

Chapter 2

Gene Expression Analysis of CCN Proteins: Whole-Mount In Situ Hybridization of *Ccn2* in Developing Calcified Tissues

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Abstract

A procedure for whole-mount in situ hybridization developed for detecting gene expression of *Ccn2* in developing calcified tissues of mouse embryos is presented. In this method, embryos are hybridized with Dig-labeled riboprobes, and the riboprobes are detected by use of the alkaline-phosphatase reaction in the presence of a 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT + BCIP) mixture. Obvious detection of positive signals for *Ccn2* in the cartilage of developing phalanges indicates that this method can be applied to gene expression analysis of other *Ccn* genes in developing calcified tissues.

Key words *Ccn2* gene expression, Whole-mount in situ hybridization, Dig-labeled riboprobe, Mouse embryo

1 Introduction

Whole-mount in situ hybridization itself is a well-known technique used to localize approximate gene expression sites of various genes; but somehow the procedure is more complicated than that of in situ hybridization on a slide glass, because each laboratory has its own technique. We introduce here the procedure used for whole-mount in situ hybridization in our laboratory, which was developed for detecting gene expression of *Ccn2* in developing calcified tissues. In this method, digoxigenin (Dig)-labeled riboprobes are used for hybridization, and the probes are detected by performing the alkaline-phosphatase reaction in the presence of a mixture of 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT + BCIP) in a refrigerator. Embryos up to E11.5 work well, whereas older embryos will only give signals at well-accessible positions such as limbs, tail, and central nervous system. Older embryos should be trimmed to get intense signals. Since CCN proteins have been suggested to be involved in embryonic

development [1–3], the method described here can be applied to investigate the roles of other CCN proteins in embryonic development as well. The fundamental procedure is based on the method developed by Bober et al. [4] and referred to by Meyer et al. [5].

In situ hybridization techniques for the detection of CCNs in formalin-fixed, paraffin-embedded sections are detailed in Chapters 26 and 41.

2 Materials

1. 10× PBS(–) (Dulbecco's PBS without CaCl_2 , 100 ml). Store at 4 °C.
2. Fixative: 2 % paraformaldehyde in a Millonig's phosphate buffer (e.g., American MasterTech, *see* **Note 1**).
3. 20× SSC. Store at 4 °C.
4. TBST: 0.14 M NaCl, 25 mM KCl, 25 mM Tris–HCl pH 7.5, 0.5 % Tween-20, 2 mM levamisole (*see* **Note 2**). Make 10× solution.
5. Dig-labeled sense and antisense riboprobes for CCN2 (*see* **Note 3**).
6. Embryonic powder: Grind E11–E14 mouse embryos after fixation with acetone and dry.
7. PBT: 1× PBS(–) with 0.1 % Tween 20.
8. Prehybridization mixture (50 ml): 50 % Ultrapure (deionized is better) formamide, 5× SSC, 0.04 % heparin (made after 100 mg/ml DW) (instead of BSA), 0.5 % tRNA (made after 11 mg/ml DW), + 1 % salmon sperm DNA (made after 10 mg/ml), 0.1 % Tween 20, add RDW to 50 ml. Store under –20 °C. Heat tRNA and ssDNA at 90 °C for 5 min to denature and then adjust pH with 1 M citrate or conc HCl to pH 4.5–5.0 (ca. 4–5 ml/50 ml mixture) before preparation of the mixture because riboprobes are unstable at alkaline pH.
9. Washing solutions:
 - Solution I: 50 % Formamide, 3× SSC, 0.1 % Tween 20.
 - Solution II: 0.5 M NaCl, 10 mM Tris–HCl pH 7.5, 0.1 % Tween 20.
 - Solution III: 50 % Formamide, 1.5× SSC, 0.1 % Tween 20.
 - Solution IV: 0.5–1.0× SSC, 0.1 % Tween 20.Concentration of SSC depends on an expected signal intensity. Use lower concentration of SSC carefully, because stringent washing might sometimes induce lower signal intensity.
10. Alkaline phosphatase (ALP) buffer: 100 mM NaCl, 100 mM Tris–HCl pH 9.5, 50 mM MgCl_2 , 0.1 % Tween 20. Use within

