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Mandibular Explant Assay for Investigating Extrinsic Stimuli on Bone and Cartilage Development

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[Abstract] A major issue in developmental biology is to determine how embryonic tissues respond to molecular signals in a timely manner and given the position-restricted instructions during morphogenesis, of which Meckel's cartilage is a classical example. The *ex-vivo* explant model is a practical and convenient system that allows investigation of bone and cartilage responses to specific stimuli under a controlled manner that closely mimics the *in vivo* conditions. In this protocol, the explant model was applied to test whether Meckel's cartilage and surrounding tissues are responsive to the Endothelin1 (Edn1) signaling molecule and whether it would rescue the phenotype of genetic mutations. The system allows a high degree of manipulation in terms of the concentrations of exogenous compounds added to the explant, time points with regards to measuring mandibular development, and the method of application of exogenous molecules and teratogens.

Keywords: *Ex-vivo* mandibular explant, Craniofacial development, Mandibular dysplasia, Mandibular hypoplasia, Agnathia, Mandibular patterning, Meckel's cartilage, Embryonic development

[Background] Craniofacial malformations are among the most frequent congenital birth defects in humans (Miettinen *et al*., 1999). Many of these malformations occur during facial morphogenesis, a complex multi-step process in which cranial neural crest cells migrate to pharyngeal arches to give rise to many facial structures (Jin *et al*., 2011).

Both variations within specific genes as well as gene-gene interactions can lead to craniofacial deformations. Mutations in *IRF6,* a gene that contributes to the formation of ectoderm and epithelium in the head and face, can result in cleft lip, cleft palate, and mandibular abnormalities. In comparison to *IRF6*, the *TWIST1* gene regulates neural tube closure during embryonic development and cranial suture fusion during skull development. Mutations in *TWIST1* can cause craniosynostosis, mandibular hypoplasia, and cleft palate (Fakhouri *et al*., 2017). Inhibition or alteration of *IRF6* and *TWIST1* expression can be done similar to the methods performed by Miettinen *et al*. (1999) with *EGF* receptors in order to examine their roles in craniofacial development. However, difficulties arise in *in vivo* experiments when the study begins to incorporate genetic interactions and rescue experiments of two or more allelic mutations. In our recent study, the genetic interaction between *Irf6* and *Twist1* causes severe mandible abnormality and cleft of the secondary palate in the mouse model (Fakhouri *et al*., 2017).

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Various studies have used *in vivo* experiments to test for the expression of various genes involved in craniofacial development. The presence of TGF-β subtypes was studied by using an *ex vivo* culture model in a serumless, chemically defined medium during mandibular morphogenesis (Chai *et al*., 1994). Similarly, a study used Alcian blue staining of cultured mandible explants to examine Meckel's cartilage during morphogenesis in *Egfr*$^{-/-}$ embryos (Miettinen *et al*., 1999). A combination of the methodologies from these studies, including an *ex vivo* mandibular explant described in this report, is useful to characterize the phenotype and signaling pathway in mammalian systems.

Materials and Reagents

1. 60 mm TC-Treated center-well organ culture dish (Corning, Falcon®, catalog number: 353037)
2. Petri dish (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 130182)
3. Test tubes (15 and 50 ml) (Denville Scientific, catalog numbers: C1012 and C1062-P, respectively)
4. Stainless steel grid (0.5 mm hole size) (Home Depot International, model: 3004107, catalog number: 1001034564)
5. Millipore type filters (0.8 µm pore size, 47 mm diameter) (Merck, catalog number: AAWP04700)
6. Sterile individually packaged pipettes (2, 5, and 10 ml) (Genesee Scientific, catalog numbers: 12-101, 12-102, 12-104C)
7. E10.5-E11.5 murine embryos
8. 70% ethanol
9. 95% ethanol
10. 6-Aminonicotinamide (Alfa Aesar, catalog number: L06692)
11. 2 mm-diameter agarose beads or Affi-Gel blue gel (Bio-Rad Laboratories, catalog number: 732-6712)
12. Endothelin1 peptide (Enzo Life Sciences, catalog number: ALX-155-001-PC01)
13. Thymol (Sigma-Aldrich, catalog number: T0501)
14. Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) (Sigma-Aldrich, catalog number: S9390)
15. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
16. Potassium chloride (KCl) (Fisher Scientific, catalog number: BP366)
17. Potassium phosphate monobasic (KH_2PO_4) (Fisher Scientific, catalog number: P286-1)
18. BGJb medium with L-glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 12591038)
19. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F0926)
20. Ascorbic acid (Fisher Scientific, catalog number: S25184)
21. Penicillin-streptomycin 100x (Caisson Laboratories, catalog number: PSL01-100ML)
22. MEM non-essential amino acid solution 100x (Sigma-Aldrich, catalog number: M7145)
23. Glacial acetic acid (Fisher Scientific, catalog number: S25118A)
24. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: P1767)

