolved in drinking water at a concentration of 2 mg/ml supplemented with 5% sucrose and provided *ad lib* to Dox treated (Dox+) animals in light-proofed water bottles for three days (the first day of treatment being considered the day of induction). Embryonic and pre-weaning pups were induced by delivering Dox to the dam. Control (Dox-) mice received standard drinking water and lab chow that had been autoclaved to inactivate any trace of tetracycline derivatives.

Analysis of Cre recombinase expression

Analysis of Cre mRNA expression—Total RNA was extracted from tissues and cDNA was prepared as previously described (Monks et al., 2007). PCR amplification of Cre was then performed as described above. An additional PCR amplification was performed using primers specific for β -actin (F 5'-TAG GCA CCA GGG TGT GAT GG-3' and R 5'-GAT GCC ACA GGA TTC CAT ACC CAA-3') to serve as a positive control.

Crosses with loxP LacZ reporter mice—To localize the Cre activity sites, HSA-rtTA/TRE-Cre double transgenic founders were crossed with homozygous ROSA26 loxP LacZ reporter mice (Soriano, 1999), obtained from Jackson Laboratories (Bar Harbor, Maine). The ROSA26 mice carry a loxP-flanked ("floxed") transcriptional "STOP" DNA sequence that prevents the transcription of the lacZ gene. Only the cells which expressed the Cre recombinant can remove the 'STOP' sequence and subsequently activate the transcription of the LacZ gene. Cells which express LacZ produce a blue color in the β -galactosidase assay (X-gal staining). HSA-rtTA/TRE-Cre/ROSA26 triple transgenic mice (referred to as triple transgenic mice hereafter) offspring were genotyped from the yolk sac or pup tail genomic DNA by PCR using the aforementioned primers. Embryos were aged according to the day when vaginal plugs were observed (which was counted as E0.5).

β -galactosidase activity assay—Embryos or neonatal pups were dissected in ice-cold 1x PBS and placed in fixative solution (2% paraformaldehyde plus 0.2% glutaraldehyde in 1x PBS (pH 7.3)) for 60–120 minutes on ice depending on the embryonic size (MacGregor et al., 1995). Fixed embryos were washed in PBS for 3 $\times$ 5 minutes and stained in the dark at 37°C with 0.1% X-gal (5-Bromo-4-Chloro-3-indolyl- β -D-galactoside) in X-gal buffer (2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 0.01% NaDC, and 0.02% NP-40 in 1x PBS (pH 7.3)) for 24 hours with one change of X-gal solution. After staining, embryos were further fixed in 4% paraformaldehyde at 4 °C overnight and then photographed using a Nikon CoolPix 995 camera attached to a Nikon SMZ800 dissection microscope.

Adult tissues (skeletal muscles, heart, bladder, liver, kidney, spleen, and spinal cord) of triple transgenic mice induced at various ages were rapidly dissected from surgically anesthetized adult mice. Tissues were frozen and stored at -80°C until cryostat sectioning or RNA extraction. Cryostat sections (12 μ m) were prepared and kept frozen at -80°C until X-gal staining. For staining, slides were equilibrated to room temperature then placed in the fixative solution described above for 15 minutes at 4°C. Following 3 washes with PBS, sections were incubated in X-gal solution described above for 24 hours at room temperature. Slides were then rinsed with PBS and stained with 0.05% Safranin prior to dehydration with graded ethanols, clearing with xylenes. Slides were coverslipped using Permount.

RESULTS

Generation of Transgenics

Five founders were identified as carrying both transgenes out of 42 offspring generated by co-injection of the two DNA fragments (HSA-rtTA and TRE-Cre). One founder produced offspring that expressed the transgene at an undetectable levels. PCR analysis of segregation patterns for five generations indicated that both transgenes co-segregated in three of the remaining four lines. Of the remaining three lines, the line with the most robust Cre expression in initial screening was chosen for in-depth characterization.

Analysis of Cre expression

Analysis of Cre mRNA expression—We first evaluated Cre mRNA expression in several tissues from induced (Dox+) and control (Dox-) transgenic mice. We found detectable expression in skeletal muscles, but not in heart, bladder, liver, kidney, spleen or spinal cord (Fig 2). Cre expression was observed in Dox- mice, although it was at the threshold of detection, suggesting that this expression is relatively weak, an interpretation supported by our observations in reporter mice described below. Similar transgene expression was observed in both male and female transgenic mice of various adult ages (data not shown). These results indicate transgene expression is specific to skeletal muscle but that detectable expression of Cre occurs in the absence of Dox.

