Isolation and Characterization of Human Adult Epithelial Stem Cells from the Periodontal Ligament

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Abstract

We report a novel method for the isolation of adult human epithelial stem cells (hEpiSCs) from the epithelial component of the periodontal ligament—the human epithelial cell rests of Malassez (hERM). hEpiSC-rich *integrin-α6*⁺ hERM cells derived by fluorometry can be clonally expanded, can grow organoids, and express the markers of pluripotency (OCT4, NANOG, SOX2), polycomb protein RING1B, and the hEpiSC supermarker LGR5. They maintain the growth profile of their originating hERM in vitro. Subcutaneous cotransplantation with mesenchymal stem cells from the dental pulp on poly-L-lactic acid scaffolds in nude mice gave rise to perfect heterotopic ossicles in vivo with ultrastructure of dentin, enamel, cementum, and bone. These remarkable fully mineralized ossicles underscore the importance of epithelial-mesenchymal crosstalk in tissue regeneration using human progenitor stem cells, which may have already committed to lineage despite maintaining hallmarks of pluripotency. In addition, we report the clonal expansion and isolation of human *LGR5*⁺ cells from the hERM in xeno-free culture conditions. The genetic profile of *LGR5*⁺ cells includes both markers of pluripotency and genes important for secretory epithelial and dental epithelial cell differentiation, giving us a first insight into periodontal ligament–derived hEpiSCs.

Keywords: enamel, cementum, Lgr5, integrin-α6, dental pulp stem cells, cell rests of Malassez

Introduction

Embryonic stem cell study and lineage tracing of stem cell fate reveal the principles of cell differentiation. Induced pluripotent stem cells are generated from somatic cells by the forced expression of markers of stem cell pluripotency, Oct4, c-Myc, Sox2, and Klf4 or Nanog (Bayart et al. 2013). Reports of genetic and epigenetic aberrations occurring during the stem cell derivation and reprogramming have raised doubts about the application of embryonic stem cells and induced pluripotent stem cells for tissue regeneration (Huch et al. 2015). Evidently human stem cells identified in tumors (Reya et al. 2001) cannot be used for regeneration. Recently, genetically unmodified, noncancer-origin adult human epithelial stem cells (hEpiSCs; Artwert et al. 2012) were successfully cultured from skin, intestinal, pancreatic, and liver stem cells, based on their expression of cell surface proteins (Calloni et al. 2013) integrin-α6 (Nowak and Fuchs 2009) and LGR5 (Huch et al. 2015). Organ regeneration through hEpiSCs requires accessibility of donor tissues, isolation, propagation, and overcoming cell death caused by loss of cell-to-cell contact (Chiarugi and Giannoni 2008). We report here the successful clonal isolation, characterization, and maintenance of *integrin-α6*⁺ cells and *LGR5*⁺ cells of the periodontal ligament (PDL) in a xeno-free, feeder layer-free tissue culture system. These cells are a by-product of orthodontic removal of wisdom teeth and exist

around all teeth, throughout adult life, therefore representing an easily accessible homologous donor hEpiSC source.

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By definition, stem cells give rise to unaltered daughter cells while also being “progenitors” of somatic cells. As distinct epithelial organs share molecular developmental mechanisms, a common profile emerges for epithelial stem cells (EpiSCs): they are slow cycling and highly enucleated; they are remnants of a developmental process; and they share patterns of gene expression with the cells to which they are precursors. Evidence of stemness consists of the presence of proliferation markers (e.g., Ki-67) and markers of pluripotency and the ability to form organoids (Calloni et al. 2013; Hynds and Giangreco 2013).

