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Construction and Characterization of a doxycycline-inducible transgenic system in *Msx2* expressing cells

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Abstract

Homeobox gene *Msx2* is widely expressed during both embryogenesis and postnatal development and plays important roles during organogenesis. We developed an *Msx2*-rtTA BAC transgenic line which can activate TetO-Cre expression in *Msx2*-expressing cells upon doxycycline (Dox) treatment. Using the *Rosa26-LacZ* (R26R) reporter line, we show that rtTA is activated in *Msx2*-expressing organs including the limb, heart, external genitalia, urogenital system, hair follicles and craniofacial regions. Moreover, we show that in body appendages, the transgene can be activated in different domains depending on the timing of Dox treatment. In addition, the transgene can also be effectively activated in adult tissues such as the hair follicle and the urogenital system. Taken together, this *Msx2*-rtTA;TetO-Cre system is a valuable tool for studying gene function in the development of the aforementioned organs in a temporal and spatially-restricted manner, as well as for tissue lineage tracing of *Msx2*-expressing cells. When induced postnatally, this system can also be used to study gene function in adult tissues without compromising normal development and patterning.

Keywords

Msx2; rtTA; appendages; urogenital; hair follicle

INTRODUCTION

Homeobox gene *Msx2* is widely expressed in a variety of organs including the heart (Chan-Thomas et al., 1993; Chen et al., 2008), hair follicles (Ma et al., 2003), female reproductive tracts (Huang et al., 2005; Yin et al., 2006), limbs (Davidson et al., 1991; Robert et al., 1991; Liu et al., 1994; Ferrari et al., 1998), external genitalia (Lin et al., 2008), craniofacial (Monaghan et al., 1991; MacKenzie et al., 1992) and neural tissues (Satokata et al., 2000) during embryonic development. It is also expressed postnatally in uterus and vagina (Huang et al., 2005; Yin et al., 2006) and hair follicles (Ma et al., 2003). The goal of this study was to

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use the *Msx2* promoter to drive inducible gene expression or gene deletion in order to study gene function in *Msx2*-expressing organs.

Temporal- and spatial-specific gene manipulation using Cre/LoxP technology has been proven to be one of the most powerful tools to study gene function *in vivo* (Sauer, 1998). Spatial control is normally achieved by the use of a tissue specific promoter, while temporal restriction depends on an inducible system. Currently, two systems are widely-used to achieve inducible gene activation: the tamoxifen-inducible ERTm system (Danielian et al., 1998) and the doxycycline-inducible tet-On/Off system (Utomo et al., 1999; Sprengel and Hasan, 2007). The use of the Tamoxifen-based ERTm system has been limited by its anti-estrogenic nature (Jordan and Murphy, 1990) and it often causes abortion when administered during pregnancy. Moreover, this system is not suited for studying reproductive processes such as embryo implantation as it interferes with the natural hormonal milieu. Thus, in this study, we adopted the doxycycline-inducible Tet-On system (Gossen et al., 1995; Sprengel and Hasan, 2007). We constructed bacterial artificial chromosome (BAC) transgenic lines that express a modified version of rtTA, rtTA-M2, under the control of the *Msx2* promoter. Upon Dox treatment, the transgene can achieve efficient gene manipulation in endogenous *Msx2*-expressing domains when coupled with an rtTA responsive TetO-Cre line (Perl et al., 2002). Finally, we report unexpected phenotypes obtained in our transgenic lines.

RESULTS AND DISCUSSION

Construction of *Msx2*-rtTA BAC transgenic lines

Our goal was to establish a transgenic system which will allow us to manipulate gene expression in endogenous *Msx2*-expressing domains. To minimize position-effect-variegation (PEV) often observed in transgenesis with smaller transgenes, and to include as many endogenous regulatory elements as possible, we adopted a BAC-based strategy. We obtained a 187 kb BAC clone containing the endogenous *Msx2* locus and flanking sequences (134 kb upstream and 47 kb downstream of *Msx2*; accession number: AC154808), and replaced part of the first exon right after the start codon with a modified rtTA gene followed by a neomycin cassette and polyA sequences using a two-step recombineering strategy (Muyers et al., 1999; Narayanan et al., 1999). In this design, we have presumably included all the *Msx2* regulatory elements while disrupting *Msx2* coding sequence to avoid *Msx2* overexpression. The modified BAC DNA was purified and subjected to pro-nucleus injection (For details, please see the Methods section and Fig. 1). We obtained six transgenic founders, three of which strongly expressed the rtTA gene as assayed by RT-PCR on tail biopsies. Founder 892 died at three months of age from unknown causes while two other founders (named 885 and 888) had a normal lifespan and were able to pass on their transgenes. Both 892 and 888 transgenic lines showed similar hair and limb phenotypes which will be described below.