β -galactosidase activity assay—We next evaluated transgene expression in Dox+ and Dox- transgenics using a functional assay for Cre expression. We crossed transgenic mice with loxP LacZ mice and induced at various developmental stages. We could not detect X-gal staining in Dox+ WT or Dox- pups, whereas appropriate staining was observed in Dox+ Tg mice (Fig 3). X-gal staining was restricted to skeletal muscle lineage tissues and corresponded well with patterns observed in HSA-LacZ mice (Monks et al., 2007). Efficient induction was achieved at all ages examined, including E10.5, E14.5, P0 (Fig 3).

We then examined transgene expression in cryostat sections of skeletal muscle, smooth muscle and cardiac muscle (Fig 4). Notably, adult induction with Dox was as effective as embryonic induction (Fig 4 A,B). In agreement with Cre mRNA measurement, we found evidence of Cre activity in skeletal muscle but not other tissues, and negligible activity in skeletal muscle in the absence of Dox (Fig 4, compare E and F). Transgene expression within adult skeletal muscle is restricted to muscle fibers. Some mosaicism is observed in transgene expression by muscle fibers, and some variation in the degree of mosaicism between skeletal muscles was observed, comparable to that observed in HSA-LacZ mice (Monks et al., 2007).

DISCUSSION

We have provided evidence that this transgenic mouse expresses Cre efficiently in skeletal muscle following Dox treatment at a variety of ages, and that expression is restricted to skeletal muscle. We have also presented evidence that, although detectable, Cre expression in this model in the absence of Dox is negligible. We did observe mosaicism in transgene expression, which is typical of this expression cassette (Monks et al., 2007). The cause of this mosaicism is not clear, although larger clones of ACTA1 appear to be expressed more uniformly (Schuler et al., 2005), suggesting that genomic elements outside of the HSA clone used are required for efficient expression in some fiber types. Nonetheless, the HSA expression cassette used in the HSA-rtTA transgene is able to affect muscle sufficiently to have obvious phenotypic consequences and has proven effective in studying gene function in skeletal muscle (Monks et al., 2007).

We have described the genesis of a novel transgenic mouse that allows for induction of *loxP*-based alleles within myocytes conditional on treatment with tetracycline or its analogs. This mouse should be of interest to those interested in studying gene function in skeletal muscle, and particularly those who wish to restrict genetic manipulation to later developmental stages, or who wish to study acute consequences of genetic manipulations. Furthermore, this model does not use an endocrine manipulation to activate the transgene, making it suitable for studies of hormonal regulation of skeletal muscle and/or interactions between muscle and motoneurons in mice.

Acknowledgments

Grant support: NINDS R01-NS51257 (DAM)

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Figure 1.

Diagram of transgenes. HSA = promoter of human skeletal actin; rtTA = reverse tet-controlled transactivator; SV 40 (A)_n = simian virus 40 polyadenylation signal; TRE = tetracycline response element; CMV, minimal human cytomegalovirus promoter; MI-I (A)_n = mouse metallothionein polyadenylation signal.

Figure 2.

Detection of Cre expression in transgenic mouse tissues by RT-PCR. Lane 1–8, tissue samples from a young adult (PND39) transgenic mice induced with Dox at E7: limb muscles (lane 1), limb muscle total RNA without reverse transcription (RT- negative control, lane 2), heart (lane 3), bladder (lane 4), liver (lane 5), kidney (lane 6), spleen (lane 7), spinal cord (lane 8); limb muscles from a non-induced adult transgenic mouse (lane 9).

Figure 3. X-gal staining of whole embryos with or without Dox induction at the age E10.5 (top row), E14.5 (middle row), and P0 (bottom row). Wildtype mice treated with Dox (WT Dox+, left column); transgenic mice induced with Dox (Tg Dox+ middle column); transgenic mice without Dox induction (Tg Dox-, right column). All Dox+ mice were treated with Dox 3 days prior to dissection.

Figure 4. Photomicrographs taken at 400× magnification of cryostat sections stained with X-gal, scale bar indicates 10 μ m. Extensor digitorum logus (EDL) muscle section of adult transgenic (Tg) mice which was induced with Dox at E14.5 (A) or P32 (B), with comparable results. Staining of sections from anterior tibialis (C) and quadriceps (D), indicate minor variability in the degree of mosaicism of transgene expression between muscles. No staining was observed in sections from heart (E) and bladder (F) of adult Tg mouse induced with Dox at E14.5. EDL muscle section of a non-induced adult Tg mouse (G), arrow indicates weak β -galactosidase activity indicative of some leaky expression. Negative control EDL section of adult wildtype mouse treated with Dox (H).