The epithelial cell rests of Malassez (ERM) structures are nested within the PDL. In oblique section, they appear like a fishnet, while in transverse section, they liken nests of highly enucleated cells (Rincon et al. 2006). They also become mitotically activated in response to injury, inflammation, or in vitro explantation and can give rise to squamous metaplastic cells, cystic lesions, and ameloblastoid odontogenic tumors (Cerri and Katchburian 2005). They are therefore suspect as the remnant dental hEpiSC niche (Nam et al. 2011). During enamel formation, integrin- $\alpha 6$ is expressed in preameloblasts (Salmivirta et al. 1996), the dental epithelia, and precursors of the ERM. Integrin binds Laminin5- $\alpha 3$ and drives odontogenesis. We therefore focused our study to the investigation of the integrin- $\alpha 6^{+ve}$ cells of the PDL as we searched for hEpiSCs. After our first reports on integrin- $\alpha 6^{+ve}$ cells (Athanassiou-Papaefthymiou et al. 2010), several others (Xiong et al. 2012; Xiong et al. 2013) reported the ERM origin, pluripotency, and plasticity of ovine integrin- $\alpha 6^{+ve}$ cells and confirmed that integrin- $\alpha 6^{+ve}$ cells are the ERM cells within the PDL. ERM cells have thus been counted among the ever-increasing variety of the integrin- $\alpha 6^{+ve}$ stem cell-rich epithelial compartments that include murine mammary EpiSC and the hair follicle, the organ that most closely resembles the tooth in its development.

EpiSCs in the developing mouse maintain quiescence, survive, proliferate, and differentiate under the control of the Wnt, Hedgehog, and Notch pathways (Reya et al. 2001). *Lgr5* has been shown to be a marker of adult murine (Sato et al. 2009) and human (Huch et al. 2015) intestinal EpiSC, with culture conditions established that can overcome anoikis. *Lgr5^{+ve}* clones isolated by trituration and cultured in matrigel organize into organoids forming complete villar structures. Subsequent discovery of LGR5 among integrin- $\alpha 6^{+ve}$ stem cells of the hair follicle and other work established LGR5 as an EpiSC supermarker (Jaks et al. 2008). Finally, *Lgr5* marked the EpiSCs of the mouse incisor (Suomalainen and Thesleff 2010) and the ERM (Handrigan et al. 2010; Oka et al. 2012), meriting further investigation into *LGR5^{+ve}* cells of the human ERM (hERM).

We report here that integrin- $\alpha 6^{+ve}$ cells can grow organoids and express the markers of pluripotency, OCT4, NANOG, and SOX2, as well as the stem cell maintenance polycomb protein RING1B and the EpiSC supermarker LGR5. *LGR5^{+ve}* cells are a subpopulation of the integrin- $\alpha 6^{+ve}$ cells, and we were able to isolate and maintain both types of cells in xeno-free culture

conditions. Based on expression of markers of pluripotency or genes important for secretory epithelial and dental epithelial function, the genetic profile of *LGR5^{+ve}* cells gives a first insight into an hEpiSC population of dental interest. Subcutaneous cotransplantation with mesenchymal human dental pulp stem cells (hDPSC) on poly-L-lactic acid (PLLA) scaffolds in nude mice gave rise to perfect ossicles in vivo, showing the ultrastructure of dentin, enamel, cementum, and bone. This remarkable fully mineralized heterotopic ossicle formation not yielded by each cell type alone underscores the importance of epithelial-mesenchymal crosstalk in tissue regeneration applications and suggests that developmental commitment has already occurred in human stem cells that maintain hallmarks of pluripotency.

Materials and Methods

Extensive experimental details are part of the Appendix.

Cell culture of ERM from PDL explants: Epithelial cell outgrowths from the PDL tissue were grown in the KnockOut DMEM system (Invitrogen, Carlsbad, CA, USA) supplemented with keratinocyte growth factor 2 (fibroblast growth factor 10).

Fluorescence-activated cell sorting: Primary PDL cultures were cell sorted with a PE-anti-Integrin- $\alpha 6$ antibody (eBiosciences, San Diego, CA, USA) and an anti-human LGR5 (Sigma-Aldrich, St. Louis, MO, USA) as described in the Appendix. Cells were harvested by fluorescence-activated cell sorting (FACS) in survival medium in tubes or plated clonally on MaxGel-ECM (Sigma-Aldrich) plates with jagged peptide.

