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Video Article

Isolation and Culture of Dental Epithelial Stem Cells from the Adult Mouse Incisor

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Abstract

Understanding the cellular and molecular mechanisms that underlie tooth regeneration and renewal has become a topic of great interest[1-4], and the mouse incisor provides a model for these processes. This remarkable organ grows continuously throughout the animal's life and generates all the necessary cell types from active pools of adult stem cells housed in the labial (toward the lip) and lingual (toward the tongue) cervical loop (CL) regions. Only the dental stem cells from the labial CL give rise to ameloblasts that generate enamel, the outer covering of teeth, on the labial surface. This asymmetric enamel formation allows abrasion at the incisor tip, and progenitors and stem cells in the proximal incisor ensure that the dental tissues are constantly replenished. The ability to isolate and grow these progenitor or stem cells *in vitro* allows their expansion and opens doors to numerous experiments not achievable *in vivo*, such as high throughput testing of potential stem cell regulatory factors. Here, we describe and demonstrate a reliable and consistent method to culture cells from the labial CL of the mouse incisor.

Video Link

The video component of this article can be found at http://www.jove.com/video/51266/

Introduction

One of the unique characteristics of vertebrates is the evolution of teeth. The tooth has become an important model system for many areas of research, as the molecular pathways and morphological specializations relevant to this organ have been investigated from several perspectives, including by developmental and evolutionary biologists[5]. More recently, the field of regenerative medicine has begun to gain valuable insights using the tooth as a model. In particular, the discovery of dental epithelial stem cells has been an important advance[6-13].

All rodents possess continuously growing incisors whose growth is fueled by stem cells, making these teeth an accessible model system to study adult stem cell regulation. Labeling experiments in the 1970s[10,11], followed by genetic lineage tracing experiments[8,9,12,14], have demonstrated that DESCs reside in the proximal region of the incisor. The stem cell progeny in the epithelial compartment on the labial side move out of the putative niche, known as the labial cervical loop (CL), and contribute to a population of cells called transit-amplifying (T-A) cells (**Figure 1**). Specifically, DESCs reside in the outer enamel epithelium (OEE) and stellate reticulum (SR)[8,9,14], and the inner enamel epithelium (IEE) gives rise to the T-A cells that progress through a limited number of cell cycles and then move distally along the length of the incisor (**Figure 1**). The differentiating ameloblasts in the mouse incisor continue to move distally along the incisor at a remarkable rate of approximately 350 µm in a single day[15,16]. As they move, the cells differentiate into mature ameloblasts and stratum intermedium (SI) cells. After depositing the full thickness of enamel matrix, many of the ameloblasts undergo apoptosis, and the remaining cells shrink in size and regulate enamel maturation[17]. The lineage of the other cell types in the labial CL, such as the SR, is less clear, and data regarding the stem cells in the mesenchyme[18] and in the lingual CL are only beginning to emerge.

Using the mouse incisor model, a number of groups have worked to elucidate the genetic pathways and cell biological processes involved in natural stem cell-based organ renewal. However, the labial CL contains a relatively small number of cells, estimated to be about 5,000 per mouse incisor, which makes working with primary cells challenging. Therefore, efforts have been made to culture and expand these cells *in vitro*[6,16,19,20] in order to open new doors to experimental approaches that are not achievable *in vivo*. The most recent study has demonstrated

that these cells can both self-renew and differentiate into amelogenin expressing cells when cultured[13]. Here, we describe and demonstrate a method for the reliable and consistent culture of cells from the mouse labial CL.

Protocol

1. Dissect Lower Hemi-mandibles from Adult Mouse

1. Prior to performing this protocol, obtain necessary institutional approval and be sure to comply with all animal care guidelines. Euthanize animal using standard IACUC approved procedures. For this work CO_2 asphyxiation was used followed by cervical dislocation.
    1. OPTIONAL: Separate head from the rest of the body using a razor blade.
2. Remove skin from the lower lip to the neckline.
3. Using a scalpel, make an incision between the two lower incisors, which are loosely connected. Upon applying pressure, the mandible will be split into two halves.
4. Wedge the scalpel between the condyle of the jaw and the temporal mandibular joint and cut alongside the jaw muscle to detach the jaw.
5. Remove all muscle, tendon, and ligament until only the bone remains.

