O9-PURIFICATION AND CHARACTERIZATION OF HUMAN DENTAL EPITHELIAL STEM CELLS

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Key words
Human Epithelial Stem Cells (hEpiSC), Dental Epithelial Stem Cells (DESC), Epithelial Cell Rests of Malassez (ERM), Periodontal Ligament (PDL), integrinα6 (cd49f), LGR5, Notch-1, CD34.

Introduction
Our **objective** is to identify and characterize epithelial stem cells that can be differentiated to enamel producing cells. Our **hypothesis** is that human epithelial cell rests of Malassez (hERM), the only remaining dental-origin epithelial cells after tooth eruption, found in the periodontal ligament (PDL) of all teeth, have stem cell properties and can be isolated, expanded and induced to form enamel in vivo. No studies exist on the regenerative potential of human dental epithelial stem cells (DESC) that could be used in enamel reconstruction, although mesenchymal stem cells have been isolated and are being studied in the context of dentin and bone tissue regeneration but can also give rise to adipocytes and nerve cells (for review Bluteau *at al.*, 2008). This missing gap is critical because dental enamel, in contrast to bone and dentin, cannot regenerate. Furthermore, without cells that can give rise to ameloblasts complete biological restoration of human dental crown can not be achieved.

Materials and Methods
hERM cells were clonally isolated from PDL using cell surface markers and flow cytometry and were expanded through inhibition of anoikis and manipulation of cell proliferation pathways. hERM clones were tested for self-renewal and karyotype stability, for the expression of stem-cell markers using immunofluorescence microscopy and PCR arrays, for their ability to express enamel specific proteins upon differentiation induction using ameloblast lineage molecular determinants and/or co-culture and in vivo co-seeding systems with dental pulp stem cells (DPSC).

Results
FACS analysis of primary periodontal ligament cell culture showed the expression of cell surface markers: LGR5 (0.9-2% depending on patient), Notch-1 (2%), epCAM (0.5%), CD34 (0.5-1%) and integrin-α6 (15%). Integrin-α6^{+ve} hERM can be maintained live in 2-D culture resembling their physiological fishnet appearance with small round nodules (nests) of cells extending to touch and connect. The cells can be passaged multiple times over prolonged time with slow proliferation rates. They grow organoids in 3-D culture, express stem cell markers *Sox-2*, *Nanog*, *Oct-4* and *Lgr5,* and the polycomb gene *Ring1B* required for stem cell maintenance. Integrin-α6^{+ve} hERM show hallmarks of pluripotency and can find application when an absolutely stem cell pure population is not required. They can also infiltrate and grow on biodegradable PLLA scaffolds.

When DPSC and hERM were co-seeded on the scaffolds and scaffolds were transplanted into nude mice a complex cell population arose including fibroblast and epithelial cells. Ameloblast-like cells appeared (6-weeks post-implantation) and hard mineralized tissue was generated (eight weeks post implantation) and TEM showed mineral depositions in crystal form characteristic of immature enamel.

LGR5 was detected by immunofluorescence in our primary culture of PDL cells and co-expressed with Ki-67, a marker of cycling cells. The differential display of genes associated with the stem cell phenotype in our LGR5^{+ve} cells as opposed to the originating cells showed enrichment in a variety of genes, including genes associated with stem cell maintenance and embryonic stem cells, cell cycle regulators and growth factors. Elevated *WNT* family member expression was noted, with the most impressive elevation for *WNT8A* and *WNT10A* which characterize epithelial commitment during embryonic development. Most notable was the complete suppression of *N-cam* a molecule associated with mesenchymal stem cell-lineage. Genes implicated in dental epithelium development such as *PitX2*, *FGF1* and *3* and *BMP1* and *2* were increased. Most pronounced was the increase of the CFTR gene, a Na/H pump which is important for ameloblast mineralization but also for

the function of mucosal epithelia. Among cells of the hPDL, cytokeratin-14 expression is unique to the hERM, and our LGR5^{+ve} cells are cytokeratin 14^{+ve} therefore of ERM origin.

Discussion

Ameloblasts, the enamel-producing epithelia undergo apoptosis at the end of tooth development (for review Simmer *et al*., 2010), but the ERM fulfill criteria for a dental epithelial stem cell niche. Distinct epithelial organs share developmental molecular mechanisms (Barker *et al*., 2007; Jaks *et al*., 2008; Sato *et al*., 2009) and a common profile emerges for epithelial stem cells (EpiSC) based on animal and human cancer data similar to hERM described here: EpiSCs have a large nuclear to cytoplasmic ratio, express integrin-α6 and cytokeratins, are remnants of a developmental process and they have some common patterns of expression with the cells to which they are progenitors. EpiSCs also maintain quiescence and can survive, proliferate and differentiate under the control of the *Wnt* and *Hedgehog* pathways as well as through communication with adjacent mesenchyme and the *Notch* pathway.

A vast volume of literature considers integrin-α6^{+ve} cells as a stem-cell rich compartment in basal epithelia. For the hair follicle, Jacks *et al* showed by flow cytometry that Lgr5, a stem cell super-marker for epithelial cells, is expressed in 0.6% of the integrin-α6 medium-to-high-expressing keratinocytes. Tooth development is closely related to hair follicle development. Laminin5-α3 is essential during ameloblast differentiation and integrin-α6, its receptor, is uniquely expressed in dental epithelia that will give rise to ameloblasts. We thus exploited the preferential expression of integrin-α6 in ERM cells to isolate progenitor/stem cells with strong potential to differentiate into ameloblasts. Our interest in integrin-α6^{+ve} ameloblast progenitor cells tied with our study of ERM LGR5^{+ve} stem cells. Both hERM cell populations (integrin-α6^{+ve} and LGR5^{+ve}) were characterized and showed stem cells properties. hERM cells were able to differentiate into enamel-forming cells.

Conclusions

Tooth organ regeneration might be possible by direct transplantation of stem cells or progenitors in the jaw *in vivo* or by implantation of an *in vitro* engineered tooth. More than cosmetic application, tooth organ regeneration is needed for patients of trauma and severe tooth abnormalities such as *Amelogenesis Imperfecta*, which may have devastating consequences in the daily life and psychology of the patient. Since ameloblasts die at the end of tooth development, our results are significant because hERMs are the only viable enamel source for human application. Our data is consistent with previous studies in animal models (Rincon *et al*., 2006; Shimonishi *et al*., 2010; Shinmura *et al*.; 2008). We are currently exploring combinations of epithelial and mesenchymal stem dental stem cells and gene delivery systems and scaffolds to guide and control enamel and dentin regeneration.

Our technique is pioneering with respect to establishing feeder-free, xeno-free culture conditions for growth of isolated human epithelial stem cells and can potentially be applied in tissues of non-dental origin for applications requiring safe self-renewal of human pluripotent stem cells from autologous-donor. DESC could also be used to regenerate non dental tissues of epithelial origin. Differentiation protocols to assess their tissue capabilities in vitro as well as in vivo are being evaluated. It is conceivable that the same pluripotent cell used for tooth regeneration might be used for skin, kidney or liver regeneration.

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