To evaluate the spatial and temporal control conferred by the inducible rtTA system, mice carrying the *Msx2*-rtTA BAC transgene were mated to TetO-Cre transgenic mice, which express Cre recombinase in a Dox-rtTA-dependent manner. Bigenic animals were then bred to the well characterized Rosa26-LacZ (R26R) reporter line (Soriano, 1999). In all studies, only hemizygotes were used. In *Msx2*-expressing cells, rtTA is expressed but does not activate TetO-Cre in the absence of Dox. In the presence of Dox, rtTA undergoes a conformational change and activates TetO-Cre expression (Perl et al., 2002), which in turn removes the floxed stop cassette 5' of the *LacZ* gene in the *R26* locus and results in β -Galactosidase (β -Gal) expression. We analyzed transgenic lines 885 and 888 and both yielded the same expression pattern. Therefore only results from line 885 which is phenotypically normal are shown.

Since robust *Msx2* expression is detected during embryonic development, we first analyzed transgene activity during embryogenesis. Dox was given to pregnant females by oral gavaging

on 9.5, 10.5 or 11.5 days post coitum at a dosage of 0.05g/kg of body weight. Embryos were collected at embryonic day (E) 12.5 and stained for β -Gal activity. Strong β -Gal activity was observed in the craniofacial region, spinal cord, mammary gland, limbs and genital tubercles of all Dox-treated but not in non-treated trigenic embryos. Embryos with earlier Dox treatment appeared to induce a stronger expression and a broader staining pattern in the craniofacial region, forelimbs and ventral ectoderm (Fig. 2a–c). In early limb bud development, endogenous *Msx2* is expressed both in the apical ectodermal ridge (AER) and in distal limb mesenchyme, especially in the anterior part (Liu et al., 1994; Ferrari et al., 1998). Later, its expression shifts to interdigital cells. Close examination of β -Gal activity in treated embryos revealed similar autopod expression in E10.5 and E11.5 treated embryos (Fig. 2e, f, h, i) with hindlimbs exhibiting a slightly smaller and more distally restricted expression domain. However, E9.5-treated embryos showed a distinct anterior staining pattern in both forelimbs and hindlimbs (Fig. 2d, g). Sections of whole mount stained limb bud revealed expression domains that included both digital and interdigital cells (Fig. 2j–l). These domains are consistent with an early *Msx2*-expressing distal mesenchymal lineage which encompasses both digital and interdigital cells. Expression in the AER was also evident by whole mount staining (insets in Fig. 2d, g). Moreover, if treated around E12.5, transgene expression was clearly detected in interdigital cells but was excluded from the proximal part of digits, which agrees with a shift in endogenous *Msx2* expression that occurs around E12.5 (Compare Fig. 2k to 2l). Endogenous *Msx2* expression was also detected in the genital tubercles (Lin et al., 2008). In the genital tubercle (GT), β -Gal activity was detected both in the urethral epithelium and in the GT mesenchyme (Fig. 3a, b, insets). Earlier Dox treatment led to a broader mesenchymal expression, whereas later Dox induction resulted in a more dorsally- and distally-restricted expression (Fig. 3a–c). In the craniofacial region, strong β -Gal staining was detected in the oral epithelium, maxilla and mandible (Fig. 3d). Distinct staining was also observed in the lens and dorsal retina (Fig. 3f). This expression pattern agrees with an early *Msx2* expression in lens surface ectoderm and the dorsal half of the optic vesicle directly apposed to the surface ectoderm (Fig. 3g–i). For all studies, embryos with only *TetO-Cre* allele were used as controls and no expression was detected in *Msx2* expression domains after Dox induction as described above. We also analyzed transgene activity in the developing heart, and found strong β -Gal staining in the myocardium and endocardial cushions including the outflow tract and the atrioventricular cushion (Fig. 3l, m). This expression pattern agrees with previously reported endogenous *Msx2* expression (Chen et al., 2008).

