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Generation and Identification of Genetically Modified Mice for BMP Receptors

Jingwen Yang[1,2] and **Yuji Mishina**[1]

[1]Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, USA

[2]State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory for Oral Biomedicine of Ministry of Education (KLOBM) School and Hospital of Stomatology, Wuhan University, Wuhan, China

Abstract

BMP signaling is critical in embryogenesis and in the development of numerous tissues. Many genetically modified (knockout and transgenic) mice have been established to study BMP function in development and disease. Mice with altered BMP receptor genes (including global knockout, conditional knockout, and conditional constitutively active transgenic mouse lines) have been particularly informative. In this chapter, we describe how the genetically modified mice were generated and introduce genotyping methods. These methods include regular PCR and genomic real-time PCR using specific primers based on different constructs in different mice strains.

Keywords

Transgenic; BMP receptors; Regular PCR; Genomic real-time PCR; Primers

1 Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily. Similar to TGF-β, BMPs signal through type I and type II transmembrane serine/threonine kinase receptors. In response to the binding of BMP ligands, type I and type II BMP receptors form a membrane-bound heterotetrameric complex. Then, the constitutively active type II receptor transphosphorylates the type I receptor at a glycine-serine rich motif (GS domain). Consequently, the Smad signal transducers are phosphorylated, and the downstream signal is propagated [1–3]. There are three type I BMP receptors (BMPR-1A or ALK3, BMPR-1B or ALK6, ACVR-1 or ALK2) and three type II BMP receptors (BMPR-2, ACVR-2A, ACVR-2B). Both type I and type II receptors are required to form a heterotetrameric complex for BMP signal transduction [1–3]. However, the mechanism of the heterotetrameric signaling complex formation can vary. For example, BMP-2 and BMP4 interact with type I receptors and recruit type II receptors, whereas BMP6 and BMP7 preferentially bind to type II receptors and recruit type I receptors [4]. More importantly, the binding of a BMP ligand to preformed receptor

complexes activate signaling pathways that differ from those activated by a receptor complex whose assembly was stimulated by BMP binding [5].

Genetic studies into the function of these complex receptors are essential for clarifying the role of BMP signaling in development and disease. As of now, several genetically modified BMP receptor mice have been established, including global knockout (KO), conditional knockout (cKO), and conditional constitutively active (ca) mice [6–17]. BMP receptor gene modifications were observed to result in embryonic lethality [18, 19] or abnormalities in many tissues, including the skeleton [20–22], craniofacial [23, 29], heart [24], vascular [25], lung [26], eye [27], and tooth [28], thus establishing that BMP signaling is critical for normal embryogenesis.

In this chapter, we first describe how the genetically modified mice were generated and then introduce genotyping methods. These methods include regular PCR and genomic real-time PCR using specific primers based on different constructs in different mice strains.

2 Materials

Unless otherwise noted, all solutions are prepared in water purified by double distillation or other methods.

2.1 Regular PCR

1. Lysis buffer: 100 mM NaCl, 1 mM Tris pH 7–8, 0.1 mM EDTA, 0.1% Triton X-100 in distilled water. Store at room temperature. Add 1/50 volume of 40 mg/mL proteinase K (0.8 mg/mL final concentration) immediately before use.
2. 40 mg/mL proteinase K.
3. PCR oil.
4. Primers for genotyping of each receptor mutations are listed in Table 1. The positions of the primers are marked in the maps of targeted alleles shown in Figs. 1 and 2.
5. 0.5 unit/μL Taq DNA polymerase.
6. 1× Taq buffer: 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl2, pH 8.3.
7. 5 mM dNTP mix: 5 mM each of dATP, dCTP, dGTP, and dTTP.
8. 25 mM $MgCl_2$.
9. Thermo cycler.
10. 10× Tris/Borate/EDTA (TBE) buffer: 1 M Tris, 0.9 M boric acid, and 0.01 M EDTA. Dilute 100 mL 10× TBE buffer in 900 mL water to make 1000 mL 1× TBE for agarose gel electrophoresis.
11. 3% agarose gel prepared in 1× TBE.
12. 10 mg/mL ethidium bromide stock.

