Video Article

Stimulation of Notch Signaling in Mouse Osteoclast Precursors

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URL: <https://www.jove.com/video/55234>

DOI: [doi:10.3791/55234](https://doi.org/10.3791/55234)

Keywords: Developmental Biology, Issue 120, Notch signaling, osteoclast, jagged1, mouse, bone, macrophages

Date Published: 2/28/2017

Citation: Kaur, G., Ahn, J., Hankenson, K.D., Ashley, J.W. Stimulation of Notch Signaling in Mouse Osteoclast Precursors. *J. Vis. Exp.* (120), e55234, doi:10.3791/55234 (2017).

Abstract

Notch signaling is a key component of multiple physiological and pathological processes. The nature of Notch signaling, however, makes *in vitro* investigation of its varying and sometimes contradictory roles a challenge. As a component of direct cell-cell communication with both receptors and ligands bound to the plasma membrane, Notch signaling cannot be activated *in vitro* by simple addition of ligands to culture media, as is possible with many other signaling pathways. Instead, Notch ligands must be presented to cells in an immobilized state.

Variations in methods of Notch signaling activation can lead to different outcomes in cultured cells. In osteoclast precursors, in particular, differences in methods of Notch stimulation and osteoclast precursor culture and differentiation have led to disagreement over whether Notch signaling is a positive or negative regulator of osteoclast differentiation. While closer comparisons of osteoclast differentiation under different Notch stimulation conditions *in vitro* and genetic models have largely resolved the controversy regarding Notch signaling and osteoclasts, standardized methods of continuous and temporary stimulation of Notch signaling in cultured cells could prevent such discrepancies in the future.

This protocol describes two methods for stimulating Notch signaling specifically in cultured mouse osteoclast precursors, though these methods should be applicable to any adherent cell type with minor adjustments. The first method produces continuous stimulation of Notch signaling and involves immobilizing Notch ligand to a tissue culture surface prior to the seeding of cells. The second, which uses Notch ligand bound to agarose beads allows for temporary stimulation of Notch signaling in cells that are already adhered to a culture surface. This protocol also includes methods for detecting Notch activation in osteoclast precursors as well as representative transcriptional markers of Notch signaling activation.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55234/>

Introduction

The mammalian Notch signaling pathway is homologous to the same pathway in *Drosophila melanogaster* and consists of four transmembrane Notch receptors (Notch1-4) and five membrane-bound ligands of the Jagged (JAG1 & JAG2) and Delta-like (DLL1, 3, & 4) families¹. Notch signaling is initiated when Notch receptor on a receiving cell is bound by ligand on a transmitting cell². During this trans-activation, the membrane-bound Notch ligand produces a stretching force on the membrane-bound Notch receptor^{3,4}. The stretching force of ligand binding induces conformational changes in the Notch receptor that facilitate extracellular cleavage of the receptor by TNFalpha converting enzyme (TACE) followed by an intracellular cleavage event mediated by a presenilin-containing gamma-secretase complex (γ-secretase). γ-secretase releases the Notch intracellular domain (NICD) which translocates into the nucleus where it forms a transcriptional activation complex with CBF-1-Su(H)-Lag-1 (CSL), mastermind-like (MAML), and cell type-specific factors to drive expression of target genes⁵.

The mechanical elements of Notch signaling activation result in the need for unique methods of Notch pathway activation *in vitro*. Soluble Notch ligands can bind to Notch receptors, but fail to produce stretching forces necessary for NICD release while at the same time competitively inhibiting binding of cell-associated Notch ligands. Thus, addition of soluble Notch ligands to culture medium can attenuate normal Notch signaling^{6,7}. Fortunately, Notch ligands can induce NICD release if they are fixed to a suitably rigid substrate^{5,8,9,10}. Seeding cells on ligand-coated culture substrates or applying ligand-coated beads to cells can both activate Notch signaling, and the choice between them depends primarily on the desired timing of Notch stimulation. For immediate, temporary Notch signaling activation, as would be desired during the midpoint of a functional or differentiation assay, Notch ligand can be bound to agarose beads, applied to cultured cells, and washed out at any time. For more sustained Notch signaling from the beginning of a culture period, tissue culture plates can be coated with ligand prior to cell seeding.

For the purposes of this protocol, methods are carried out using mouse osteoclast precursors, but the methods and variations on the methods described here are applicable to a wide variety of cell types^{6,11,12,13,14}. Osteoclasts are terminally differentiated hematopoietic lineage cells that are responsible for the resorption of bone tissue, and they are implicated in multiple disorders of bone loss¹⁵. Thus, *in vitro* study of the differentiation of osteoclasts from their monocyte/macrophage-lineage precursors and the molecular mechanisms controlling their function is essential to better understanding of osteoclasts and development of new bone-regenerating therapies. While it is now generally accepted that Notch signaling plays a positive role in the differentiation and function of osteoclasts, variations in both Notch signaling stimulation and osteoclast precursor culture and differentiation led to initially contradictory findings^{16,17,18,19}. Closer examination of the differences in methods and use of genetic models have greatly clarified the role of Notch signaling in osteoclastogenesis, but application of standardized Notch stimulation and culture methods could prevent such controversies in future studies of Notch signaling in other cell types^{20,21,22,23}.

There are multiple methods for culturing and differentiating mouse osteoclast precursors, and, as with varying methods for stimulating Notch signaling, the best method will depend upon the experimental question. Herein, our preferred method of culturing adherent and non-adherent fractions of marrow cells flushed from mouse long bones will be presented. This method has the advantage of requiring essentially no specialized equipment and producing cells that are applicable to a variety of differentiation methods.

Protocol

All research involving vertebrate animals was performed in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

1. Culture Media Preparation

1. Prepare α -MEM by dissolving minimum essential medium (MEM) powder and 1.9 g sodium bicarbonate in 900 ml H₂O and supplement with 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 100 ml heat-inactivated fetal bovine serum. Sterilize using a 0.22 μ m polyethersulfone (PES) filter.
2. Prepare macrophage medium by supplementing α -MEM with 35 ng/ml recombinant mouse Macrophage-Colony Stimulating Factor (M-CSF)
3. Prepare osteoclast medium by supplementing macrophage medium with 100 ng/ml RANKL

2. Bone Marrow Cell Isolation

1. Fill one 10 ml syringe with a 25% gauge needle per mouse with 10 ml α -MEM.
2. Euthanize mice via 10 min exposure to CO₂ at a flow rate of 1.3 L/min. Ensure animal death by following CO₂ exposure with cervical dislocation before proceeding with dissection.
3. **Using clean technique, dissect out and separate the tibiae and femora. Disinfect mouse skin with 70 % ethanol before making an incision along the anterior surface of each hind limb to expose the tibia and femur.**
  1. Cut through the knee joint to free the distal end of the femur, and cut through the femur as close to the pelvis as possible. Remove the femur and clean as much soft tissue as possible. To remove the tibia, cut through the ankle and remove as much soft tissue as possible.
4. Hold the bone with forceps and flush marrow into a 15 ml conical tube with 2.5 ml room temperature α -MEM combining marrow flushes from a single mouse into a single tube.
NOTE: A successful marrow flush will change the coloration of the bone from red to beige.
5. Allow debris to settle to the bottom of the tube and transfer supernatant to a clean 15 ml conical tube taking care to avoid transferring any debris.
NOTE: Some cells may also settle with the marrow debris. If a greater number of cells is required, marrow flushes can be filtered through a 70 μ m cell strainer into a clean 15 ml tube.
6. Centrifuge tube(s) at 300 x g for 5min at room temperature to pellet cells.
7. Vacuum aspirate the supernatant and resuspend cell pellet in 0.5 ml Ammonium-Chloride-Potassium (ACK) lysing buffer and incubate at 37 °C for 3 min to lyse red blood cells.
8. Add 10 ml PBS directly to ACK-cell solution, and centrifuge at 300 x g for 5 min.
NOTE: The previously red cell pellet should now be beige.
9. Vacuum aspirate supernatant and resuspend pellet in 10 ml α -MEM, and plate in a 100 mm tissue culture-treated dish. Incubate cells overnight at 37 °C in a humidified tissue culture incubator.
NOTE: Counting cells is not necessary for this step. During this time, adherent cells from the bone marrow will attach to the culture surface; non-adherent bone marrow cell populations will remain in suspension. MCSF is not present in the culture medium during this initial incubation.

3. Enrichment of Osteoclast Precursors

1. After overnight incubation of marrow flushes, transfer culture supernatant containing non-adherent cells to a 50 ml conical tube.
NOTE: Culture dishes with adherent cells, which include bone marrow mesenchymal progenitor cells, can be discarded or fed with fresh α -MEM, if desired for other experiments.
NOTE: Osteoclasts can be differentiated directly from these non-adherent bone marrow cells by plating them into tissue culture-treated dishes at a density of 4×10^5 cells/cm² in osteoclast medium and refreshing medium every other day for 5 days.
2. Add α -MEM to non-adherent cell solution to a final volume of 18 ml and add M-CSF to a final concentration of 35 ng/ml.
3. Add 3 ml of cell suspension each to 6 60 mm suspension culture dishes.

4. Incubate plates overnight at 37 °C in a humidified tissue culture incubator. During this time, M-CSF will stimulate differentiation of macrophages, which can adhere even to non-tissue culture-treated surfaces.
5. After incubating overnight, re-feed osteoclast precursors with 3 ml fresh macrophage medium. This will remove non-adherent cells that haven't differentiated into macrophages.
6. Continue to culture osteoclast precursors at 37 °C and 5 % CO₂ for 2 days or until cultures reach roughly 70% confluence.

4. Osteoclast Differentiation

1. Wash osteoclast precursors with 5 ml PBS and treat cells with 1 ml marine-origin proteolytic/collagenolytic enzyme at room temperature until the cells can be dislodged by gentle tapping of the plate.
NOTE: Osteoclast precursors can only be lifted if they are cultured in suspension dishes; precursors attach too firmly to tissue culture-treated surfaces to be lifted. Trypsin can activate osteoclast precursors and should be avoided.
2. Add 4 ml α -MEM to each dish and transfer cells to a 50 ml conical tube. Count cells using a hemocytometer and dilute cells to a density of 5×10^4 cells/ml and add M-CSF to a final concentration of 35 ng/ml and RANKL to a final concentration of 100 ng/ml.
3. Seed cells at a density of 2.6×10^4 cells/cm² into tissue culture-treated plates and incubate at 37 °C in a humidified tissue culture incubator for 2 days. Differentiation is typically carried out in 24-well plates, where 5×10^4 cells are seeded into each well.
4. 2 days after the start of the differentiation, re-feed cells by replacing medium with 1 ml fresh osteoclast medium.
NOTE: Osteoclast differentiation will usually be complete within 3 days. Resulting osteoclasts can be TRAP-stained for easy quantification and morphological analysis.

5. Continuous Stimulation of Notch Signaling with Jagged1-coated Surface

1. Suspend goat anti-human IgG Fc antibody in PBS at a concentration of 10 μ g/ml (1:1,000 dilution of 10 mg/ml stock). Add antibody solution to culture surface at a density of 1.3 μ g/cm² and incubate at room temperature for 1 hr. In the case of 24-well plates, add 250 μ l (2.5 μ g) per well.
2. Vacuum aspirate wells and refill with 1 ml PBS. Repeat this step 2 times.
3. Suspend recombinant Jagged1-Fc fusion protein in PBS at a concentration of 10 μ g/ml. Add Jagged1-Fc solution to the antibody-coated culture surface at a density of 1.3 μ g/cm² and incubate at room temperature for 2 hr. Culture surfaces coated with antibody only can be used as controls.
4. Vacuum aspirate wells and refill with 1 ml PBS. Repeat this step 2 times. Keep PBS in wells until just prior to cell seeding to prevent them from drying.
5. Seed 2.6×10^4 osteoclast precursors/cm² onto coated surfaces. Notch signaling will be continuously stimulated for at least 3 days.