Msx2 is also expressed in the urogenital tract and hair follicles during late embryogenesis. To investigate whether Dox can efficiently induce gene activation during late stages of development, we treated females with Dox at either E14.5 or E16.5 and examined β -Gal expression at later time points. Notably, Dox treatments at a dosage of 0.05g/kg, although occasionally caused abortion, did not have any overt effects on pregnancy in more than eighty percent of pregnant females (15 out of 18, 2 aborted, and one delivered pre-maturely). The trigenic pups exhibited distinct β -Gal activity in the genitourinary tract and the skin. At E16.5, whole-mount stained embryos (Dox-treated at E14.5) showed exclusive hair follicle-specific staining in the epidermis (Fig. 4i). In the urogenital system, strong and homogeneous staining was detected in the epithelial lining of the ureter, bladder and urethra in both sexes (Fig. 4f–h). In addition, β -Gal staining was found uniformly in the uterine and vaginal epithelia in females (Fig. 4d, e). No transgene activity was ever detected in the ovary and oviduct (Fig. 4b, c).

One important feature of *Msx2* is that its expression is maintained postnatally in a variety of organs. To test whether this *Msx2*-rtTA transgene can be used to delete or activate genes in adults, we analyzed β -Gal activity in *Msx2* expressing organs of three month old animals 48 hours after they were treated with Dox. As expected, robust β -Gal activity was detected in the epidermal component of the hair follicle (Fig. 4l) and in bladder epithelium (Fig. 4m). Since

Msx2 expression in the bladder has not been documented, we performed indirect immunofluorescence to examine MSX2 protein expression. We detected distinct nuclear staining in wild-type bladder epithelium (Fig. 4j), whereas only weak and diffuse cytoplasmic background staining was observed in *Msx2*^{-/-} bladder epithelium (Fig. 4k), indicating that the transgene recapitulated endogenous *Msx2* expression in the bladder. Next, we carefully examined β -Gal expression in the uterus. Unexpectedly, we only detected very weak staining in the luminal epithelium and moderate staining in the glandular epithelium (Fig. 4n). Neither increasing Dox dosage nor altering the treatment regimen helped to increase β -Gal expression in the adult uterus. This result does not seem to agree with the high endogenous *Msx2* expression found in the uterine epithelium (Huang et al., 2005). One possibility is that the promoter activity of either *Rosa26* or *TetO-Cre* in adult uterus might not be as strong as in other tissues. Alternatively, other regulatory elements outside the *Msx2* BAC might be required for uterine *Msx2* expression. Whether this system can be used for implantation studies needs to be further investigated.

