Induction of Rhesus Keratinocytes into Functional Ameloblasts by Mouse Embryonic Dental Mesenchyme

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Abstract Fast progresses in stem cell-based tooth tissue engineering have been achieved in recent years in several animal models including the mouse, rat, dog, and pig. Moreover, various postnatal mesenchymal stem cells of dental origin have been isolated and shown capable of differentiating into odontoblasts and generating dentin. Meanwhile, human keratinocyte stem/progenitor cells, gingival epithelial cells, and even iPSC-derived epithelium have been demonstrated to be able to differentiate into functional ameloblasts. Translational medicine studies in the nonhuman primate are irreplaceable steps towards clinical application of stem cell-based tissue engineering therapy. In the present study, we first examined the epithelial stem cell markers in the rhesus skin using immunostaining. Keratinocyte stem cells were then isolated from rhesus epidermis, cultured *in vitro*, and characterized by epithelial stem cell markers. Epithelial sheets of these cultured keratinocytes, which were recombined with E13.5 mouse dental mesenchyme that possesses odontogenic potential in the presence of exogenous FGF8, were induced to differentiate into enamel-secreting ameloblasts. Our results demonstrate that in the presence of appropriate odontogenic signals, rhesus keratinocytes can be induced to gain odontogenic competence and are capable of participating in odontogenesis, indicating that rhesus keratinocytes are an ideal epithelial cell source for further translational medicine study of tooth tissue engineering in nonhuman primates.

Keywords Rhesus macaque · Keratinocytes · Tooth tissue engineering · Ameloblasts · Stem cell

1 Introduction

With the rapid advance of stem cell-based tissue engineering, repairing and replacing lost or damaged tissues or even organ in humans would be realized in the near future [1, 2]. Tooth loss and damage caused by periodontal and carious diseases, or genetic disorders are common and frequently occurring dental defects in humans. Although artificial dentures made of high polymer materials, ceramics, and noble metal have been successfully used to

treat these defects in clinical practice, there are disadvantages as compared with normal teeth, including limited working life, amenity, damage to periodontal tissues and inducing inflammation. Attempts have been made to establish approaches for bio-engineering of whole tooth in decades. Studies in several model animals including the mouse, rat, dog, and pig have provided wealthy data on stem cell-based bio-engineering of tooth [3–9]. Remarkably, Ikeda and colleagues reported that bioengineered tooth germs reconstituted by re-aggregated mouse embryonic molar cells, after being transplanted into the oral cavity of adult mice, were able to develop into fully functioning teeth with correct structure, sufficient hardness of mineralized dentin and enamel, appropriate revascularization in the pulp, proper periodontal connection, and root formation in the lost tooth region of adult mice [10, 11]. These landmark studies support the concept that a

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successful replacement of a complete and fully functioning tooth organ in an adult oral cavity could be achieved by transplantation of bio-tooth germ reconstituted through manipulation of stem cells with odontogenic fate *in vitro*.

Mammalian tooth development is regulated by reciprocal and sequential tissue interactions that are mediated by diffusible growth factors between the dental epithelium and the adjacent dental mesenchyme [12]. Both of these tissue components are obligated to participate in tooth development. Odontogenic potential represents an instructive capability of a tissue to initiate tooth formation, while odontogenic competence defines the capability of a tissue to respond to odontogenic signals and to participate into tooth development [13]. In mice, it is demonstrated that the odontogenic potential is initially resided in the dental epithelium prior to embryonic day 12 (E12) and is able to induce non-dental mesenchymal tissue to participate in tooth formation. After E12, this potential shifts to the dental mesenchyme that becomes able to induce non-dental epithelial tissue to participate in tooth development [14]. Our previous study reported that human dental mesenchyme at the bell stage also possesses such tooth inducing capability and is able to induce human keratinocytes to differentiate into enamel-secreting ameloblasts [15]. Based on such developmental principle, bio-engineering of a transplantable tooth germ requires both epithelial and mesenchymal cell components with either of them possessing tooth inducing capability.