2.2. Genomic Real-Time PCR

1. TaqMan® Universal PCR Master Mix (Thermo Fisher, Cat: 4334437).
2. TaqMan primer sets of each receptor mutations are listed in Table 2.
3. Optical 96-well reaction plates compatible with your PCR machine.
4. Optical adhesive film.
5. Real-time PCR system.

3 Methods

3.1 Regular PCR

1. Collect small piece (less than 1 mm^3) of tissues from the ear (ear notch), tail, or any organs. For embryos, the yolk sac or amniotic membrane may be used (do not use the placenta for genotyping). Place tissues into 96-format PCR tubes (do not cap).
2. Add 50 μL lysis buffer in each tube, overlaid with PCR oil.
3. Incubate at 55 °C for 6 h or more, then incubate at 85 °C for 30 min to inactivate proteinase K.
4. Take 4 μL DNA solution to mix with 76 μL of water to dilute samples, then use 4 μL of the diluted samples to set up 10 μL PCR reaction. Reaction mixture will be made as follows:

10× Taq buffer	1.0 μL
5 mM dNTP mix	1.0 μL
0.5 unit/μL Taq DNA polymerase	1.5 μL
25 mM $MgCl_2$	1.5 μL
50 μM 5′ primer (Table 1)	0.1 μL
50 μM 3′ primer (Table 1)	0.1 μL
Water	0.8 μL
Template DNA (20× diluted)	4.0 μL

The conditions for thermal cycling are as follows (*see* Note 1):

Initial denaturation for 94 °C for 5 min followed by 30 to 40 cycles of denaturation at 94 °C for 30 s, annealing at 50–70 °C for 30 s, and extension at 72 °C for 1 min (30–40 cycles), then ending with 72 °C for 5–10 min followed by a cool down.

1. Samples for which genotypes are known should be used as controls. For these primers and most others, these conditions work adequately. If not, try the following: (1) change the dilution of template DNA. The reaction will not work when the DNA concentration is too high; (2) optimize the annealing temperature; (3) change Taq DNA polymerase to Taq hot start DNA polymerase; or (4) purify DNA further.

The number of cycles and annealing temperatures for different primer sets is shown in Table 1.

5. Run 3% agarose gel at 250–300 V, stain the gel with ethidium bromide, and photograph.

3.2 Genomic Real-Time PCR for In Vivo Deletion Efficiency

The in vivo deletion efficiency of conditional knockout by Cre-recombinase and other recombinases can vary. This protocol describes the quantification of *Alk2* or *Alk3* deletion in conditional knockout mice by genomic real-time PCR using custom-designed primer set [30, 31]. 5′ primers and 3′ primers are designed to amply the flox regions (exon 7 for *Alk2*, exon 4 for *Alk3*). FAM-labeled probes are designed within the PCR amplicons for detection (*see* Table 2).

1. Extract genomic DNA as in of Subheading 3.1, **steps 1–3**.
2. Mix the Gene Expression Master Mix thoroughly by swirling the bottle. Thaw Alk2 or Alk3 frozen primer set (Table 2) and templates DNA on ice. When thawed, vortex and then centrifuge the tubes briefly (*see* Note 2).
3. Prepare the PCR reaction mix (20 μL reactions):

TaqMan PCR Master Mix (2×)	10 μL
Primer set (20×)	1 μL
Template DNA (20x diluted)	5 μL
Water	4 μL

Perform three replicates of each reaction. Then vortex the tubes briefly to mix the solutions, centrifuge the tubes briefly to spin down the contents, and eliminate any air bubbles from the solutions.

4. Transfer 20 μL of each reaction mixture to each well of an optical plate.
5. Cover the plate with an optical adhesive film. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.
6. Run the plate on a real-time PCR instrument using the following thermal cycling conditions: initial incubation at 50 °C for 2 min then denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min, then ending with a cool down.
7. Determine the threshold cycles (CT) for the amplification curves. Use the comparative CT method to analyze target gene levels normalized to *Gapdh* level as described by the manufacturer of the instrument.

2. Protect all reagents from light in the freezer until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.

3.3 Genomic Real-Time PCR to Determine Homozygosity vs. Heterozygosity of Conditional Constitutively Active Transgenes

To generate conditional constitutively active transgenic mice, the EGFP cassette was inserted into the constructs (Fig. 2d) [10]. Therefore, the copy number of target genes can be quantified based on the copy number of *Egfp* via real-time PCR. This is a common strategy for the caALK2, caALK3, and caAlk6 mouse lines. The *Egfp* primer set is shown in Table 2.