As mentioned previously, two transgenic founders have similar limb and hair phenotypes. Founder 892 died at three month of age but did give birth to a litter containing only one transgenic pup, which showed severe growth retardation, complete absence of body hair and died two weeks after birth. Founder 888 appeared to have a normal life span and was capable of transmitting the transgene. All animals from line 888 showed consistent limb and hair phenotypes. Specifically, the digits of the forelimbs were shorter (Fig. 5d) and interdigital webbing was evident especially in hindlimbs (Fig. 5e). This phenotype was consistent with a lack of cell death in the interdigital region of limb buds on E13.5 (Fig. 5g). Moreover, the vibrissae of the transgenic animals were shorter and curly while pelage hairs were wavy (Fig. 5a). The fact that two independent transgenic lines exhibited these phenotypes argues against a possible gene disruption by transgene integration as a potential cause. Although *Msx2* is expressed both in the hair follicle and in the interdigital region of limb buds, it is unlikely that these phenotypes resulted from *Msx2* overexpression because exon one of *Msx2* was deleted in the BAC construct. Although theoretically *Msx2* exon2, which does contain an inframe ATG, could be transcribed and translated to generate a homeodomain-only form of MSX2, it is highly unlikely because a polyA sequence has been inserted after rtTA. To further rule out the possibility of read-through transcripts and/or alternative splicing, we used an antibody against the C-terminal domain of MSX2 protein to perform Western blots using proteins extracted from both the skin and E13.5 limbs of the transgenic mice. We detected a 28 kd band corresponding to the wild type MSX2 protein as expected, but fail to detect any truncated or fusion proteins (data not shown). Together these data strongly argue against overexpression of truncated MSX2 as the underlying basis for these observed phenotypes. Thus, we reasoned that the phenotype was most likely caused by overexpression of another gene (s) on the BAC. The fact that these phenotypes occurred in *Msx2*-expressing domain suggests that this gene (s) may share temporal and spatial regulatory elements with *Msx2*. Besides *Msx2*, six additional genes were predicted in the BAC clone RP24-512K19 by the online software GENSCAN (<http://genes.mit.edu/GENSCAN.html>) (gene positions are marked in Fig. 1b). Three of them were in the same orientation as *Msx2*. However, nothing is known about the expression or function of those genes. Whether and how these hypothetical genes may contribute to the phenotype needs further investigation. Finally, one caveat about this system is that although line 885 does not show any phenotypic anomaly in any *Msx2*-expressing organs, minor molecular changes may have occurred as a result of overexpression of other genes on the BAC, which may or may not complicate the use of this transgenic system.

In summary, we generated a new *Msx2-rtTA;TetO-Cre* transgenic system that should be useful to perform conditional gain- and loss of function studies in the development of body appendages, skin, and urogenital system. Dox treatment at different time points induced gene expression in different domains of body appendages, which provided a novel tool in lineage-

tracing and knockout studies. This system should be valuable for studying embryonic patterning and differentiation of the reproductive tract, particularly the uterus, as no uterine epithelial-specific Cre line has been reported. Another unique advantage of this system is that it can achieve efficient recombination in adult hair follicles, bladder epithelium and uterine glandular epithelium. This feature will be very useful for studying gene function in adult tissues without disturbing normal embryonic development. It will also allow us to study gene function at a particular stage in the hair cycle.

METHODS

Animal Maintenance and Treatment

All animals were maintained in the Washington University animal facility and handled according to National Institutes of Health guidelines. R26R mice were purchased from Jackson Laboratory (Bar Harbor, MN). TetO-Cre transgenic mouse was described previously (Perl et al., 2002). 1200 hours on the day of vaginal plug was designated as E0.5. Doxycyclin (Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water at a concentration of 20 mg/ml. 0.05g/kg of body weight Dox was delivered by oral gavaging to pregnant females at different times during gestation or to adult animals at three months. Primers used to genotype the transgenic lines are *Msx2*-GF1 (5'-ATC GCC TAA TAA CAA CTC TGC TGA CTG CTC C) and *Msx2*-GR1 (5'-ACT CCC AGC TTT TGA GCG AGT TTC CTT GTC G), which will amplify a 425bp fragment in transgenic animals (Fig. 1b, c).

Construction of the *Msx2*-rtTA BAC transgene

To make the transgenic construct, rtTA with a polyA sequence was excised from pUHRt62-1 (Urlinger et al., 2000) and subsequently cloned into the XhoI site of pSV-Flp, which contains a FRT-flanked pSV-Neo/Kan cassette (Fig. 1a). A 0.5kb PCR amplicon immediately upstream of the starting ATG of *Msx2* was inserted into the PmeI site of pSV-Flp, with the 3' end proximal to the rtTA2^S-M2 fragment. A 0.5kb PCR product amplifying the first intron of *Msx2* was cloned into the SalI site of the backbone, with the 5' end proximal to the pSV40 promoter (Fig. 1a). Following this, a targeting construct was released by AscI-PmeI double-digestion and subjected to BAC recombineering using a two-step recombineering strategy (Fig. 1b). Briefly, a recombinase-expressing plasmid, pGETrec was transformed into E. coli cells containing *Msx2* BAC (AC154808). Bacteria clones containing both pGETrec and BAC were selected. Subsequently, the targeting construct was transformed into these cells. This allowed homologous recombination to occur on both sides of the *Msx2* locus on the BAC, and resulted in a construct in which the rtTA gene is placed immediately downstream of the *Msx2* promoter (Fig. 1b). BAC DNA was then purified and subjected to pro-nuclear injection.