Several types of human dental tissue derived postnatal stem cells, including dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth, periodontal ligament stem cells, dental follicle progenitor cells, and stem cells from the apical papilla (SCAP), have been isolated and characterized thus far [16]. These stem cells exhibit the capability to differentiate into dental tissue *in vitro* and *ex vivo*. While none of them possesses odontogenic potential, these stem cells are regarded promising cell sources with the potential of clinical practice for future tooth replacement and regenerative therapy. Although epithelial dental stem cells do not exit due to apoptosis of ameloblasts upon tooth eruption, our and others' previous studies have shown that human keratinocyte stem/progenitor cells [17], gingival epithelial cells [18], and even iPSC-derived epithelium [19] are able to differentiate into functional ameloblasts and produce enamel with identical running pattern of prism and comparable mechanical property to human ones.

Nonhuman primates share significant genetic and physiological features with humans and have served as an invaluable model for translational medicine of tissue engineering [20–22]. The rhesus dental pulp stem cells and epidermal progenitor cells have been isolated and evaluated for therapeutic potential of neurological disease and

ocular surface diseases respectively [23, 24]. However, no previous attempts have been made to use stem cells from nonhuman primate origin for tooth bio-engineering. In this study, we isolated rhesus keratinocyte stem/progenitor cells and recombined them with mouse E13.5 dental mesenchyme that possesses odontogenic potential to test if cell sources of nonhuman primate origin could be induced to become odontogenic fate and to participate in tooth formation.

2 Materials and methods

2.1 Rhesus skin tissues

Rhesus skin tissues used in this study were provided by the Fuzhou Key Laboratory of Technical Evaluation of Fertility Regulation for Non-human Primates, National Health and Family Planning Commission of People's Republic of China. The tissues were fixed, dehydrated, embedded in paraffin, and sectioned at 8 μm for immunohistochemical staining or isolate rhesus keratinocytes.

2.2 Isolation and culture of rhesus keratinocytes

Primary rhesus keratinocytes were isolated and cultured following the previous reports with some modifications [24, 25]. Briefly, about 1 cm^2 of the back skin from a 6-month old rhesus macaque, euthanized for clinical reasons, was washed in phosphate-buffered saline (PBS, pH 7.3) with 100 units/mL penicillin and 100 units/mL streptomycin (Sigma, St. Louis, MO, USA) for several times immediately after collection. The skin tissues were sliced into small pieces (0.2 $\text{cm} \times 1 \text{ cm}$) with scissors. The epidermal layer was separated from the dermis by overnight incubation of the skin tissues with 2 U/mL Dispase II (R&D System) at 4 $^{\circ}\text{C}$. The separated epidermis was digested in 0.25% trypsin/0.05% EDTA at 37 $^{\circ}\text{C}$ for 15 min and dispersed with a pipette into single cell suspension. About 1×10^6 suspended cells were plated onto a 6-cm cell culture dish in keratinocyte serum-free medium (KSFM) (Gibco). Unattached cells were discarded by medium replacement after 24 h culture. Attached cells were continuously cultured, formed colonies, and before colonies grew to confluence, cells were passaged. Passage 2 cells were used for recombination experiments. An intact epithelial cell sheet was removed from the dish using 2 U/mL Dispase II (BD), and was sliced into about 1- mm^2 size, then stored in culture medium on ice for subsequent tissue recombination experiments.

2.3 Tissue recombination and subrenal culture

Tissue recombination and mouse subrenal culture were carried as described previously [9]. Briefly, mouse E13.5 embryos were collected from timed pregnant mice. Molar tooth germs were dissected from mandibles and incubated in 2 U/mL Dispase II at 37 °C for 20 min. The dental epithelium was removed from the dental mesenchyme with fine forceps. A piece of mouse dental mesenchyme, implanted with several agarose beads soaked with FGF8 (125 ng/μL, R&D Systems), was recombined with a piece of prepared rhesus keratinocyte cell sheet. Recombinants were cultured in Trowell-type organ culture for 24 h before being subjected to subrenal culture in adult nude male mice. Samples were harvested after 2 weeks in subrenal culture and proceeded for histological and immunohistochemical analysis.

