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Isolation of Epithelial Cells from Human Dental Follicle

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TITLE:

Isolation of Epithelial Cells from Human Dental Follicle

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KEYWORDS: cell isolation, dental follicle, odontogenic epithelial cells, dental follicle cells, heterogeneous cell population, serum-free medium

SUMMARY:

The dental follicle contains an epithelial population and mesenchymal cells. The epithelial population was selected from the heterogeneous dental follicle cell population by providing a distinct culture medium. Epithelial cells survived and formed colonies in a serum-free medium.

ABSTRACT:

The dental follicle (DF) was harvested during the removal of an impacted third molar by an oral maxillofacial surgeon. Epithelial cell isolation was performed on the day of DF harvest. The DF was washed three times with DPBS and then dissected with tissue scissors until the tissue had a pulpy or squishy consistency. Single-cell populations were pelleted by centrifugation and washed with keratinocyte serum-free medium. Heterogeneous cell populations were distributed in a culture dish. Keratinocyte serum-free medium was used to select the epithelial cells. The culture medium was changed daily until no floating debris or dead cells were observed. Epithelial cells appeared within 7–10 days after cell population distribution. Epithelial cells survived in serum-free medium, while α-modification minimal essential medium supplemented with 10% fetal bovine serum allowed the proliferation of mesenchymal-type cells. The DF is a tissue source for the isolation of dental epithelial cells.

The purpose of this study was to establish a method for the isolation of epithelial cells from human DF. Periodontal ligament (PDL) was used for the isolation of human dental epithelial cells. Procuring epithelial cells from human PDL is not always successful due to the small tissue volume, leading to low numbers of epithelial cells. DF has a larger volume than PDL and contains more cells. DF can be a tissue source for the primary culture of human dental epithelial cells. This protocol is easier and more efficient than the isolation method using PDL. Procuring human dental epithelial cells may facilitate further studies of dental

epithelial–mesenchymal interactions.

INTRODUCTION:

Tooth formation begins with the invagination of the oral epithelium[1]. According to the tooth developmental stage, the oral epithelium has different names, including inner and outer enamel epithelium, cervical loop, and Hertwig's epithelial root sheath (HERS). The epithelial compartments communicate with the surrounding mesenchymal cells. Epithelial–mesenchymal interactions regulate tooth formation and tissue regeneration. Procuring dental epithelial cells, such as oral keratinocytes and Hertwig's epithelial root sheath cells (HERSCs), is crucial for the study of dental epithelial-mesenchymal interactions[2].

Rodent-derived dental epithelial cells are isolated from the epithelial structure, such as the HERS. Li and colleagues isolated and immortalized rat molar-derived HERSCs after harvesting the apical portion of the developing tooth germs from 8-day-old rats[3]. The HERS was separated from apical tissue under magnification. Considering the tooth developmental stage and age, harvesting the HERS from humans is nearly impossible because of ethical issues:, a developing tooth germ needs to be removed from a young child to harvest the human HERS. Immature tooth germs are rarely extracted. Human dental epithelial cells can be isolated from the gingiva and periodontal ligament (PDL). Epithelial structure-derived cells participate in tooth formation together with mesenchymal components and might be more suitable for the study of dental epithelial–mesenchymal interactions than oral keratinocytes. Epithelial cell rests of Malassez (ERM) are HERS-derived epithelial remnants and reside in small numbers in the PDL[4]. Studies report the isolation of human HERSCs from PDL[5]. However, harvesting human HERSCs from PDL tissue is not always successful because of the scarcity of the epithelial population in this location[5,6].

Although rodent-derived HERSCs are maintained in serum-containing media[3,7], human DF-derived epithelial cells are cultured with serum-free media similar to other human epithelial cells, such as normal human epidermal keratinocytes and normal human oral keratinocytes[8,9]. This implies physiologic or functional differences between rodent dental epithelial cells and human dental epithelial cells. Understanding the mechanism regarding dental epithelial–mesenchymal interactions might contribute to the development of clinical applications, including periodontal reattachment during replantation, periodontal regeneration in periodontal disease, pulp–dentin complex regeneration, and bio-tooth generation. Considering the characteristics of translational research, human dental epithelial cells may be more appropriate than rodent dental epithelial cells for the study of epithelial–mesenchymal interactions.