6. Temporary Stimulation of Notch Signaling with Jagged1-coated Beads

1. Combine the following in an appropriately sized tube: 0.5 μ g/cm² Jagged1-Fc, 21 μ l/cm² protein G agarose beads, and PBS to a final volume of 1.5 ml. To prepare an adequate number of Jagged1 beads for 1 well of a 24 well plate, combine 1 μ g Jagged1-Fc, 40 μ l protein G agarose beads, and PBS to 1.5 ml.
2. Incubate tube with rotation at 4 °C for 2 hr.
3. Centrifuge tube at 300 x g for 1 min to pellet beads. Resuspend beads in 1.5 ml PBS. Repeat this wash 2 more times.
4. Resuspend Jagged1 beads in 130 μ l/cm² culture medium and apply to cultured cells.
5. Incubate cells with beads at 37 °C and 5 % CO₂ for desired period. Remove beads with several washes of PBS or culture medium.
6. Verify Notch signaling activation via measurement of Notch target gene transcripts¹⁰.

Representative Results

The aim of this method is to culture and stimulate Notch signaling in osteoclast precursors. When properly cultured, osteoclast precursors exhibit a primarily elongated spindle-shaped morphology with smooth cytoplasm (**Figure 1A**). Care should be taken to avoid immunological activation of the osteoclast precursors. Upon activation, precursors spread and become flattened with foamy cytoplasm (**Figure 1B**). These "fried egg" cells are resistant to RANK signaling and do not efficiently differentiate into mature osteoclasts. Under correct culture and differentiation conditions, enriched osteoclast precursors will differentiate and fuse into large TRAP-positive multinuclear osteoclasts within three days (**Figure 1C**).

Seeding osteoclast precursors onto Jagged1-Fc coated surfaces up-regulates Notch target genes within 24 h (**Figure 2**). Specific genes up-regulated by Notch signaling vary by cell type. In the case of osteoclast precursors, the most predominant gene up-regulated by Jagged1 is Hes1, though Hey2 and Myc are moderately up-regulated, as well. Expression of other Notch target genes such as Hey1 and p21 are not significantly altered by Jagged1 stimulation of osteoclast precursors.

When osteoclast precursors are cultured in 24-well plates, treatment with beads coated with as little as 600 ng Jagged1-Fc demonstrate a significant increase in Hes1 expression within 24 h (**Figure 3**). Forty-eight h treatments show a similar increase in Hes1 expression, though to a lesser degree.

Figure 1: Culture and differentiation of osteoclast precursors. (A) Naïve osteoclast precursors cultured with 35 ng/ml MCSF and without activation stimuli have a smooth, spindle-shaped morphology. Scale bar = 25 μ m. (B) Upon immunological activation, osteoclast precursors adopt a flattened, foam-filled morphology and will not differentiate into osteoclasts. To induce activation, osteoclast precursors were treated with 10 ng/ml lipopolysaccharide for 16 h. Scale bar = 25 μ m. (C) Immunologically naïve adherent osteoclast precursors cultured with 35 ng/ml MCSF and 100 ng/ml for 3 days differentiate into mature osteoclasts. Mature osteoclasts are large, multinuclear, and TRAP-positive. Scale bar = 200 μ m. [Please click here to view a larger version of this figure.](#)

Figure 2: Up-regulation of Notch target genes in osteoclast precursors plated on immobilized Jagged1-Fc. Osteoclast precursors were plated on immobilized Jagged1-Fc and cultured for 24 hr. During the culture period, expression Hes1, Hey2, and Myc mRNA was increased. Other Notch target genes, Hey1 and p21 were not altered. Error bars are standard error of the mean, significance was evaluated using student's 1-tailed t test. *, $p < 0.05$. [Please click here to view a larger version of this figure.](#)

Figure 3: Up-regulation of Hes1 mRNA in osteoclast precursors treated with Jagged1-Fc beads. 600 ng Jagged-Fc bound to protein G agarose beads up-regulates Hes1 expression in osteoclast precursors cultured in the well of a 24-well plate within 24 h of exposure. Hes1 induction by Jagged1-beads was comparable to Hes1 levels induced by Jagged1-Fc-coated plates. Other Notch target genes which were not strongly induced by Jagged1-Fc-coated plates were not assessed. Error bars are standard error of the mean, significance was evaluated using student's 1-tailed t test. *, $p < 0.05$. [Please click here to view a larger version of this figure.](#)

