CELL REGENERATION

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Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells

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Abstract

Background: Tooth is vital not only for a good smile, but also good health. Yet, we lose tooth regularly due to accidents or diseases. An ideal solution to this problem is to regenerate tooth with patients' own cells. Here we describe the generation of tooth-like structures from integration-free human urine induced pluripotent stem cells (ifhU-iPSCs).

Results: We first differentiated ifhU-iPSCs to epithelial sheets, which were then recombined with E14.5 mouse dental mesenchymes. Tooth-like structures were recovered from these recombinants in 3 weeks with success rate up to 30% for 8 different iPSC lines, comparable to H1 hESC. We further detected that ifhU-iPSC derived epithelial sheets differentiated into enamel-secreting ameloblasts in the tooth-like structures, possessing physical properties such as elastic modulus and hardness found in the regular human tooth.

Conclusion: Our results demonstrate that ifhU-iPSCs can be used to regenerate patient specific dental tissues or even tooth for further drug screening or regenerative therapies.

Keywords: Human urine, Integration-free iPSCs, Recombinant tooth, Bioengineered tooth, Dental epithelium

Background

The goal of regenerative medicine is to regenerate fully functional tissues or organs that can replace lost or damaged ones occurred during diseases, injury and aging [1,2]. The advent of iPSCs should speed up the application of regenerative tissues or organs such as tooth in the clinic [3]. While iPSC-derived cells have been tested in animal models [4-7], no solid organs or tissues such as tooth have been generated with human iPSCs. The tooth represents one of the best experimental models in organogenesis [8,9], and is easily accessible for human replacement therapy [10]. Tooth is formed by reciprocal interactions between epithelium and mesenchymal cells derived from the cranial neural crest [11,12]. Developmentally, the odontogenic potential shifts from the dental epithelium to dental mesenchyme at bud stage (Embryonic day 12, E12) [13,14]. Then, the epithelium differentiates into ameloblasts and finally forms the enamel, while the mesenchyme differentiates into the dentin, cementum and dental pulp [15]. Tooth stem cells such as dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), and gum stem cells (GSCs) have been isolated and investigated for tooth regeneration [16-18]. Recently, Arakaki and colleagues reported that mouse iPSCs (miPSCs) could be differentiated into ameloblasts via interactions with dental epithelium and these miPSCs derived epithelial cells were positive with the epithelial cell markers p63, cytokeratin-14 (K14), and ameloblast markers ameloblastin and enamelin [19]. Shortly after, another group differentiated miPSCs into neural crest-like cells to generate odontoblasts that express dental mesenchyme markers Msx1, Pax9, Lhx6, and odontoblast marker dentin sialoprotein (DSP) [20]. Recently, miPSCs were mixed with dissociated mouse

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dental epithelial and mesenchymal cells and found to contribute to odontogenesis *in vivo* [21]. However, human iPSCs have not yet been explored for tooth regeneration.

We developed a chimeric culture system for tooth regeneration from human iPSCs (Additional file 1). We have a collection of both H1 hESCs as a control and iPSCs derived in our laboratories, mostly from human urine cells (hU) by oriP/EBNA episomal vectors carrying a combination of reprogramming factors Oct4, Sox2, SV40LT, Klf4 and miR302/367 through electroporation [22,23]. We hypothesized that hESCs or hiPSCs can be induced to produce epithelial sheets capable of replacing the E14.5 mouse dental epithelium in a reconstitution process (Additional file 1). The reconstituted explants can then be cultured *in vitro* for 1–2 days and transplanted beneath mouse subrenal capsule for 3 weeks for tooth regeneration (Additional file 1).