Immunofluorescent immunocytochemistry: Immunofluorescent cytochemistry was performed at the Microscopy and Image Analysis Laboratory at the University of Michigan.

PCR arrays on *LGR5^{+ve}* cells: *LGR5^{+ve}* cell RNA was prepared (PCR RNAqueus Kit, Ambion; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Superscript III (Invitrogen)-generated cDNAs were verified for beta-actin, GAPDH, and CD44 expression (Platinum PCR; Invitrogen) and SABiosciences *WNT* and Stem Cell (Qiagen, Valencia, CA, USA) human polymerase chain reaction (PCR) microarrays were used with FastStart SYBR Green mix (Roche, Pleasanton, CA, USA) on a 7900H ABI machine (Thermo Fisher Scientific) for real-time PCR.

In vivo experiments: Experiments were performed in accordance with the code of ethics for animal research at the University of Michigan. DPSCs (10^3) were coseeded with integrin- $\alpha 6^{+ve}$ cells (10^3) on PLLA nanoscaffolds and implanted subcutaneously in the back of nude mice (Jackson Laboratory, Bar Harbor, ME, USA), 1 in each limb quadrant. Mice were sacrificed at 6- and 10-wk time points and analyzed.

Results

EpiSC Properties among the Cells of Human PDL

We verified the presence of hEpiSC-like cells in the PDL with preliminary experiments utilizing a previously characterized population (Xu et al. 2009) depleted of both mesenchymal and endothelial stem cells (*STRO1*^{−ve}/*CD146*^{−ve} cells) and thus lacking the corresponding markers *STRO1* and *CD146* (Fig. 1a). Apart from the ERM, the only other epithelial cells in the PDL are endothelial cells that line capillaries, the *CD146*^{−ve} cells. *CD146*^{−ve} cells that are epithelia, can only be ERM. These cells were cultured in selective LHC-9 medium (Shinmura et al. 2008; Shimonishi et al. 2010), and the resulting population expressed the epithelial cell marker E-cadherin, coexpressing amelogenin, an ameloblast marker, in osteogenic induction media (Fig. 1b, c). LHC-9 selected cells could also grow organoids (Fig. 1a).

Subsequent studies involved isolating the epithelial cell component from the PDL of healthy third molars from human subjects. To establish a xeno-free culture favoring epithelia, without introducing components from an extratumoral environment, instead of Matrigel, we provided human extracellular matrix and components of human origin interacting with dental epithelia in vivo: collagen IV, laminin (epithelial-binding component), fibronectin (essential for stem cell migration). Passage led to vigorous growth of high cell numbers in primary cultures (Appendix Fig.). Within PDL cells of morphological heterogeneity, islands of rapidly growing epithelial cells, even embryonic stem cell-like cells, could be seen (Fig. 1d–f). Ring cloned colonies from the epithelial cell islands gave cells with epithelial morphology, forming nest-like, net-like structures.

Epithelial cells from the PDL are particularly sensitive to handling during FACS, but we optimized cell survival during the washes, incubation with antibody, and isolation under proprietary conditions. FACS experiments showed that the *integrin-α6*^{−ve} cells were a subpopulation of keratin-expressing cells (Alam et al. 2011), and *LGR5*^{−ve} cells were shown to be a subpopulation of *integrin-α6*^{−ve} cells (Nam et al. 2011). Here we show that *integrin-α6*^{−ve} clones from the hERM can be maintained live in 2-dimensional culture resembling their physiologic fishnet appearance with nests of cells extending to touch and connect as if in a nest (Fig. 2a–c). The cells can be passaged multiple times over prolonged periods and can infiltrate PLLA nanoscaffolds. In their physiologic state, ERM cells maintain cell numbers via equal rates of cell proliferation and apoptosis. The slow growth of the clonally selected cells in the absence of mesenchyme is consistent with their in vivo profile. Conditioned media from hDPSC culture alone was not sufficient to produce enhanced proliferation, either because mesenchymal and epithelial cell contact is necessary or because an unidentified, nonsoluble extracellular matrix component produced by the mesenchyme is critical for the proliferation or differentiation of the epithelial cells. *Integrin-α6*^{−ve} hERM also grow organoids when seeded in Matrigel at clonal density (Fig. 2f).

Integrin-α6^{−ve} and *LGR5*^{−ve} Cells Show Profiles of hEpiSC

Our *integrin-α6*^{−ve} cells (Fig. 2d, e) grow organoids in 3-dimensional culture (Fig. 2f) and express stem cell markers SOX2, NANOG, OCT4, and LGR5 and the polycomb protein RING1B, which is required for stem cell maintenance (Fig. 2g–n). They thus show hallmarks of pluripotency. As the *integrin-α6*^{−ve} cell population has been widely established as stem cell rich, we consider the *integrin-α6*^{−ve} hERM, an hEpiSC-rich PDL population.

We further examined the expression of stem cell markers in *LGR5*^{−ve} hERM cells (Fig. 3). *LGR5* was detected by immunofluorescence in our primary culture of the PDL and was coexpressed with Ki-67, a marker of cycling cells (Fig. 3d–g). We succeeded in live sorting of *LGR5*^{−ve} cells among the PDL (Fig. 3m) and in identifying factors critical for cell survival. Live *LGR5*^{−ve} cells account for 0.8% and up to 2% of early passage cells (passages 0 to 3). *LGR5* is being expressed in *integrin-α6*^{−ve} cells (Fig. 2l). Other stem cell markers, such as epCAM, NOTCH, and CD34, are also expressed among our PDL tissue culture. By immunocytochemistry of cultures of live-sorted *LGR5*^{−ve} cells from hERM, we found them positive for Ki-67, for markers of pluripotency OCT4 and NANOG, and for p63, which is at the heart of epithelial cell and tooth development (Laurikkala et al. 2006). As expected by their expression of *integrin-α6*, our *LGR5*^{−ve} cells also express cytokeratin 14, another marker of Malassez origin.

The RNA of *LGR5*^{−ve} cells and matched donor-origin control PDL cells was then used to test for the expression of 165 genes using the SABiosciences *WNT* and Stem Cell PCR arrays. The differential display of genes associated with the stem cell phenotype in our *LGR5*^{−ve} cells (Fig. 4), as opposed to the originating cells, showed enrichment in a variety of genes, including genes associated with stem cell maintenance and expressed in embryonic stem cells, as well as cell cycle regulators and growth factors. Elevated *WNT* family member expression was general as expected by the role of Wnt in tooth development (Suomalainen et al. 2010) with highest for *WNT11* and *FRIZZLED1* as well as *WNT8A* and *WNT10A*, which characterize ectodermal origin during embryonic development (Niehrs 2012) and are essential regulators of mesenchymal morphogenesis. *WNT10A* deletion leads to tooth agenesis (Yang et al. 2015). This result is consistent with our finding that full mineral formation in vivo occurs only when the hEpiSC-rich cells are coimplanted with hDPSC. In addition to the expression of *OCT4* and *NANOG*, which was verified with immunocytochemistry, the arrays showed enrichment of *MYC* and *SOX2*. Not only is *SOX2* an inducer of pluripotency, but it also marks epithelial competence to generate teeth in mammals (Juuri et al. 2013). *NCAM*, a molecule associated with mesenchymal stem cell lineage, was completely suppressed. We were surprised to see the muscle differentiation gene *MYOD* also enriched, and it is worth exploring whether *integrin-α6*^{−ve} hEpiSCs can give rise to myoepithelial cells. Genes implicated in dental epithelium development were

Figure 1. Isolation and initial characterization of human epithelial cell rests of Malassez derived from periodontal ligament (PDL). (**a–d**) PDL depleted of mesenchymal (Stro⁺) and endothelial (CD146⁺) markers contained epithelial stem cells (ESCs) and mineralizing epithelia (**a**). (**b**) Epithelial STRO⁺/CD146⁺ cells from human PDL were selected in LHC-9 medium and showed stem cell-like organoid growth when seeded in BD Matrigel hESC-qualified Matrix. PDL cells (5×10^4) that had undergone double selection and removal of STRO-1 positive cells were seeded in the Matrigel and suspended in epithelial selective LHC-9 media. Control mixed PDL cells did not form organoids but grew in single-cell suspension. hERM, human epithelial cell rests of Malassez. (**c, d**) The same STRO⁺/CD146⁺ cells were LHC-9 selected and exposed to mineralization induction media. After 2 d, E-cadherin expressing cells (**c**) also coexpressed amelogenin, a mineralization marker. Note the cytoplasmic and membrane localization of E-cadherin and the Golgi, cytoplasmic, and extracellular/secretory localization of amelogenin. Antibodies: anti-amelogenin rabbit antibody and Texas red conjugated anti-rabbit secondary; anti E-cadherin mouse antibody and FITC-labeled anti-mouse secondary. Human dental pulp stem cells show no E-cadherin reactivity. (**e–j**) Epithelial morphology of PDL-explanted cells grown on extracellular matrix (ECM). Cells with epithelial morphology were observed among a mixed population of periodontal ligament cells from tissue explants (white-dashed lines) (**e–g**) and on isolated ring-cloned colonies culture growing on feeder-free in Knockout DMEM system (**h–j**). Note the nest-like arrangement of cells within PDL cultures at lower densities (**e**), the ESC-like border and growth of highly enucleated cells observed in dense cultures (**f**), and stem cell-like focus formation (**g**). Ring-cloned cells from within ESC-like growths showed characteristics of epithelial and nest-like ERM arrangements (**h–j**). Bars: 10 μ m. This figure is available in color online at <http://jdr.sagepub.com>.

Figure 2. Isolation, clonal expansion, in vitro stem cell properties and in vivo mineralization potential of *integrin-α6*⁺ epithelial cell rests of Malassez. (a–c) Light microscopy of *integrin-α6*⁺ cells growing in special survival media and showing nest-like morphology. (d) Immunofluorescent cytochemistry of *integrin-α6*⁺ cells (red). Nuclear DAPI (4',6-diamidino-2-phenylindole) counterstain (blue). (e) Fluorescence-activated cell sorting histograms of *integrin-α6*⁺ human epithelial cell rests of Malassez cells labeled with PE-conjugated CD49f antibody (blue histogram) as opposed to isotype control (red histogram). High *integrin-α6*-expressing cells isolated correspond with the P3 gate as indicated. (f) Organoid of *integrin-α6*⁺ cells in Matrigel. (g–l) Immunofluorescent cytochemistry of *integrin-α6*⁺ cells with stem cell markers. Stem cell markers in *integrin-α6*⁺ cells included proliferation markers BMI1 (g) and Ki-67 (h) and pluripotency markers OCT4 (i), SOX2 (j), LGR5 (k), and SEA-44 (l). Each field was stained with a different antibody as described in Materials and Methods. *Integrin-α6* coexpression is shown with LGR5 (m) and with the stem cell maintenance marker Ring-1B (n). DAPI was added. (n) Cells were incubated with 0.1% Triton X-100, and Image-it FX signal enhancer was added prior to staining. DAPI was contained in Prolonged Gold Anti-Fade Mounting Medium (n). (m) No Triton X was used (no nuclear antigens, and DAPI is diffusible). Bars: a–l = 5 μm; m, n = 2 μm. This figure is available in color online at <http://jdr.sagepub.com>.

increased, such as *PITX2*, an exclusive marker of dental epithelium *FGF1* and *FGF3* and *BMP1* and *BMP2*. Most pronounced was the increase of the *CFTR* gene, a Na/H⁺ pump that is important for ameloblast mineralization but also for the function of mucosal epithelia. In addition to *OCT4* and *NANOG*, the arrays showed enrichment of *MYC*, *SOX2*, and *T* (brachyury). Notably, brachyury also acts in concert with

Wnt8. While *NCAM*, *SIGMAR*, and *GUSB* were completely suppressed, *MYOD* was surprisingly enriched, but this was consistent with our later observation of myoepithelial cells of human origin in studies of *integrin-α6*⁺ dental hEpiSCs implanted in nude mice. Also surprising was the increase in *COL1A1*, but like *WNT8* expression, it is consistent with active transcription from chromosome 17 alterations that are

not subjected to mineralization clearly showed ossicle formation in the coimplants of *integrin-α6*⁺ cells and hDPSCs. All 4 coimplants and 0 of 12 controls at the 10-wk time point developed ossicles. When separated from the softer tissue, the ossicles had a shiny outer appearance consistent with the presence of an enamel layer. The harder enamel was breaking off of the thin sections during sectioning. Half the implants (in some cases, half of each implant) were sent for transmission electron microscopy (TEM) and were not subjected to demineralization. (q) TEM magnification: 1,400,000×. Dense enamel-like mineralization (E) in the ossicle is shown in panel s with enamel crystals marked by yellow arrows. (r) TEM magnification: 34,000×. Mineralized areas of varying textures and densities in the ossicle are shown in panel s. Dentin-like processes mark dentin (D); fibers and thicker mineral density mark cementum (C); even more dense mineral marks enamel, while a trabecular structure of mineralized tissue marks bone (B). Extensive presentation of TEM results is the subject of a separate publication. b–l, n–p: Demineralized implants. Bars: b, d, k, m = 20 μm; c, e, f, h, i, l, p, o = 10 μm; g, j, s = 5 μm; q = 200 nm; r = 100 nm. This figure is available in color online at <http://jdr.sagepub.com>.

Figure 4. Cotransplantation of *integrin- $\alpha 6^{+ve}$* cells and human epithelial stem cells (hEpiSCs) leads to formation of fully mineralized ossicles. **(a)** Experimental schema: 2 poly-L-lactic acid nanoscaffolds were prepared with 10^3 dental pulp stem cells and incubated with survival media overnight; then 10^3 *integrin- $\alpha 6^{+ve}$* cells were coseeded on 1 of the scaffolds ($\alpha 6^{+ve}$ HERM + hDPSC sample) or seeded alone ($\alpha 6^{+ve}$ -only control). hDPSC, human dental pulp stem cell; hERM, human epithelial cell rests of Malassez. Scaffolds were left another 48 h in culture with change of survival medium, before being implanted subcutaneously in the back of nude mice, 1 in each limb quadrant. Four scaffolds were implanted per mouse: the sample, controls for each cell type alone, and an acellular control. Four mice were generated for each of 2 time points with the 4 scaffolds rotated clockwise on each lumbar quadrant to control for positional effects before mice were sacrificed. **(b–j)** Cellular histochemistry of implants at the 6-wk time point. **(k–p, s)** Cellular histochemistry of implants at the 10-wk time point. **(b, c, e, f, h, i)** Hematoxylin and eosin stained, demineralized implants at 6 wk. **(k, l)** Hematoxylin and eosin stained, demineralized implants at 10 wk. **(d, g, j)** Immunofluorescent histochemistry of 6-wk implants with anti-human nuclei antibody. **(n–p)** Immunofluorescent histochemistry of 10-wk implants with anti-human nuclei antibody marking nuclei of cells of human origin. Epithelial structures (m) were seen in the implants, and columnar epithelia in the periphery (o) or hDPSC cells (p) within the demineralized area were observed as marked. All slides were counterstained with DAPI. Negative control was immunofluorescence with secondary antibody alone. Anti-human antibody controls also included processing of native mouse tissues to ensure no cross-reactivity. **(m, s)** Toluidine blue staining of samples that were

