# Characterization of mesenchymal-fibroblast cells using the Col1a2 promoter/enhancer

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Abstract

Excessive deposition of extracellular matrix (ECM) is a common hallmark of fibrotic diseases in various organs [1]. Chiefly amongst this ECM are collagen types I and III, secreted by local fibroblasts, and other mesenchymal cells recruited for repair purposes. In the last two decades, the search for a fibroblast-specific promoter/enhancer had intensified in order to control the regulation of ECM in these cells and limit the scarring of the fibrotic process. In our previous work, we characterized an enhancer region 17kb upstream of the *Col1a2* gene transcription start site. This enhancer in transgenic mice is expressed mainly in mesenchymal cells during development and in adults upon injury. When driving beta-galactosidase or luciferase, this construct acts as an informative reporter of collagen transcription and predictive of collagen type I deposition. In this chapter, we provide detailed protocols for identifying similar enhancers, use the sequence to generate a construct for, transfection and generation of transgenic animals. We also provided information on the use of luminescence in transgenic mice, tissue processing as well as using cre/lox system to obtain conditional gain and loss of function in mice.

Key words

Collagen type I, mesenchymal cells, transfection, enhancer, transgenic mice, transgene.

Running head

The Col1a2 promoter/enhancer

## Introduction

 The origin of scar-producing cell(s) in tissues appeared to be different depending on the tissue involved and the experimental injury model. In general, myofibroblasts are thought to be the cell type that generates and deposits collagen-I and collagen-III rich pathological extracellular matrix (ECM) leading to irreversible fibrosis and causing organ dysfunction. Antibodies against the intermediate filament alpha-smooth muscle actin (α-SMA) have been widely used as a marker for collagen producing myofibroblasts, and other less-widely accepted markers such as the transcription factor S100A4 or fibroblast-specific protein (FSP) as it was termed, specifically in kidney, [2], as well as vimentin [3] have been reported to label myofibroblasts. However none of these markers were unique to fibroblasts or myofibroblasts as they are expressed on many other cell types [4]. Furthermore, α-SMA is not necessary for myofibroblast formation and function and for wound closure as other filaments such as γ-actin and skeletal muscle α-actin may be able to functionally compensate for the lack of α-SMA in myofibroblasts [5].

 One unique characteristic of fibroblasts is that they synthesize and secrete a number of ECM molecules including collagen type I, a fibril-forming collagen. Collagen type I is a heterotrimer formed from two α1-chains and one α2-chain. These chains are synthesized as precursor molecules termed pro-α1(I) and pro-α2(I). The genetic sequences coding for these precursors have been found to be located on separate chromosomes for a number of species. Our approach was to look for a fibroblasts-specific element within the collagen type I gene regulatory elements since collagen is the terminal differentiating marker of fibroblasts. However, collagen type I is expressed in many other cell types such as osteoblasts [6,7], smooth muscle cells [8], odontoblasts [9], tenocytes [10], etc.

 Starting with DNase I hypersensitivity assay, we showed that separate cis-acting elements in the murine *Col1a1* gene drive expression in different cell lineages [9]. Similarly, we identified hypersensitive sites (HSs) in of the mouse *Col1a2* gene and a cluster of three hypersensitive sites some 17 kb upstream of the transcription start site are found to be associated with an enhancer that expresses mainly in mesenchymal cells during development [11] (**Figure 1A**). This region was later found to be similar in humans but was shifted further towards the 5’ end at -21 kb [12]. Other HSs were found in *Col1a1* [13] (shown as HS4 and 5 in **Figure 1B**) Transgenic mice carrying a GFP transgene were used to show that these elements control the expression pattern including hepatic stellate cells [14], and kidney fibroblasts [15].

 The *Col1a2* 17 kb driving beta-galactosidase and luciferase [11] was shown to be an informative reporter of collagen transcription and predictive of collagen deposition in development. More importantly, when collagen type I is normally downregulated in adult fibroblasts, it is reactivated by this enhancer in adult tissue following injury [16]. We have used this mouse as a read out for collagen transcription and therefore fibrosis to understand the pathways involved [17,18]. Furthermore, we used transgenes to track bone marrow cells *in vivo* after transplantation into recipient mice to show the extent of their participation in repair or fibrosis in injury models [19-21]. Similarly, we tracked fetal cells during gestation and post pregnancy to document the cell types actively transcribing collagen type I genes in fibrotic models or in response to injury [22-25] (**Figure 2**).

