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Coppe, Carolyn

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A Study of Dental Pulp Cells

by

Carolyn M Coppe

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

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in the

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of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by
Carolyn M Coppe

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A Study of Dental Pulp Cells from Primary Teeth.

Carolyn M Coppe

ABSTRACT: **Purpose:** The purpose of this study was to characterize dental pulp cells from human primary teeth, and determine their ability to induce differentiation of oral epithelial cells. **Methods:** Dental pulp cells were isolated from freshly extracted primary incisors, digested with 4mg/ml collagenase/dispase and grown in DMEM supplemented with 10% fetal bovine serum. Stem cell populations were identified by immunocytochemical staining for STRO-1 and CD146 and fluorescence activated cell sorting. The pulp cells were grown in co-culture with human fetal oral epithelial cells. After 3 days, the co-cultured cells were collected, and analyzed for amelogenin expression by real time-PCR and immunocytochemical staining. **Results:** STRO-1+/CD146+ cells were immunolocalized in pulp cell cultures. Fluorescence activated cell sorting of STRO-1+ cells, showed this stem cell population to be approximately 1.7% of the total pulp cell population. Oral epithelial cells grown in co-culture with growth arrested primary pulp cells showed positive expression of amelogenin by immunocytochemistry and real time-PCR. Oral epithelial cells alone were amelogenin immunonegative. **Conclusions:** Primary tooth dental pulp cells contain less than 2% stem cells. Cells within the primary tooth pulp can promote oral epithelial cell differentiation toward an ameloblast phenotype, as indicated by up-regulation of amelogenin expression.

Introduction

The dental pulp of primary teeth differentiates from dental mesenchyme, beginning at the sixth week in utero. It is during the initial stage, the bud stage, that the mesenchymal cells begin to proliferate and form the dental papilla, which gives rise to both the dentin and the pulp tissues. Some cells derived from the dental papilla differentiate into odontoblasts, which go through several stages of differentiation as they form dentin. As the tooth continues to form the pulp organ forms, which consists mostly of fibroblast cells, undifferentiated mesenchymal cells, and a few larger blood vessels and nerves associated with these vessels, traversing the pulp [1].

Primary teeth may be a resource of stem cells that can be used to promote tooth tissue engineering. In 2003, Miura et al, from Dr. Songtao Shi's laboratory, identified stem cells from exfoliated primary teeth. These stem cells are referred to as "stem cells from exfoliated deciduous teeth" or SHED [2]. The source of these stem cells and their role in primary tooth odontoblast formation remains to be determined. Along with stem cells, other mesenchymal cells contained within the dental mesenchymally-derived pulp may retain the ability to direct dental epithelium to differentiate into the enamel forming ameloblasts. This question is of particular interest in future studies of tooth tissue engineering.

The purpose of our study is two fold. First we will characterize primary dental pulp cells (pDPCs) from human primary teeth. Specifically dental pulp cells isolated from primary teeth will be characterized for the presence of collagen and non-collagen proteins, as well as for the relative percentage of stem cells. Secondly we will determine whether pDPCs can induce differentiation of fetal oral epithelial cells (OECs) to cells with an ameloblast phenotype. pDPCS will be co-cultured with OECs to determine their role in promoting ameloblast differentiation.

BACKGROUND

Dental pulp cells:

The dental pulp is a loose vascular connective tissue that is surrounded by dentin and consists of a population of heterogeneous cells, including fibroblasts, odontoblasts, blood cells, Schwann cells, endothelial cells and undifferentiated mesenchymal cells. In teeth, the pulp is confined to the pulp chamber and the root canals, which on average consists of 0.02 cc volume of pulp tissue, with the molars having four times the volume as the incisors [1]. The most abundant cells in the pulp are the stellate-shaped fibroblast cells. Odontoblasts are a group of differentiated mesenchymal pulp cells that are responsible for the synthesis and secretion of the fibers and extracellular matrix of the predentin and the biomineralization of the dentin. Type I collagen is the major protein that is produced by odontoblasts [1].

A number of studies have shown that dental pulp mesenchymal cells can differentiate into odontoblasts to repair damage done to the dentin, such as occurs in caries or trauma [3, 4]. Cells from the dental pulp, mostly fibroblasts and undifferentiated mesenchymal cells, are able to grow on culture dishes and have been used to study pulp cell differentiation into odontoblast-like cells. When these cells are grown in the presence of ß-glycerolphosphate, they synthesize markers of odontoblasts [5-9]. These differentiation markers include fibronectin, which is secreted in the initial stage of odontoblast differentiation [10-12], type I collagen[13-15], and osteonectin which is expressed in young odontoblasts but not in all pulp cells [16, 17]. Dentin sialophosphoprotein (DSPP), as well as DMP1, are markers of differentiated odontoblasts [14, 18, 19], and dentin sialoprotein (DSP) a cleavage products of DSPP [20] and alkaline phosphatase activity, are both late markers for committed odontoblasts [21].

Dental pulp stem cells:

Miura et al., showed that remnants of dental pulp derived from exfoliated primary teeth contain a multipotent stem cell population, which they named SHED (Stem cells from Human Exfoliated Deciduous teeth) [2]. SHED were found to express Stro-1 and CD146, two early mesenchymal stem cell markers that were previously found in bone marrow stromal stem cells and dental pulp stem cells from permanent teeth [2]. Human postnatal stem cells have a great potential to be used in stem cell mediated clinical therapies and tissue engineering. Postnatal stem cells have been isolated from a variety of human tissues such as: bone marrow, peripheral blood, neural tissue, skeletal muscle, dental pulp, periodontal ligament, and the pulp of primary teeth. Recent studies have shown that adult stem cells have the ability to differentiate into more than just their tissue of origin. Different adult stem cells have been able to contribute to blood, skeletal muscle, liver, neuronal tissue, PDL, and bone [2, 22, 23].

Primary teeth are uniquely different from permanent teeth in their developmental processes, tissue structure, and function. The stem cells from their pulps are also different, with their higher proliferation rates, increased cell-population doublings, sphere-like cell-cluster formation, osteoinductive capacity *in vivo*, and failure to reconstitute a dentin-pulp-like complex [2]. Implantation of pulp cells from primary human teeth subcutaneously planted into rat muscle resulted in the formation of a bone-like tissue, unlike the dentin-pulp complex that was formed in similar experiments with permanent teeth. SHED represent a population of multipotent stem cells that may be particularly suited to tooth related tissue regeneration.

Ameloblasts:

The formation of enamel begins with the differentiation of the inner enamel epithelial cells into ameloblasts. During the bell stage of tooth formation, reciprocal interactions between the dental epithelium and mesenchyme lead to cusp morphogenesis and cytodifferentiation [1]. Mesenchymal cells lining the dental papilla differentiate into dentin-producing odontoblasts, and the dentin matrix proteins and growth factors from dental mesenchyme induce the differentiation of inner enamel epithelial cells. These inner enamel epithelial cells differentiate into preameloblasts, then secretory ameloblasts, followed by mature ameloblasts. These cells secrete matrix proteins and self-assemble to form the enamel matrix on the dentin surface [24-28].