X-Gal Staining

Embryos or adult tissues were dissected, rinsed in PBS and briefly fixed in 4% paraformaldehyde (PFA) on ice for 45 minutes to an hour. After washing, embryos were stained in 0.1% X-Gal solution containing 0.01% deoxycholate, 0.02% NP40, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆ and 5 mM K₄Fe(CN)₆ at 37°C overnight. Embryos/tissues were rinsed in PBS, fixed in PFA, dehydrated through graded ethanol washes and followed by either light microscopy for whole mount pictures, or embedding in paraffin and sectioning at 10µm for histological analysis.

In situ Hybridization

Whole mount in situ hybridization was performed as described (Wilkinson, 1992) using a 1 kb digoxigenin labeled *Msx2* antisense probe. Embryos were permeabilized by three washes with RIPA buffer and a brief treatment (3 minute) with proteinase K (10 µg/ml) to avoid

disruption of the surface ectoderm. Embryos were sectioned at 50 μ m using a Leica VT 1000M/E vibrating microtome, mounted in 100% glycerol and photographed with a Zeiss Axiophot microscope using Nomarski optics.

Immunofluorescence

Indirect immunofluorescence was performed as previously described (Huang et al., 2005; Yin et al., 2006). Rabbit polyclonal antibody against MSX2 (sc-15396) was purchased from Santa Cruz Biotech (Santa Cruz, CA, 95060).

Nile blue analysis

E13.5 embryonic limbs were dissected and stained with 0.05% Nile Blue (Sigma, St. Louis, MO) in PBS at 37°C for two hours. Tissues were then washed three times in PBS on ice and examined by light microscopy.

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REFERENCES

- Chan-Thomas PS, Thompson RP, Robert B, Yacoub MH, Barton PJ. Expression of homeobox genes *Msx-1* (Hox-7) and *Msx-2* (Hox-8) during cardiac development in the chick. *Dev Dyn* 1993;197:203–216. [PubMed: 8106021]
- Chen YH, Ishii M, Sucov HM, Maxson RE Jr. *Msx1* and *Msx2* are required for endothelial-mesenchymal transformation of the atrioventricular cushions and patterning of the atrioventricular myocardium. *BMC Dev Biol* 2008;8:75. [PubMed: 18667074]
- Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* 1998;8:1323–1326. [PubMed: 9843687]
- Davidson DR, Crawley A, Hill RE, Tickle C. Position-dependent expression of two related homeobox genes in developing vertebrate limbs. *Nature* 1991;352:429–431. [PubMed: 1677742]
- Ferrari D, Lichtler AC, Pan ZZ, Dealy CN, Upholt WB, Kosher RA. Ectopic expression of *Msx-2* in posterior limb bud mesoderm impairs limb morphogenesis while inducing BMP-4 expression, inhibiting cell proliferation, and promoting apoptosis. *Dev Biol* 1998;197:12–24. [PubMed: 9578615]
- Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995;268:1766–1769. [PubMed: 7792603]
- Huang WW, Yin Y, Bi Q, Chiang TC, Garner N, Vuoristo J, McLachlan JA, Ma L. Developmental diethylstilbestrol exposure alters genetic pathways of uterine cytodifferentiation. *Mol Endocrinol* 2005;19:669–682. [PubMed: 15591538]
- Jordan VC, Murphy CS. Endocrine pharmacology of antiestrogens as antitumor agents. *Endocr Rev* 1990;11:578–610. [PubMed: 2292243]
- Lin C, Yin Y, Long F, Ma L. Tissue-specific requirements of {beta}-catenin in external genitalia development. *Development*. 2008
- Liu YH, Ma L, Wu LY, Luo W, Kundu R, Sangiorgi F, Snead ML, Maxson R. Regulation of the *Msx2* homeobox gene during mouse embryogenesis: a transgene with 439 bp of 5' flanking sequence is expressed exclusively in the apical ectodermal ridge of the developing limb. *Mech Dev* 1994;48:187–197. [PubMed: 7893602]