2.4 Histological staining and immunostaining

For histological staining and immunostaining analysis, samples were fixed in 4% paraformaldehyde (PFA) overnight, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 1 week, embedded in paraffin, and sectioned at 8 μm. Sections were processed for histological analysis with Azan dichromic staining or subjected to immunostaining. For immunostaining, slides were boiled in 10 mM sodium citrate (pH 6.0) for 20 min to recover antigen, washed in TBS, blocked in serum blocking solution for 1 h, and then subjected to incubate with primary antibodies at 4 °C overnight following the instructions provided by the manufacturers: anti-human K14 (ZA-0540, ZSGB-Bio), anti-human K15 (SC80860, Santa Cruz), anti-human p63 (SC8431, Santa Cruz), anti-human integrin β1 (SC9970, Santa Cruz), anti-human MHC (ab133789, ABCam), anti-mouse DSP (SC18328, Santa Cruz) and anti-human Ameloblastin (SC33102, Santa Cruz). Slides were rinsed with TBS, incubated with horseradish peroxidase-conjugate secondary antibodies (Santa cruz) and then were visualized by incubation with DAB for immunohistochemical staining or incubated with Alexa Fluor 488/594 IgG (H + L) secondary antibody (Life technology), together with DAPI (Life technology) counterstaining, directly observed and photographed under laser scanning confocal microscope (LSM780, Zeiss) for immunofluorescence staining.

Cultured rhesus keratinocytes at 30% confluence were subjected to immunofluorescence analysis. Cells were fixed with 4% PFA in PBS for 10 min, treated with 1% Triton X-100 for 15 min, blocked in serum blocking solution for 45 min, and then subjected to incubate with the anti-human K14, K15, p63, and integrin β1 primary antibodies at 4 °C overnight. Cells were rinsed with TBS, incubated with

Alexa Fluor IgG (H + L) secondary antibodies, counterstained, and photographed under laser scanning confocal microscope. The primary antibodies were omitted in negative controls.

3 Results

3.1 Expression of epithelial stem cell markers in the rhesus skin

Despite the expression patterns of stem cell markers and the location of epithelial stem cells have been intensively studied in humans, little is known in rhesus. For instance, in human, K14 is correlated with the degree of pluripotency of the basal cells in the stratified epithelium and is down-regulated when cells of most stratified epithelium differentiate [26]. K15, p63, and integrin β1 are known as markers of epithelial stem cells in the stratified epithelium [27–29]. In order to unveil the location of epithelial stem cells in rhesus skin, we examined the expression of K14, K15, p63, and integrin β1. K14 was expressed in the basal epidermal layer (Fig. 1B) and hair follicle except the dermal papilla (Fig. 1C). Expression of K15 was intensive in the basal epidermal layer (Fig. 1E) and root sheath of hair follicle with slightly weaker in the hair follicle matrix (Fig. 1F). Localization of p63 expression revealed that p63 was highly enriched in cell nuclei of the basal epidermal layer (Fig. 1H) and presented intermittently along the interfollicular epidermis (Fig. 1I). Integrin β1 exhibited rather similar pattern to K15, being present in the basal epidermal layer (Fig. 1K) and root sheath with a much lower level in the matrix (Fig. 1L). The comparable expression patterns of these markers with that in human skin suggest the existence of epithelial stem cells in the epidermal layer of rhesus skin with similar physiological function as to humans.