2. Isolate the Incisor

1. Using a #15 scalpel, carefully shave off the bone at a 45° angle from the distal end to the proximal end of the membranous region. Use the tip of the scalpel to pick away any remaining bone including the bone fragments at the edge. Slowly start to pick away at the lingual cortical plate of the mandible, starting just below the 3rd molar.
2. Continue until the incisor is clean, working carefully towards the area containing the CL.
3. Remove inferior alveolar nerve bundle.
4. Make a clean cut to first remove the bone and tissues proximal to the CL.
5. Make a second cut distally with the scalpel, roughly below the 2nd molar. The proximal incisor region is then dissected out by inserting the scalpel between the bone and the medial surface of the proximal incisor. This must be carefully done, so as not to tear the tissue. The incision motion is proximal-distal.

    ALTERNATIVE STEP:

    Remove as much bone as possible along the area above the incisor. Once area is cleared, carefully remove entire incisor using a size 5 forceps to handle the enamel portion closest to CL region.
6. Place dissected tissue (either isolated CL as in **Figure 3E**, or entire incisor as in alternative Step 2.5) in 2% collagenase in 1X PBS for 3-4 hr at 4 °C in a low adherence plate. For 1-10 incisors, use 100 µl per well in a 12-well plate. For greater than 10 incisors, use 500 µl per well in a 6-well low adherence plate.

3. Microdissect the Labial Cervical Loop

1. Remove CL region from the collagenase and place in cold DMEM/F12 media.
2. Pull gently at the lower end of the CL using either size 5 forceps or an insulin syringe (1 cc, 28 G ½); the mesenchyme will fall apart while the epithelium remains intact. The CL and the adjacent epithelium that extends laterally as a “wing-like” structure will be visible.
3. Remove the apical bud from the adjacent epithelium by making a V-shaped incision on either side, and immediately place in cold DMEM/F12 on ice.
4. Repeat for all CLs, collect in 1.5 ml microfuge tube.
5. Spin down microfuge tube with specimens, remove media, replace with 100 µl cell detachment solution.
6. Incubate tissues in cell detachment solution for 30 min at 37 °C.
7. Dissociate the tissues to produce a single cell suspension by gently pipetting up and down using a 1,000 µl low-adhesion pipette tip. Cells can also be placed through a sterile cell strainer to achieve dissociation.
8. Count cells with hemocytometer, plate on tissue culture plastic or other desired material.

4. Primary Culture of DESCs

1. Plate cells in a 6-well plate at 1-6 x 10^4 cell/ml in DESC media, which consists of DMEM/F12 supplemented with mouse EGF recombinant protein at a concentration of 20 ng/ml, FGF recombinant protein at a concentration of 25 ng/ml, 1X B27 supplement, and 1% antibiotic solution (penicillin, 100 U/ml, streptomycin, 50 µg/ml).
2. Grow cells plated on a 6-well plate at 37 °C in 5% CO_2 in 1 ml of DESC media. Do not disturb initial cultures for 5 days after plating to allow cell adhesion and colony formation.
3. For the first media change, replace half of the volume (500 µl) of old media with half volume (500 µl) of new media. Check colonies under microscope when changing media.
4. After the first media change, continue to change media (1-2 ml) every 2 days. Check colonies under microscope when changing media.

5. Passage DESCs

1. Aspirate media and wash cells 3x with pre-warmed PBS.
2. Add pre-warmed 0.25% Trypsin-EDTA in saline solution and incubate at 37 °C for 10-15 min for the DESCs to detach.

3. Add DESC media to neutralize the trypsin, gently pipette along tissue culture plate to collect the cells, and place in a 15 ml conical tube.
4. Spin down cells at 800 x g for 2 min.