The human DF is a loose connective tissue and often resides in an impacted tooth. The DF contains mesenchymal precursors[10]. However, to our knowledge, no study has reported the isolation of epithelial cells from dental follicles before 2021. Oh and Yi reported the isolation of epithelial cells from human DF in 2021[8]. The epithelial phenotype was confirmed by western blotting and morphologic analysis. Analysis of the origin of DF-derived epithelial cells demonstrated similar results with other studies. DF-derived epithelial cells were neither endothelial nor hematopoietic[5,11], and Oh and Yi suggested naming these cells as DF-HERSCs. The DF has a larger volume than the PDL, and more epithelial cells can be

isolated from the DF. This enhances the emergence of epithelial colonies and results in a high success rate in harvesting epithelial cells from DF. This study suggests using the DF as a tissue source for the isolation of dental epithelial cells.

In the present study, single cells were isolated from the DF according to previously described procedures[10,12]. The DF contains heterogeneous cell populations, and several cell types could be present at the early stage of the procedure. Morsczek and colleagues isolated DF-derived mesenchymal stem cells[10]. We hypothesized that the DF contains epithelial cells and that only epithelial cells can survive under serum-free conditions. This study differs from that of Morsczek et al. in terms of the selection of the epithelial population and the inhibition of mesenchymal cells. The selection was performed using keratinocyte serum-free media (SFM), which allows epithelial cell proliferation and inhibits mesenchymal cell proliferation. This study originated from a report by Oh and Yi[8]. The aim of this study was to describe details of the method used in that report for the isolation of epithelial cells from human DF.

PROTOCOL:

This study was approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong (IRB approval no. KHNMC 2017-06-009).

1. Collect DF

NOTE: Patients gave informed consent before surgery for the removal of a mature or immature impacted third molar. Patients with the following disease were excluded: diabetes, hypertension, tuberculosis, hepatitis, acquired immunodeficiency syndrome. Pregnant women were also excluded.

1.1. Harvest DF during the surgical extraction of impacted third molars (**Figure 1A**).

NOTE: Surgery was performed by an oral maxillofacial surgeon. Cooperation is required with a dentist for tissue collection.

1.2. Store DF in Dulbecco's phosphate-buffered saline (DPBS) with 3% penicillin-streptomycin at 4 °C before use. Perform isolation procedures on the day of DF harvest (**Figure 1B**).

2. Isolate single-cell populations from DF

2.1. Prepare stock solutions of collagenase type I and protease in DPBS so that final concentrations of collagenase type I and protease are 1 mg/mL and 2.4 mg/mL, respectively.

2.2. Prepare three 50 mL washing tubes with 20 mL of DPBS per tube. Label the tubes as 1, 2, and 3.

2.3. Wash the DF in the washing tubes sequentially. Hold the DF with tweezers and place

the DF in tube 1. Take the DF out of tube 1 and place it in tube 2. Take the DF out of tube 2 and place it in tube 3. Gently shake DF 10–15 times in each tube. After washing three times, place the DF in a 60 mm culture dish (**Figure 2A**).

2.4. Mince the DF with tissue scissors (**Figure 2B**). Transfer only a small portion to the culture dish to minimize tissue loss. Repeat the cutting until the DF has a pulpy or squishy appearance (**Figure 2C**).

2.5. Mix 1 mL of collagenase type I and 1 mL of protease solution in a 15 mL conical tube to obtain final concentrations of 1 mg/mL and 2.4 mg/mL, respectively.

2.6. Transfer the minced DF into the 15 mL conical tube from step 2.5. Gently shake and incubate the tube for 1 h at 37 °C.

2.7. Add 5 mL of 0.05% trypsin-EDTA to the tube, shake, and incubate the tube for 15 min at 37 °C.

2.8. Prepare keratinocyte medium containing keratinocyte SFM 10% fetal bovine serum (FBS). Add 3 mL of keratinocyte medium into a new 50 mL conical tube. Place a 40 µm strainer on top of this tube and use a 10 mL serological pipette to aspirate the supernatant from step 2.6 and pass it through the strainer.

NOTE: Minimize the incorporation of digested tissue remnants while taking the supernatant. Remove the tissue remnants with a 40 µm strainer to minimize failure. Tissue remnants may pass through 70 µm strainers and hinder colony formation. FBS in the keratinocyte medium will inactivate the enzymes.