Discussion

Critical steps within the protocol

Culture and *in vitro* differentiation of osteoclast precursors provides a useful platform for investigation of molecular mechanisms of osteoclastogenesis and identification of therapeutic targets for bone regeneration and preservation of bone mass. When culturing mouse osteoclast precursors, the most critical element is maintenance of precursors in a naïve state. As macrophage-like cells, osteoclast precursors are primed to respond to bacterial components and pro-inflammatory cytokines. Upon activation, osteoclast precursors will no longer efficiently differentiate into osteoclasts. The primary cause of precursor activation is presence of inflammatory cytokines such as TNF α or improperly inactivated complement in fetal bovine serum. For this reason, sera lots must be tested for their ability to support osteoclastogenesis prior to use in experiments. In addition, all sera used in osteoclast precursor culture must be heat inactivated to denature complement that can activate the osteoclast precursors.

Modifications and troubleshooting

In this method, Jagged1-Fc is used to stimulate Notch signaling in osteoclast precursors. The Fc fusion with Jagged1 facilitates both immobilization on culture surfaces with anti-Fc antibodies and coupling to protein G beads. Other Notch ligands fused to Fc, such as Delta-like1-Fc, can be used in a similar fashion. It should be noted that not all suppliers provide statements of recombinant Notch ligand activity. Therefore, while recombinant Notch ligands can be obtained from multiple suppliers, care should be taken to obtain biological activity information from the supplier or tests of ligands in known Notch ligand-responsive cells should be performed.

Using the protocols described in this method allows for constitutive and temporary stimulation of Notch signaling in cultured osteoclast precursors. The standard method for detecting activation of Notch signaling is quantification of Notch target genes via quantitative reverse-transcription PCR (RT-qPCR). Target genes upregulated by Notch signaling vary by cell, so when Notch stimulation is performed on a previously uncharacterized cell type, expression of a panel of canonical Notch target genes such as members of the Hes and Hey family of transcription factors should be determined. In a shorter time frame, Notch activation can potentially be detected via Western blot assessment of Notch Intracellular Domain (NICD). Care should be taken, however, as there are four Notch receptors and NICDs from the different receptors in different cell types are not released to the same degree following ligand stimulation. NICD level in Notch-stimulated cells should also be compared to levels in non-stimulated cells to account for baseline NICD release.

Limitations of the technique

While this method is theoretically applicable to all Notch ligands, to date it has only been consistently applied using Jagged1 and Delta-like1^{10,20,21}. Further testing will be required to determine whether additional Notch ligands are amenable to this method. This method is also unspecific in its Notch receptor activation; assessment of specific Notch receptor activation following stimulation using this method is necessary to ascribe cellular responses to particular Notch receptors.

Significance of the technique with respect to existing/alternative methods

Osteoclast differentiation is dependent up on both osteoclast precursor number and osteoclast precursor differentiation potential. Evaluation of osteoclast differentiation of mixed bone marrow populations cannot distinguish between alterations in osteoclast precursor number and cell autonomous differentiation potential. Enrichment of osteoclast precursors prior to differentiation controls for differences in precursor numbers and allows closer interrogation of the osteoclast differentiation process. Given that Notch signaling exerts different effects at different points during osteoclastogenesis, temporal control of both Notch signaling and osteoclast differentiation are essential for proper investigation of the molecular pathways it governs.

Future applications after mastering this technique

Using this protocol, assessment of roles for Notch signaling can be performed not only in osteoclast precursors, but potentially in other cell types, as well. By varying length of Notch stimulation and time of Notch signaling initiation, potential multi-phasic roles of Notch signaling in a variety of cellular process can be defined.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This work was supported by the University of Pennsylvania Center for Musculoskeletal Disorders (5 P30 AR050950-09), a grant from the AO Foundation (S-16-12A), the Philadelphia VA Medical Center Translational Musculoskeletal Research Center, and an intramural orthopaedic surgery departmental research development fund. JWA is supported by the University of Pennsylvania Postdoctoral Opportunities in Research and Teaching (PENN-PORT) fellowship funded by the National Institute of General Medical Sciences Institutional Research and Career Development Award (IRACDA; 5 K12 GM081259-08).

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