Results

Epithelial sheets generated from hESCs and iPSCs

We first must devise a way to obtain dental epithelia from hESCs or ifhU-iPSCs and decided on a stage-specific approach based on retinoic acid (RA) and bone morphogenetic protein 4 (BMP4) in N2 medium [24]. To this end, we obtained epithelial cells with keratinocyte-like morphology at D7 (Figure 1A). When passaged with a ratio at 1:3, these cells became definitive keratinocytes in a defined keratinocyte serum-free medium (DSFM) at D42 (Figure 1A). However, these cells survive poorly after passage. We then allowed the cells to grow as epithelial sheets without passage, and they became denser at D14, and detached slightly with some cell death at D21 (Figure 1A). The differentiating cells at D7, 14, 21, 28 were harvested and investigated for the expression of pluripotent and keratinocyte progenitor's markers by qPCR and Western blot. During epithelial differentiation, H1-ESCs and ifhU-iPSCs behaved similarly as shown with markers examined at RNA level (*Oct4*, *K18*, *p63*, *K19*, *CD29*, and *K14*), showing up regulated expressions in epithelial markers and down-regulation of pluripotent marker *Oct4* (Figure 1B). Similar trends were also observed for protein expression (Oct4, K18 and p63, Figure 1C). Moreover, the expression of p63 and K14 were verified by immunofluorescence. p63 was detected earlier at D7 and continuously expressed at D21, while K14 expression was detected later at D21 (Figure 1D). As a result, we obtained homogenous layers of epithelial cells as sheets from both hESCs and ifhU-iPSCs at D7 (Figure 1D). These sheets harvested at D7 were tenacious and flexible, showing the flat and smooth surface at the apical side as observed by scanning electronic microscopy (SEM) (Figure 1E). The sheets became rugged with prominent nuclei at D14 (Figure 1E). Under transmission electron microscopy (TEM), the desmosomes could be observed clearly between the epithelial cells at both D7 and D14 (Figure 1E). Together, these results suggest that the epithelial sheets generated at D7 from hESCs or ifhU-iPSCs have desired properties for being induced for tooth regeneration.

Tooth-like structures generated from hESC and iPSC derived epithelial sheets

We then harvested D7 epithelial sheets and recombined them with the mouse dental mesenchyme before transplantation into mouse subrenal capsule (Additional file 1). After 3 weeks, we observed tooth-like structures with the fibrous cysts in the kidney (Figure 2A). We isolated individual tooth-like structures by removing them from the fibrous cysts and the surrounding bone (Figure 2A, left columns). We found that the tooth-like structures always appeared with the presence of fibrous cysts. The tooth-like structure contained dental pulp, dentin, enamel space, and enamel organ (Figure 2A, middle columns). The enamel organs have elongated ameloblasts with a ruffled border-like structure and papillary layer (Figure 2A, middle columns). We also observed the expression of Ameloblastin (Amel) located in the layer of ameloblasts and its papillary layer (Figure 2A). We confirmed the human origin of the epithelial component in cross sections of recombinant tooth prior to isolation by immunostainings with human specific antibodies against human leukocyte antigen-I (HLA-I) and human nucleus antigen (hNA) (Figure 2B). Both antibodies stained negatively in the dental pulp, cartilage, surrounding bone-like structures, which were developed from mouse dental mesenchyme (Figure 2B). As expected, both human specific antibodies stained positively for the ameloblasts (Figures 2B1 and 2B4), papillary layer besides (Figures 2B2 and 2B4), and squamous epithelial cells in the cyst (Figure 2B3). Furthermore, positive HLA-I staining was localized in the cytoplasm (Figures 2B1-2B3), while hNA was complementarily localized in the nucleus (Figures 2B4 and 2B5). As control, without recombination with hESCs or ifhU-iPSCs derived epithelial sheets, mouse dental mesenchymes transplanted under identical conditions formed bone-like structures instead (n=10/10), as confirmed by positive staining of bone sialoprotein (BSP) in the whole bone-like structure embedded with osteocytes (Figure 2C).

We then analyzed the hardness and elastic modulus of human adult teeth (human group), 3-week mouse teeth developed from tooth germs under kidney capsule (Mouse WT group), and the regenerative teeth from H1-ESCs and ifhU-iPSCs groups by Nano-indentation (Figure 2D). The harness and elastic modulus of dentin and enamel in above five groups showed similar properties (Figure 2D). In the case of enamel, the hardness and elastic modulus of H1-ESCs and two ifhU-iPSCs groups were lower than those of human and Mouse WT groups (Figure 2D,