2.9. Repeat steps 2.7 and 2.8.

2.10. Transfer the collected suspension to a 15 mL conical tube.

2.11. Centrifuge the 15 mL conical tube at 288 × *g* for 3 min at 4 °C. Remove the supernatant.

NOTE: Be careful to avoid any accidental removal of pelleted cells, which are mostly invisible.

2.12. Add 3 mL of keratinocyte SFM and wash the cells twice with a 1 mL pipette with centrifugation as in step 2.11.

2.13. Plate the single-cell population into a 60 mm culture dish using keratinocyte SFM. Maintain the culture dish with a final volume of 5 mL in a humidified atmosphere of 5% CO_2 at 37 °C. Change the culture medium daily until no tissue or cell debris are observed.

NOTE: Remove floating debris in the culture medium by changing the medium. Delayed removal of tissue debris or dead cells has an unfavorable effect on the primary culture.

2.14. Examine the plate from step 2.13 to check the growth of epithelial cells (**Figure 3A**).

NOTE: Epithelial cells grow within 7–10 days after plating single-cell populations.

2.15. Expand the epithelial colonies and subculture the cells.

2.15.1. Aspirate the medium and wash the bottom of the culture plate once with 3 mL of keratinocyte SFM.

2.15.2. Add 2 mL of cell dissociation protease and incubate in a humidified atmosphere of 5% CO_2 at 37 °C for 3 min.

NOTE: Repeat step 2.15.2 if cell detachment is not effective.

2.15.3. Add 2 mL of keratinocyte medium to the culture plate and transfer the contents in a 15 mL conical tube.

NOTE: Adding more keratinocyte medium is not required if step 2.15.2 is repeated.

2.15.4. Centrifuge the 15 mL conical tube at 288 × *g* for 3 min at 4 °C.

2.15.5. Remove the supernatant and wash the cell pellet two times with 3 mL of keratinocyte SFM.

2.15.6. Plate the cells in a 100 mm culture dish using keratinocyte SFM (final volume of 10 mL per 100 mm dish).

2.16. Prepare and store cell stocks in a liquid nitrogen tank.

2.16.1. Harvest cells as described in steps 2.15.1–2.15.5.

2.16.2. Distribute the cells into cryovials in 1 mL of freezing medium (80% keratinocyte SFM, 10% dimethylsulfoxide, 10% FBS).

2.16.3. Place the vials in a freezing container and store them at -80 °C for 24 h.

2.16.4. Transfer the vials from the deep freezer to a liquid nitrogen tank.

REPRESENTATIVE RESULTS:

DF harvesting

Surgery was performed by an oral maxillofacial surgeon. Human-derived materials, including the tooth fragment, gingival tissue, and DF, were collected by a surgeon (**Figure 1A**). The DF might be attached to the tooth fragment. An oral maxillofacial surgeon will be able to identify the DF. Cooperation and communication with the surgeon are required for tissue collection. The DF is an irregularly shaped membrane-like tissue. Gingival tissue has a keratinized surface and can be distinguished from the DF. Pulp tissue resides in the pulp chamber and is rarely separated from teeth during surgery. The DF was stored in DPBS with

3% penicillin-streptomycin at 4 °C before use (**Figure 1B**).

Mechanical treatment of DF

Tissue debris and blood were removed by washing the DF with DPBS. Three conical tubes were prepared with washing solution. After washing, the DF was transferred onto a 60 mm or 100 mm culture dish (**Figure 2A**). The DF should be minced with scissors until the tissue has a pulpy or mushy appearance (**Figure 2B, C**).

Emergence of epithelial population

After plating heterogeneous cell populations, many cells and small debris floated in the culture medium. The number of floating cells gradually decreased with successive medium changes. Delayed medium change may cause a turbid culture environment. At the initial stage, different cell types appear at the bottom of the culture dish but disappear when maintained in keratinocyte SFM. Cells with epithelial morphology appear within 7–10 days after plating single-cell populations (**Figure 3A**). The number of cobblestone-shaped cells ranges from 1 to 10 at the time of the emergence of epithelial cells, which expand into colonies over time (**Figure 3B**).

The mincing procedure was repeated to increase DF contact area with 0.05% trypsin-EDTA to promote cell dissociation. More single cells were harvested from DF pulp than intact DF. The stickiness of the DF pulp indicated the probability of success of the isolation of epithelial cells. Daily medium changes prevented the keratinocyte SFM from becoming turbid, which is important for epithelial cell survival.

FIGURE AND TABLE LEGENDS

Figure 1: Human-derived materials. (**A**) Human-derived materials were collected by an oral maxillofacial surgeon. The yellow arrow indicates the DF. Black arrows indicate the tooth fragment and tooth-attached soft tissue. (**B**) The DF was stored in PBS supplemented with 3% penicillin-streptomycin at 4 °C. Isolation procedures were performed within 24 h of collection. Abbreviations: DF = dental follicle; PBS = phosphate-buffered saline.

Figure 2: Treatment of dental follicles. (**A**) The DF was transferred to a 60 mm culture dish after washing with DPBS three times. (**B**) The DF was minced with tissue scissors. (**C**) The minced DF had a pulpy or mushy appearance.

Figure 3: **Emergence of epithelial cells.** (**A**) Cells with epithelial morphology appear within 7–10 days after plating single-cell populations. (**B**) *Ex vivo* expansion of DF-derived epithelial cells by subculture. Scale bars = 100 µm. Abbreviation: DF = dental follicle.

DISCUSSION:

This protocol includes critical steps. Harvesting single-cell populations is essential for the successful isolation of epithelial cells from DF. We sought to isolate epithelial cells from the DF based on our hypothesis that there are more epithelial cells in the DF. The mincing procedure enhances the detachment and release of cells from the DF. The mincing procedure was improved, and mincing repeated until the DF appeared pulpy to facilitate the release of single cells. Obtaining the maximum number of single cells increases the

probability of the emergence of the epithelial population. Contamination frequently occurs during the isolation of epithelial cells from the DF. The use of 40 µm strainers minimizes the inclusion of tissue debris in single-cell populations, as tissue remnants can pass through 70 µm strainers and inhibit epithelial cell colonization.

Epithelial cells seem to be more vulnerable to certain environments than mesenchymal cells, and the daily changing of the culture medium also provided a clean environment for the emergence of the epithelial population. Losing cells due to frequent medium change may be a limitation of this protocol. However, daily changing of the medium was performed after troubleshooting for this protocol, and consequently, epithelial cells appeared and expanded. Delaying the medium change can affect the cultures. As the cell pellet after centrifugation is nearly invisible, the narrow tip of a 15 mL conical tube increases the ease of supernatant removal without losing the cell pellet. Therefore, this protocol suggests transferring the contents of the 50 mL conical tube to a 15 mL conical tube.

The DF provides a larger volume of tissue and more single cells than the PDL. Hence, the DF can be used instead of the PDL as a tissue source for the isolation of human dental epithelial cells. Selection and inhibition of cell types in the DF can be done simply through a selective culture medium. SFM induces epithelial growth, whereas serum-containing medium selects for mesenchymal cells. This methodology allows easy accessibility to human dental epithelial cells. Morsczek and colleagues previously isolated DF-derived mesenchymal precursors. This study differs from theirs by the selection of epithelial populations and the inhibition of mesenchymal cells.

The regulatory role of dental epithelial cells during tooth formation is of great interest in dental research. Studying epithelial–mesenchymal interactions during tooth development may suggest clues for the resolution of clinical problems[13]. Tissue regeneration by combining epithelial and mesenchymal components has been attempted[14]. Dental epithelial cell-derived factors, such as an enamel matrix derivative, have been evaluated as therapeutic targets aiming at periodontal regeneration, pulp-dentin complex regeneration, and periodontal reattachment[2,15]. The combination of regeneration factors, including stem cells, epithelial-mesenchymal cell sheets, cell-derived factors, and biomaterials, may generate bio-teeth in case of tooth loss or agenesis[16]. Tooth agenesis-associated genes, such as *PAX9*, *MSX1*, *AXIN2*, *EDA*, *EDAR*, and *WNT10A* genes, are potential candidate key regulatory genes for epithelial–mesenchymal interactions[17]. Further, next-generation sequencing method-derived data from tooth agenesis-associated studies may similarly propose putative novel target genes regulating molecular control during dental epithelial–mesenchymal interactions.

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DISCLOSURES:
The authors declare that they have no conflicts of interest.