Representative Results

The mouse hemi-mandible comprises one continuously growing incisor and three rooted molars (**Figure 1A**). All teeth consist of dentin and enamel, the two mineralized components of the tooth crown (**Figures 1A** and **1A'**). The incisor houses two stem cell niches called the labial CL and lingual CL, and enamel is formed exclusively on the labial side (**Figure 1A'**). DESCs are responsible for incisor enamel formation and are housed in the labial CL, specifically the OEE and SR (**Figure 1A"**)[8,9]. The labial CL also contains the IEE, T-A cells, and the SI (**Figure 1A"**). Our technique is focused on the dissection and isolation of DESCs of the labial CL (**Figure 1A"**). *Krt14*-Actin GFP marks all epithelial-derived cells of the hemi-mandible (**Figure 1B**), and the labial CL can be clearly seen through the mandible (**Figure 1B'**, white arrow). It should be noted that all cell types can be identified under higher magnification (compare **Figures 1A"** and **B"**).

The procedure for the isolation of DESCs from the labial CL is summarized in **Figure 2.** Briefly, the hemi-mandible is first removed from the animal, mandibular bone is removed to expose the labial CL, and then the labial CL is treated with collagenase to separate the epithelium from the surrounding mesenchyme. The CL is microdissected, treated with cell dissociation buffer, and plated in tissue culture plates. Separation of the labial CL from the underlying jaw bone (**Figure 3**) and addition of proper volume of media are essential for isolation and the growth of the colonies, respectively. Small, tight epithelial colonies formed using this technique are visible by 7 days (**Figure 4A**), and these grow into large colonies within 2-3 weeks (**Figure 4B**).