3.2 Isolation and identification of rhesus keratinocyte stem cells

Since the rhesus skin exhibits similar structure and localization of epithelial stem cells with the human one (Fig. 1), we employed the established isolation and culture methods [25] and commercialized culture medium [17] for human keratinocyte stem cells to isolate and culture rhesus keratinocyte stem cells. In such a condition, the rapid adherent primary cells exhibited extensive cell proliferation and colony formation capability characterized by typical epithelial cell morphology with a small round or oval appearance (Fig. 2A). Cells were passaged after 2–3 weeks before colonies became confluent (Fig. 2B). Healthy cell morphology was well maintained in the Passage1 (P1) and

Fig. 1 Expression of epithelial stem cell markers in the rhesus skin. **A–C** K14 is expressed in the basal epidermal layer, root sheath, and matrix of hair follicles in rhesus skin. **B, C** Higher magnifications of rectangles in **A**. **D–F** K15 is expressed in the basal epidermal layer, root sheath, and matrix of hair follicles in rhesus skin. **E, F** Higher magnifications of rectangles in **D**. **G–I** p63 is expressed in the basal epidermal layer, root sheath, and matrix of hair follicles in rhesus

skin. **H, I** Higher magnifications of rectangles in **G**. **J–L** Integrin $\beta 1$ is expressed in the basal epidermal layer and root sheath of the hair follicles in the rhesus skin. **K, L** higher magnifications of rectangles in **J**. **M–O** negative control. **N, O** higher magnifications of rectangles in **M**. Red arrows point to matrix. (*bl* basal epidermal layer, *rs* root sheath, *mx* matrix, *dp* dermal papilla). Bars 500 μ m (**A, D, G, J, M**), 100 μ m (**B, C, E, F, H, I, K, L, N, O**)

Passage2 (P2) cells (Fig. 2C, D). However, when passaged to 3rd generation, the cells became flat and hardly proliferated, exhibiting senescence characteristics (data not shown).

To further confirm the keratinocytes that we isolated from rhesus skins were stem cells, immunofluorescent staining was performed to characterize P2 cells with anti-human K14, anti-human K15, anti-human p63, and anti-

Fig. 2 Culture of rhesus keratinocytes. **A** Colony formation of cultured primary rhesus keratinocytes. **B** Cellular morphology of the cultured primary rhesus keratinocytes 10 days after plating. Primary cells (P0) were characterized by typical epithelial cell morphology with a small round or oval appearance. **C, D** Healthy cellular morphology was well maintained in the passage 1 (P1; **C**) and passage 2 (P2; **D**) rhesus keratinocytes. Bars 50 μ m

human integrin β 1 antibodies. As shown in Fig. 3, K14, K15, p63, and integrin β 1 were present abundantly in almost all of the cultured keratinocytes. Among them, K14, K15, and integrin β 1 were expressed in the cytoplasm (Fig. 3A–F, J–L), whereas p63 was notably present in the nucleus (Fig. 3G–I). The immunostaining results demonstrate that keratinocytes isolated from rhesus skins are stem/progenitor cells and their stemness is still well maintained in P2 cells.

3.3 Induction of rhesus keratinocytes into enamel-secreting ameloblasts

Our previous study has reported that the E13.5 mouse dental mesenchyme serves as an ideal applicable source to induce human keratinocytes to differentiate into enamel-secreting ameloblasts [17]. In this study, we also employed the E13.5 mouse dental mesenchyme to test if rhesus keratinocytes could be induced into ameloblasts by odontogenic signals. Histological analysis of chimeric teeth that were grown in the kidney capsule of nude mice for 2-week showed the formation of tooth-like structures with dentin (in blue color) and enamel (in red color) deposition in about 20% of cases (Fig. 4A; 3/15). To rule out the possibility of tissue contamination, we conducted immunohistochemistry using anti-human MHC antibody on the tissue recombinants to distinguish between mouse and rhesus tissues. Specific positive staining in ameloblasts but not in dental mesenchyme including odontoblasts

confirmed that the epithelial component was indeed derived from rhesus keratinocytes (Fig. 4B). Immunohistochemical assays further confirmed the odontogenic differentiation of rhesus keratinocytes, assessed by the present of ameloblastin, a molecular marker for differentiating ameloblasts, in the elongated epithelial cells of tooth-like structure (Fig. 4D) and DSP (dentin sialoprotein) expression in the mesenchymal component in the chimeric tooth (Fig. 4C). Our results demonstrate that rhesus keratinocytes, like human keratinocytes, are able to participate in tooth formation and to be induced into functional enamel-secreting ameloblasts.