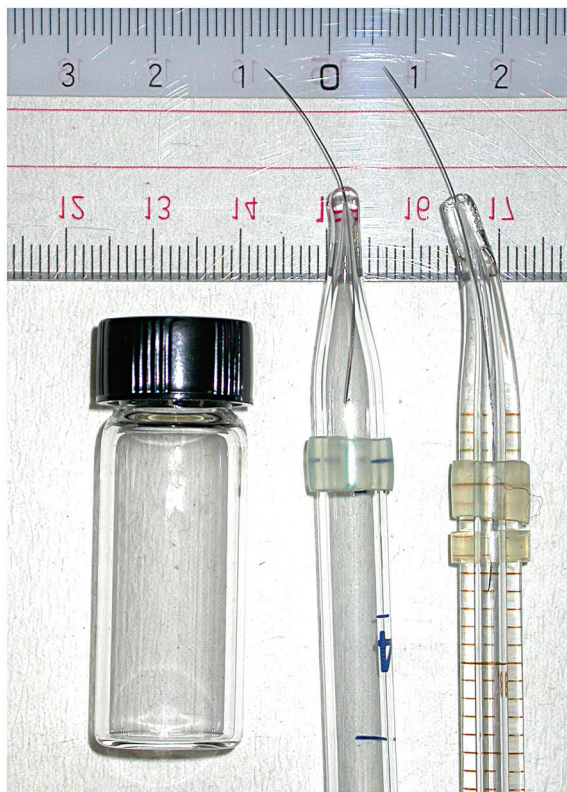


Fig. 1 Photograph shows sharp tungsten needles and a glass sample vial. Prepare adequate size glass vial for use, such as the one in the figure, and sharpen the needles by a finest sandpaper for convenient use

a week. 2 mM Levamisole is not necessary only in 4 °C. Tween 20 might block RNase activity in each sol'n during ISH.

11. Anti-Dig-ALP conjugate.
12. NBT + BCIP mixture (Boehringer Mannheim, Germany).
13. Glass sampling tubes (size: 1 ml) (Fig. 1).

3 Methods

The modified methods described here are based on the method by Bober et al. [4] and require at least 5 days to perform them.

3.1 Fixation and Dehydration

On the first day, fixation and dehydration are performed as follows:

1. Fix whole embryos at 4 °C by immersing embryos up to E12 in the fixative (*see* Subheading 2, **item 2**) at pH 7.3. However, embryos over E13 should be perfused with same fixative

through the left cardiac ventricle at 37 °C and then placed in a sample tube filled with the same fixative at 4 °C.

2. Irradiate the fixed embryos on ice in the same fixative mentioned above with six cycles of 5-s irradiation at 600 W and 15-s interval to cool down without irradiation by use of a kitchen microwave oven (*see Note 4*). This step is required for better fixation. Also, this microwave irradiation method is available not only for the fixation but also for dehydration up to 70% methanol or 70% Et-OH, immunoreactions, histochemical reactions, and washing (*see Note 5*).
3. Perform additional fixation after irradiation by keeping the samples in the same fixative for 30 min–3 h (depending on the sample size) at 4 °C on a shaker to obtain better morphology.
4. Wash samples 2×30 min with PBT (1× PBS(–) with 0.1% Tween 20).
5. Make punctures in samples (fixed embryos or organs) in PBT (or during 50–70% methanol) dehydration by a very sharp, thin needle, to obtain a better reaction (Fig. 1) (*see Note 6*).
6. Dehydrate samples by passing them through an ascending methanol series (50%, 70%, 80%, 90%, 95%, 100%) on ice for at least 15 min for each concentration of methanol (embryos can be stored in 100% methanol at –20 °C for 3 months) or overnight at 4 °C.

3.2 *In Situ* Hybridization

On the second day of the procedure, in situ hybridization should be performed as follows:

1. Generate Dig probe (*see Note 3*).
2. Transfer samples to room temperature (RT) from 4 °C.
3. Bleach samples with a solution of methanol:H₂O₂=5:1 for 1 h at RT with shaking.
4. Rehydrate the samples by passing them through a descending methanol series (100%, 95%, 90%, 80%, 70%, 50%) on ice.
5. Rinse with PBT 3×5 min at RT.
6. Etch samples with proteinase K (1–20 µg/ml PBT, use DNase- and RNase-free grade only) for 10–30 min at RT (*see Note 7*).
7. Refix samples for 20 min at RT with freshly made 2% paraformaldehyde (PFA) and 0.2% glutaraldehyde (GIA) in adequate buffer (dilute 25% GIA that was frozen at –20 °C:2% PFA=1:125).
8. Rinse with PBT for 3×5 min at RT. If desired, acetylate samples twice for 20 min each time at RT to reduce the background (*see Note 8*). Rinse acetylated samples with PBT for 10 min at RT.