1. Same as Subheading 3.2, **steps 1** and **2**.
2. Prepare the PCR reaction mix (20 μL reactions):

TaqMan PCR Master Mix (2×)	10 μL
Egfp primer set (20×)	1 μL
Template DNA (20× diluted)	5 μL
Water	4 μL

Perform three replicates of each reaction. Then vortex the tubes briefly to mix the solutions, centrifuge the tubes briefly to spin down the contents, and eliminate any air bubbles from the solutions. Wild-type, *caAlk* het, and *caAlk* homo samples, which genotypes are known, should be used as controls.

3. Same as Subheading 3.2, **steps 4–7** (*see* Note 3).

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3.The calculated copy number of the *Egfp* in *caAlk* homozygous mice should be about twice as that of heterozygous mice. Homozygosity also can be genetically confirmed by crossing the mice to wild-type mice. All F1 pups should carry the transgene.

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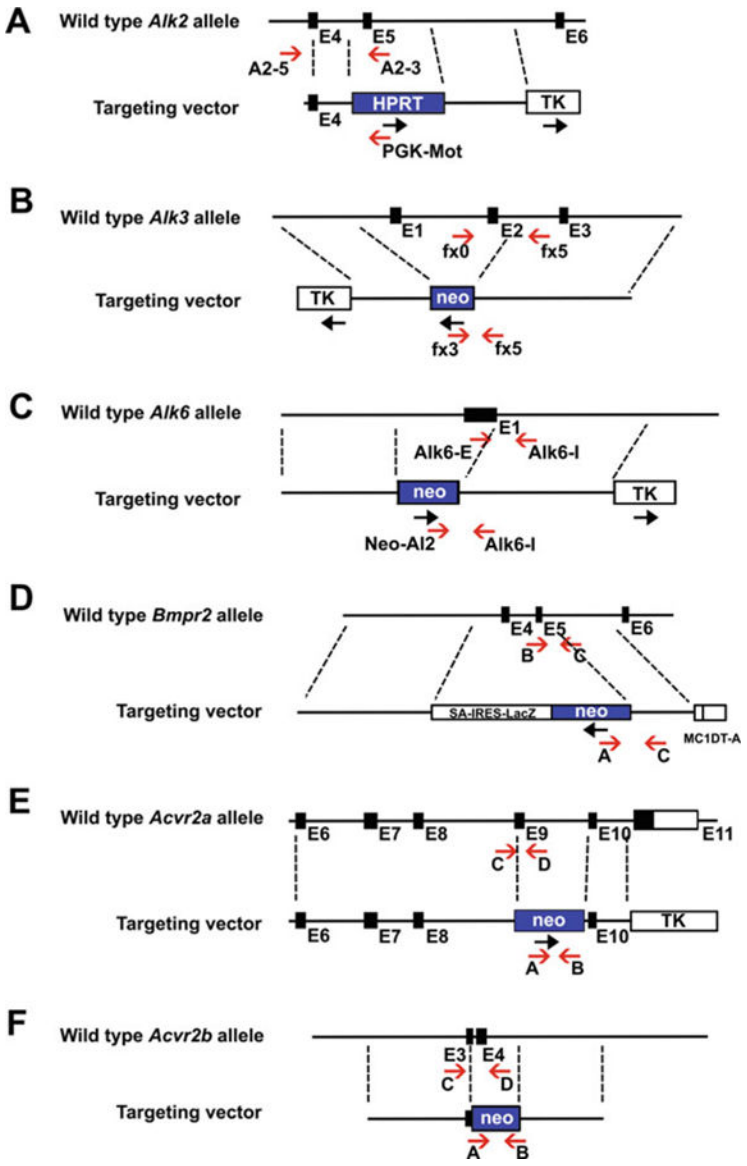

Fig. 1.

Structure representation of each BMP receptor KO mutation and PCR genotyping strategy. Schematic diagrams showing the wild-type locus of the *Alk2* (**a**), *Alk3* (**b**), *Alk6* (**c**), *Bmpr2* (**d**), *Acvr2a* (**e**), and *Acvr2b* (**f**) gene, and the targeting vector for each gene. The positions (red arrows) of PCR primers for genotyping are indicated below the locus

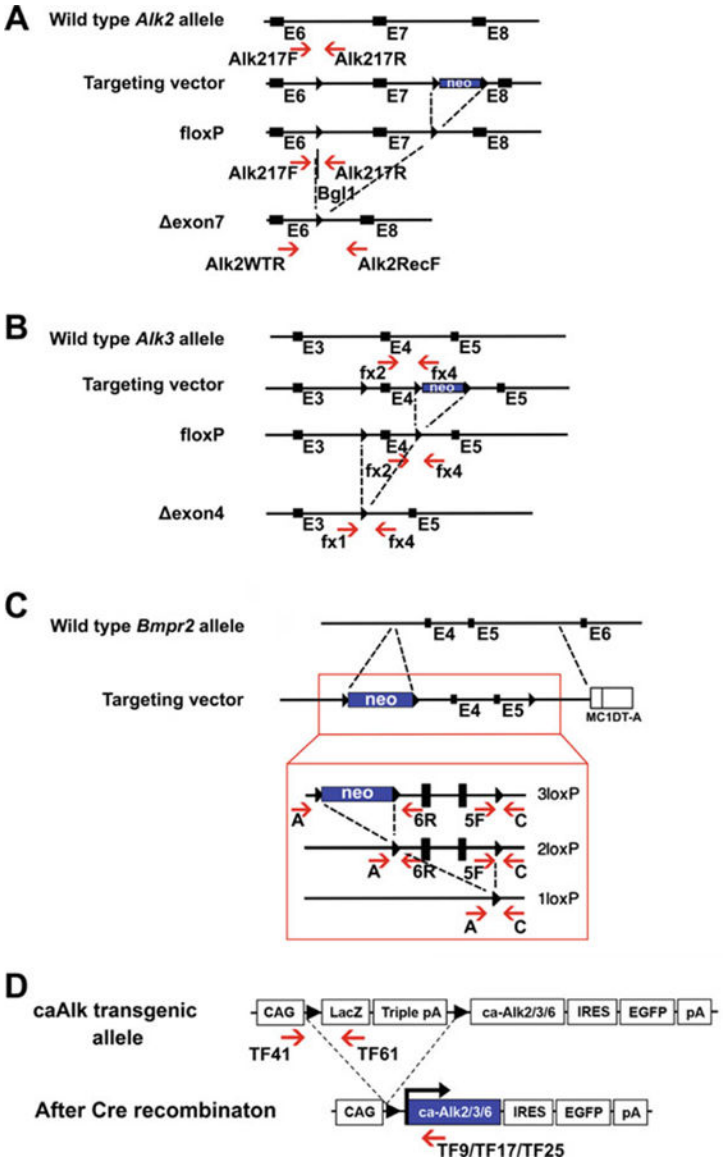

Fig. 2.

Structure representation of each BMP receptor cKO mutation and ca transgene and PCR genotyping strategy. (**a–c**) Schematic diagrams of the wild-type *Alk2* (**a**), *Alk3* (**b**), and *Bmpr2* (**c**) locus, targeting vector, and mutant alleles after recombination. The positions of primers for genotyping by PCR are indicated by red arrows. (**d**) Schematic representation of the ca transgene of *Alk2*, *Alk3*, or *Alk6*. The primers used for genotyping are shown by red arrows below the locus

