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Phenotypic Analyses of Genetically Modified Mice for BMP Receptors

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Abstract

BMPs play important roles in the development, disease, and regeneration of many tissues. Genetically modified mice with altered BMP receptor genes are particularly informative for clarifying the role of BMP signaling. In this chapter, we introduce several selected protocols for in vivo functional characterization of BMP receptors in genetically modified mice, including immunohistochemistry of BMP downstream signaling (P-Smad1/5/9 or others), histological analysis, whole-mount skeletal staining for cartilage and bone tissues, and whole-mount cartilage staining.

Keywords

Transgenic; BMP receptors; Immunohistochemistry; Histological analysis; Whole-mount skeletal staining; Whole-mount cartilage staining

1 Introduction

Bone morphogenetic proteins (BMPs) are multifunctional paracrine growth factors that belong to the transforming growth factor β (TGF β) superfamily [1–3]. BMPs play important roles in the development, disease, and regeneration of many tissues, including craniofacial [4], heart [5], vascular [6], lung [7], eye [8], and tooth [9], especially cartilage [10–12] and bone [13–17]. BMPs signal through the interaction with BMP-specific type I (BMPR-1A or ALK3, BMPR-1B or ALK6, ACVR-1 or ALK2) and type II (BMPR-2, ACVR-2A, ACVR-2B) serine/threonine kinase receptors and then start the signaling cascade via both Smad-dependent (canonical) and Smad-independent (noncanonical) signaling pathways. In the Smad-dependent signaling pathway, BMPs activate receptor-specific Smads, Smad1, Smad5, and Smad9 (P-Smad1/5/9), at a C-terminal SSXS motif. Then P-Smad1/5/9 forms a complex with Smad4, and this complex translocates to the nucleus where it functions as a transcription factor with coactivators and corepressors to regulate gene expression [1–3].

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 [18, 19]. Therefore, different receptors may have similar or totally different functions in transducing BMP signals. Genetically modified mice with altered BMP receptor genes are particularly informative for clarifying the role of BMP signaling in development, disease, and regeneration. During embryo development, mice lacking *Alk2*, *Alk3*, or *Bmpr2* show defects in embryogenesis at the gastrulation stage [20–22]. In cartilage tissues, mice lacking *Alk3* and *Alk6* show achondroplasia in vivo [10, 11], while nasal cartilage morphogenesis is inhibited in mice with conditional constitutively active *Alk3* (*caAlk3*) in the neural crest [12]. In bone, mice lacking *Alk6* show decreased bone mass [13], whereas loss-of-function of *Alk2* in osteoblasts increases bone mass [14]. A mice model with constitutively active *Alk2* (*caAlk2*) that requires signaling complexes with type II receptors leads to ectopic bone formation [15]. During tissue regeneration, *caAlk2* in hair follicle stem cells attenuates cutaneous wound healing [23], and *caAlk2*-expressing adipose-derived stem cells significantly enhance bone regeneration [16]. The phenotypic analyses of such complex responses in mice with genetically modified BMP receptors require numerous methods.

In this chapter, we introduce several selected protocols that we use for phenotypic analysis of BMP receptors in genetically modified mice, including immunohistochemistry of BMP downstream signaling (P-Smad1/5/9, or others), histological analysis, whole-mount skeletal staining for cartilage and bone tissues, and whole-mount cartilage staining. We previously provide the detailed methods for in situ *hybridization* (both whole mount and section); thus, for people who are interested in gene expression studies, please refer to the previous chapter [24]. In this chapter, we will focus on immunohistochemistry and other phenotypic analyses.

2 Materials

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

2.1 Immunohistochemical Examination of P-Smad1/5/9

1. 4% paraformaldehyde fixative.
2. 30% sucrose: 30 g sucrose dissolved PBS (phosphate-buffered saline, 8.0 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄, 1.8 g KH₂PO₄ dissolved in water to 1000 mL, pH 7.4.), to a final volume of 100 mL.
3. Cryopreservative solution (OCT compound).
4. Cryostat.
5. Small artist's paint brushes.
6. Positively charged microscope slides.
7. 0.1% PBST (0.1% Triton-X 100 in PBS): dissolve 1 mL Triton-X 100 in PBS to make 1000 mL solution.

8. Citrate buffer: 10 mM sodium citrate, adjusted to pH 6.
9. Blocking buffer: 500 μ L normal donkey serum or 500 μ g bovine serum albumin diluted in 0.1% PBST to a final volume of 10 mL.
10. Primary antibody: Rabbit anti-P-Smad1/5/9 antibody.
11. Alexa fluor 488-anti-Rabbit secondary antibody.
12. Coverslips.
13. Antifade Mountant with DAPI.
14. Fluorescence microscope.