4 Discussion

In the present study, we show that keratinocyte stem cells isolated from the rhesus skin could serve as an epithelial cell source for tooth tissue engineering in translational medicine study. The mammalian skin is composed of epidermis, the outmost layer of the skin, and dermis, the layer of skin beneath the epidermis. Keratinocytes at different differentiation stages are resided in the epidermis and connective tissues are present in the dermis. Epithelial stem cells are responsible for the skin self-renewal throughout life span and have been reported to be an important stem cell source in terms of repairing and replacing lost or damaged skin tissues. In humans, epithelial stem cells include the keratinocyte stem cells that

Fig. 3 Expression of stem cell related genes in cultured rhesus keratinocytes. **A–L** Immunofluorescent staining with antibodies against human K14 (**A–C**), K15 (**D–F**), p63 (**G–I**), and integrin β 1 (**J–L**) in the cultured rhesus keratinocytes. K14, K15, integrin β 1

are abundantly present in the cytoplasm while p63 is expressed in the nucleus. **M–O** Negative control by staining with DAPI and omitting of primary antibodies. Bars 100 μ m

are located in the basal layer of the epidermis and the hair follicle stem cells that reside at the bulge of outer root sheath of the dermis [30–32]. K14, K15, p63, and integrin β 1 have been used as accepted markers to precisely locate the epithelial stem cells in the human skin [25, 31].

Surprisingly, as an important animal model for translational medicine study, no data is available in the literatures regarding rhesus skin histology and distribution of epidermal stem cells. Herein, we employed the above four human epithelial stem cell markers to determine the

Fig. 4 Induction of rhesus keratinocytes into enamel-secreting ameloblasts by the bud-stage mouse dental mesenchyme. **A** Azan dichromatic staining shows a tooth crown structure formed in a recombinant of rhesus keratinocytes and E13.5 molar mesenchyme. **B** Immunohistochemistry using the anti-human MHC antibody stained ameloblasts of rhesus origin in a recombinant after 13 days

in subrenal culture. **C** Immunohistochemical staining with anti-mouse DSP antibody stained the dental pulp cells of mouse origin in the dental mesenchyme of the recombinant. **D** Immunofluorescent staining with anti-human ameloblastin antibody shows well-differentiated enamel-secreting ameloblasts in a recombinant (*am* ameloblast, *d* dentin, *e* enamel, *od* odontoblast). Bars 50 μ m

location of epithelial stem cells in the rhesus skin. Our data revealed very similar expression patterns of these four markers in the rhesus skin as compared to that in humans. K14 is synthesized in the basal layer of the interfollicular epidermis and outer root sheath of hair follicles [33]. K15 is produced in basal keratinocytes and synthesized in the outer root sheath of hair follicles [33], and is down-regulated when the basal keratinocytes begin to differentiate [28]. p63 is highly expressed in the proliferative basal cell layer of the skin [34] and bulge of the hair follicle, with lower level of expression in the interfollicular epidermis and the matrix [29]. Integrin β 1, a basal cell marker associated with certain stem cell properties, is detected in the basal keratinocytes and outer root sheath of the hair follicle [27]. Similar expression patterns of epithelial stem cell markers in the human and rhesus skin suggest existence and distribution of epithelial stem cells in the epidermal layer of the rhesus skin.

Lu and colleagues reported a protocol for isolation and culture of rhesus keratinocyte stem cells with complicated steps including 3 passage culture in collagen IV coated dishes and gradient centrifugation to eliminate fibroblast during each passage [24, 35]. By comparison, the commercialized protocol for human keratinocyte stem cell