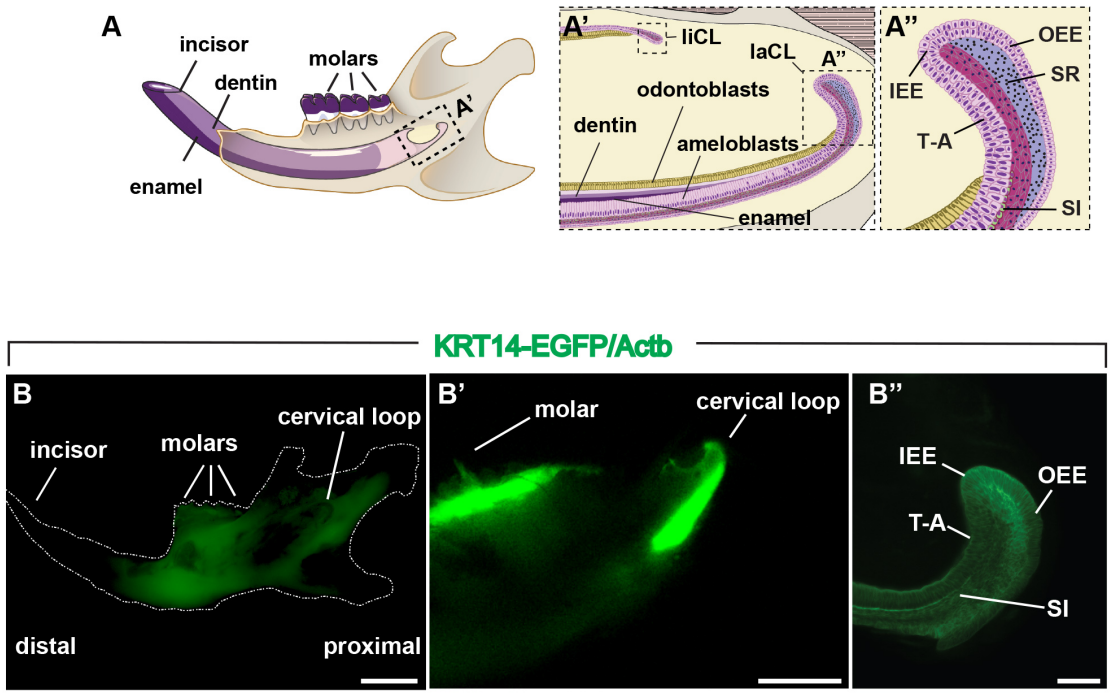

Figure 1. Illustrations of the adult mouse incisor. (A) The adult mouse hemi-mandible showing the mineralized components, enamel and dentin, and the two types of teeth, molars and incisors. The proximal incisor region where the DESCs are housed is highlighted in A'. **(A')** Sagittal view of the proximal incisor showing the 2 stem cell niches, the labial and lingual cervical loop (laCL and liCL), ameloblasts that generate enamel, and the odontoblasts that form dentin. The laCL, which exclusively generates ameloblast progenitors cells and ultimately form incisor enamel is highlighted in A". **(A")** The laCL showing the outer enamel epithelium (OEE), stellate reticulum (SR), inner enamel epithelium (IEE), transit-amplifying (T-A) region, and the stratum intermedium (SI). The SR is represented in blue and dark pink to reflect the subpopulations with differing densities. **(B)** Visualization of the cervical loop using a fluorescent reporter mouse, KRT14-EGFP/Actb. Bone is relatively autofluorescent, and removal of the bone exposes the cervical loop **(B')** and the rest of the epithelium, which can then be easily excised. **(B")** All structures of the cervical loop including the OEE, IEE, T-A and SI can be easily visualized with the reporter gene. Scale bars B', B" = 2 mm, B" = 50 µm. Please click here to view a larger version of this figure.