Table 1

Primers used for genotyping of different BMP receptors genetically modified mice

	Ref	Modified gene	Primers	Primer ratios	PCR products	Annealing
Global knockout mutation						
Fig. 1a	[6]	*Alk2* null	A2–5 5′-ATGCTAGACCTGGGCAGCCATA-3′ PGK-Mot 5′-CGTGTGTAAGGTGTAGGTG GCC-3′	A2–5:PGK-Mot: A2–3 = 2:1:1	195 bp (*Alk2* null)	65 °C, 40 cycles
			A2–5 5′-ATGCTAGACCTGGGCAGCCATA-3′ A2–3 5′-CATGCTAGCAGCTCGGAGAAAC-3′		371 bp (wild type)	
Fig. 1b	[18]	*Alk3* null	fx3 5′-AGACTGCCTTGGGAAAAGCGC-3′ fx5 3′-GGACTATGGACACACAATGGC-3′	fx3:fx5:fx0 = 1:2:1	190 bp (*Alk3* null)	65 °C, 40 cycles
			fx5 5′-GGACTATGGACACACAATGGC-3′ fx0 5′-CTCTGAATTTCTAGTCCACATCTGC-3′		280 bp (wild type)	
Fig. 1c	[7]	*Alk6* null	Alk6-I 5′-TGGTGAGTGGTTACAACAAGATC AGCA-3′	Neo-Al2: Alk6-I: Alk6-E = 1:2:1	300 bp (*Alk6* null)	65 °C, 40 cycles
			Neo-Al2 5′-GAAAGAACCAGCTGGGGCTC GAG-3′			
			Alk6-I 5′-TGGTGAGTGGTTACAACAAGATC AGCA-3′		350 bp (wild type)	
			Alk6-E 5′-CTCGGCCCAAGATCCTACGTTG-3′			
Fig. 1d	[14]	*Bmpr2* null	A 5′-GCTAAAGCGCATGCTCCAGACTGCC TT-3′	Primer A:B:C = 2:1:1	260 bp (*Bmpr2* null)	
			C 5′-AGGTTGGCCTGGAACCTGAGGAAATC-3′			
			A 5′-GCTAAAGCGCATGCTCCAGACTGCC TTG-3′		200 bp (wild type)	
			B 5′-TCACAGCATGAACATGATGGAGGCGG-3′			
Fig. 1e	[16, 32]	*Acvr2a* null	A 5′-TGGGAAGACAATAGCAGGCATGC-3′	1:1:1:1	900 bp (*Acvr2A* null)	70 °C, 30 cycles
			B 5′-GCAGAGTGTGACCCGTACCCAC-3′			
			C 5′-GTTGGTACCCGGAGGTATATGGC-3′		140 bp (wild type)	
			D 5′-CCCTTACCATCTGCAGCAGTGCA-3′			
		Acvr2b null	A 5′-ATGAACTGCAGGACGAGGCAGCG-3′	1:1:1:1	600 bp (*Acvr2B* null)	
			B 5′-GGCGATAGAAGGCGATGCGCTG-3′			
			C 5′-CCGACAGCCCCCACCCTGCTCA-3′		241 bp (wild type)	
			D 5′-GGCCCACCAGAGGGGATGGGGG-3′			
Conditional knockout mutation						
Fig. 2a	[29]	*Alk2* flox	Alk217F 5′-CCCCCATTGAAGGTTTAGAG AGAC-3′	Alk217F: lk217R2 = 1:1	160 + 90 bp (flox) (*see* Note 4), 250 bp (wild type)	65 °C, 40 cycles
			Alk217R2 3′-CTAAGAGCCATGACAGA GGTTG-3′			

	Ref	Modified gene	Primers	Primer ratios	PCR products	Annealing
		Alk2-recombined	Alk2RecF 5′-GAATTGCTAGAAGCCCATA GGC-3′	Alk2RecF:Alk2WTR: Alk217F = 1:2:1	625 bp (recombined)	60 °C, 40 cycles
			Alk2WTR 5′-TGAGATTGTTCTAGCACTGC CC-3′			
			Alk217F 5′-CCCCCATTGAAGGTTTAGAGA GAC-3′		530 bp (wild type)	
			Alk2WTR 5′-TGAGATTGTTCTAGCACTGC CC-3′			
Fig. 2b	[8]	*Alk3* flox	fx2 5′-GCAGCTGCTGCTGCAGCCTCC-3′	fx2:fx4 = 1:1	230 bp (flox)	65 °C, 40 cycles
			fx4 3′-TGGCTACAATTTGTCTCATGC-3′		150 bp (wild type)	
Fig. 2b		*Alk3*-recombined (*see* Note 6)	fx1 5′-GGTTTGGATCTTAACCTTAGG-3′	fx1:fx4:BMP2-A: BMP2-B = 1:1:1:1	180 bp (recombined)	55 °C, 40 cycles
			fx4 5′-TGGCTACAATTTGTCTCATGC-3′			
			BMP2-A 5′-AGCATGAACCCTCATGTGTT GG-3′		322 bp (wild type)	
			BMP2-B 5′-GTGACATTAGGCTGCT GTAGCA-3′			
Fig. 2c	[15]	*Bmpr2* flox	5F 50-GGCAGACTCTGACTTTGACGCTAG-3′		315 bp (2loxP and 3loxP)	60 °C, 40 cycles
			C 5′-TTATTGTAAGTACACTGTTGCTGTC-3′			
			A 5′-CACACCAGCCTTATACTCTAG ATAC-3′		260 bp (wild type)	
			6R 5′-CACATATCTGTTATGAAACTTGAG-3′		350 bp (2loxP)	
			A 5′-CACACCAGCCTTATACTCTAG ATAC-3′		270 bp (wild type)	
			C 5′-TTATTGTAAGTACACTGTTGCTGTC-3′		500 bp (1loxP)	
Conditional constitutively active transgenic line						
Before recombination						
Fig. 2d	[12]	*caAlk*	TF41 5′-GTGCTGGTTATTGTGCTGTCTC-3′	TF41:TF61:LbnFR1: LbnRev3 = 1:1:1:1	580 bp (*caAlk* transgene)	65 °C, 40 cycles
			TF61 5′-GACGACAGTATCGGCCTCAGGAA-3′			
			LbnFR1 5′-GAGGACGCAGTCCAGTACCT-3′		334 bp (Internal control)	
			LbnRev3 5′-TAGCCTCTGCCTCACGCCCT GC-3′			
After recombination						
Fig. 2d	[11]	*caAlk2*	TF41 (common) 5′-GTGCTGGTTATTGTG CTGTCTC-3′	TF41:TF9 = 1:1	750 bp (*caAlk2* transgene)	65 °C, 40 cycles
			TF9 (Alk2) 5′-CGAACACTACAGAGAGAAT AATG-3′			
	[12]	*caAlk3*	TF41 (common) 5′-GTGCTGGTTATTGTGC TGTCTC-3′	TF41:TF17 = 1:1	300 bp (*caAlk3* transgene)	
			TF17 (Alk3) 5′-CGGCGTAGCTGGGCTTTT GGA-3′			
		caAlk6	TF41 (common) 5′-GTGCTGGTTATTGTGC TGTCTC-3′	TF41:TF25 = 1:1	300 bp (*caAlk6* transgene)	

Ref	Modified gene	Primers	Primer ratios	PCR products	Annealing
		TF25 (Alk6) 5′-GACATCCAGAGGTGACAA CAG-3′			

4. PCR amplification generates a 250 bp from both flox and wild-type alleles. Bgl1 digestion, which uniquely digests the flox gene product into 160 bp and 90 bp fragments, can be used to distinguish flox and wild-type PCR products.

6. The primers BMP2-A and BMP2-B are for an internal control at the *Bmp2* locus. Please refer reference [33] for the specific positions of those primers.

Table 2

TaqMan primers used for genomic real-time PCR of different BMP receptors genetically modified mice

Ref	Modified gene	Primers
Conditional knockout mutation		
[31]	*Alk2* (exon 7) (*see* Note 5)	AIKAL5S_F 5′-CTCACTACTCTGGATACGGTTAGCT _3′, AIKAL5S_R 5′-GGGTCCCAAATATCTCTATGTGCAA-3′, AIKAL5S_M FAM 5′-CTATGGACAGTACAATCCG-3′
[30, 31]	*Alk3* (exon 4) (*see* Note 5)	AI89LJ8_F 5′-GACCAGAAGAAGCCAGAAAATGGA-3′, AI89LJ8_R 5′-TGTCCTGAGCAATAGCACTTTAAGAA-3′, AI89LJ8_M FAM 5′-CCTCTGGTGCTA AAGTC-3′
Conditional constitutively active transgenic line		
	Egfp (*see* Note 5)	5′-GAGCGCACCATCTTCTTCAAG-3′, 5′-TGTCGCCCTCGAACTTCAC-3′, FAM 5′-ACGACGGCAACTACA-3′

5.All three primers are pre-mixed into one tube by the manufacturer.

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