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25. Alcian Blue 8GX (National Diagnostics, catalog number: HS-504)
26. Alizarin Red S (Acros Organics, catalog number: 400480250)
27. Glycerol (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 17904)
28. Nuclear fast red (Acros Organics, catalog number: 211980050)
29. 10x phosphate-buffered saline (PBS) (see Recipes)
30. Mandibular explant media (see Recipes)
31. 3% acetic acid solution (see Recipes)
32. 2% KOH solution (see Recipes)
33. Alcian blue solution pH 2.5 (see Recipes)
34. Alizarin red solution (see Recipes)
35. 1% KOH / 20% glycerol solution (see Recipes)

Equipment

1. Surgical scissors (World Precision Instruments, catalog number: 501225)
2. Sterile cell culture hood (NuAire, model: NU-545)
3. Stereomicroscope (Nikon Instruments, model: SMZ800N)
4. Forceps (World Precision Instruments, catalog number: 15915)
5. Autoflow IR Direct Heat CO_2 incubator (NuAire, model: NU-5510)
6. Ceramic hot plate (VWR, catalog number: 97042-602)
7. Pipette pump (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 9511)
8. Autoclave (Future Health Concepts, Primus, catalog number: PRPSS8-A)

Software

1. NIS Elements AR software (Nikon Instruments) used for the stereomicroscope

Procedure

A. Embryos (E10.5-E11.5) extraction
   1. Perform euthanization via CO_2 followed by cervical dislocation on a pregnant female mouse (see Note 1). All animal procedures were approved by the Animal Welfare Committee (AWC-16-0068) at the University of Texas Health Science Center at Houston and followed the National Institute of Health guidelines as described by Metwalli *et al*., 2017.
   2. Transfer the animal to a clean procedural room.
   3. Spray ethanol (70%) on the abdominal area of the female.
   4. Use scissors to cut the abdominal skin from the lower abdomen to the epigastric region. Perform the cut at the midline of the abdomen.

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5. Pull the uterus out of the body and cut the connective tissue. This will separate the uterus from the fallopian tubes and ovaries.
6. Keep all embryos together in the uterus sack.
7. Place the sack in a Petri dish with cold 1x PBS (15-20 ml, see Recipe 1).
8. In a sterile hood, separate the embryos by cutting the uterus between the embryos.
9. Transfer all embryos to a new Petri dish with 1x PBS (15-20 ml) at RT to wash and remove excess blood.
10. Open the amniotic sac and separate the placenta from the embryos, severing the umbilical cord near the embryo.
11. Place the embryo in a 15 ml test tube.
12. Wash each embryo twice with 5 ml of 1x PBS (see Note 2).

B. Mandibular extraction

1. In a sterile cell culture hood, dissect the mandibular processes of the first pharyngeal arch under a stereomicroscope while in BGJb culture medium at RT.
2. Pre-warm BGJb culture medium and mandibular explant media (see Recipe 2) at 37 °C. Add 1-2 ml of PBS at RT to the exterior portion of the center-well organ dish and 0.5 ml of the mandibular explant media to the center-well of the organ dish.
3. Orient the embryos in a supine position. Using a scalpel, cut through the labial commissure of the mouth at each corner of the mouth in order to separate the mandibular process from the head of the embryo (see Figure 1).

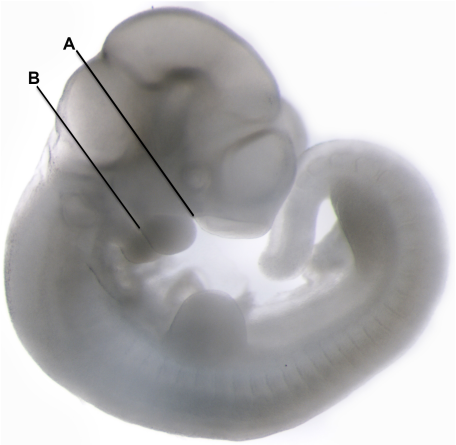

Figure 1. A murine embryo at E11.5 used for dissecting the mandibular process for *ex-vivo* assay. The first cut was done at the commissure of the mouth all the way to the hind-brain (A). The second cut was performed at the top of the neck to separate the mandibular process from the rest of the body (B).