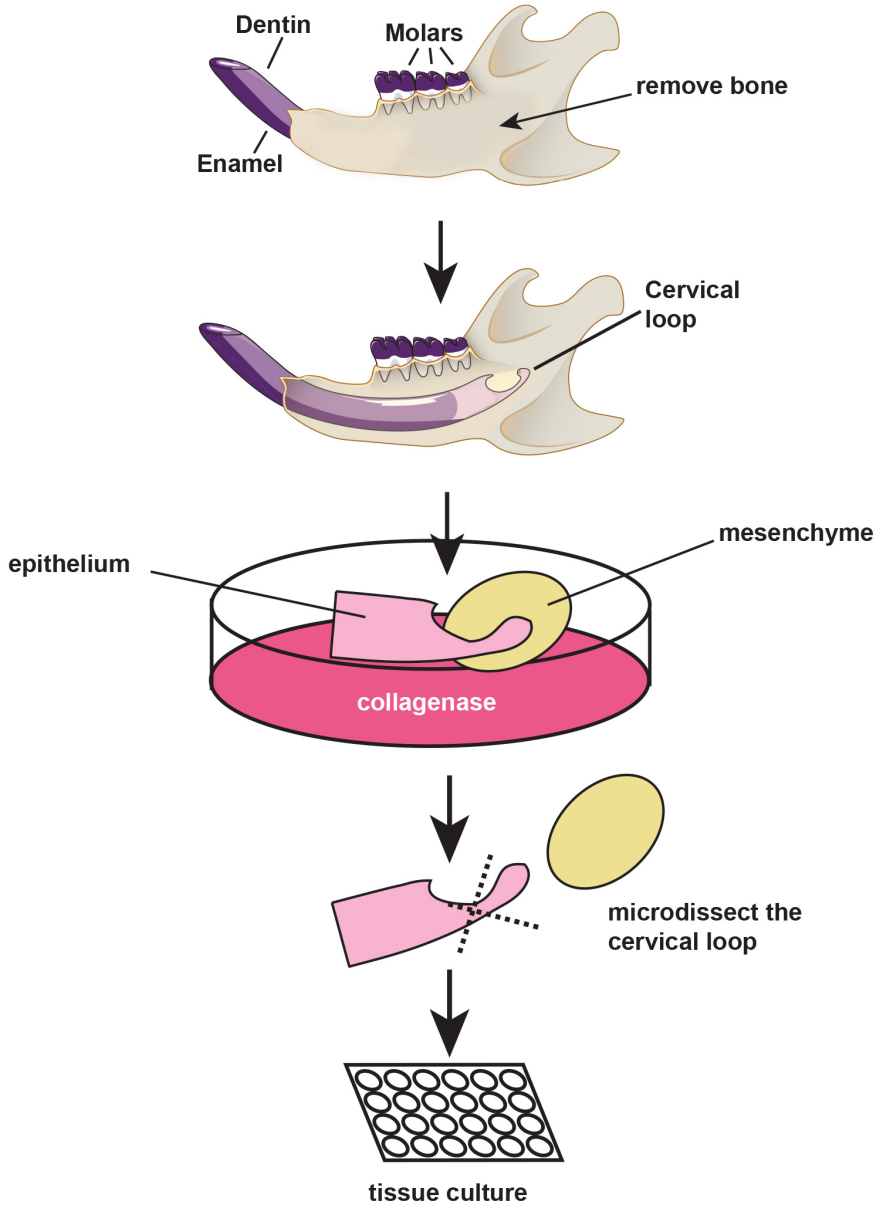

Figure 2. Overview of the dissection and isolation of the CL of the mouse incisor. Schematic representation of the procedure. The CL is located at the proximal end of the mandibular incisor. The first step is to remove surrounding jaw bone to expose the incisor with CL intact. Next, the tooth organ is isolated and placed in 2% collagenase to separate epithelium from mesenchyme. After 4 hr incubation, the CL is manually excised, dissociated into single cells, and cultured atop standard tissue culture plates.

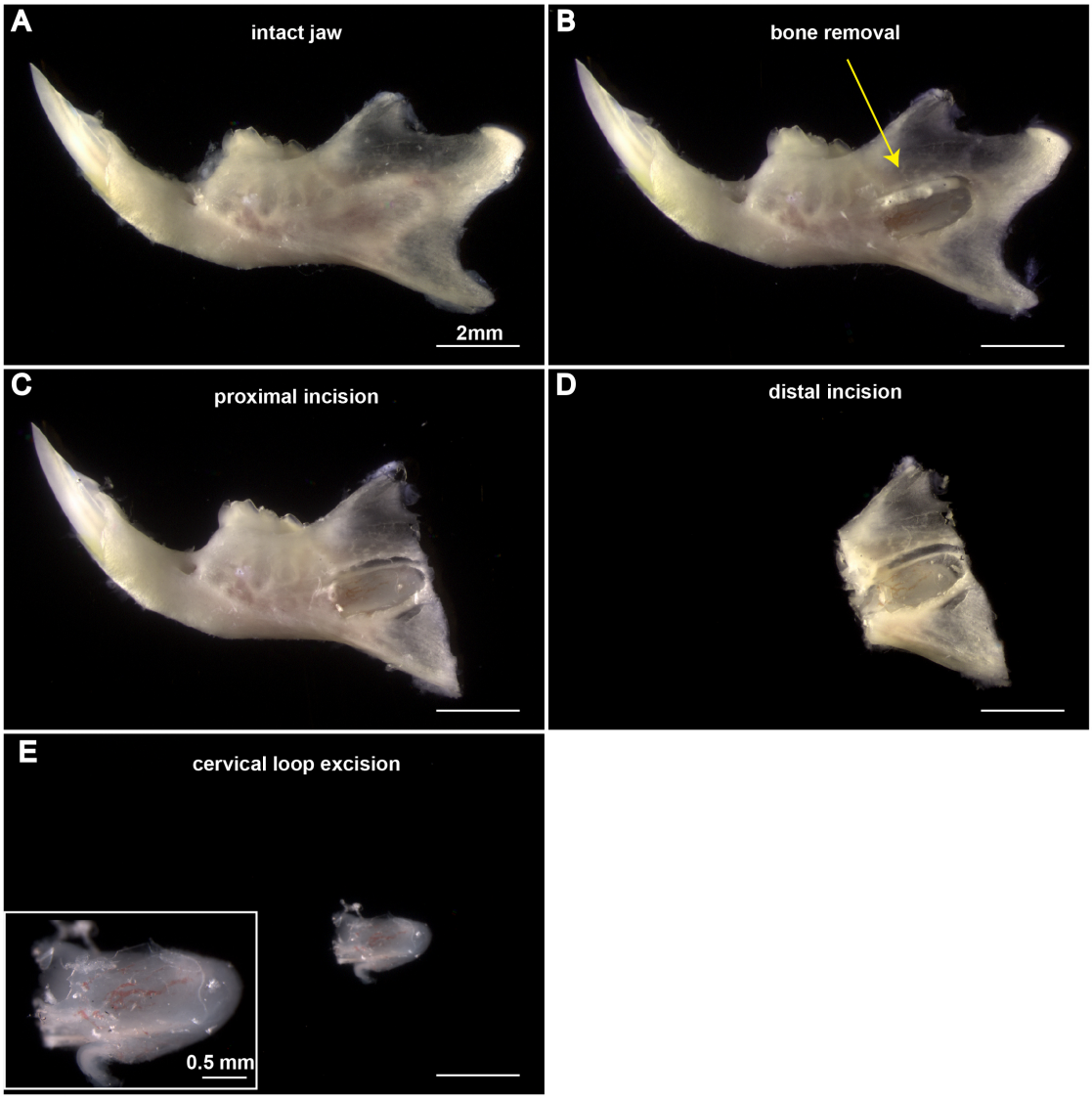

Figure 3. Bone removal of the mouse mandible to expose the underlying CL region. Visual checkpoints for the bone removal of the dissection process are shown. **(A)** Shows the hemi-mandible after Step 1.5, once the muscle, tendon and ligament are removed from the bone. **(B)** Indicates the area to begin removing bone, starting from just below the 3rd molar, moving proximally towards the region containing the CL. Next, all bone proximal to the CL is removed (**C**), as noted in step 2.4. The next incision to remove the rest of the bone as stated in Step 2.5 is shown in **(D)**. Finally, the CL is removed from the remaining bone as stated in Step 2.6 (**E**), magnified view (inset).

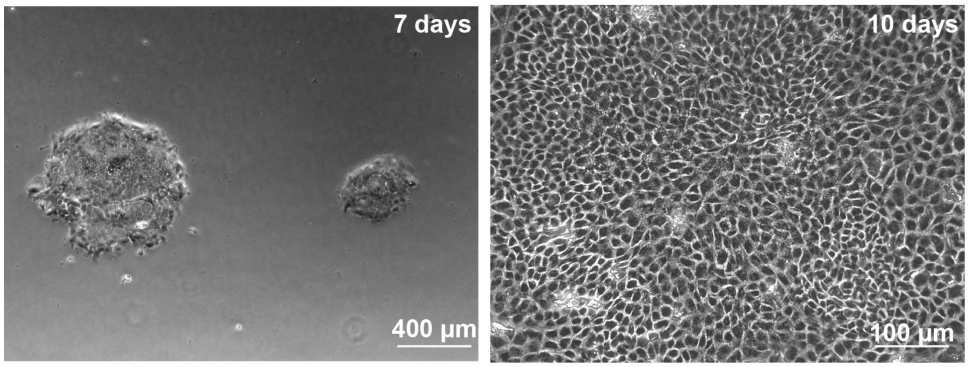

Figure 4. Representative results of successful colony formation *in vitro*. A. Phase contrast microscopy of DESCs, after plating in culture for 7 days. Tight colony formation indicates successful epithelial isolation with no mesenchymal contamination. Scale bar = 400 µm. **B.** After 10 days of incubation, colonies are larger in size and cells have a typical epithelial cobblestone morphology. Scale bar = 100 µm. Please click here to view a larger version of this figure.

Discussion

Epithelial cells were first successfully cultured over 40 years ago[21-24], and more recently, successful isolation of epithelial stem cells[25-27] has advanced our knowledge of epithelial biology. We report a protocol for isolating the DESCs of the adult mouse incisor, a relatively understudied population of stem cells that has the potential to yield important insights into dental biology and enamel formation. This protocol was initially based on previous reports of epithelial stem cell isolation from the hair follicle[28]. Whereas many protocols use feeder layers and serum to maintain epithelial stem cells, our method is a feeder free, serum free system. However, our growth media does require the use of a cocktail of EGF, FGF2 and B27 supplement. EGF has long been used to maintain undifferentiated cells [29]. A cocktail of EGF and FGF2 along with B27 was used previously to culture hair follicle stem cells[27], and B27 is widely used to maintain neural stem cells in culture[30,31]. We have also previously measured the viability and growth rate characteristics of DESCs atop tissue culture plastic and on a variety of ECM substrates, and no differences were detected in the rates of proliferation[19]. Cells could be maintained for up to 10 serial passages[19].