2.2 Phenotypic Examination of Mice with Genetically Modified BMP Receptors

2.2.1 Hematoxylin and Eosin Staining

1. Ethanol (*see* Note 1).
2. 7 g/L hematoxylin (Sigma, HHS32–1L).
3. 1% acid alcohol solution: hydrochloric acid 1 mL, add 70% ethanol to 100 mL.
4. 0.2% ammonia water solution (bluing): ammonium hydroxide (concentrated) 2 mL, add water to 1000 mL.
5. 0.1% (w/v) eosin (Sigma, HT110316–500ML).
6. Xylene.
7. Permount mounting medium.
8. Light microscope.

2.2.2 Safranin O Staining

1. Weigert's iron hematoxylin solution: stock solution A (1 g hematoxylin, 100 mL 95% ethanol), stock solution B (4 mL 29% ferric chloride in water, 95 mL water, 1 mL hydrochloric acid). Mix equal parts of stock solution A and B to make Weigert's iron hematoxylin working solution (*see* Note 2).
2. 0.02% fast green solution: 0.2 g fast green dissolved in water to make a final volume of 1000 mL.
3. 1% acetic acid solution: 1 mL glacial acetic acid, add water to 100 mL.
4. 0.1% safranin O solution: 0.1 g safranin O dissolved in water to a final volume of 100 mL.

2.3 Whole-Mount Skeletal Staining

1. 95% ethanol.

¹·Molecular sieve can be used to remove trace amount of water to maintain 100% purity of ethanol. This greatly improves penetrancy of xylene.

²·Weigert's iron hematoxylin working solution is stable for about 4 weeks.

2. 0.015% Alcian Blue solution: 0.015 mg/mL Alcian Blue 8GX, 20% acetic acid, 80% ethanol (*see* Note 3).
3. 2% KOH (use plastic container).
4. 0.015% alizarin red S solution: 0.015 mg/mL alizarin red sodium sulfate in 1% KOH (use plastic container).
5. 1% KOH, 20% glycerol: 10 g KOH, 200 mL glycerol, add water to 1000 mL (use plastic container).
6. 47.5% ethanol, 50% glycerol: 475 mL 100% ethanol (or 500 mL 95% ethanol), 500 mL glycerol, add water to 1000 mL.
7. Filter paper.
8. Stereo microscope.

2.4 Whole-Mount Cartilage Staining

1. Bouin's solution: 300 mL saturated picric acid, 100 mL formaldehyde, and 20 mL glacial acetic acid. Stable for 1 year.
2. 0.1% NH₄OH: 1 mL 30% NH₄OH (open in the fume hood) in 210 mL of 100% ethanol, add water to 300 mL.
3. 5% acetic acid: Take 10 mL of 100% acetic acid (open in the fume hood) and add water to 200 mL.
4. 0.05% Alcian Blue solution: 25 mg Alcian Blue 8 GX dissolved in 5% acetic acid.
5. Methanol.
6. BABB solution: 100 mL benzyl alcohol mixed with 200 mL benzyl benzoate (use glass container and handle in fume hood).

3 Methods

3.1 Immunohistochemical Examination of P-Smad1/5/9

3.1.1 Dissection—For the analysis of whole embryos, euthanize the pregnant mother quickly by CO₂ inhalation followed by induction of bilateral pneumothorax or other IACUC approved methods of euthanasia. Remove whole uterus with embryos, and wash the embryos with cold PBS. Separate individual embryos, remove all membranes covering them, wash briefly in cold PBS, and transfer to fixative. Remove the skin from embryos older than 17.5 days before fixation.

3.1.2 Fixation, Embedding, and Sectioning

1. Fix tissue in 4% paraformaldehyde at 4 °C with gentle shaking for 4 h (*see* Notes 4 and 5).

³. Alcian Blue staining solution should be used within 3 weeks.

2. Rinse tissue in PBS at 4 °C with gentle shaking for 12 h.
3. Cryoprotection with sucrose: transfer the samples into 30% sucrose at 4 °C with gentle shaking until the samples sink to the bottom.
4. Embedding: transfer the samples into an embedding mold filled with OCT. Use forceps to adjust the location and direction of the samples. The trimmed side of the samples must be facing to the bottom of the embedding mold. Place the mold on dry ice until the OCT solidifies.
5. Cut 10 µm thick sections and place on microscope slides.
6. Air-dry the sections. Proceed to staining or store at −80 °C (*see* Note 6).