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4. Make another cut through each embryo's neck using a scalpel to completely separate the mandibular process from the rest of the embryo.
5. Place a single stainless steel mesh across the center-well organ dish, so it is submerged in the mandibular explant media (see Figure 2 and see Note 3).

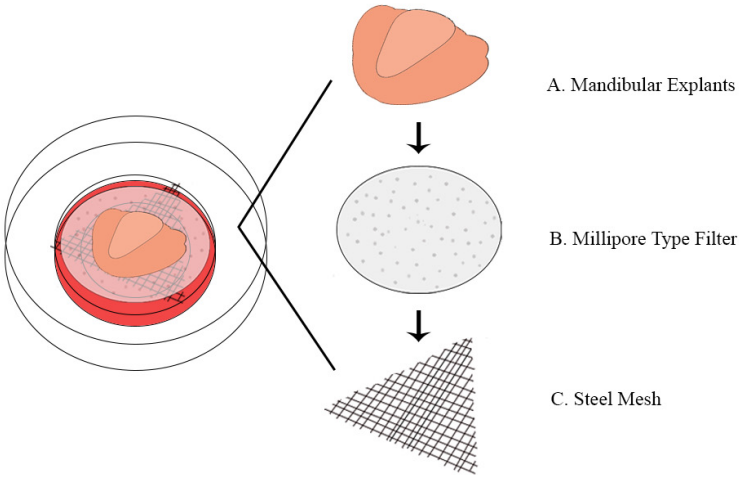

Figure 2. Design of mandibular explant on organ-well culture plate

6. Lay filter paper on top of the metal mesh sheet and allow the filter paper to be submerged in the mandibular explant media (see Note 4).
7. Place explants in the center of the filter paper.
8. Place the Petri dish in a CO_2 incubator at 37 °C.
9. Replace the media (0.5 ml) in the center-well with pre-warmed (37 °C) fresh mandibular explant media every 12 h for the first 24 h and then once a day after the first 24 h.
10. After 12 h of incubation, treat the mandibular sections with 100 mg/ml of 6-aminonicotinamide in mandibular explant media for 3 h. In our study, the 6-aminonicotinamide treatment was used in the experimental samples as a teratogen to the explant (see Note 5) in order to disturb proper tissue development.
11. After 3 h treatment, replace the media containing the teratogen with fresh pre-warmed (37 °C) mandibular explant media.
12. In our case, after the teratogen treatment, the experimental mandible samples were treated with 2 mm-diameter agarose beads containing 0.1 µg/ml of endothelin-1 (Edn1) peptide for 8 h in the CO_2 incubator at 37 °C (see Note 6). The Edn1 was applied to test whether Meckel's cartilage and the surrounding tissues are responsive to this signaling molecule and if it would rescue the phenotype of genetic mutations.
13. After the 8 h treatment, remove the media and the agarose beads that contained Edn1 peptide.
14. Replace the media (0.5 ml) in the center-well with pre-warmed (37 °C) fresh mandibular explant media. After the first 24 h, replace the media once a day (see Note 7).

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15. After 24-120 h, fix explants with 95% ethanol for 1-2 days at RT.
16. Replace the 95% ethanol with 2% KOH (see Recipe 4) for 2-4 h until the explant tissue becomes transparent.
17. Replace 2% KOH with Alcian Blue staining overnight at RT (see Recipe 5). Alcian Blue stains for acidic glycosaminoglycans found in cartilage.
18. Replace staining with 95% ethanol for 12-24 h at RT.
19. Replace the 95% ethanol with Alizarin Red staining (see Recipe 6) for 6 h (Figure 3) at RT. Alizarin Red will stain for calcium containing osteocytes.
20. Submerge explants in 20% glycerol and 1% KOH (see Recipe 7) to remove excess staining.
21. Replace solution with 100% glycerol to store at 4 °C. An antifungal such as Thymol may be added to the storing solution (optional).