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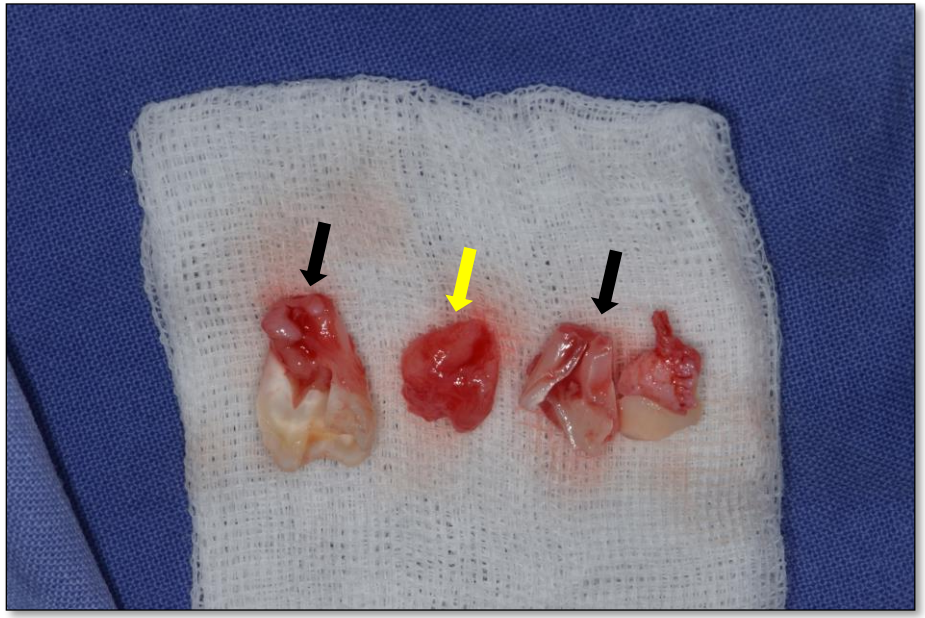
Figure 1

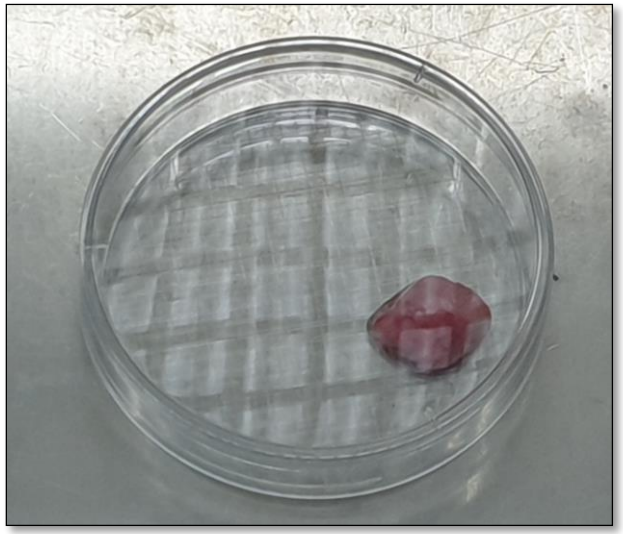
A



B

Figure 2

A



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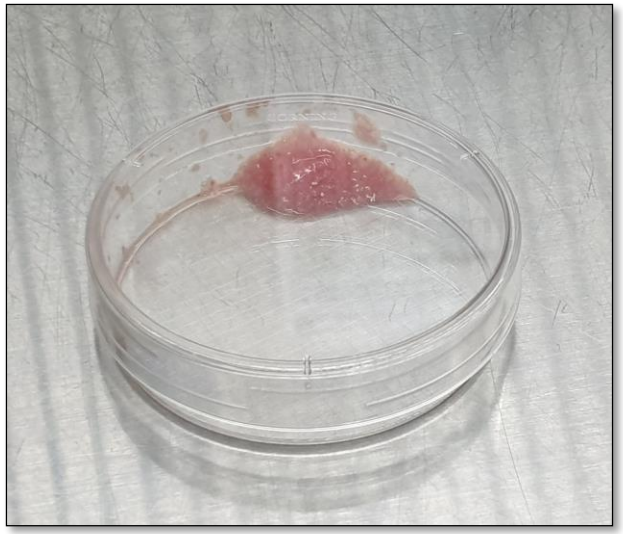