associated with cancer development in many epithelial tissues. Expression of collagen genes found on other chromosomes was not enriched. In in vitro cultures, epithelial cells secrete their own extracellular matrix and assemble a laminin-rich basal matrix to establish the basal cue that determines polarity (Manninen 2015). Several genes implicated into cell cycle control showed significant changes in their expression levels: 1) *CCND1*, which is required for the G1-to-S transition, was remarkably reduced; 2) *CDC2*, which is also required for a coherent S phase and for the G2-to-M transition, was increased; and 3) *CDC42*, which transduces signals from integrins, *GPCRs*, and growth factors to p21 family kinases, was highly enriched. *PARD6* was also elevated—a semi-cd42 gene controlling asymmetric cell division and polarity and playing a role in epithelial-to-mesenchymal transitions.

In Vivo Potency of hERM as hEpiSC: Epithelioid Organelle Formation or Mineralization—A Matter of Cross Signaling

Integrin-α6⁺ hEpiSCs (10³) were coseeded with an equal number of hDPSCs on PLLA nanoscaffolds, which were implanted into nude mice subcutaneously, 1 in each limb quadrant. Four scaffolds were implanted per mouse: the sample and 3 controls. Control implants in each mouse included an acellular implant, an *integrin-α6*⁺ cell only (Fig. 4e–g), and an hDPSC-only control of 2 time points at 6 and 10 weeks with the 4 scaffolds rotated clockwise on each lumbar quadrant to control for positional effects (Fig. 4a; cotransplants shown on Fig. 4b–d, k–r). Histologic analysis of explants from sacrificed mice at the 6-wk time point (Fig. 4b–j) showed acinar, secretory epithelial-like structures but no mineralized tissue in the hERM-only control (Fig. 4f, g). Staining with anti-HuNu antibody identified the nuclei of human origin cells (Fig. 4d, g, j, n–p). At the 10-wk time point, 4 of 4 coimplants and 0 of 12 controls developed remarkable well-formed ossicles, surrounded by dark blue cartilage when stained with Toluidine blue for histologic analysis (Fig. 4m, s). Histologic analysis of the same explants demineralized for histology (Fig. 4k–s) and stained with anti-HuNu antibody showed columnar human epithelial cells lining the demineralized space (Fig. 4o), where hDPSCs were also found (Fig. 4p). Transmission electron microscopy (TEM) showed mineral depositions characteristic of dentin, cementum, enamel, and bone (Fig. 4r, s) as well as profiles of secretory epithelia secreting mineralized fibers, secretory vesicles, mineralization of areas rich in collagen fibers, acellular spicules within mineralizing tissue, as well as trabecular structures of bone (TEM is the subject of another report). The formation of dentin was expected by the inclusion of hDPSCs. Ameloblasts and cementoblasts respectively derive from the enamel organ (committed to epithelial lineage) and the dental papilla (considered mesenchymal). The formation of these different types of mineralized tissue in heterotopic ossicles is seen here for the first time. Because we consider *integrin-α6*⁺ cells multipotent but committed to an epithelial

lineage, we propose that signaling from the *integrin-α6*⁺ cells was required for this route of differentiation. The cementoblast potential of the ERM has also been hypothesized (Nam et al. 2011). Further experimentation is needed to understand the epithelial and mesenchymal crosstalk in tooth regeneration as well as the origin of the fibroblasts around the explants.

Discussion

The PDL is a readily accessible, promising source for human therapy. With the additional incentive that the dental hEpiSC niche remains largely unexplored, the search for hEpiSCs in the ERM becomes particularly exciting.

Teeth are highly mineralized organs resulting from sequential and reciprocal interactions between the oral epithelium and the underlying cranial neural crest-derived mesenchyme (Mitsiadis et al. 2003; Wang et al. 2007), important at each stage of tooth development. The program of hair follicle morphogenesis is similar to that of tooth morphogenesis; the onset of both morphogenetic processes is heralded by the formation of the ectodermal placode. The molecular mechanisms regulating the development of distinct epithelial organs are shared to a great extent (Pispa and Thesleff 2003).

Several studies suggest that the biochemical pathways that lead to differentiation of DPSCs into functional odontoblasts are similar to differentiation pathways of BMSCs into osteoblasts (Huang et al. 2009). The odontogenic potential of hDPSCs has been assessed by a number of organ, explant, and cell culture methods, noting the ability of such cultures to mineralize, at least in part (Gronthos et al. 2000; Sonoyama et al. 2006; Rosa et al. 2012). However, DPSCs alone form sparse and dense calcified nodules and fail to develop lipid-laden adipocytes. In our studies, the combination of hDPSCs with hEpiSCs leads to a fully formed osseous explant with the ultrastructure of cementum, dentin, enamel, and bone, surrounded by cartilage-producing fibroblasts (Fig. 4m, r, s; Appendix, TEM studies). Abundant lipid-laden adipocytic clusters are also uniquely seen. Such results were not seen in our control implants with only hERM or only hDPSCs and have not been reported elsewhere, possibly indicating that a single type of “stem” or progenitor cell is insufficient for tissue regeneration. In contrast, a combination of cells would produce a fully functional organ. *Grem1*⁺ cells, for example, were shown to be required to provide a functioning lamina for intestinal organelles produced by the *Lgr5*⁺ cells (Worthley et al. 2015). We propose that mesenchymal-epithelial signaling is necessary for the full formation of mineralized tissues (also suggested by Zhang et al. 2010). Our results raise the possibility that to exploit the genetic potential of human stem cells to regenerate organized tissues, architectural engineering of the supportive extracellular microenvironment may need to be combined with organized cell printing.

This article describes an examination of the hEpiSC properties of hERM *integrin-α6*⁺ and *LGR5*⁺ cells obtained from human subjects (appropriate use statement in Materials and Methods in Appendix).

We have maintained *integrin- $\alpha 6^{+ve}$* and *LGR5 $^{+ve}$* cells in xeno-free and feeder-free conditions, and we believe that our methods and findings are of general interest in work with hEpiSCs or progenitor cells of human origin and have potential in nondental tissue application. *Integrin- $\alpha 6^{+ve}$* clones show ERM-like growth patterns in 2-dimensional culture and grow organoids in matrigel. They express markers of pluripotency and grow organoids (Fig. 2). *LGR5 $^{+ve}$* cells, a subpopulation of *integrin- $\alpha 6^{+ve}$* cells, expressed *OCT4*, *NANOG*, *MYC*, and *SOX2*, the 4 genes that have been shown sufficient for the creation of induced pluripotent stem cells and stem cell pluripotency. An exclusive marker of dental epithelium in the orofacial cavity, the transcription factor *PITX2* showed the highest enrichment, confirming the ERM origin of *LGR5 $^{+ve}$* cells. Additionally, the *CFTR* gene and *S100B* genes, necessary for the differentiation of dental epithelia during enamel tissue formation (Josephsen et al. 2010) and ameloblast differentiation, were highly elevated. *LGR5 $^{+ve}$* cells also showed suppression of genes associated with a neurogenic phenotype and enrichment of genes that maintain an undifferentiated phenotype and control the cell cycle. Differentiation protocols to assess their tissue regeneration capabilities in vitro as well as in vivo are being evaluated.

Our laboratory focuses on the biology of enamel tissue formation. Our long-term objective is to integrate the use of hEpiSCs within the current efforts for tooth organ regeneration. The potential for tissue regeneration achieved with this report constitutes an advance toward the successful tissue engineering of complex mineralized tissue with the cooperation of mesenchymal-epithelial progenitor cell populations that mediate tooth development. The dental enamel structure cannot be replaced nor repaired, because the cells that synthesize the enamel matrix are lost during tooth eruption. The establishment of a novel therapy to engineer enamel would provide an important therapeutic option for reversal of enamel loss.

Author Contributions

M. Athanassiou-Papaefthymiou, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; P. Papagerakis, contributed to conception, design, and data analysis, critically revised the manuscript; S. Papagerakis, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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