9. Add prehybridization mixture, wait for 5 min (floating samples should settle to the bottom of the tube within 5 min), and then incubate in fresh prehybridization mixture for 0.5 h at 50 °C with gentle rotation.
10. Hybridize samples with 7 µl (0.7 µg) of Dig-riboprobe/2 ml of hybridization mixture/bottle overnight at 55–70 °C with gentle rotation in an incubator. Temperature could depend on the size of the probe. Denature probes for 5–10 min at 80 °C before use.

3.3 Immunological Reaction

On the third day of the experiment, the immunological reaction should be performed as follows:

1. Wash samples 3×60 min with prewarmed solution I with shaking at the same temperature as used for the hybridization.
2. Wash samples with shaking with a mixture of solution I:solution II=1:1 for 10 min at the same temperature used for the hybridization.
3. Wash with solution II, 3×10 min at RT with shaking (only for RNaseA treatment).
4. Treat with RNaseA/solution II (100 µg/2 ml solution II) for 30 min at 37 °C with shaking (*see Note 9*).
5. Rinse with shaking with solution III, 3×60 min at the same temperature as used for hybridization.
6. Rinse with solution IV for 15–30 min at RT (shaking is optional).
7. Wash samples with TBST 3×10 min at RT; in the meantime, heat inactivate a small amount of embryo powder (enough to stick to the tip of a Gilson's yellow tip) in 2 ml of TBST for 30 min at 56 °C.
8. Block embryos with heat-inactivated 1–3% normal goat serum in TBST for 1 h at RT; in the meantime spin down the embryo powder quickly, discard the supernatant (SNT), cool on ice, and add 1:2000–10,000 dilution of an anti-Dig-ALP conjugate in 1% heat-inactivated normal goat serum in 1× TBST; disperse powder well and incubate for 1 h at 4 °C.
9. Spin down the embryo powder for 5 min at 10,000×g, and then dilute the SNT 1:4 with 1% heat-inactivated normal goat serum in 1× TBST.
10. Remove the blocking solution and then add 2 µl of the diluted anti-Dig-ALP solution per tube, mix well, and incubate at 4 °C overnight with rotation.

3.4 Washing

On the fourth day of the experiment, washing is performed as follows:

1. Remove anti-Dig-ALP solution by washing with TBST 3×10 min at RT on a shaker.

2. Wash all day with TBST at RT, change TBST several times during this time, and continue to wash with it overnight at 4 °C with shaking.

3.5 Color Reaction

On the fifth day, start color reaction with the NBT + BCIP mixture in alkaline phosphatase (ALP) buffer at 4 °C (not at RT), for about 1 day–1 week. This lower temperature blocks mammalian endogenous nonspecific ALP activity.

1. Wash with freshly made ALP buffer 2 × 60 min at RT with shaking.
2. Start the color reaction in glass vials, with 2 ml of ALP buffer, 1 mM Levamisol, and 1/10–1/20 concentration of NBT + BCIP mixture (for instance, 100 µl mixture/100 ml ALP buff.) in the dark at 4 °C for about 1 day–1 week (see **Note 10**).
3. Place vials in the dark (cover with aluminum foil); staining might start in about 20 min after the addition of the color reaction mixture and may continue for 1 week. But generally the color reaction on whole-mount samples will be finished within 1 day. Monitor S/N (signal/noise) ratio under a microscope.
4. Stop the reaction when the background has increased, rinse with PBT + 1 mM EDTA, three times, and store at 4 °C (if the color reaction is not adequate, try **steps 1–3** again).
5. Refix with 2 % PFA with 0.2 % GIA in adequate buffer for 30 min at RT.
6. To take photographs, whole-mount samples are cleared in 70 % glycerol and 30 % 1× PBS (Figs. 2 and 3, and also see Refs. 6, 7). Store samples in PBT at 4 °C.

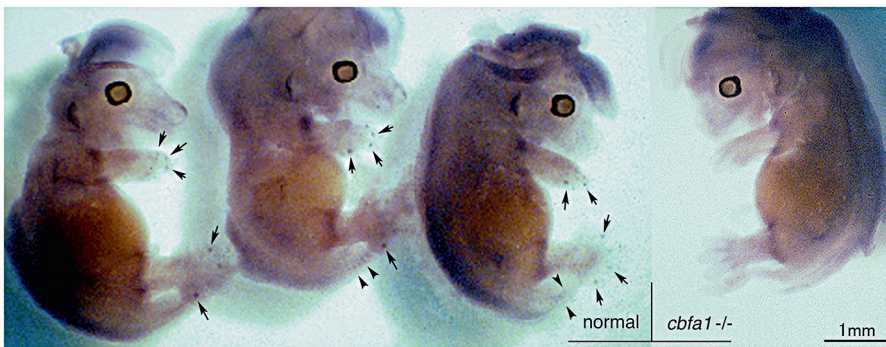


Fig. 2 Sample of gene expression in mouse embryos at E16.5. The gene expression of *Ccn2* is observed in the fingers (arrows) and tails (arrowheads) of wild-type embryos (left 3), but not in those of *cbfa1*-null mutants. Embryos are cut into half on the median line after perfusion for better fixation

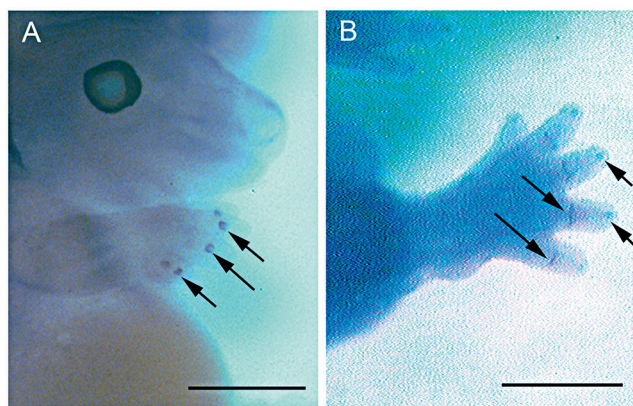


Fig. 3 Sample of gene expression (*Ccn2*) in the phalanges of wild-type mouse embryos at E15.5 (*left*) and E16.5 (*right*). Arrows indicate the sites of the gene expression of *Ccn2* observed in distal phalanxes indicated dark blue in E15.5 (**a**). In E16.5 embryo (**b**), the gene expression of *Ccn2* is also observed in interphalangeal joints, as indicated by the arrows. Bars in (**a**) and (**b**), 1 mm

4 Notes

1. Millonig's fixative is good for electron microscopy observation. The fixative composed of a higher concentration of the buffer (440 mOsm) maintains the true morphology of the embryos.
2. Levamisole is essential to decrease the background when performing ISH on an endogenous alkaline phosphatase-rich tissue or cell, as liver, kidney, testis, ovary, placenta, adrenal gland, small intestine, salivary glands, osteoblast, endothelial cell, lymphocyte, etc. in this system.
3. We prepared a noncoding region of mouse *Ccn2* as a probe for hybridization, which was amplified from cDNA by using the following primers: 5'-GGGTAAGGTCCGATTCCTACC-3' (sense) and 5'-CTAGAAAGGTGCAAACATG-TAAC-3' (antisense).

The mouse CCN2 fragment inserted into the pGEM-T plasmid easy was cut by Pst I restriction enzyme for sense and by Nco I restriction enzyme for antisense template. Dig-labeled riboprobes are generated as follows by using a DIG RNA labeling kit (Roche, Mannheim, Germany; *see* Refs. 8, 9): Briefly, mix 10× transcription buffer (2 µl), Dig-NTP (2 µl), template (linearized) DNA (1 µl), RNA polymerase (2 µl), HPRI (RNase inhibitor, 1 µl), and sterilized DW (12 µl). Incubate the mixture for 1 h at 37 °C for T7 polymerase (sense) and at 36 °C for Sp6 polymerase (antisense). After that, add 1 µl of DNase I and incubate for 0.5 h at 37 °C before precipitating with 4 M LiCl and 100% ET-OH. Dissolve precipitate in a mixture

comprising 8 μ l DEPC-treated DW, 1 μ l HPRI, 1 μ l 1 mM DTT, and 10 μ l of formamide, and store at -70°C . Use glass sampling tubes for ISH; never use plasticwares. Plasticwares may cause adsorption of probes.

4. Irradiation with microwaves gives better fixation.
5. Authors recommend to use methanol instead of ethanol during dehydration to obtain better signal contrast.
6. This procedure is very important to obtain fine signals.
7. Time of treatment and concentration of proteinase K are highly dependent on the position of the expected signal in the embryo; for example, for the *met*, *sf/hgf* signaling stream in the dermis of E10.5 embryos, 2 $\mu\text{g}/\text{ml}$ PBT for 10 min is required, whereas in the case of the sympathetic system, 20 $\mu\text{g}/\text{ml}$ PBT for 30 min is needed.
8. Prepare 10 ml of DW+25 μ l of HCl+150 μ l of triethanolamine+25 μ l of acetic anhydride (add and shake just before use).
9. This step is not necessary in many cases.
10. The color reaction should be performed at 4°C with dilute reagent (1/10–1/20) to block endogenous mammalian (mouse) alkaline phosphatase activity.

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