culture is much simpler. Based on the strikingly similar expression pattern of epithelial stem cell markers in human and rhesus skin, we employed the aforementioned commercialized protocol to isolate and culture rhesus keratinocyte stem cells [17]. Rhesus keratinocyte stem cells exhibit strong cell proliferation capability and colony formation until passage to 2nd generation. We further identified the keratinocyte stem cells with stem cell markers. Integrin β 1 is served as the basis characteristic marker to identify keratinocyte stem cells, since its high expression is required for the maintenance of keratinocyte stem cells in an undifferentiated state [36, 37]. K15, p63 and K14 are other markers that have been used to identify the keratinocyte stem cells [25, 31]. In our present research, the cultured P2 rhesus keratinocytes show high expression levels of integrin β 1, K15, p63 and K14, exhibiting characteristic stemness of keratinocyte stem cell (Fig. 3). Compared to human keratinocyte stem cells that can be cultured for 5–6 generations, healthy cell morphology of rhesus keratinocyte stem cells could only be maintained to P2, but the stemness is still well maintained (Fig. 3). We therefore used rhesus keratinocyte stem cells at 2nd generation under the current condition as a non-human primate epithelial source in the following tooth regeneration

experiments. We believe that the limited passages of rhesus keratinocyte stem cells could be attributed to the culture medium that is specific for human keratinocyte stem cell culture but may not be appropriate for rhesus keratinocyte stem cell growth. In addition, our future work will devote to improving the culture medium to make it more suitable for rhesus keratinocyte stem cells growth.

After successful culture of rhesus keratinocyte stem cells, we used the cells as epithelial sheets to recombine with E13.5 mouse dental mesenchyme and found that rhesus keratinocyte stem cells were also able to differentiate into enamel-secreting ameloblasts after subrenal culture. It confirms that the rhesus keratinocyte stem cells were very similar in physiological function of human keratinocyte stem cells, which could also be induced to differentiate into ameloblasts by the bud-stage mouse dental mesenchyme in our previous studies. Our current studies are the first attempt of using nonhuman primate epithelial cell source in tooth regeneration studies, and demonstrate that rhesus keratinocyte stem cells are an appropriate source for tooth tissue bio-engineering studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Use of rhesus tissues in this study was approved by the Fujian Provincial Family Planning Institute of Science and Technology (Approval No. FJJHSY2014-202), and use of mice was approved by the Animal Ethical and Welfare Committee of Fujian Normal University (Approval No. IA15134).

References

- Otsu K, Kumakami-Sakano M, Fujiwara N, Kikuchi K, Keller L, Lesot H, et al. Stem cell sources for tooth regeneration: current status and future prospects. *Front Physiol.* 2014;5:36.
- Oshima M, Tsuji T. Functional tooth regenerative therapy: tooth tissue regeneration and whole-tooth replacement. *Odontology.* 2014;102:123–36.
- Duailibi MT, Duailibi SE, Young CS, Bartlett JD, Vacanti JP, Yelick PC. Bioengineered teeth from cultured rat tooth bud cells. *J Dent Res.* 2004;83:523–8.
- Honda MJ, Ohara T, Sumita Y, Ogaeri T, Kagami H, Ueda M. Preliminary study of tissue-engineered odontogenesis in the canine jaw. *J Oral Maxillofac Surg.* 2006;64:283–9.
- Young CS, Terada S, Vacanti JP, Honda M, Bartlett JD, Yelick PC. Tissue engineering of complex tooth structures on biodegradable polymer scaffolds. *J Dent Res.* 2002;81:695–700.
- Zhang W, Abukawa H, Troulis MJ, Kaban LB, Vacanti JP, Yelick PC. Tissue engineered hybrid tooth-bone constructs. *Methods.* 2009;47:122–8.
- Hu B, Nadiri A, Bopp-Küchler S, Perrin-Schmitt F, Lesot H. Dental epithelial histomorphogenesis in vitro. *J Dent Res.* 2005;84:521–5.
- Kuo TF, Lin HC, Yang KC, Lin FH, Chen MH, Wu CC, et al. Bone marrow combined with dental bud cells promotes tooth regeneration in miniature pig model. *Artif Organs.* 2011;35:113–21.
- Song Y, Zhang Z, Yu X, Yan M, Zhang X, Gu S, et al. Application of lentivirus-mediated RNAi in studying gene function in mammalian tooth development. *Dev Dyn.* 2006;235:1334–44.
- Ikedo E, Morita R, Nakao K, Ishida K, Nakamura T, