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Analysis of CCN4 Function in Osteogenic and Osteoclastic Cells Using Gain and Loss of Function Approaches

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Abstract

Analysis of CCN4 function in bone was assessed using both gain and loss of function approaches. In mice this was done by genetic engineering and homologous recombination to create mice with complete ablation of the protein. For human skeletal cells adenoviral gene transfer and shRNA were used for gain and loss of function respectively. Here we describe procedures used to make and then analyze osteogenic and osteoclastic cells with or without CCN4 to determine its role in osteogenic differentiation.

Keywords

CCN4; Gene knockout; Osteogenic differentiation; Osteoprogenitor cell

1 Introduction

The ability to manipulate gene expression either by total ablation or by over expression has had tremendous impact on understanding specific protein function. For the “loss of function” approach, genes are recombined in murine embryonic stem (ES) cells rendering them deficient in a specific protein’s expression. ES cells injected into blastocysts can then incorporate into adult mice and when populated into germ cells can even be transferred to offspring making new mouse lines deficient in the protein of interest. Such genetically engineered mouse lines are often referred to as “knockout” (KO) mice [1]. These new strains of mice are then examined at the tissue and cellular levels to determine the role of the loss of the protein on skeletal function. Our lab generated mice deficient in CCN4 [2] and this chapter explains in detail how the cells from these mice were used to show how CCN4 affects bone-derived cell functions. We did this using: (1) bone marrow stromal cells (BMSCs) that form new bone and control osteoclast formation and (2) osteoclast progenitors

that carry out bone resorption. We describe in this chapter how the osteogenic capabilities of mouse BMSCs are determined both in vitro and in vivo using induced differentiation assays and a transplant system [3] respectively.

Different approaches were used to study CCN4 in osteogenic and osteoclastic human skeletal cells. For a loss of function studies BMSCs are transfected with lentivirus encoding shRNA (short hairpin RNA) that “knocks down” gene expression in transduced cells [4, 5]. For gain of function adenoviruses are used because they are particularly effective for human material giving transient but highly efficient and abundant protein production [5, 6]. The effectiveness of targeted knockdown or overproduction is determined by measuring mRNA and/or protein for CCN4 [4].

In summary, in this chapter, methods are described for isolating and analyzing osteoprogenitors from mouse and human sources to study the role of CCN4 in skeletal function.

2 Materials

Ccn4-KO mice, mouse bone marrow stromal cells (mBMSCs), primary osteoclasts, human bone marrow stromal cells (hBMSCs).

2.1 Genotyping

2.1.1 DNA Extraction

1. Tail biopsies from *WT* or *Ccn4*-KO mice.
2. Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA): Extraction solution, Tissue preparation solution, Neutralization Solution B.
3. Block incubator or PCR machine.

2.1.2 PCR

1. Oligonucleotides at 0.1 µg/µL corresponding to wild type and *Ccn4*-KO alleles [2].
2. GoTaq® Master Mix (Promega, Fitchburg, WI, USA).
3. DNA template (see Subheading 3.1.1).
4. Distilled DNase free water (DW).
5. PCR machine

2.2. In Vitro Osteogenic Differentiation Assay Using Mouse Bone Marrow Stromal Cells (mBMSCs)

2.2.1 mBMSCs Culture

1. 6- to 8-week-old *WT* or *Ccn4*-KO mice.
2. mBMSCs culture media: α-MEM, 20 % FBS (see **Note 1**), 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 55 µM 2-mercaptoethanol.

3. Hank's balanced salt solution (HBSS).
4. 19, 23, and 26G needles.
5. 1 and 20 mL syringes.
6. Tissue culture treated dish.
7. Cell incubator (37 °C, 5 % CO₂).

2.2.2 Plating mBMSCs for the Experiment

1. Cell preparation solution: 175 U/mL of Type IV collagenase, 1 U/mL of dispase, Hank's balanced salt solution (HBSS) (*see Note 2*).
2. HBBS.
3. 0.05 % trypsin-EDTA.
4. mBMSCs culture media (*see Note 3*, Subheading 2.2.1).
5. Cell incubator (37 °C, 5 % CO₂).

2.2.3 Osteogenic Differentiation of mBMSCs

1. mBMSCs (*see Subheading 3.2.1*).
2. Osteoblastic differentiation medium: mBMSC culture medium (*see Subheading 2.2.1, item 2*), 10⁻⁸ M dexamethasone, 100 µM ascorbic acid 2-phosphate, and 2 Mm, β-glycerophosphate.
3. 6-well culture plate.

2.2.4 Alizarin Red S Staining

1. BMSCs cultured until mineralized nodules are observed (*see Subheadings 2.2.3 and 3.2.3*).
2. Staining solution: 2% alizarin red S (pH 4.2; Sigma-Aldrich), DW.
3. 0.22 µm filter.
4. PBS, PH 7.4.
5. 60 % isopropanol.

2.2.5 Quantification of the Alizarin Red S Staining

1. Eluting solution: 0.5 % SDS, 0.5 N HCl.
2. 96-well plate.
3. Microplate reader (405 nm filter).

2.3 In Vivo Osteogenic Differentiation Assay

2.3.1 Transplants

1. mBSMCs (*see Subheading 2.2.1*).

2. Immunocompromised mice (*see* **Note 4**).
3. Gelfoam™ (Pfizer, New York City, NY, USA): cut in uniform size of $5 \times 5 \times 7$ mm.
4. Two sheets of sterilized filter paper.
5. Culture dishes.
6. Sharp-point tip straight sterile tweezers.
7. Sterile scissors.
8. Wound clip/tissue glue/suture.

2.3.2 Analysis

1. Micro-CT (*see* **Note 5**).
2. Histology (*see* **Note 6**).

2.4 In Vitro Bone Resorption Assay

2.4.1 Primary Osteoclasts Culture

1. 6- to 8-week-old *WT* or *Ccn4*-KO mice.
2. Primary osteoclast culture medium: α -MEM, 10 % FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2.5 U/mL amphotericin.
3. 19, 23, and 26G needles.
4. 1 and 20 mL syringes.
5. Tissue culture treated dishes or flasks.
6. 96-well plates.
7. 20 ng/mL of M-CSF (R&D Systems, Minneapolis, MN, USA).
8. 30 ng/mL of sRANKL (R&D Systems).

2.4.2 Bone Resorption Assay

1. Primary osteoclastic precursor cells (*see* Subheading 2.4.1).
2. OsteoAssay™ 96-well plates (Nunc, Rochester, NY, USA).
3. Primary osteoclast culture media (*see* Subheading 2.4.1, **item 2**).
4. 10 % bleach solution (dilute sodium hypochlorite with double distilled water).

2.5 In Vitro Analysis Using Human Bone Marrow Stromal Cells (hBMSCs)

2.5.1 hBMSCs Culture—hBMSCs are isolated from human bone marrow which were obtained from healthy adult donors under sufficient informed consent or purchased from Lonza (Basel, Switzerland).

1. hBMSC culture medium: α -MEM, 20 % lot-selected FBS, 2 mM glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin sulfate (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA).
2. Tissue culture treated dishes or flasks.
3. Humidified cell incubator (37 °C, 5 % CO₂).

2.5.2 Gain of Function

1. Adenoviruses encoding human CCN4: generate using the BamHI/NotI fragment of full-length human CCN4 cDNA, cloned into the adenovirus vector VQ ad5 DMV K-NpA (Viraquest) (*see Note 7*).
2. hBMSC culture media (*see Subheading 2.5.1, item 1*).
3. Tissue culture treated dishes or flasks.
4. Humidified cell incubator (37 °C, 5 % CO₂).

2.5.3 Loss of Function

1. shRNA lentivirus targeting human CCN4 or GFP control virus (OpenBioSystems; GE Dharmacon, Lafayette, CO, USA) (*see Note 8*).
2. 2 μ g/mL of puromycin.
3. hBMSC culture medium (*see Subheading 2.5.1, item 1*).
4. Tissue culture treated dishes or flasks.
5. Humidified cell incubator (37 °C, 5 % CO₂).

2.5.4 In Vitro Osteogenesis Assay

1. Osteogenic differentiation medium: hBMSC culture medium (*see Subheading 2.5.1, item 1*), 10–8 M dexamethasone, 100 μ M ascorbic acid 2-phosphate, and 2 mM β -glycerophosphate
2. Adenovirus-infected hBMSCs (*see Subheading 2.5.2*).
3. Tissue culture treated dishes or flasks.
4. Humidified cell incubator (37 °C, 5 % CO₂).

2.5.5 In Vivo Osteogenesis Assay

1. Hydroxyapatite–tricalcium phosphate (HA/TCP).
2. 8-week-old immunocompromised mice (Athymic Nude-*FoxInu*, Charles River).
3. Virus-infected hBMSCs (*see Subheading 2.5.2*).
4. Tissue culture treated dishes or flasks.
5. Humidified cell incubator (37 °C, 5 % CO₂).

3 Methods

3.1 Genotyping

3.1.1 DNA Extraction

1. Cut tail (about 3–4 mm) and put into test tube.
2. Add 60 μL of extraction solution and 15 μL of Tissue preparation solution and mix well.
3. Incubate for 10 min at 28 °C, 10 min at 37 °C, 5 min at 98 °C, then cool down to 4 °C.
4. Add 60 μL of neutralization solution B to each DNA sample.

3.1.2 PCR

1. Mix Materials 1–4 (1 μL each of oligonucleotides, 10 μL of GoTaq® Master Mix, 1 μL of DNA template, 6 μL of DW; total 20 μL) to prepare PCR template.
2. Run PCR with “touch down” protocol (*see below*)

	94 °C	5 min
2 cycles {	94 °C	30 s
	X °C	30 s
	72 °C	30 s
“Touch down”: X = 68, 66, 64, 62, 60, 58, 56, 54, 52, 50		
20 cycles {	94 °C	30 s
	48 °C	30 s
	72 °C	30 s
	72 °C	3 min
	4 °C	Fold (Fig. 1)

3.2 In Vitro Osteogenic Differentiation Assay Using Mouse Bone Marrow Stromal Cells (mBMSCs)

3.2.1 mBMSCs Culture

1. *Prepare the mouse.* Euthanize a mouse and keep it on ice. Soak the mouse in 70 % ethanol for sterilization.

2. *Dissect bones:* Skin the mouse and dissect out the hind limb. It must be kept in HBBS (*see Note 3*) prior to starting the next step. After sanitizing the workspace, separate a femur from a tibia and remove muscles as much as possible. Cut off both ends of the femur and tibia and keep it in the culture media (*see Note 3*). Make sure that the marrow cavity is exposed.
3. *Flush marrow cells from the bone shaft:* Flush bone marrow into new 50 mL test tube from the bone shaft with culture medium using a 23 G needle. Flushing with culture media, move needle up and down to wash the inside of the bone shaft. Change to 16 G needle and suspend slowly to separate the cells in the 50 mL test tube.
4. *Spin down the cells:* at $277 \times g$ (RCF) for 8 min.
5. *Cell seeding:* Seed cells into $1 \times T-75$ cm² flasks (marrow from two femurs + two tibias). First medium change will be after 7 days.

3.2.2 Plating mBMSCs for the Experiment

1. Prepare cell preparation solution.
2. Wash the cells (it is best to use primary cells) with 15 mL of HBSS one time.
3. Incubate the cells with 10 mL of cell preparation solution for 1 h at 37 °C.
4. In the meantime, prepare ice and 15 mL of mBMSC culture medium in 50 mL test tube.
5. Collect the cell preparation solution into the Falcon tube and keep on ice.

Add 10 mL of 0.05 % trypsin–EDTA into the flask and incubate for another 5–10 min at 37 °C.

1. Tap the flask several times, then add 5 mL of mBMSC culture medium and wash the inside the flask with the trypsin medium to get as many cells as possible. Place the trypsin medium into the Falcon tube kept on ice.

*Repeat **steps 4–5** if many cells remain on the culture flask.

1. Add 10 mL of mBMSC culture medium into the flask and wash, then transfer to the test tube.
2. Centrifuge at $277 \times g$ (RCF) for 8 min. Aspirate most of the liquid without touching the pellet.
3. Add 10 mL of media and count the number of the cells.

3.2.3 Osteogenic Differentiation of mBMSCs

1. Culture mBMSCs in 6-well plate with mBMSC culture medium until 80 % confluent.
2. Change medium with osteogenic differentiation medium 2–3 times a week for 3–6 weeks or more.

3. When the mineralized nodules are observed (phased bright), stain with alizarin red S (*see* Subheadings 2.2.4 and 3.2.4).
4. RNA and protein collection is at 1 or 2 weeks after induction (Fig. 2).

3.2.4 Alizarin Red Staining

1. Make the staining solution by dissolve 2 g of alizarin red S in 100 mL of distilled water.
2. Filtrate staining solution through 0.22 μ m filter and store at RT.
3. Wash the BMSCs culture dish with PBS two times.
4. Fix the cells with 60 % isopropanol for 1 min at RT.
5. Rehydrate with distilled water for 2–3 min
6. Stain with staining solution for 3 min at RT.
7. Wash the dish with distilled water several times.
8. Dry and observe under the microscope

3.2.5 Quantification of the Alizarin Red S Staining

1. Make the eluting solution.
2. Add 500 μ L/well (6-well plate) of eluting solution and wait 30 min at R/T.
3. Transfer 150 μ L of stained solution to 96-well plate. It is recommended to prepare triplicate for each sample to adjust for technical error.
4. Measure with microplate reader at 405 nm.

3.3 In Vivo Osteogenic Differentiation Assay

3.3.1 Transplants

1. *Preparation of cells:* Trypsinize cells and count them (*see* Subheadings 2.2.2 and 3.2.2). Resuspend to a concentration of 2.0×10^6 cells/mL and put a 1.0 mL aliquot into 1.5 mL test tube. Spin down the cells at $277 \times g$ (RCF) for 5 min, then aspirate the media down to about 400 μ L (the volume between each tube needs to be equal).
2. *Preparation of the Gelfoam™:* Soak Gelfoam™ into the culture media (in the new culture dish) to take all air bubbles out. Fold sterilized paper in the middle and place it onto the new sterile 15 cm culture dish. Sandwich a piece of Gelfoam™ between sterilized paper to remove the culture media and air bubbles as much as possible.
3. Mix the cell suspension, and then gently put the Gelfoam™ into it to saturate it with the all the cells.

—Move to an animal procedure room—

1. Disinfect the skin of the back with povidone–iodine and alcohol.

2. Make a 1/2 to 1 in. incision and make a pocket by opening and closing scissors. Put a piece of Gelfoam™ into the subcutaneous tissue pocket on both sides of the upper/lower back, then close the wound with autoclips, suture, or tissue glue.
3. Wait for 6–8 weeks and then harvest.

* The transplant will feel hard if it is mineralized (Fig. 3)

3.4 In Vitro Bone Resorption Assay

3.4.1 Primary Osteoclast Culture

1. Male mice 9–11 weeks old are euthanized via isofluorane (chloroform is not good for osteoclasts), the long bones [femur and tibia] are harvested and the marrow is flushed using α -MEM medium *with no serum* into 50 mL tubes. In order to break up all cell clusters and get a single cell suspension the cells are triturated using a syringe with a long 19 G needle followed by 23 and 26G needles.
2. Centrifuge the tube at $493 \times g$ (RCF), RT for 10 min.
3. Remove the supernatant and resuspend in a volume of α -MEM medium containing 10 % FBS that corresponds to the desired volume based on the size of the dish to be used (i.e., 10 mL per 10 cm dish).
4. Add the medium and add cells in treated culture dish. In general, use the same number of dishes as the number of mice.
5. After 3 h collect the supernatant and add it to a new treated culture dish and incubate overnight.
6. After the overnight incubation, collect the supernatant and put it into a 50 mL tube.
7. Centrifuge at $373 \times g$ (RCF), RT for 5 min.
8. Discard the supernatant and resuspend the cells according to the pellet size (usually 1 mL for cells pelleted from one animal).
9. Count the cells using a 1:20 dilution (add 25 μ L of the cell suspension into 475 μ L of α -MEM + 10 % FBS medium).

3.4.2 Bone Resorption Assay

1. Plate 5.0×10^5 cells/cm² (1.65×10^5 cells per single well of 96-well plate) with 30 ng/mL of sRANKL and 20 ng/mL of M-CSF. To reduce an edge effect, fill surrounding experimental wells with liquid.
2. Change medium every other day until giant multinucleated cells (osteoclasts) appear (after ~3–5 days).
3. Add 100 μ L/well of 10 % bleach solution.
4. Incubate at RT for 5 min.

5. Aspirate bleach solution and wash wells with 150 μ L/well of double distilled water twice.
6. Air-dry at RT for 3–5 h.
7. To visualize the resorbed area, stain with Von Kossa or 1 % toluidine blue.

3.5 In Vitro Analysis Using Human Bone Marrow Stromal Cells (hBMSCs)

3.5.1 hBMSCs Culture

1. Culture cells with hBMSC culture media until 80 % confluent.
2. Use hBMSCs from third to seventh passage because BMSCs are heterogeneous populations and gradually begin to lose the ability to differentiate after repeated and extensive passaging.

3.5.2 Gain of Function

1. Culture hBMSCs with culture medium until 80 % confluent.
2. Change media to contain adenovirus (5×10^3 /particles/cell) to allow for transduction of adenovirus into the BMSCs.
3. Cells can be used for analysis 72 h after transduction.

3.5.3 Loss of Function

1. Culture hBMSCs with culture medium until 80 % confluent.
2. Change medium containing 2 μ g/mL of puromycin for positive selection.
3. Add shRNA lentivirus targeting human *CCN4* or *GFP* control virus to the cells.
4. Cells were used for analysis 48 h after transduction

3.5.4 In Vitro Osteogenesis Assay

1. Culture cells with hBMSC culture medium until confluent.
2. Switch medium to osteogenic differentiation medium and change twice a week for 3–6 weeks or more.
3. When the mineralized nodules are observed, stain with alizarin red S and visualize phase bright in an inverted microscope.
4. RNA and protein collection is at 1 or 2 weeks after induction (for details *see ref.* 4).

3.5.5 In Vivo Osteogenesis Assay

1. $2.0\text{--}3.0 \times 10^6$ of virus-infected hBMSCs were mixed with 40 mg of sterilized HA/TCP powder.
2. Incubate them at 37 °C for 90 min on a shaker.
3. Spin down $193 \times g$ (RCF) for 1 min.

4. Implant it under the skin on the backs of 8-week-old immuno- compromised mice as described above.
5. After 6 weeks, harvest the transplants and fix with 4 % PFA and embedded in paraffin.
6. 6- μ m sections are stained with hematoxylin and eosin.
7. Quantification of de novo bone formation was performed using a microscope equipped with an analyzer (Fig. 4).

4. Notes

1. To use “lot selected FBS” is necessary for BMSCs culture.
2. Cell preparation solution must be protected from light and good through 1 month at 4 °C.
3. It is advised to keep the HBBS or culture media on ice.
4. The immunocompromised mice can also be purchased from Harlan and Taconic and using the strain: Nude-Fox1nu.
5. The precise method for analyzing the transplants by micro-CT will depend on the type of machine you are using. After the transplants are fixed they can be scanned in the micro-CT and then processed for histology (*see Note 6*). Many parameters can be assessed using the including Bone Volume/Total Volume, trabecular number and trabecular spacing. Because of the potential variation in the experimental outcome it is sug- gested that comparisons between *WT* and *Ccn4*-KO be made within the same animal.
6. The BMSCs form a hard tissue within the transplant can be first fixed then subject to micro-CT scanning and then subse- quently de-calcified prior to embedding and processing. One standard means of decalcifying is to soak the tissue in a solu- tion of 10 % EDTA in 1 $\times$ PBS. The efficacy of demineraliza- tion is determined by X-ray: the decalcified tissues will have no radiopaque (white) areas in the X-ray. Standard histology is performed after sectioning using H&E staining. The bone is characteristically pink and quantified by tracing the bone area/total area.
7. The concentration of virus used should be titrated for each exper- iment to determine the optimal dose to use. In our hands 5 $\times$ 10³/ particles per cell were sufficient to give robust CCN4 expression in hBMSCs. Note that the adenovirus transduction provides a transient but efficient level of protein production. The maximum expression of a transgene after transduction will be from 3–5 days and then tapers off until 2 weeks post-transduction.
8. In general, several different clones of lentivirus should be tested to check for their efficacy. For CCN4 knockdown, five clones were tested and ranged in their ability to reduce CCN4 expression from ~30 % of controls (the least effective) to 90 % (the most effective). The efficiency of knockdown was deter- mined by

measuring the relative CCN4 mRNA expression/ S29 (a ribosomal gene) in the infected BMSCs. Note also that the lentivirus provides a stable integration of the shRNA into the host cell genome.

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Fig. 1.

Genotyping of *WT* and *Ccn4*-KO mice. (a) A small biopsy is removed from the tail and DNA extracted and subject to PCR using oligonucleotides that specifically amplify the recombined (targeted) *Ccn4* allele. A positively recombined ES cell clone (3H4) was used as control (*last lane*). One mouse, #34 contained the recombined allele while another (#36) did not. (b) BMSCs from mouse #34 were isolated and expanded in culture and mRNA extracted for real-time PCR analysis using oligonucleotides specific for *Ccn4*. *** $p < 0.001$. Mice with the successfully altered allele were devoid of *Ccn4* expression making them a true knockout (KO) mouse line

Fig. 2.

(a) Outline of the temporal sequence of BMSC cell culture and analysis in weeks (W). (b) Representative alizarin red staining of BMSCs from *WT* (+/+) or *Ccn4*-KO (-/-) mice. (c) Quantitative assessment of the relative level of alizarin red staining in WT and *Ccn4*-KO at 1 and 3 weeks of culture. At both time points the *Ccn4*-KO cells accumulate less calcium that binds to alizarin red S. This is used as an indirect measure calcification and osteogenic differentiation. * $p < 0.05$

Fig. 3.

(a) Diagram of the methods used to expand and analyze BMSC growth in vivo in subcutaneous transplants. (b) Micro-CT analysis of representative transplants from *WT* (+/+) and *Ccn4*-KO mice. *Top panel* shows a view of half the transplant and the bottom panels show axial, sagittal, and coronal views showing that in all cases the transplants formed from the *Ccn4*-KO cells have less radiodense bone formation (reproduced from [4] with permission from John Wiley and Sons)

Fig. 4.

(a) Transplants of hBMSCs transduced with control (adCMV) that does not contain an overexpressing transgene, adCCN4, adBMP-2 that overexpress CCN4 and or BMP-2 respectively. (b) Quantitation of the bone area in the transplants shown in a *** $p < 0.001$ between control adCMV and adCCN4 or adBMP-2, ###*** $p < 0.001$ between adBMP-2 and adCCN4 plus adBMP-2. (c) Transplants from hBMSC infected with control lentivirus (GFP) or lentivirus encoding shCCN4 RNA. (d) Quantitation of the bone area made by control lentivirus compared to shCCN4 lentivirus. *** $p < 0.001$ between control GFP and shCCN4 (reproduced from [4] with permission from John Wiley and Sons)