 In order to identify the shortest element capable of driving this enhancer, we deleted some sequences under HS5 and found that a 1.5 kb between -17.0 and -15.5 kb in the mouse [26] is the shortest sequence that is functional. The expression of a reporter in transgenic mice starts at E10.5, in aorta and pericardium, heart valve and near otic region in the head. As organogenesis expands, the transgene is seen in many tissues including and strongest in the developing dermis. The epidermis is always devoid of expression. By E15.5, the meninges surrounding the brain and along the spinal cord are positive, the developing soft tissues such as lung, kidney, spleen and most but not all skeletal muscles interstitial cells expressed the transgene. In addition, the bladder wall and muscular layers of stomach and intestines show expression of the transgene. The hard tissues, including long bones do not express, the only osteoblasts that show expression belong to the intramembranous type, including the calvaria, mandible and clavicles, as well as some chondrocytes in developing ribs show staining but to a lesser extent than other mesenchymal cells. Importantly, we have shown that in all of the data outlined above, the transgene expression mirrors the *in situ* hybridization results of the *col1* gene [16].

 In adult mice, the expression is significantly diminished but injury re-activates the expression [16]. In particular, several kidney injury models have shown by in situ hybridization that the epithelial cells of the tubular network transcribe collagen type I genes early on after injury. This was confirmed by transgene expression [16], by in situ hybridization of the transgene compared with the endogenous gene [21], and further by mRNA levels of cells using laser capture technique [27] suggesting that these cells contribute *in situ* to deposition of collagen in fibrosis.

 In addition, the enhancer was used to drive expression of other genes to produce models of fibrosis. For example overexpression of CCN2 using this enhancer showed clear fibrosis in skin, lung and kidney but this was concentration dependent as only homozygote mice showed a phenotype [28]. Similarly this enhancer was used to overexpress transforming growth factor (TGF)-β receptors to generate other fibrotic models [29-31] [32] and also to generate a skin model for dystrophic epidermolysis bullosa [33].

 We originally generated an inducible cre fused to a modified estrogen receptor that responds to tamoxifen and not estrogen (Cre ERT), using 6 kb enhancer -19.5 to -13.5 kb [34]. Another non-inducible Cre mouse was created using a large (100 kb) *Col1a2* gene [35]. Having established the minimal sequence that drives mesenchymal cells, we generated a 1.5 kb enhancer driving tamoxifen inducible cre recombinase (Cre ERT2). Lineage tracing experiments can be accomplished using this *Col1a2 creERT2*mouse mated with mice harboring a reporter gene preceded by a stop signal flanked by LOXP sites. **Figure 3** shows soft tissues from a dual fluorescent proteins reporters B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (Jax Stock No: 007676) [36]. The mice were given tamoxifen at 6 weeks of age and sacrificed at 12 weeks and we looked at the cells expressing collagen type I. As we have seen before during development, the floxed cells express EGFP in fibroblasts of the interstitium, around blood vessels of many soft tissues and in the eye. We describe below our current established protocols for vector cloning, transfection, generation of transgenic animals, and tissue processing and analysis.

## Materials

### Cloning

1. High purity genomic DNA as template
2. Nuclease-free water
3. Tris-EDTA (TE) buffer
4. pβgal-Basic vector (Clontech)
5. HSP68-LacZ-Gateway™ vector (Addgene plasmid #37843)
6. Luciferase based vector (we use the pgl4.10[*luc*] vector from Promega)
7. Taq polymerase
8. Platinum™ Pfx DNA polymerase
9. Thermal cycler
10. Monarch® PCR & DNA clean up kit (NEB) or similar kit
11. QIAquick gel extraction kit (Qiagen) or similar
12. pCR™8/GW/TOPO® TA cloning kit (Invitrogen, K2500-20)
13. 50 μg/ml streptomycin-containing LB-agar plates
14. 100 μg/ml ampicillin-containing LB-agar plates
15. Gateway™ LR Clonase II enzyme mix (Life Technologies, 11791-020)
16. rSAP or Antarctic phosphatase
17. lambda DNA-HindIII ladder
18. UV transilluminator with a wavelength of 312 nm
19. T4 DNA ligase
20. T4 polynucleotide kinase
21. TOP10 Chemically Competent E.coli (we use the One Shot® format from Invitrogen)
22. JM109 Competent cells
23. Ultra-pure water for embryo transfer
24. PVDF 0.22 μm centrifuge filters
25. Inducible Cre plasmid pCreERT2 [37]
26. Restriction enzymes
27. Bacterial shaking incubator

### In vitro analysis of enhancer regions

1. High glucose (4,500 mg/l) DMEM
2. Complete DMEM: high glucose DMEM supplemented with L-Glutamine, 10% fetal bovine serum (FBS), 100 IU/ penicillin and 100 μg/ml streptomycin. Alternatively use complete media for the specific cell type of interest
3. Opti-MEM® Medium (Gibco)
4. Lipofectamine 2000 (Invitrogen)
5. Renilla luciferase control vector (we use pRL-TK from Promega)
6. Dual- Luciferase® Reporter Assay system (Promega)
7. 48 well clear bottom tissue culture treated plates
8. Cell culture material (biosafety cabinet, cell culture incubator, sterile pipettes, etc.)
9. White 96-well plate that can be used in luminometers
10. Luminometer that accommodates 96-well plates (we use the Promega Glomax®-Multi Detection system)

### ß-Galactosidase staining

1. 1M sodium phosphate (NaPi) buffer pH 7.4:
	1. Dissolve 138g of sodium dihydrogen phosphate (NaH2PO4 –H2O) in 1 liter of ddH2O and adjust pH to 7.4;
	2. Dissolve 142g sodium phosphate dibasic (Na2HPO4) in 1 liter of ddH2O, and adjust pH to 7.4.
	3. Mix 423 ml of NaH2PO4 –H2O and 577 ml of Na2HPO4 to make 1 l of 1 M NaPi buffer.
2. X-gal Fixative (FIX solution): 0.2% (v/v) glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.3), 5 mM Ethylene glycol-bis(2-aminoethylether)-*N, N, N’, N’*- tetraacetic acid (pH 8.0), 2 mM MgCl2 and 2% (v/v) formalin.
3. Rinse solution: 0.1 M sodium phosphate buffer, 2 mM MgCl2, 0.1% (w/v) sodium deoxycholate, 0.2% (w/v) NP40 (or substitute).
4. STAIN solution: 1 mg/ml 5-bromo-4-chloro-3-indolyo-β-D-galactoside (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1% sodium deoxycholate, 0.2% (w/v) Nonidet P-40, 2 mM MgCl2 and 0.1 M NaPi buffer. Add the X-gal fresh before use (stored in the dark).
5. 10% (v/v) neutral buffered formalin: 100 ml 37-40% (v/v) formaldehyde, 4 g sodium phosphate monobasic, 6.5 g sodium phosphate diabasic anhydrous in 1 liter of water.
6. 1% (w/v) potassium hydroxide (KOH) in water
7. 100% (w/v) glycerol
8. Tube rotator that can hold 15ml and 50ml centrifuge tubes

### In vitro ß-Galactosidase staining

1. 1X Phosphate-buffered saline
2. 4% paraformaldehyde
3. STAIN solution with X-gal substrate at 1mg/ml (from section 2.3.4)
4. Foil
5. Laboratory rocker or 37°C incubator

### Processing of stained tissue

1. Ethanol
2. Xylene
3. Paraffin wax for histology
4. Tissue processor (such as from TP1020 Leica Biosystems)

### In vivo bioluminescent imaging

1. 150mg/kg ᴅ-luciferin in PBS
2. 30G x 8mm needle + syringes
3. 2.5% isoflurane
4. In vivo imager (we use the IVIS spectrum in vivo imager system from Perkin-Elmer)

## Methods

### Identification of a specific enhancer in a given gene

 In the 1990’s when we first investigated enhancers in *Col1a2*, we knew that enhancers are able to act independently of distance and orientation in relation to their target gene and are generally highly conserved in evolution. DNaseI hypersensitive sites were one of few indicative experiments for possible enhancer sites, since areas of active enhancers are generally void of nucleosomes allowing these DNA regions to be accessible to DNaseI. Such sites therefore coincide with DNaseI hypersensitivity which can be used as a part of a criterion to identify possible enhancers [38]. Today, we know that enhancers are marked by certain histone modifications to show a permissive chromatin such as acetylation of the 3rd histone at the 27th lysine (H3K27ac) and H3K4me1 [39,40]. Most importantly, if you are starting fresh, we would recommend that you start with the Encyclopedia of DNA Elements project (ENCODE), which provides publically available data sets on functional elements of the genome:

1. Open the University of California at Santa Cruz genome browser webpage (<https://genome.ucsc.edu/>) [41].
2. Under “Genomes” in the upper left part of the page and select the the mouse assembly July 2007 mm9 (NCBI37/mm9) and search for Col1a2 as target gene in the box that reads “enter position, gene symbol or search terms.”
3. In the tab “Expression and Regulation” which is the 7th tab down the page, click ChIP-Seq from the ENCODE Ludwig Institute for Cancer Research (ENCODE/LICR) project track data and select the epigenetic signatures H3K4me1, H3K27ac, H3K4me3 in E14.5 mouse limb and in mouse embryonic fibroblasts (MEFs) (**Figure 4**).
4. Select the DNaseI hypersensitivity in E11.5 mouse fibroblasts and the immortalized mouse fibroblast cell line (NIH3T3) from the ENCODE project University of Washington (UW DNaseI HS) (**Figure 4)**.
5. Ensure the conservation throughout vertebrate genomes is selected, this can be found in the 8th tab of the genome browser under “comparative Genomics” (you can pick the genomes of particular interest by clicking on the tabs).
6. Look for regions that have high conservation, good histone peaks and DNase1 peaks, you can use other tracks as exclusion criteria’s but generally, these marks are good indicators for enhancers without needing to conduct ChIP-seq or DNaseI hypersensitivity experiments **(Figure 5)**.
7. Identify these sequences with a 100bp extra nucleotides from both side, and clone them out in an expression vector for in vitro and in vivo analysis as described in section 3.2.

### Cloning for in vivo and in vitro analysis and inducible systems

#### Producing the insert by PCR

1. Design primers for PCR that are ±100 bp from the regions identified in the ENCODE, ChIP or DNase1 analysis (*see* **Note 1**).
2. Resuspend primers in nuclease-free water or TE buffer to a concentration of 100 pmol/μl to create a stock and then used in a 1 in 10 dilution in nuclease-free water.
3. Amplify DNA fragments by PCR from high purity genomic DNA (for mouse Promega, G3091), cosmids or genomic clones. There are two methods used in this lab to generate plasmids for examining enhancer function *in vivo*: cloning the minimal promoter and enhancer directly in the pβgal-Basic, or just the enhancer into the HSP68-*LacZ*-Gateway [42]. For *in vitro* analysis enhancers and minimal promoters are cloned into the pGl4.10-*luc* (but other luciferase based vectors can be used). The pβgal-Basic and luciferase vectors are cloned using standard cloning methods (*see* **Notes 2 and 3**) while the HSP68 vector is cloned using Gateway™ (*see* **Note 4 and 5**).
4. All PCR reactions should be verified by running 5 μl on 1% agarose gel electrophoresis.
5. After verification clean-up the remaining PCR reaction using the Monarch® PCR & DNA clean up kit (*see* **Note 6**).
6. Clone PCR fragment into vector as described in section 3.2.2 “Gateway cloning” or 3.2.3 “standard cloning”.
7. Once cloned into the vector it is advised to perform sequencing to verify the inserted regions.

#### Gateway cloning

 Fragments are first inserted into the pCR™8/GW/TOPO® vector using the pCR™8/GW/TOPO® TA cloning kit according to manufacturer’s instructions as follows.

1. Place 2 μl of DNA freshly extracted DNA product, 1 μl salt solution, 0.5 μl TOPO® vector with 2.5 μl dH2O in a 1.5 ml centrifuge tube, touch down centrifuge to ensure all reagents are together and then incubate at room temperature for 30 minutes for short fragments of up to 1.5 kb, and 1 hour for fragments longer than 1.5 kb.
2. Transform 4 μl of the TOPO® reaction into TOP10 E.Coli bacteria and incubate overnight at 37 °C on streptomycin LB- agar plates. Include a negative control and the pCR™8/GW/TOPO positive control that is provided in the kit.
3. The following day select colonies and perform colony PCR. Colonies that contain the insert can then be used to inoculate starter cultures for minipreps. Cloning success and correct orientation of insertion should be checked and verified by restriction enzyme digestion and Sanger sequencing.

 The enhancer sequences are transferred from the pCR8/GW/enhancer entry vector to the HSP68/LacZ/Gateway® destination vector using Gateway LR Clonase II Enzyme mix.

1. Set up a reaction of 1 μl of HSP68/LacZ/Gateway® (37ng/μl) with 1 μl of the TOPO entry vector (37ng/μl) in a 1.5 ml centrifuge tube.
2. Thaw the LR Clonase II enzyme for 2 minutes on ice then vortex for 2 seconds twice.
3. Add 0.5 μl of LR Clonase II to the reaction mix, mix and then incubate at room temperature for 1 hour or up to 16 hours for larger fragments.
4. Stop the reaction by the addition of 0.25 μl of 2 μg/μl Proteinase K solution and incubate at 37 °C in a water bath for 15 minutes.
5. Transform the entire reaction into TOP10 chemically competent E.coli bacteria and plate onto ampicillin selection plates. Incubate at 37 °C overnight.
6. The following day, perform colony PCR and create starter cultures for minipreps; verify constructs using restriction enzyme digestion and Sanger sequencing.

#### Standard cloning

1. Digest the vectors and inserts (containing restriction enzyme recognition sites on the 5’ and 3’ ends) with the desired restriction enzyme overnight at the recommended temperature.
2. Dephosphorylate the vector using rSAP or Antarctic phosphatase (*see* **Note 7**). Add the phosphatase to the reaction at 1-5 units per pmol of DNA ends based on the following calculation:

$$μg DNA × \frac{pmol}{600pg} × \frac{10^{6}pg}{1μg} ×\frac{1}{N} ×2 × \frac{kb}{1000bp} =pmol DNA ends $$

N is the nucleotides in kilobases, 660 pg/mol is the average molecular weight of a nucleotide pair, 2 is the number of ends, Kb/1000 bp is the conversion factor.

1. Incubate the reaction for another hour at 37 °C for rSAP and 30 minutes for Antarctic phosphatase
2. Heat inactivate at 65 °C for 5 minutes for rSAP or 80 °C for 2 minutes for Antarctic phosphatase. If possible this will denature the restriction enzyme too, if the enzyme cannot be heat inactivated perform gel electrophoresis and gel purification or column based purification.
3. Run 2 μl of the insert and vector on 1% agarose gel alongside 0.5 μg and 1 μg of lambda DNA-HindIII digest ladder.
4. Estimate the concentration based on the HindIII ladder or use programs to estimate the concentration such as the BioRad Image Lab™ software. Additional verification by UV spectroscopy is also possible.
5. Carry out ligations at a 4:1 molar ratio of insert to vector, (*see* **Notes 8**):
	1. Determine the amount of insert in a 10 μl ligation mix based on the calculation:

$$mass of insert \left(g\right)=desired\frac{insert}{vector}molar ratio ×mass of vector \left(g\right) ×ratio of insert to vector lengths $$

* 1. Carry out the ligation in the following reaction: 1 μl 10X T4 ligase buffer, 1 μl vector, X µl insert, 1 μl T4 DNA ligase (NEB), complete to 10µl with ddH2O (*see* **Note 9, 10 and 11**).
	2. Incubate at 37 °C for 1 hour.
	3. Heat inactivate at 65 °C for 20 minutes in a thermal cycler.
1. Incubate ligations at 16 °C overnight.
2. Heat inactivate at 65 °C for 10 minutes.
3. Use 5 μl of ligation mix to transform competent bacteria (we use JM109 from Promega) and plate onto ampicillin selective plates.
4. After growing selected clones into ~5 ml LB broth with ampicillin, extract DNA with minipreps kit following manufacturer’s instructions.
5. Check vectors for correct insertion using restriction enzyme digestion and Sanger sequencing.

### In vitro transfection

 We use a fibroblast cell line to determine the activation of the enhancer but different cell types can be used based on the expression pattern or predicted expression of the enhancer. We use the murine embryonic fibroblast, NIH3T3, maintained in complete DMEM.

1. Seed the NIH3T3 at a density of 1.75x104 cells/well in a 48 well tissue culture treated plate and incubate overnight (around 12 hours) in a cell culture incubator. Only conduct transfection when cells are ~80% confluent.
2. Co-transfect the cells in at least triplicates with the enhancer constructs and any Renilla luciferase control vector to allow normalization of transfection efficacy. Blanks, luciferase vector with no insert and pRL-TK alone are included in each experiment as controls.
3. For each well set up the following two reaction tubes:
	1. Tube 1: 80 μl Opti-MEM®, 2 μg DNA, 120 ng pRL-TK vector;
	2. Tube 2: 80 μl Opti-MEM®, 3 μl Lipofectamine® 2000.
4. Incubate 5 minutes at room temperature.
5. Add 80 μl from tube 2 to tube 1 and mix by flicking.
6. Incubate at room temperature for 25 minutes.
7. Add 40 μl of the mix to each well (so the final concentration of DNA is 500ng with 0.75 μl of Lipofectamine and 30 ng of TK- vector).
8. Assay the cells after 24-48 hours.

### Luciferase assays

 We conduct luciferase assays using the Dual-Luciferase® Reporter Assay system. Reagents are prepared fresh for each experiment, with the exception of the Luciferase Assay Reagent II (LARII).

1. Prepare the passive lysis buffer by adding 1 volume of 5X concentrate into 4 volumes of distilled water and invert to mix.
2. Prepare the LARII by adding 10 ml of luciferase assay buffer II to the lyophilized luciferase assay substrate and store in aliquots of 1 ml at -80 °C.
3. Add 1 volume of Stop & Glo® substrate to 50 volumes of Stop & Glo® buffer and invert to mix (*see* **Note 12**). Wrap in foil to protect from light.
4. Equilibrate cells to room temperature for 10 minutes.
5. Add 65 μl of Passive lysis buffer to each well and incubate at room temperature for 15 minutes while rocking.
6. Add 50 μl of LARII to each well of a white 96-well plate and perform a background reading in a luminometer.
7. Transfer 10 μl of cell lysate into the plate and mix by pipetting 6 times
8. Read the plate within 10 minutes of mixing.
9. Add 50 μl of Stop & Glo® Reagent mix by pipetting 4 times and read the Renilla luminescence.
10. Normalize the luminescence to the control vector and calculate the relative response ratio (RRR) using the following formula:

$$RRR= \frac{(experimental sample ratio)-(negative control ratio)}{(positive control ratio)-(negative control ratio)}$$

### X-gal staining in cells

 Cells that are transfected with a β-gal based vector or primary cells taken from mice harboring a β-gal transgene, can be stained for β-galactosidase.

1. Remove cells from cell culture incubator and allow to equilibrate to room temperature.
2. Remove growth medium and wash with ice cold 1X PBS twice.
3. Fix cells in 4% paraformaldehyde on ice for 2-5 minutes.
4. Wash cells twice with ice cold 1X PBS.
5. Add enough stain solution with X-gal to cover the cells and incubate in the dark at 37 °C for 8 hours (longer incubations can be done). Alternatively, cover in foil and incubate at room temperature with agitation or on a laboratory rocker for 16 hours (or overnight).
6. Wash in PBS and image cells (**Figure 6**).

### In vivo bioluminescence imaging

 *In vivo* imaging of transgenic mice that harbor a luciferase gene can be used as a non-invasive longitudinal monitoring of the gene expression. To visualize the expression *in vivo* we use the following method.

1. Inject ᴅ-luciferin intraperitoneally into the mice using 30G needle (*see* **Note 13**).
2. Anesthetize mice using 2.5% isoflurane. If looking at specific tissues, cull the animal and dissect the tissue of interest.
3. Place mouse or tissue into an in vivo imager.
4. Collect data depending on study parameters. Adjust camera settings to obtain a signal level of 600 to 60,000 counts / easy with auto exposure setting. Make sure to determine plateau of constant luciferase activity rather than peak of activity (***see* Notes 14**). You may need to shade high expressing areas to see other low levels (*see* **Notes 15 and 16**).

### X-gal staining in embryos and adult tissues

#### X-gal staining of mouse embryos

1. Transgenic mouse embryos can be collected at different time during gestation. Embryos taken after E15.5 will require de-skinning or opening up along the vertical axis from head to tail to allow penetration of the fix/stain solution. After dissection, keep the yolk sac and placenta for genotyping.
2. Rinsed samples in ice-cold 1X PBS and fix embryos in FIX solution at 4 °C. Recommended fixation times relative to gestation stages are shown in **Table 1**. [Table 1 near here]
3. Rinse embryos twice in PBS (*see* **Note 17**).
4. Place embryos into 50ml tubes with 25-40ml of STAIN solution; cover the tubes with foil paper and incubate overnight in the dark at room temperature.
5. Rinse in PBS.
6. At this stage you can photograph the embryos then fix overnight in 4%PFA at 4 °C (if >E15.5 fix for 1-2 hours) for further histology (see section 3.7.3).

#### X-gal staining of adult tissues collected from mice

1. Incubate tissues in 6 times their volume of FIX solution for 2 hours at room temperature whilst rotating.
2. Continue from step 3 of section 3.7.1.

#### Paraffin embedding for histology of X-gal stained samples

 After overnight fixation samples can be processed to allow histological interrogation. The lacZ staining may be reduced significantly with xylene. We have adopted a processing procedure of LacZ stained embryos to minimize exposure to xylene in the processing (*see* **Note 18**). Alternatively, samples can be cryo preserved and then cryo-sectioned.

 For embryos up to E14.5, dehydrate and paraffin-embed fixed samples by successively immersing them in ethanol, xylene and wax (strictly follow reagents and times indicated in **Table 2**).

 For larger LacZ stained embryos (E14.5 to E18.5) and soft tissue, open the embryos down the center to allow better infiltration and follow reagents and times indicated in **Table 3**.

### Generation of a fibroblast-specific Cre inducible strain

 For inducible systems, we use the plasmid pCreERT2, which contains the cDNA for the protein Cre (causes recombination) with a modified estrogen receptor (ERT2) [37]. The pCreERT2 is digested with the enzymes *SfiI* and *PvuII*, blunted with T4 DNA polymerase and dephosphorylated with Antarctic phosphatase. Cloning into the vector of interest is performed following the cloning procedures detailed in sections 3.2.2 or 3.2.3. The 1.9 kb fragment of the Col1a2 enhancer (-17.0/-15.45kb) and the mouse minimal proximal promoter (SDV150) [26] are prepared by *BamHI* excision and gel extraction using the QIAquick gel extraction kit. The insert is ligated to the linear pCreERT2 by T4 DNA ligase and verified by restriction enzyme digest and Sanger sequencing. For oocyte injection, the vector is digested with *FspI* and the 6181 bp fragment is gel purified. This mouse model can be used in different strategies to target fibroblasts in gain and loss of function experiments as illustrated in **Figure 7**.

## Notes

1. Primers can be generated using a web based program primer blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/> ). Generally, primers should be 18-30 nt with an annealing temperature of 60 °C with a difference of ±2 °C (for anything greater, a touch down PCR may be required). Standard desalt or high purity salt free purification can be used with no deleterious effects to cloning, the GC content of the primers should be about 40-60% with no more than 3 similar nucleotides between primer pairs (inter-homology) to reduce primer dimers and no more than 4 repeats. For sticky end cloning add restriction site to the end of the primers with 4 or 3 bases between restriction enzyme recognition sites and the end of the primer is generally advised to allow successful cleavage.
2. For standard cloning, proofreading Platinum™ *PfX* DNA polymerase is the *Taq* polymerase we use. If PCR fails or the temperature between primer pairs are greater than 5°C, a touch-down PCR was conducted starting at 5 °C higher than the lowest temperature of the primer pairs can be. The standard three step cycling is carried out by an initiate denaturing step at 94°C for 5 minutes, then 35-40 cycles of 94°C for 15 seconds, 55°C for 30 seconds and an extension step of 68°C at 1kb/min, the reaction can then be maintained at 4°C or frozen and stored at -20°C for future use. Alternatively a two-step PCR of 94 °C for 5 minutes, then 30 cycles of 94 °C for 15 seconds extension carried out at 68 °C, at 1kb/min. The *PfX* taq polymerase produces a blunt ended product that can be used in blunt ended cloning if no restriction enzymes are suitable.
3. For Gateway™ based cloning a non- high-fidelity DNA *taq* polymerase, in our case REDExtract™-N-Amp ReadyMix™ (Sigma, E3004), is used to utilize the ability of the *taq* polymerase to perform A’ tailing to allow for TA cloning. Reaction parameters of 94 °C for 3 minutes to denature the DNA, 30 seconds at the specific primer annealing temperature (58 °C - 62 °C), extension of 2 minutes at 72 °C for 30 cycles a prolonged final extension time of 10 minutes (to allow A- tailing) and a soak step at 4 °C.
4. The advantage of the gateway cloning is that once you generate a recipient vector, many more enhancers can be directly cloned in. The disadvantage is the use of proprietry LR clonase II and specific bacteria. To propagate a new destination vector that has not been used in a LR reaction or has no insert a bacteria that is able to withstand the *ccdB* gene product such as the *ccd*B Surival™ 2 T1R Chemically competent Cells from invitrogen
5. If the PCR yields multiple bands, but a band of the correct size is seen, the correct band can be extracted from the gel using kits such as the QIAquick gel extraction kit.
6. rSAP can be added directly to the restriction mix whereas Antarctic phosphatase buffer should supplement the restriction mix and the volume brought up to 30 μl.
7. You can use different ratios of 1:1, 2:1, 5:1, 10:1 or even 1:10 if there are problems with ligation.
8. If using blunt ended cloning treat the purified PCR product using T4 polynucleotide kinase (T4 PNK) in the following reaction in 0.2 ml PCR reaction tube, if using a different buffer than the T4 ligase, add 10mM ATP to the mix to allow the T4 PNK to work. Set up the reaction as follows: up to 1 μg PCR product, 2 μl 10X T4 ligase buffer, 1 μl T4 PNK, dH2O up to 20 µl.
9. Phosphatased inserts can be used in blunt end cloning in blunted vector (i.e. that has been digested with a restriction enzyme that blunts), or sticky ends that have been filled in by DNA polymerase I large (Klenow) fragment (Promega) or by T4 DNA polymerase (NEB).
10. Blunt end ligations can be carried out in the presence of the blunt end enzyme to prevent vector re-ligation and increase efficiency of the ligation.
11. The yellow color should be uniform throughout the solution.
12. Luciferin has to distribute evenly inside animal before measuring luciferase activity (in our experience 10 minutes)
13. In our 17 kb black mouse, tail, periodontal ligaments and ears are seen as baseline expression without shaving, **Figure 2A** or with shaving to obtain low activity as in bone marrow contribution to repair **Figure 2B**.
14. Calibrated units are Photons per Second, representing the flux radiating omni-directionally from a user defined region of interest (ROI)
15. Factors that will affect imaging of luciferase include: Relative placement of animal versus camera, fur color and presence (may need to shave), imaging time point, luciferin dose per mouse (keep weight record of mice and inject constant dose), biological factors (e.g. circadian rhythm, surgery, implantation).
16. The larger the embryos, the more rinse they require.
17. The xylene steps have been reduced to minimize the leeching of the X-gal staining.

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Tables

Table : Embryo fixation time relative to gestation stage

|  |  |
| --- | --- |
| **Gestation state** | **Fixation time** |
| E7.5-8.5 | 10 mins |
| E8.5-9.0 | 20 mins |
| E9.5 | 30 mins |
| E10-11.5 | 45 mins |
| E12.5 | 1hr |
| E13.5 | 2.5-2hr |
| E14.5 | 2.5hr |
| E15.5  | 3hr |
| E16.5-17.5 | 4hr |

Table : Dehydration and paraffin-embedding protocol for β-gal stained embryos less than E14.5

|  |  |
| --- | --- |
| Reagent | Immersion Period (Minutes) under vacuum |
| 70% Ethanol | 30 |
| 90% Ethanol | 30 |
| 100% Ethanol | 10 |
| 100% Ethanol | 10 |
| 100% Ethanol | 10 |
| 100% Ethanol | 10 |
| Xylene | 10 |
| Xylene | 10 |
| Wax | 100 |
| Wax | 100 |

Table : Dehydration and paraffin-embedding protocol for β-gal stained large embryos (E.14.5 to E18.5) and adult tissues

|  |  |
| --- | --- |
| Reagent | Immersion Period (Minutes) under vacuum |
| 70% Ethanol | 30 |
| 90% Ethanol | 30 |
| 100% Ethanol | 10 |
| 100% Ethanol | 10 |
| 100% Ethanol | 10 |
| 100% Ethanol | 10 |
| Xylene | 30 |
| Xylene | 30 |
| Wax | 120 (2hours) |
| Wax | Overnight |

Figure Legends

**Figure 1. Schematic diagram of the collagen alpha 2 gene with location of hypersensitive sites (HS) and proximal promoter (pp). Illustration of where the elements are expressed *in vivo.* A) Constructs used to generate mice expressing either transgene or cre recombinase are depicted with references at the top. B) Transcriptional elements in the *Col1* genes are illustrated with HS sites (black arrows) and S1 nuclease sensitive sites (orange arrows) depicted on the linear map with specific expression in mesenchymal cells. Reference to the GFP mouse generated using rat promoter.**

**Figure 2. Bioluminescence of 17kb fibroblast enhancer in C57bl6 mice. A) Bioluminescence expression in tail, paws and ears. Note the increase in collagen transcription in the tail injection site of the mouse on the left that was carried out three days earlier. In this example we used bioluminescence as a genotyping method. B) C57bl6 WT mice were injected with bone marrow from Col1a2 luc (left) and WT (right). They were then subjected to full skin thickness excisional wound (red circle). Showing that bone marrow from *Col1a2 luc* (left) and wild type (right). Mice were then subjected to full skin thickness excisional wound (red circle). This example shows that the bone marrow from *col1a2 luc* is transcribing the *col1a2* gene and participated in wound repair of the recipient mouse.**

**Figure 3. Col1a2 fibroblasts enhancer driving Cre ERT2 mated with dual fluorescent protein reporter [36] with tamoxifen given at six weeks of age and tissue collected at 12 weeks showing fibroblasts expressing GFP in tissues that are expressing *Col1a2.* Scale bar =32 µm. *A)* In skin, GFP is detected in the dermis but not epidermis of hair follicles (A, f) compared with no tamoxifen (B). C) In kidneys, interstitial fibroblasts are green around tubules (t) and vessels (v). E) In the heart, there are small cells outside some of the fascicles but most of the staining is seen in vessels (v) along the length (arrows). G) In the lungs, GFP is expressed in fibroblasts surrounding vessels (v) and alveoli (a). D) In the liver, most of the diffuse staining is seen around the central vein (v). H, F) In the eye, GFP is detected in the choroid (ch) and in cells within splenic nodules (F, round circles) and blood vessels (arrow).**

**Figure 4: UCSC window for selection of enhancer criteria. A) Browser window showing “Expression and Regulation” tracks after scrolling down to the 7th tab of the mouse/mm9. B) Display window after clicking on the LICR Histone, from this you can select the cell type and the different histone modifications that mark enhancers and other regulatory elements (for the Col1a2 we select MEFs and limb specific modifications (red boxes)). Once submitted, they will appear as tracks in the browser. C) The window presented to the user once UW DNaseI Hs is selected. You can select different cell types that may be of interest but for the *Col1a2* fibroblast and the cell line NIH3T3 was used. For the purpose of searching for enhancers the ENCODE Ludwig Institute for Cancer Research (ENCODE/LICR) tracks for histone modifications and ENCODE project University of Washington (UW DNaseI HS), highlighted in red boxes, can be selected.**

**Figure 5. ENCODE read out showing histone methylation and acetylation in two types of fibroblasts and MEF cells showing peaks of activity in acetylation and methylation of K4m1 that represent enhancers (orange box) in the 5’ sequences of the *Col1a2* mouse gene. The peaks correspond to high homology of sequence from humans to chicken.**

**Figure 6:** **X-gal staining after cell transfection** in fibroblasts expressing control vector (A) and Col1a2 transgene (B). Positive staining is blue. **Scale bar =10µm**

**Figure 7: Uses for Col1a2 CreERT2. A)** Strategy for using fibroblast-specific enhancers to over express a specific gene in mesenchymal cells. **B)** Gain of function experiment where two transgenic mice are generated. One mouse expresses cre recombinase, the other expressing a gene of interest (in this case GFP) with a transcriptional STOP site flanked by LoxP sites. In the presence of tamoxifen (or in non-inducible cre lines) the recombinase enzyme will flox out the transcriptional stop to express GFP only in mesenchymal cells. **C)** In loss of function experiments, a floxed exon on a specific gene mated with a mouse expressing cre recombinase in mesenchymal cells will delete this gene only in mesenchymal cells.