3.1.3 Immunohistochemistry of P-Smad1/5/9

1. Soak slides in 0.1% PBST, change solution every 5 min for three times (3 × 5 min).
2. Antigen retrieval (optional): Preheat steamer or water bath with staining dish containing citrate buffer until temperature reaches 95–100 °C. Immerse slides in the staining dish. Place the lid loosely on the staining dish and incubate for 10 min. Turn off steamer or water bath and remove the staining dish to room temperature and allow the slides to cool for at least 20 min (*see* Note 7) [25].
3. Rinse sections in 0.1% PBST, 3 × 3 min.
4. Apply 200 µL blocking solution to each slide and incubate at room temperature for 30 min (no rinse) (*see* Note 8).
5. Apply 200 µL primary antibody (P-Smad1/5/9, 1:100 diluted in blocking buffer) to each slide, and incubate slides at 4 °C overnight in a moisturized sealed container.
6. Rinse sections in PBS, 3 × 3 min.
7. Apply 200 µL secondary antibody (1:200 diluted in PBS) to each slide, and incubate slides at room temperature for 1 h (protect from light from this step forward).
8. Rinse sections in PBS 3 × 3 min.

⁴The duration for fixation should differ according to the size of the samples. Longer fixation is needed for larger samples. However, longer fixation may mask antigenic sites, which will increase false-negative results of immunohistochemistry signals. If this occurred, antigen retrieval before blocking might be required.

⁵Here we describe cryosectioning followed by immunohistochemical detection of P-Smad1/5/9, which does not require antigen retrieval. Histological sections prepared after paraffin embedding also work for P-Smad1/5/9 detection; however, antigen retrieval is required.

⁶A lower temperature maintains better morphology and antigenicity of sections.

⁷Enzymes (e.g., trypsin, pepsin) and heat can be used for antigen retrieval. The concentration and treatment times of enzymatic retrieval must be tested and optimized to avoid damaging section. For the heat-induced antigen retrieval methods, the most commonly used buffers are 10 mM sodium citrate pH 6, Tris-EDTA pH 9, or EDTA pH 8. Heat-induced antigen retrieval is most often performed using a pressure cooker, microwave, steamer, or water bath. Unless the antigen retrieval method is stated on the antibody data sheet, the optimal method for each antibody/antigen combination must be determined.

⁸Perform avidin/biotin blocking with 3% H₂O₂ if necessary.

9. Apply 1–2 drops mountant with DAPI to each slide, and cover the slide with coverslip.
10. Keep slides in cold room before taking pictures under a fluorescent microscope (Fig. 1a, b).

3.2 Phenotypic Examination of Mice with Genetically Modified BMP Receptors

3.2.1 Hematoxylin and Eosin Staining (See Note 9)

1. Place slides in a slide rack and wash under running tap water for 2 min.
2. Stain in 7 g/L hematoxylin for 2 min. Wash under running tap water for 2 min.
3. Place in acid alcohol bath for 10 seconds (s) to remove nonspecific blue staining. Wash under running tap water for 2 min.
4. Place in 0.2% ammonia water solution for 20 s to develop the remaining specific blue color. Wash under running tap water for 2 min.
5. Place in 0.1% eosin for 3 s, then immediately wash under running tap water for 20 s.
6. Dehydrate in sequential 70%, 80%, 95%, 100%, and 100% ethanol solutions for 20 s each.
7. Place in xylene for 5 min. Repeat twice.
8. Mount each slide with Permount and a coverslip before taking pictures under a light microscope. The method will stain nuclei in blue, while the acidophilic cytoplasm in red, basophilic cytoplasm in purple, and erythrocytes in cherry red (Fig. 1c, d).

3.2.2 Safranin O Staining (See Note 9)

1. Hydrate frozen sections with water.
2. Stain in Weigert's iron hematoxylin for 5 min.
3. Rinse in water for 3 min.
4. Dip in 1% acid alcohol to destain hematoxylin.
5. Rinse in water for 3 min.
6. Stain in 0.02% fast green for 1 min (do not rinse).
7. Stain in 1% acetic acid for 10 s (do not rinse).
8. Stain in 0.1% safranin O for 20 min (do not rinse).
9. Rinse in 95% ethanol and then in 100% ethanol to dehydrate.
10. Rinse in xylene, then mount coverslip with Permount before taking pictures under a light microscope. This method will stain nuclei in black, cytoplasm in

⁹Hematoxylin and eosin staining and Safranin O staining work well for both paraffin and cryosections.

bluish green, and cartilage, mucin, mast cell granules in orange to red (Fig. 1e, f).

3.2.3 Whole-Mount Skeleton Staining (See Note 10)—This protocol covers the staining of the pre- and postnatal mouse skeleton using Alcian Blue and alizarin red to identify cartilage and bone, respectively.