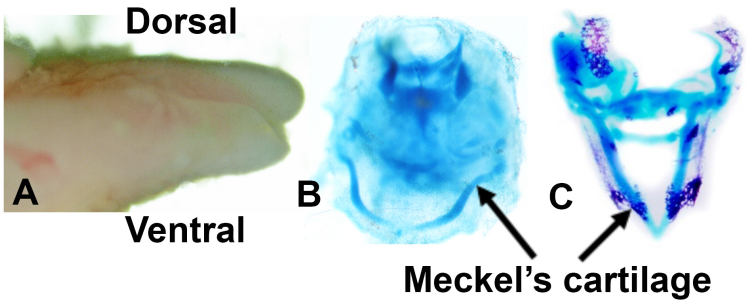

Figure 3. *Ex-vivo* mandibular explant models at E11.5. A. Embryonic mandibular explants extracted at E11.5 and cultured for two days. The image was taken from a side view. B. Alcian Blue stained mandibular explants for Meckel's cartilage two days post-incubation. The image was taken from the top view of the mandible. C. Alcian blue and Alizarin red staining of mandibular explants after 4 days of incubation.

Data analysis

Skeletal staining was performed on the explants and observed through the stereomicroscope using the software NIS Elements AR. For our study, we examined changes in cartilage and bone formation. In this case, we observed variations including a lack of symmetry, arrested growth of the cartilage, and the shape and thickness of the Meckel's cartilage. It is recommended to use at least five technical replicates, meaning five embryos from the same litter, for each experimental treatment to account for any intrinsic variation.

Notes

1. The age of embryos desired to be extracted can be determined by the date of breeding with male and the presence of a copulation plug in the pregnant female.

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2. Each embryo should be washed at least twice. If blood remains, the embryos should be washed until all the blood is removed. The embryos must remain wet during all manipulation steps.
3. The steel mesh was cut into 1 cm by 1 cm triangles by a guillotine in order to be placed on top of the media in the inner well of the organ culture dish. In our case, the steel mesh was sterilized by dousing it in ethanol and placing it on a fire.
4. The Millipore filter paper was cut into 1 cm squares, in order to fit within the inner well of the organ culture dish.
5. 6-Aminonicotinamide was directly added to the medium prior to treatment with Edn1 ligand. Explants without pre-treatment of 6-aminonicotinamide and only treated with Edn1 were included as controls.
6. Edn1 was delivered by direct pipetting on 2-mm agarose beads and incubated for 5 min. The beads containing Edn1 were placed on either side of the mandibular explants by forceps, and left for 8 h in order for the molecules to diffuse out.
7. The mandibular explants may be kept in culture for up to one week without any noticeable necrosis or deterioration.
8. All the tools and solutions used in this protocol should be sterile.

Recipes

1. 10x phosphate-buffered saline (PBS)
   25.6 g $Na_2HPO_4 \cdot 7H_2O$
   80 g NaCl
   2 g KCl
   2 g KH_2PO_4
   Bring to 1 L with distilled H_2O and dissolve the salts by using the ceramic plate and magnetic stir bar
   For 1x PBS, dilute 100 ml of 10x PBS in 900 ml of distilled H_2O. Sterilize by autoclaving
2. Mandibular explant media
   47.5 ml of BGJb medium supplemented with L-glutamine
   3% FBS (1.5 ml/50 ml medium)
   7 mg of ascorbic acid
   0.5 ml of streptomycin-penicillin solution (100x)
   0.5 ml of MEM non-essential amino acid solution (100x)
3. 3% acetic acid solution
   3 ml glacial acetic acid
   97 ml distilled water
4. 2% potassium hydroxide (KOH) solution
   2 g potassium hydroxide
   100 ml distilled water

Mix well by using the ceramic plate and the magnetic stir bar

5. Alcian blue solution (pH 2.5)
   1 g Alcian blue, 8GX
   100 ml 3% acetic acid solution
   Mix well by using the ceramic plate and magnetic stir bar. Adjust pH to 2.5 using acetic acid
6. Alizarin red solution
   0.5 mg Alizarin red S
   100 ml 1% potassium hydroxide
   Mix well by using the ceramic plate and magnetic stir bar. Final concentration is 0.005% (w/v) alizarin red in 1% potassium hydroxide
7. 1% KOH/20% glycerol solution
   20 ml glycerol
   50 ml KOH (2%)
   30 ml distilled water
   Mix well by using the ceramic plate and magnetic stir bar

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