Figure 3

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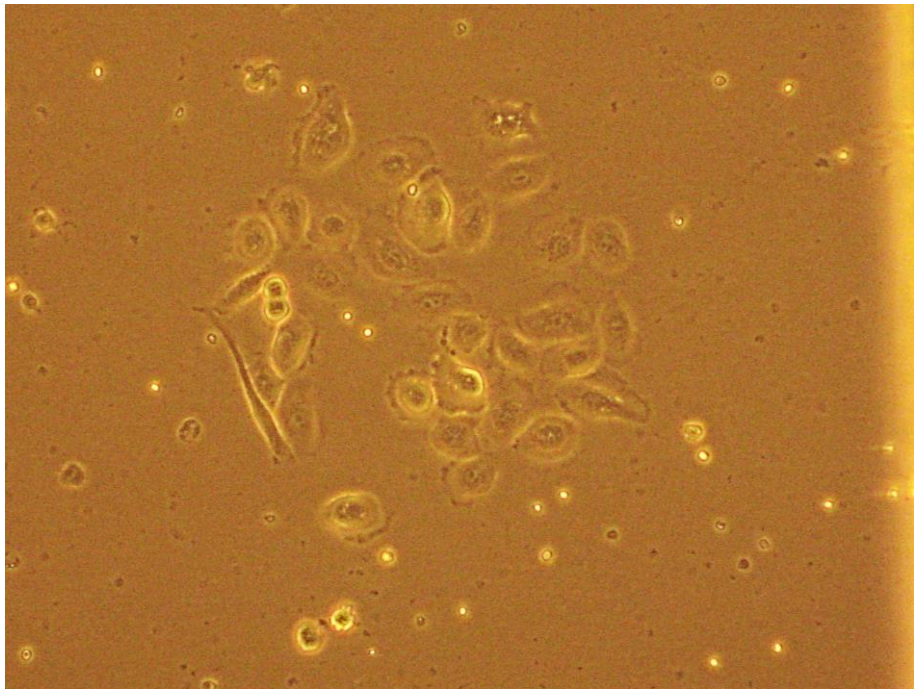

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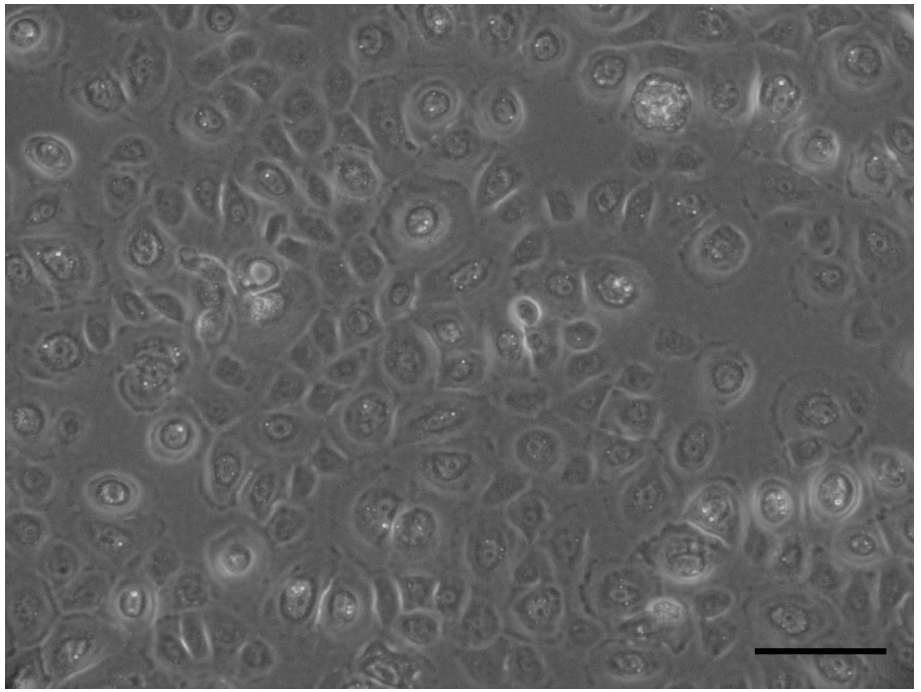

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