At the end of secretion, ameloblasts shorten into transition stage ameloblasts, and begin to synthesize KLK-4, a matrix proteinase that hydrolyzes the enamel matrix proteins as mineralization occurs. Maturation stage ameloblasts differentiate between a smooth-ended and ruffle-ended cell that function to complete the removal of proteins from the matrix, forming the final highly mineralized enamel structure. Maturation stage ameloblasts control pH fluctuations that occur as mineral is deposited in the matrix, with ion exchange pumps, including anion exchanger member 2 [29]. As the tooth matures, the ameloblasts undergo apoptosis and finally disappear as the tooth erupts [30-35]. Any disruption in the amount and timing of matrix protein secretion dramatically affects enamel formation.

The factors that control cell fate decisions in the enamel organ, as well as the continued differentiation of ameloblasts are not known and the issue of accessibility of ameloblasts limits the studies available. The knowledge of ameloblast differentiation and function has been mostly derived from *in vivo* analyses or organ culture studies. The few studies that have

reported growing ameloblast-like cells *in vitro*, use different cell types and mixed cells cultures. In our study we hypothesize that dental pulp cells could induce another source of epithelial cells into dental epithelial cells. This idea comes from the work of Mina and Kollar [40] that found that recombination of mouse dental mesenchyme with non-dental epithelia resulted in the formation of a tooth like structure. The studies proposed in this application to evaluate pulp cell-ameloblast interactions, will do much to advance our knowledge of the role of dental mesenchyme in the differentiation of oral epithelium.

Oral epithelial cells:

The oral cavity contains many different types of stratified squamous epithelia. The tissue that covers the buccal area is known as the lining mucosa and is a nonkeratinized stratified squamous epithelium. These oral epithelial cells and the epithelial cells of the early forming dental lamina are cells derived from the same cell source. The dental lamina epithelium induces differentiation of the underlying mesenchyme, which reciprocally induces further differentiation of the dental epithelium, including ameloblasts [36-40]. This reciprocal interaction between the dental epithelium and mesenchyme continues throughout the various stages of tooth formation [41]. In the studies described in this thesis, we sought to determine whether an activated dental mesenchyme from primary pulp tissue could induce the differentiation of oral epithelium to ameloblast lineage cells, as characterized by an upregulation of the enamel matrix protein, amelogenin.

Significance:

Primary teeth are an accessible source of cells, which could be stored for future tissue engineering. In this study, we characterized the cells contained within the primary tooth

dental pulp. We further determined whether these cells retained their ability to promote differentiation of epithelium, which is required for enamel formation.

This study will lay the ground work for future studies to isolate populations within the dental pulp, including SHED and to see how they affect ameloblast differentiation, in the context of tooth tissue engineering.

METHODS AND MATERIALS

Identification and characterization of primary dental pulp cells

Pulp cell isolation and culture: The following protocol was used for cell isolation and characterization of pulp cells of primary teeth (pDPCs). All cells were obtained with permission from the UCSF Committee on Human Research. Primary teeth that were extracted for reasons other than research were obtained from the UCSF Pediatric Dental Clinic. The tooth surface was cleaned with povodine iodine and cut around the cementum-enamel junction to reveal the pulp chamber. In order to prevent bacterial and fungal contamination, the pulp tissue was gently separated from the crown and root and placed in 10% penicillin/streptomycin/fungizone in 4˚C overnight. It was then digested in 4 mg/ml collagenase/dispase (Roche) for 4 hour at 37°C with rotation. Single-cell suspensions were obtained by passing the cell mass through a 70-μm strainer. These cells were grown on Falcon tissue culture dishes (Becton/Dickinson) with Dulbecco's modified Eagle's medium low glucose supplemented with 10% FBS and 1% penicillin/streptomycin.

PCR: Total RNA was extracted from cultured pDPCs using RNeasy Mini Kit (QIAGEN, Valencia). The mRNA was reverse transcribed into cDNA with oligo (dT) primer and Superscript reverse transcriptase (Invitrogen). The primers for amplifying DSPP and ALP

gene were designed according to sequences published in NCBI. PCR amplification was performed by using *Taq* DNA polymerase (Invitrogen). The following primers from humans were used: ALP (gacccgtcactctccgagatg/ctgcgcctggtagttgttgtg), DSPP (atggcatccagggacaagtaa/ccaggccagcatcttctccag), synthesized by ElimBio (Hayward, CA). The PCR products were separated on 1% agarose gel. The bands were imaged using a digital camera system and compared semi-quantitatively by NIH Image software.

Immunocytochemistry: First passage cells were grown on chamber slides until 80% confluent. The cells were fixed in pre-cooled 4% paraformaldehyde (PFA) for 10 min and washed three times with PBS. The cells were then incubated with 0.1% TritonX-100/PBS for 15 min at room temperature before blocking with 3% bovine serum albumin (BSA) in PBS. Rabbit anti-human antibodies against STRO-1 (1:200 dilution, R&D Systems), CD146 (1:100 dilution, R&D Systems), collagen type-I (1:100 dilution, Santa Cruz), laminin (1:100, Sigma), Ki67 (1:50, Santa Cruz) and amelogenin (1:1000 dilution) were used to incubate with cells overnight in 4°C. Amelogenin antibody (1:1000 dilution) was generated from recombinant human amelogenin immunized rabbits. After thorough washing, the cells were stained by fluorescence labeled secondary antibodies for one hour, and nuclei were counterstained with 1μg/ml Hoechst 33342 (Molecular Probes), and photographed using a Nikon Eclipse 300 microscope and digital imaging system (QIMAGING digital camera and SimplePCI software).

Fluorescence Activated Cell Sorting (FACS): Primary tooth pulp tissue was gently separated from the crown and root and placed in 10% penicillin/streptomycin/fungizone in 4°C overnight. The pulp tissue was then digested in 4 mg/ml collagenase/dispase (Roche) for 4 hours at 37°C. Single cells were isolated and incubated with STRO-1 antibody for 1 hour, then followed by

incubation with FITC labeled secondary antibody for 1 hour. The cells were sorted using a FACS machine and the percentage of Stro-1 positive cells was determined.

Identification and culture of oral epithelial cells

Oral epithelial cell (OEC) isolation and culture: Buccal mucosa was isolated from 18 to 22 week human fetal tissue, obtained under guidelines set by University of California at San Francisco. The tissue was digested in 2 mg/ml collagenase/dispase for 1 hour at 37˚C, washed with PBS, followed by further digestion with STV (0.05% trypsin, 0.025% versene) for 5 minutes. The cells were pelleted and washed with PBS, and $1x10^5$ cells were plated on a 100 mm Primaria tissue culture dish (Becton Dickinson Labware, Franklin Lakes) and fed with supplemented keratinocyte media (KGM-2) (Cambrex, Walkersville) with 0.05 mM calcium. This medium is selective for epithelial cells, and inhibits growth of fibroblasts. The medium was changed every other day.

Immunocytochemistry: The cells were fixed by incubating the cells in pre-cooled 4% PFA for 10 min and washed three times with PBS. Antibodies against CD34 (1:100 dilution, Ab Cam), cytokeratin 14 (1:200 dilution, Chemicon), amelogenin (1:1000 dilution) were used to incubate with cells overnight in 4˚C. Amelogenin antibody was generated from recombinant human amelogenin immunized rabbits. After thorough washing, the cells were stained by FITC labeled secondary antibodies, and nuclei were counterstained with Hoechst 33342 (Molecular Probe, Eugene). The sections were photographed using a Nikon Eclipse 300 microscope and digital imaging system (QIMAGING digital camera and SimplePCI software).

Co-culture of dental pulp cells and oral epithelial cells

pDPCs were growth arrested by gamma radiation. Briefly, initial passage (P0) pDPCs were grown to 80% confluence in DMEM low glucose supplemented with 10% FBS and 1%

penicillin/streptomycin. The cells were trypsinized with STV, rinsed with PBS and gamma irradiated at 5000 rad. The irradiated pDPCs were plated on Petri dishes ($2x10^5$ cells) and chamber slides ($1x10^5$ cells) with DMEM low glucose supplemented with 10% FBS and 1% penicillin/streptomycin. Twenty-four hours later, first passage OECs were plated onto the same Petri dishes and chamber slides. The co-culture cells were grown in modified DMEM containing low calcium concentration of 0.05 mM calcium to enhance epithelial cell growth, and supplemented with 10% FBS and 1% penicillin/streptomycin. The medium and was changed every two days.

As controls, pDPCs were plated alone and OECs were plated alone and grown in the same modified DMEM for the same length of time. On day 3, the dishes of control cells and the co-cultured OECs and irradiated pDPCs were collected and prepared for real time-PCR. The chamber slides were all fixed with pre-cooled 4% PFA and washed 3 times with PBS in preparation for immunostaining.

Real time-PCR: Total RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN, Valencia). The mRNA was transcribed into cDNA with random primer and Superscript reverse transcriptase (Invitrogen). Amplification with amelogenin gene specific primers (Applied BioSystems, location) was done a using Applied BioSystems real time PCR machine, model #7500. 18s RNA was used as a control.

Immunocytochemistry: The cells were fixed by incubating the cells in pre-cooled 4% PFA for 10 min and were washed three times with PBS. Following 15 min permeabilization and one hour blocking, rabbit antibodies against Amelogenin (1:1000 dilution) were used to incubate with cells overnight in 4°C. Amelogenin antibody was generated from recombinant human amelogenin immunized rabbits. After thorough washing, the cells were double

stained with mouse cytokeratin 14 (1:200 dilution, Chemicon), antibody for 1hour at room temperature. After thorough washing, the cells were stained by secondary antibodies FITC labeled anti-rabbit antibody, Alexa labeled anti-mouse antibody, and nuclei were counterstained with Hoechst 33342 (Molecular Probe, Eugene). The sections were photographed using a Nikon Eclipse 300 microscope and digital imaging system (QIMAGING digital camera and SimplePCI software).

RESULTS

Culture of and characterization of pDPC showed the presence of stem cells.

In our initial experiments to grow pDPC from extracted carious teeth, the cultures were frequently contaminated by bacteria. To prevent bacterial contamination, particularly for the decayed extracted primary teeth, we added the additional step of placing the dissected pulp tissue in 10% penicillin/streptomycin/fungizone in 4°C overnight. This modification allowed us to use teeth extracted due to caries as a source of primary dental pulp tissue.

The primary (P0) cells took approximately 4-5 weeks to reach confluence. Once the cells were passaged however, they grew at a much faster rate, taking 7-10 days to reach confluence (Figure 1). P0 cells had a stellate appearance (Figure 2), where as later passage cells had a more fibroblast–like phenotype. This stellate-shaped appearance is phenotypically characteristic of stem cells.

PCR of dental pulp cells showed that the pDPCs were positive for both ALP and DSPP (Figure 3). ALP expression was negative for the initial passage of cells and was positive after the first passage of cells.

Immunocytochemistry showed that pDPCs contained a small population of cells that expressed the early mesenchymal stem cell markers STRO-1 and CD146. The majority of cells were

immunopositive for collagen type I, one of the major proteins made by odontoblasts. Amelogenin expression was undetectable in our cell population. The cells did stain positive for laminin, a mesenchymal cell marker and for Ki67, which is a marker for cell proliferation (Figure 4).

Fluorescence activated cell sorting of P0 cells identified 1.67% of the primary culture cells to be STRO-1 positive (Figure 5). An attempt was made to grow the Stro-1 positive cells, but with no success, most likely due to the small number of cells.

Culture of OECs

Fetal OECs could be successfully grown in KGM 2 media (Figure 6). We found that a more pure population of OECs could be obtained by removing the cell population released after the first minute of trypsinization. The epithelial cells adhered more tightly to culture plates, while contaminating fibroblasts were more readily released during the trypsinization step. This modification to our culture method resulted in OECs without evidence of fibroblasts to plate on top of the pDPCs.

Immunocytochemistry of OECs showed positive staining for cytokeratin 14, a marker for all lining epithelial in the human oral cavity. The cells were also positive for CD34, and only slightly positive for amelogenin (Figure 7).

Co-culture of OEC with pDPC upregulated amelogenin expression.

After several trials, we found that gamma radiation of the P1 cells at a 5000 rad dose for 19 minutes, prevented dental pulp cell proliferation while maintaining cell viability. When the OECs were grown in co-culture on P1 growth-arrested pDPCs, the cells formed cell clusters that were immunopositive for amelogenin (Figures 8 and 9). Double staining with both cytokeratin 14 and amelogenin, showed again that the cells organize and stain positive for amelogenin (Figure 10). It is interesting to note that the positive staining for cytokeratin

14 overlapped with the amelogenin, suggesting that the oral epithelial cells are producing the amelogenin. Real time-PCR detected showed a strong upregulation of amelogenin mRNA expression in the co-cultured cells (Figure 11), whereas there was minimal to no amelogenin expression in either dental pulp or oral epithelial cells cultures alone.

DISCUSSION

In this study we used fluorescence activated cell sorting to identify the relative number of STR0-1 positive cells contained in pulp tissue from extracted primary teeth. The teeth used in our study were mostly primary incisors that were extracted due to caries or an inability to restore the tooth. This tooth source differs from the exfoliated teeth that were used in the study by Miura and co-workers [2]. The pulp in exfoliated teeth consists primarily of that remaining in the tooth crown, as the root has been resorbed. Because of the difficulty in obtaining freshly exfoliated teeth with a viable pulp, we identified our source as teeth that were extracted during the UCSF Pediatric Dental Clinic's general anesthesia cases.

Carious extracted teeth presented a challenge to us as initially our cultures were overgrown with bacteria and fungus. To overcome this problem, we first soaked the teeth in 10% povidone iodine for 5 minutes prior to excavation of the pulp. Once the pulp was removed, it was placed in 10% penicillin/streptomycin/fungizone in 4°C overnight and the following morning the tissue was digested.

This protocol overcame the bacterial contamination problem. However, teeth with large carious lesions are known to have significant inflammatory infiltrates in the pulp [42-44], which further compromised the number of cells available for study. Not surprisingly, we

found that the teeth that were the most desirable for our study were the ones that had been extracted due to trauma or for orthodontic reasons and therefore had no caries or the ones in which the caries were further from the pulp. These findings are supported by the article by Raslan and Wetzel "Exposed human pulp caused by trauma and/or caries in primary dentition: a histological evaluation" which found that the inflammatory infiltration was reduced in the traumatized teeth as opposed to the caries infected teeth and that the inflammatory infiltrate of a carious primary incisor reached to the middle third of the root canal [45].

The dearth of studies on human primary tooth pulp, following the initial study by Miura et al. [2] is likely to be due in part to the difficulties inherent in collecting intact primary tooth pulp cells. Jing et al reported that stem cells from dental pulp and deciduous teeth are "difficult, even impossible to harvest in clinic" [46]. However with appropriate tooth selection, and culture we were able to relatively easily obtain cells from primary tooth pulp for our studies.

In our study the P0 cells grew at much a slow rate as compared to P1 cells. Some of the P0 cells had a star-shaped appearance phenotypically characteristic of stem cells. The appearance of these cells and their generally slower grown may suggest a more undifferentiated population of P0 cells as compared to cells grown at later passages.

The dental pulp cells were positive for both ALP and DSPP by PCR analysis. ALP expression was negative for the initial passage of cells (P0) and was positive at P1. This data supports the hypothesis that as the cells are passaged they differentiate to odontoblasts and lose their stem cell properties.

The immunocytochemistry results further confirmed the presence of stem cells amongst the pDPCs. A small percentage of cells in the primary passage stained positive for both Stro-1 and CD146, two early mesenchymal stem cell markers [47] [2]. The presence of laminin also confirms that these cells are mesenchymal, while the high expression of type I collagen confirms that these cells are dental pulp cells. Ki67 is highly positive showing a high proliferation rate, which is expected in these cells. Amelogenin mRNA could not be detected in the dental pulp cells.

Although primary human teeth contain stem cell populations, this is a minor component of the pulp cell population. We found only 1.7% of the total primary (P0) cell population to be STRO-1 positive, with similar results by immunostaining for the mesenchymal stem cell markers, STRO-1 and CD146. This percentage is lower than the 9% stem cells population identified by Miura et al. [2]. The different source of cells (carious extracted teeth in our study vs. exfoliated teeth in the Miura study), may be an explanation for the differences. However, in either case, these results point to a relatively small population of stem cells within the primary tooth pulp, reducing enthusiasm for this possible stem cell source. After cell sorting, we were unable to grow the STRO-1+ cells, probably due to the low numbers. Though it was difficult to isolate pure stem cell populations from the primary tooth pulp, these cells may still have a role in promoting a differentiation of other cells, such as OEC.

In order to grow the pDPCs and OECs in co-culture we had to arrest the growth of the pulp cells or they would overgrow the culture. Initially we attempted to use mytomysin C, but found this technique to be unsuccessful. After several attempts, we were successful in identify the correct dose of radiation that would arrest cell growth, while still allowing these cells to be maintained in culture. The irradiation works by causing damage to the spindle fibers and thereby preventing cell growth, but allowing the cell to live [48]. It seems that the

irradiation also does damage to the cells RNA. This became apparent when we tried unsuccessfully a number of times to collect the RNA from the irradiated cells.

The OECs were collected from the buccal mucosa of aborted 18-22 week old human fetal tissue. These cells were easily digested and easily grown in culture. The protocol was similar to that of ameloblast isolation and culture [49], but the digestion time was less, one hour versus 2-4 hours, for the OECs. Unlike ameloblast, which have been found to be difficult to culture [49], the oral epithelial cells were easily grown.

The positive immunostaining of these cells by cytokeratin 14 and CD34 confirmed that both the cells were epithelial and that they contained relatively undifferentiated cells. Cytokeratins are a group of intermediate filaments specifically expressed by epithelial tissues and all lining epithelial in the human oral cavity express cytokeratin 14 and cytokeratin 5. The buccal mucosa specifically expresses cytokeratin 14, along with a few others [50]. CD34 is a marker for primitive pluripotential stem cells of both stromal and hematopoietic lineages. It identifies a cell surface antigen expressed by most primitive stromal other than hematopoietic stem cells and it is gradually lost after differentiation of lineage-committed progenitors [51].

To enhance our co-culture we attempted to obtain a more pure culture of oral epithelial cells. When the oral epithelial cells were initially plated there was evidence of fibroblast contamination. In order to remove these fibroblasts, we utilized our observation that the epithelial cells adhered more strongly to the tissue culture dishes than did the fibroblasts. Therefore, after P0 cells were plated, prior to passaging, we added trypsin to the dish for 1 minute, and then removed the trypsin cell suspension. Since the fibroblasts were more

susceptible to the trypsin, this method allowed for a more pure passage of oral epithelial cells overlying to plate on the dental pulp cells in co-culture.

The upregulation of amelogenin in co-cultured cells indicated the transformation of the oral epithelial cell to dental specific epithelium. Some authors have shown amelogenin mRNA in cells from other tissues that enamel [52]. However, these low levels of amelogenin expression are not evident by immunohistochemical staining. In addition to upregulated amelogenin expression, the cells formed structures, and these structures were not uniformly amelogenin positive. These findings show that primary dental pulp cells can induce dental epithelial formation. This novel finding will open the door to future studies of tooth and enamel tissue engineering. Future studies will determine the specific cells in the dental pulp that can direct dental epithelial differentiation.

CONCLUSION

In conclusion, the dental pulp cells from primary teeth are a unique, heterogeneous population of cells that include a small population of stem cells. The heterogeneous population of cells in the dental pulp can promote differentiation of fetal oral epithelial cells into ameloblast lineage cells. In the future it will be of interest to further characterize the stem cell population within the dental pulp. Specifically it will be necessary to isolate these cells and determine what makes them different from the pulp cells of permanent teeth and to isolate what factors are promoting the differentiation of the oral epithelial cells

Figure 1

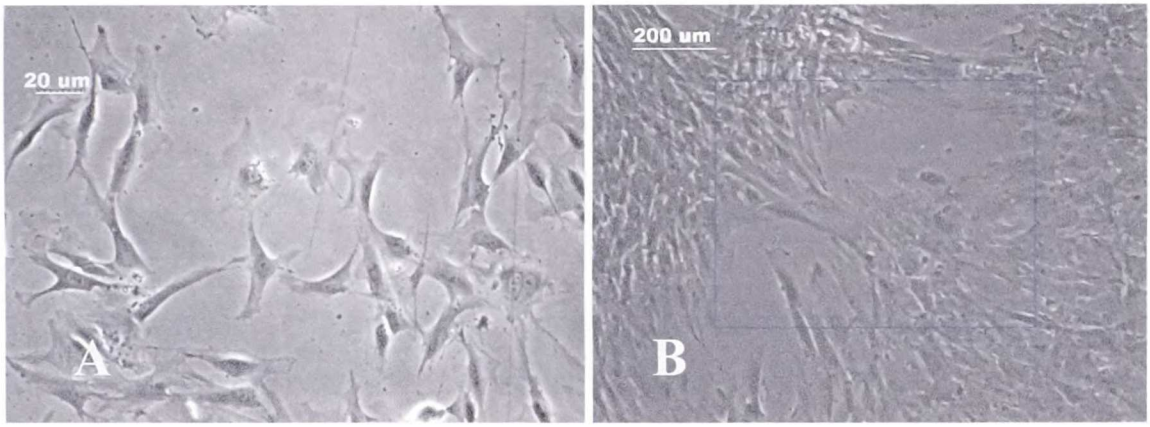


Figure 1: Phase contrast image of dental pulp cells from primary teeth grown in DMEM. (A) passage0 cells (B) passage1 cells. Note the change in morphology of the more rapidly growing P1 cells.

Figure 2

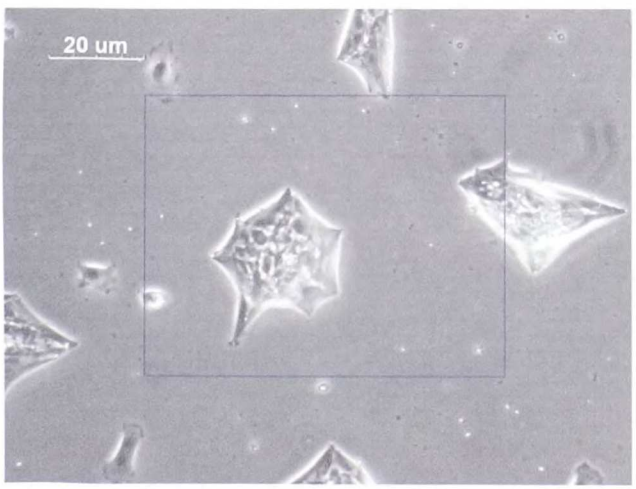


Figure 2: Phase contrast image of pDPC grown in DMEM. Note the stellate shape cell.

Figure 3

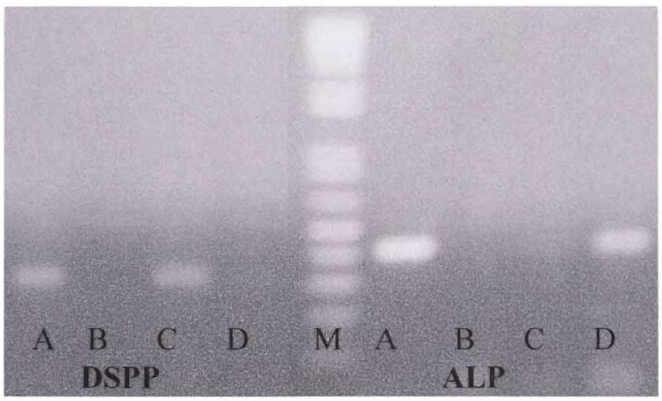


Figure 3: PCR of pDPC showing expression of DSPP in pDPC passage0 and expression of ALP in pDPC passage1. (A) positive controls (B) negative controls (C) pDPC passage0 (D) pDPC passage1 (M) marker

Figure 4

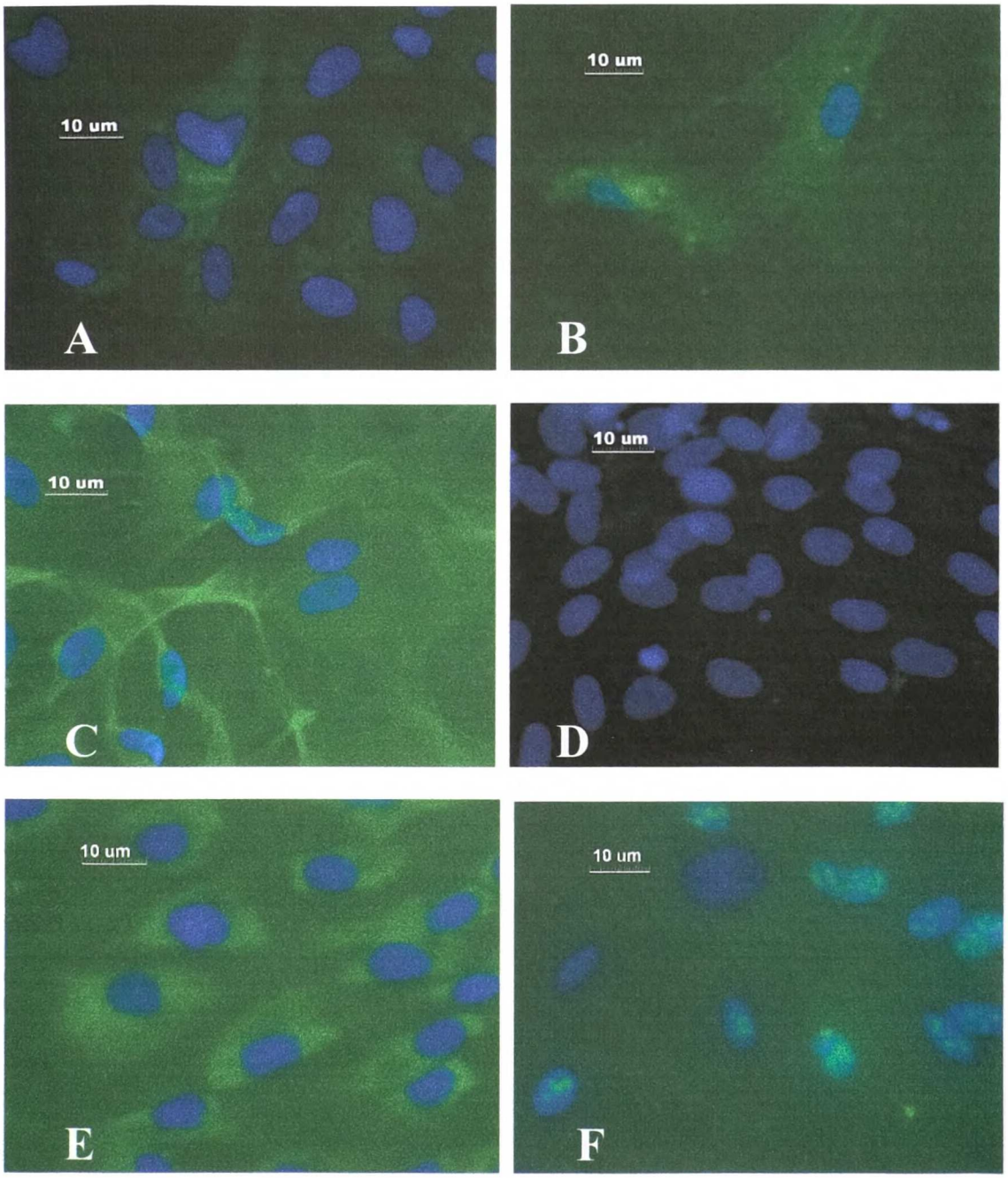
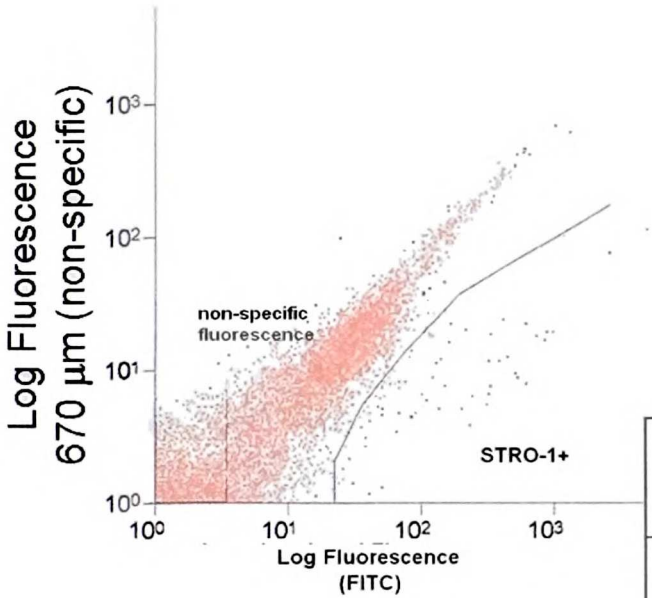


Figure 4: Immunostaining of P1 pDPCs. Green fluorescence shows relatively few cells with stro-1 (A) and CD146 (B), stem cell markers. Type I collagen (C) stains both cells and matrix with no amelogenin (D) staining observed. Laminin (E) and Ki67 (F) stains positive as well.

Figure 5



	Count	%
Total Cells	5913	100
STRO-1+ Cells	99	1.67

Figure 5: Fluorescence activated cell sorting of primary culture (P0) of pDPCs showing a small percentage of Stro-1+ cells. The cells above the line on the graph fluoresce equally at both wavelengths, indicating non-specific auto-fluorescence. The fewer cells below the line show specific fluorescence at 525 µm, indicating FITC labeled STRO-1 cells. The STRO-1 labeled cells are 1.67% of the total number of cells.

Figure 6

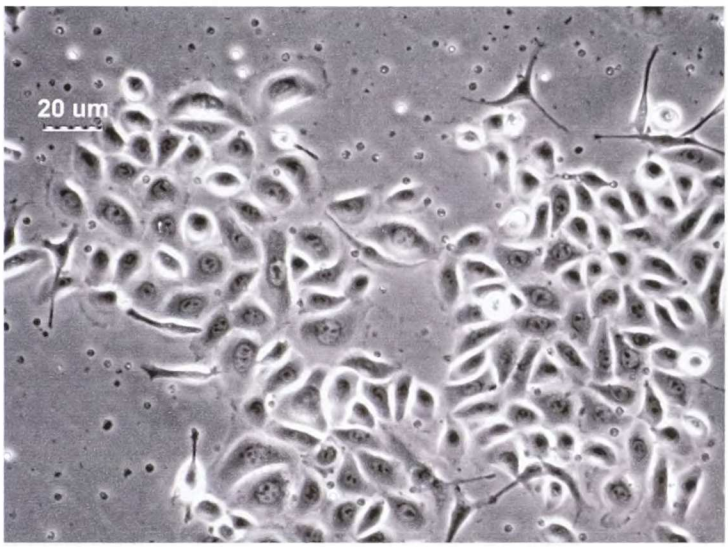


Figure 6: Phase contrast image of OECs from fetal buccal mucosa grown in KGM. Note the characteristic cobblestone appearance of the epithelial cells and the few interspersed fibroblast cells.

Figure 7

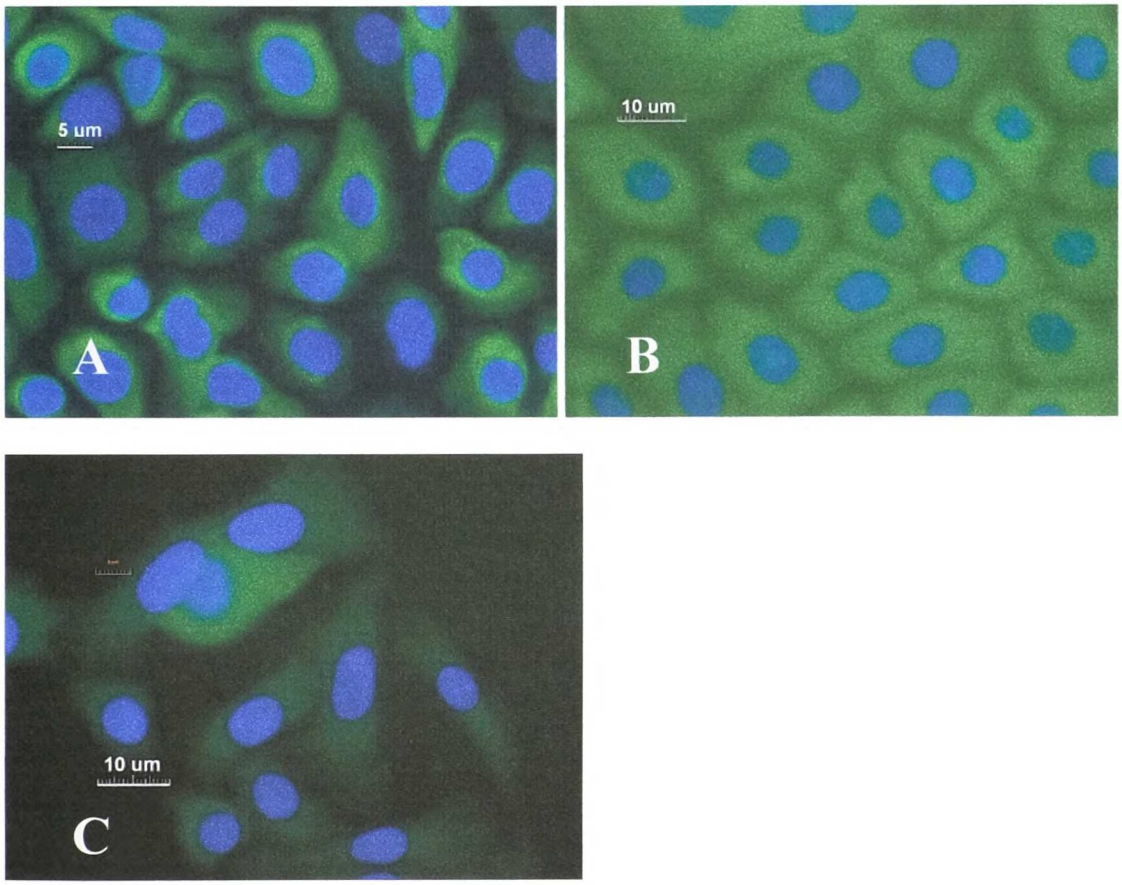


Figure 7: Immunofluorescence staining of OECS. Green fluorescence shows most cells to be positive for cytokeratin14 (A) and all cells to be positive for CD34 (B). The OECs are slightly positive for amelogenin (C).

Figure 8

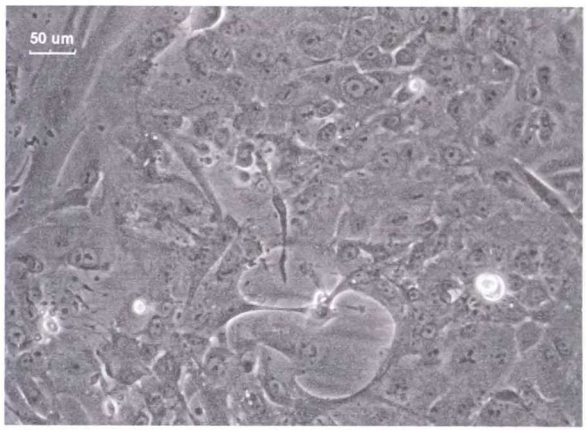


Figure 8: Phase contrast image of OECs co-cultured with pDPCs. Note the clusters of the OECs and pDPCs.

Figure 9

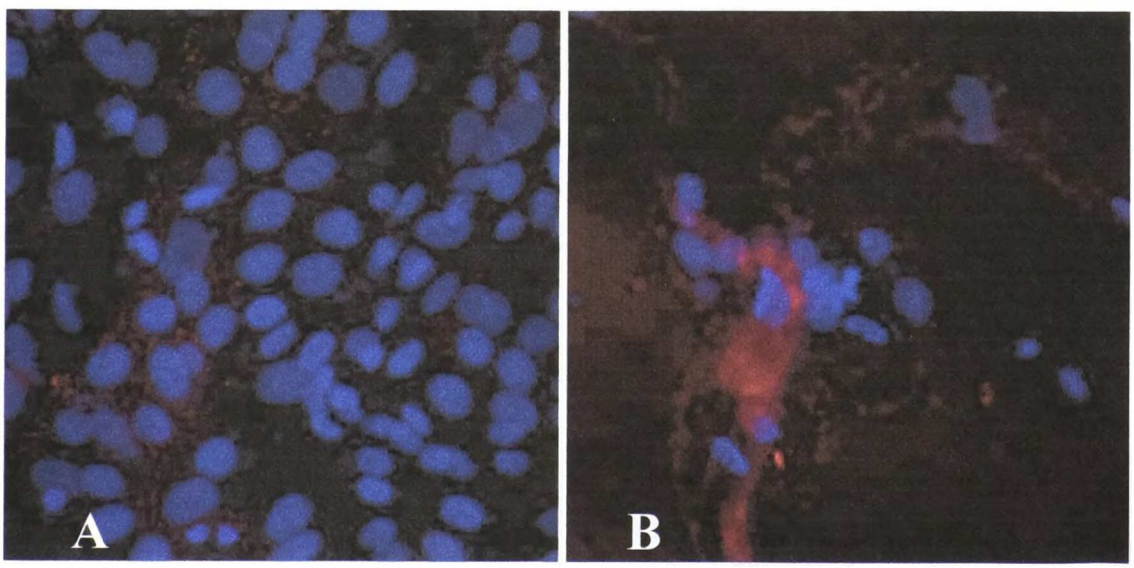


Figure 9: Immunofluorescence staining for amelogenin (red) shows no immunostaining in OECs alone (A). When OECs are co-cultured with pDPCs, the cells appear to organize, forming a cell cluster and express amelogenin (B).

Figure 10

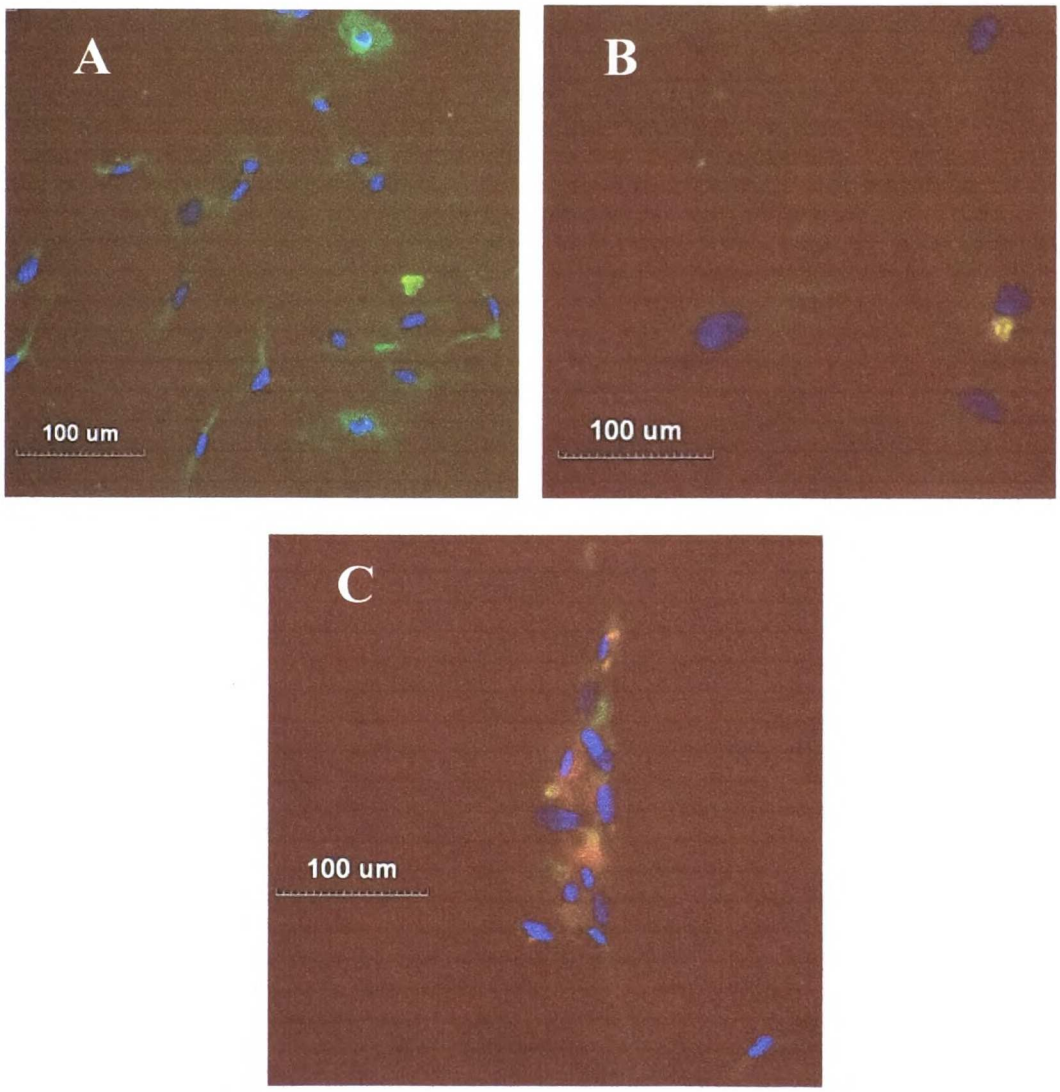


Figure 10: Double immunofluorescence staining with amelogenin (red) and cytokeratin14 (green) shows cytokeratin positive OECs (A). pDPCs are negative for both amelogenin and cytokeratin14 (B). When OECs and pDPCs are grown together, the cells organize to form a cell cluster that is positive for both amelogenin and cytokeratin 14 (C).

Figure 11

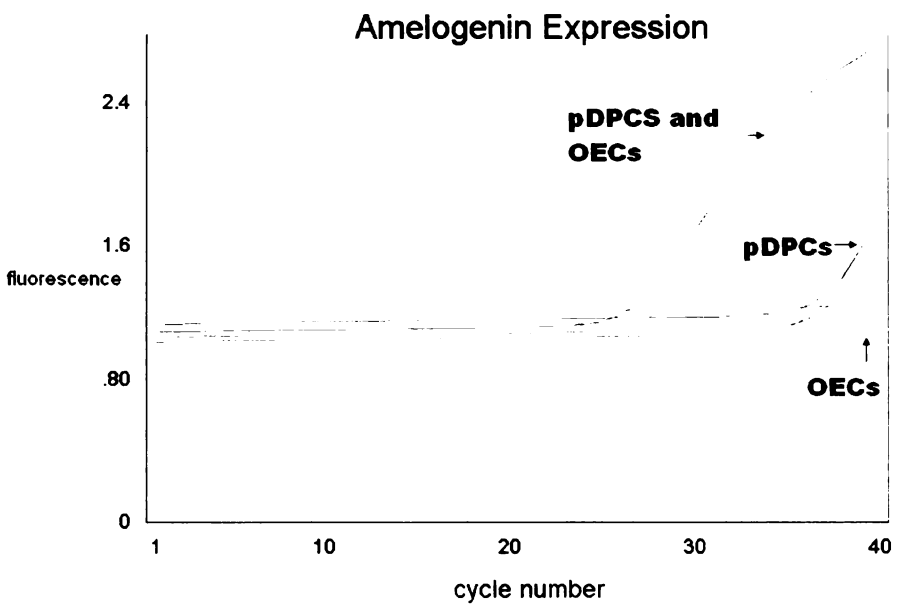


Figure 11: Real time-PCR results showing an upregulation of amelogenin expression in the co-cultured pDPCs and OECs. For the co-cultured cells, the expression is the strongest and starts to be detected at cycle #25. pDPCs alone showed relatively little amelogenin expression consistent with studies of amelogenin mRNA expression in adult dental pulp cell culture. There is no evidence of amelogenin expression in OECs.

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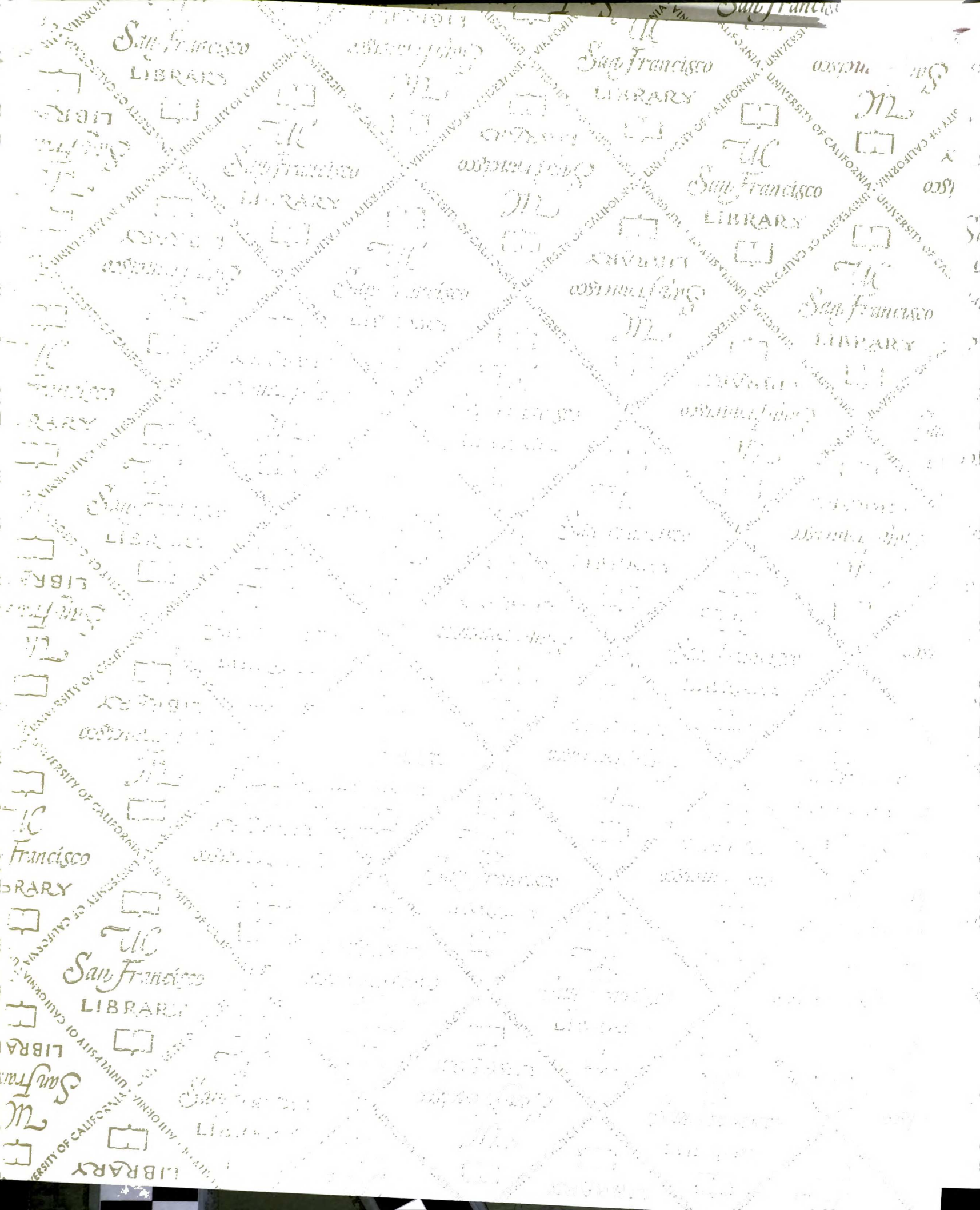
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