1. Remove skin and internal organs. If possible, remove big muscles from shoulders, back, and limbs.
2. Fix remaining carcass overnight in 95% ethanol.
3. Stain in 0.015% Alcian Blue solution overnight for cartilage.
4. Put back in 95% ethanol for at least 3 h.
5. Treat with 2% KOH for 24 h or longer for adult mice (*see* Note 11).
6. Stain overnight in 0.015% alizarin red S solution for the bone.
7. Clear skeleton in 1% KOH, 20% glycerol solution for 2 days, or more (*see* Note 12).
8. Store the skeleton in a 1:1 mixture of glycerol and 95% ethanol before taking picture under a stereomicroscope. This method will stain bone tissue in red and cartilage tissue in blue (Fig. 2).

3.2.4 Whole-Mount Cartilage Staining (See Note 10)

1. Dissect embryos in cold PBS and remove extraembryonic membranes and skin (*see* Note 13).
2. Fix in Bouin's solution for 2 h or overnight.
3. Rinse with solution 1% NH_4OH diluted in 70% ethanol over a course of 24 h until embryos appear white. The solution may need to be changed six to eight times.
4. Equilibrate with 5% acetic acid for 1 h. Repeat twice.
5. Stain with 0.05% Alcian Blue solution for 2 h.
6. Rinse twice with 5% acetic acid, 1 h for each.
7. Dehydrate the samples by incubating in 100% methanol for 1 h. Repeat twice.

¹⁰Both fixatives for whole-mount skeletal (ethanol) and cartilage (Bouin's solution) work for Alcian Blue staining to reveal cartilage. The differences between those two methods are that (1) skeletal staining uses ethanol for fixation, allowing a sequential staining with alizarin red after Alcian Blue, while cartilage staining uses Bouin's solution for fixation, which is not compatible for alizarin red, (2) and samples for skeletal staining are cleared in KOH, while those for cartilage staining become transparent in BABB solution.

¹¹Do not move samples since the junction of bone parts becomes very fragile. Carefully remove the 2% KOH solution. If the tissues become very soft, removing the solution completely is not necessary.

¹²If muscles are not cleared after 2 days, incubate in 1% KOH until they are cleared, or use forceps to remove muscles or other soft tissues.

¹³In older embryos (E17 or older), removing the skin may be necessary.

8. Clear the samples in BABB solution before taking picture under a stereomicroscope (see Note 14). This method will stain cartilage in blue color (Fig. 3a, b).

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14. Cartilage staining may last several years; however, photo documentation should be done as soon as possible. Use glass container when taking pictures, since plastic container cannot tolerate organic solvents.

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Fig. 1.

Example of increased BMP signaling and embryonic phenotype in mice with a Cre-recombinase activated receptor gene. Conditional constitutively active *Alk3* (*caAlk3*) mice were crossed with *P0-Cre* mice (Mutant) to increase BMP signaling in neural crest cells (NCC) and NCC-derived cells. Control embryos had only the *P0-Cre* transgene. **(a, b)** P-Smad1/5/9 staining pattern in the skull of control **(a)**, or mutant **(b)** embryos. **(c, d)** Hematoxylin and eosin staining (Subheading 3.2.1) of the sections from control **(c)** and mutant **(d)** heads, showing the disappearance of the suture (black arrows) in the mutant skull. **(e, f)** Safranin O staining (Subheading 3.2.2) of the sections from control **(e)** and mutant **(f)** nasal cavities, showing the nasal cartilage (red) and a failure of fusion between the nasal septum and the secondary palate in the mutant nasal cavity **(f, red arrow)**

Fig. 2. Example of whole-mount skeletal staining of heterotopic ossification (HO). HO was induced by injection of Adenovirus Cre with cardiotoxin to the hind limb muscle of mice bearing a conditional constitutively active *Alk2* (*caAlk2*) gene. (a) X-ray taken 10 days after induction, showing the increased density of tissue (white arrows) around the tibia. (b) After removing the skin, the limb was stained with Alcian Blue as described in Subheading 3.2.3. The black arrows show the ectopic cartilage tissue in the muscle. (c) The same tissue was further treated with KOH and stained with alizarin red as described in Subheading 3.2.3 to reveal heterotopic ossification in the muscle (black arrows)

Fig. 3.

Example of whole-mount cartilage staining of embryos with a Cre-recombinase activated receptor gene. Conditional constitutively active *Alk2* (*caAlk2*) mice were crossed with *Prx1-Cre* mice (Mutant) to increase BMP signaling in early limb bud mesenchyme cells (LBMCs) and LBMC-derived cells. Control embryos had only the *caAlk2* transgene. Embryos were stained with Alcian Blue and cleared in BABB solution as described in Subheading 3.2.4. Despite minimal differences in the autopod (red boxes (a) in A' and B'), mutant embryos showed major defects in the zeugopod (b), stylopod (c), and scapula (d) cartilage of the forelimb compared with control embryos