We use the lower incisors for this procedure because of the ease of removal as compared to the upper incisors. The most important steps within our protocol during isolation are the complete removal of the CL area from the mandibular bone before placing in collagenase and a clean microdissection of the CL. Because the proximal incisor is very delicate, it is important not to damage it during dissection, which could result in loss of the labial CL. Incomplete removal will lead to remnants of the labialCL and a lower yield of DESCs. In addition, it is important to avoid contamination with non-CL epithelial cells. Thus, after the epithelium has been separated from the mesenchyme, a clean V-shaped incision should be made to remove the labial CL from the neighboring epithelium. Finally, it is essential to plate these cells at a concentration of over 1 x 10^4 cells per ml and leave half of the conditioned media for the first media change.

The major benefit of this protocol to dental research is that it allows efficient production and manipulation of DESCs *in vitro.* Although the mouse incisor is established as an *in vivo* model[7-9,12,32-34], the development of an *in vitro* system advances the dental research field by opening the doors to experiments that are difficult to perform *in vivo.* Advantages of this system include the ability to readily manipulate the cells in various culture conditions, easy targeting of single or even multiple signaling pathways, and inhibition or activation of targeted molecules using siRNA or growth factors. Downstream applications of this *in vitro* system include using the above listed techniques as well as other cutting edge techniques to conduct functional and/or mechanistic studies, particularly at the single cell level.

Overall, information gleaned from DESC *in vitro* culture can help to unravel the intricacies of stem cell based tooth renewal.

Disclosures

The authors have no conflicts of interest to disclose.

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References

1. Sharpe, P. T., & Young, C. S. Test-tube teeth. *Sci Am.* **293**, 34-41 (2005).
2. D'Souza, R. N., & Klein, O. D. Unraveling the molecular mechanisms that lead to supernumerary teeth in mice and men: Current concepts and novel approaches. *Cells Tissues Organs.* **186**, 60-69, doi:Doi 10.1159/000102681 (2007).
3. Jernvall, J., & Thesleff, I. Tooth shape formation and tooth renewal: evolving with the same signals. *Development.* **139**, 3487-3497, doi:Doi 10.1242/Dev.085084 (2012).
4. Yen, A. H., & Yelick, P. C. Dental Tissue Regeneration - A Mini-Review. *Gerontology.* **57**, 85-94, doi:Doi 10.1159/000314530 (2011).

5. Jheon, A. H., Seidel, K., Biehs, B., & Klein, O. D. From molecules to mastication: the development and evolution of tooth development. *WIREs Dev Biol*. **2**, 165–182 (2013).
6. Wang, X. P. *et al.* An integrated gene regulatory network controls stem cell proliferation in teeth. *PLoS Biol.* **5**, e159 (2007).
7. Harada, H. *et al.* Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *J Cell Biol*. **147**, 105-120 (1999).
8. Juuri, E. *et al.* Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. *Dev Cell*. **23**, 317-328, doi:10.1016/j.devcel.2012.05.012 S1534-5807(12)00239-0 [pii] (2012).
9. Seidel, K. *et al.* Hedgehog signaling regulates the generation of ameloblast progenitors in the continuously growing mouse incisor. *Development*. **137**, 3753-3761, doi:137/22/3753 [pii] 10.1242/dev.056358 (2010).
10. Smith, C. E., & Warshawsky, H. Cellular renewal in the enamel organ and the odontoblast layer of the rat incisor as followed by radioautography using 3H-thymidine. *Anat Rec*. **183**, 523-561 (1975).
11. Smith, C. E., & Warshawsky, H. Quantitative analysis of cell turnover in the enamel organ of the rat incisor. Evidence for ameloblast death immediately after enamel matrix secretion. *Anat Rec*. **187**, 63-98 (1977).
12. Parsa, S. *et al.* Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors. *Development*. **137**, 3743-3752, doi:10.1242/dev.051672 137/22/3743 [pii] (2010).
13. Chang, J. Y. *et al.* Self-renewal and multilineage differentiation of mouse dental epithelial stem cells. *Stem Cell Res*. **11**, 990-1002, doi:S1873-5061(13)00081-0 [pii] 10.1016/j.scr.2013.06.008 (2013).
14. Biehs, B. *et al.* Bmi1 represses Ink4a/Arf and Hox genes to regulate stem cells in the rodent incisor. *Nat Cell Biol*. **15,** *846-52* (2013).
15. Hwang, W. S., & Tonna, E. A. Autoradiographic Analysis of Labeling Indices and Migration Rates of Cellular Component of Mouse Incisors Using Tritiated Thymidine (H3tdr). *J Dent Res*. **44**, 42-53 (1965).
16. Li, C. Y. *et al.* E-cadherin regulates the behavior and fate of epithelial stem cells and their progeny in the mouse incisor. *Dev Biol*. **366**, 357-366, doi:S0012-1606(12)00147-9 [pii] 10.1016/j.ydbio.2012.03.012 (2012).
17. Smith, C. E. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med*. **9**, 128-161 (1998).
18. Lapthanasupkul, P. *et al.* Ring1a/b polycomb proteins regulate the mesenchymal stem cell niche in continuously growing incisors. *Dev Biol*. **367**, 140-153, doi:S0012-1606(12)00218-7 [pii] 10.1016/j.ydbio.2012.04.029 (2012).
19. Chavez, M. G. *et al.* Characterization of dental epithelial stem cells from the mouse incisor with two-dimensional and three-dimensional platforms. *Tissue Eng Part C Methods*. **19**, 15-24, doi:10.1089/ten.TEC.2012.0232 (2013).
20. Kawano, S. *et al.* Establishment of dental epithelial cell line (HAT-7) and the cell differentiation dependent on Notch signaling pathway. *Connect Tissue Res*. **43**, 409-412 (2002).
21. Briggaman, R. A., Abele, D. C., Harris, S. R., & Wheeler, C. E., Jr. Preparation and characterization of a viable suspension of postembryonic human epidermal cells. *J Invest Dermatol*. **48**, 159-168 (1967).
22. Fusenig, N. E. Isolation and cultivation of epidermal cells from embryonic mouse skin. *Naturwissenschaften*. **58**, 421 (1971).
23. Fusenig, N. E., & Worst, P. K. Mouse epidermal cell cultures. I. Isolation and cultivation of epidermal cells from adult mouse skin. *J Invest Dermatol*. **63**, 187-193 (1974).
24. Rheinwald, J. G., & Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell*. **6**, 331-343 (1975).
25. Barrandon, Y., & Green, H. Three clonal types of keratinocyte with different capacities for multiplication. *Proc Natl Acad Sci U S A*. **84**, 2302-2306 (1987).
26. Blanpain, C. *et al.* Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*. **118**, 635-648, doi:10.1016/j.cell.2004.08.012 S0092867404007895 [pii] (2004).
27. Toma, J. G. *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol*. **3**, 778-784, doi:10.1038/ncb0901-778 ncb0901-778 [pii] (2001).
28. Nowak, J. A., & Fuchs, E. Isolation and culture of epithelial stem cells. *Methods Mol Biol*. **482**, 215-232, doi:10.1007/978-1-59745-060-7_14 (2009).
29. Rheinwald, J. G., & Green, H. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature*. **265**, 421-424 (1977).
30. Brewer, G. J., Torricelli, J. R., Evege, E. K., & Price, P. J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res*. **35**, 567-576, doi:10.1002/jnr.490350513 (1993).
31. Lesuisse, C., & Martin, L. J. Long-term culture of mouse cortical neurons as a model for neuronal development, aging, and death. *J Neurobiol*. **51**, 9-23, doi:10.1002/neu.10037 [pii] (2002).
32. Felszeghy, S., Suomalainen, M., & Thesleff, I. Notch signalling is required for the survival of epithelial stem cells in the continuously growing mouse incisor. *Differentiation*. **80**, 241-248, doi:S0301-4681(10)00067-8 [pii] 10.1016/j.diff.2010.06.004 (2010).
33. Fujimori, S. *et al.* Wnt/beta-catenin signaling in the dental mesenchyme regulates incisor development by regulating Bmp4. *Dev Biol*. **348**, 97-106, doi:S0012-1606(10)01073-0 [pii] 10.1016/j.ydbio.2010.09.009 (2010).
34. Klein, O. D. *et al.* An FGF signaling loop sustains the generation of differentiated progeny from stem cells in mouse incisors. *Development*. **135**, 377-385, doi:dev.015081 [pii] 10.1242/dev.015081 (2008).