- Ma L, Liu J, Wu T, Plikus M, Jiang TX, Bi Q, Liu YH, Muller-Rover S, Peters H, Sundberg JP, Maxson R, Maas RL, Chuong CM. 'Cyclic alopecia' in *Msx2* mutants: defects in hair cycling and hair shaft differentiation. *Development* 2003;130:379–389. [PubMed: 12466204]
- MacKenzie A, Ferguson MW, Sharpe PT. Expression patterns of the homeobox gene, *Hox-8*, in the mouse embryo suggest a role in specifying tooth initiation and shape. *Development* 1992;115:403–420. [PubMed: 1358591]
- Monaghan AP, Davidson DR, Sime C, Graham E, Baldock R, Bhattacharya SS, Hill RE. The *Msh*-like homeobox genes define domains in the developing vertebrate eye. *Development* 1991;112:1053–1061. [PubMed: 1682128]
- Muyrers JP, Zhang Y, Testa G, Stewart AF. Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res* 1999;27:1555–1557. [PubMed: 10037821]
- Narayanan K, Williamson R, Zhang Y, Stewart AF, Ioannou PA. Efficient and precise engineering of a 200 kb beta-globin human/bacterial artificial chromosome in *E. coli* DH10B using an inducible homologous recombination system. *Gene Ther* 1999;6:442–447. [PubMed: 10435094]
- Perl AK, Wert SE, Nagy A, Lobe CG, Whitsett JA. Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc Natl Acad Sci U S A* 2002;99:10482–10487. [PubMed: 12145322]
- Robert B, Lyons G, Simandl BK, Kuroiwa A, Buckingham M. The apical ectodermal ridge regulates *Hox-7* and *Hox-8* gene expression in developing chick limb buds. *Genes Dev* 1991;5:2363–2374. [PubMed: 1684333]
- Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S, Peters H, Tang Z, Maxson R, Maas R. *Msx2* deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet* 2000;24:391–395. [PubMed: 10742104]
- Sauer B. Inducible gene targeting in mice using the Cre/lox system. *Methods* 1998;14:381–392. [PubMed: 9608509]
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999;21:70–71. [PubMed: 9916792]
- Sprengel R, Hasan MT. Tetracycline-controlled genetic switches. *Handb Exp Pharmacol* 2007;1:49–72.
- Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* 2000;97:7963–7968. [PubMed: 10859354]
- Utomo AR, Nikitin AY, Lee WH. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat Biotechnol* 1999;17:1091–1096. [PubMed: 10545915]
- Wilkinson, DG. *In Situ Hybridization: A Practical Approach*. Oxford University Press; London: 1992.
- Yin Y, Lin C, Ma L. *MSX2* promotes vaginal epithelial differentiation and wolffian duct regression and dampens the vaginal response to diethylstilbestrol. *Mol Endocrinol* 2006;20:1535–1546. [PubMed: 16513791]

Fig. 1.

Construction of the *Msx2*-rtTA transgene. (a) Map of the pSV-Flp plasmid. rtTA and polyA sequence was cloned into the XhoI site, while 500 bp 5' *Msx2* promoter sequence and 500 bp of intron 1 sequence were inserted into the PmeI and Sall sites, respectively. The construct was then released by AscI-PmeI double-digestion. (b) The targeting construct was introduced into *E. Coli* cells containing the *Msx2* BAC and subjected to homologous recombination. *Msx2* coding region is located in the 187 kb BAC clone RP24-512K19 (AC154808) which contains approximately 134kb upstream and 47kb downstream genomic sequences. Arrows on the BAC schematic map indicate the position and orientation of putative genes predicted by GENSCAN. Primers used for transgene genotyping are also indicated. (c) PCR analysis using primers indicated in B showed a band of the expected size in transgenic animals.

Fig. 2.

Embryonic activity of the *Msx2*-rtTA;TetO-Cre system. Embryos carrying *Msx2*-rtTA, TetO-Cre and R26R alleles (hereafter referred to as trigenics) were stained for β -Gal activity. (a–c) Whole mount stained E12.5 trigenic animals exposed to Dox at different times. Expression in limbs, neural tube, mammary glands (arrow in b and inset), and craniofacial region was evident in all three embryos, whereas ventral ectodermal staining was detected only in those treated at E9.5 and E10.5 (arrow heads). (d–f) Close examination of forelimbs showed similar expression in E10.5- and E11.5-treated embryos, while E9.5-treated limbs had stronger expression in the zeugopod. Inset in d clearly showed AER staining in an E11.5 mouse forelimbs (9.5-treated). (g–i) E9.5-treated hindlimbs showed anterior restricted staining, while E10.5- and E11.5-

treated hindlimbs showed autopod LacZ activity. Inset in g clearly showed AER staining in E11.5 mouse hindlimbs (9.5-treated). (j–l) Sectioning of E13.5 hind limbs treated at different stages. E9.5 treatment led to anterior restricted staining in both digital and interdigital regions (j), E11.5 treatment led to global distal staining in both digital and interdigital regions (k), while E12.5 treatment led to expression in the interdigital cells (arrows in k), whereas the proximal part of the digits were not stained (arrowheads in l). In all panels anterior aspect is left. Bars in i–l represent 250um.

Fig. 3.

(a–c) Whole mount stained E12.5 genital tubercles (GT). (a) E9.5-treated embryos showed a more global LacZ expression pattern, whereas the most proximal-lateral part of the genital tubercle (GT) was LacZ-negative in E10.5-treated embryos (arrows in b). The expression domain of E11.5-treated GT was restricted more distally compared with that of the E10.5-treated GT (c). (d–f) Coronal sections of E10.5-treated E12.5 embryonic heads revealed expression in the maxilla, mandible, oral epithelium, vibrissa follicles, neural tissues, lens and upper neuronal retina (Arrows in d–f). (g–i) In situ hybridization analysis showing endogenous *Msx2* expression in the developing eye. At E9.5 *Msx2* is expressed in the lens surface ectoderm (arrowheads in g) and the dorsal aspect of the optic vesicle (arrows in g). As eye development

progresses, *Msx2* is detected in the invaginating lens placode (E10.0) (h) and the lens pit (E10.5) (i). *Msx2* is also expressed in the dorsal and ventral peri-optic mesenchyme from E9.5–10.5. (Dorsal aspect is up). (j–m) β -Gal stained E11.5 embryonic heart (treated on E9.5). (j, l) Whole mount staining showing β -Gal activity in the outflow tract and A–V cushions. Sectioning of stained heart showing both endocardial cushion expression (arrows in k, m) and myocardial expression (arrowheads in k, m). Bars in e–i, k and m represent 100 μ m. The bar in d represents 500 μ m.

Fig. 4.

Expression of transgene in female genital urinary tract. (a) Whole mount stained P6 female (Dox treatment on E16.5) urogenital system. (b–h) Cross section of the urogenital tract revealed homogeneous LacZ staining in the epithelium of the uterus (Utr) (d) and vagina (Vgn) (e); bladder (Bld) (g), ureter (Urt) (f) and urethra (Urth) (h). No staining was found in the ovary (Ov) (b) and oviduct (Ovd) (c). (i) A whole mount stained E16.5 embryo (Dox treatment on E14.5) showing exclusive hair follicle staining in the epidermis. (j, k) Indirect immunofluorescence using an anti-MSX2 polyclonal antibody. Clear nuclear staining (white arrowheads in j) was observed in wild-type bladder epithelium (j), but not in that of a littermate *Msx2*^{−/−} mutant. (l–n) Transgene expression in a three-month-old female 48 hours after Dox

treatment. (l) Strong *LacZ* expression was detected throughout the epidermal part of the hair follicle but not in the dermal papilla. (m) Strong β -Gal staining was observed in the bladder epithelium. (n) Weak β -Gal staining was detected in glandular epithelium of the uterus (arrow). Bars in b–h and j–n represent 50 μ m. The bar in l represents 100 μ m.

Fig. 5. Phenotype of transgenic line 888. (a) Shortened and curly vibrissae (arrow) and wavy body hairs were observed in transgenic animals. (b–e) Comparison between wild-type (b, d) and transgenic animals (c, e) showing truncated forelimbs (c) and interdigital webs (asterisks in e) in hindlimbs in the transgenics. (f, g) Nile blue analysis on E13.5 limbs revealed dead cells in wild-type interdigital region but not in transgenics (asterisks in g).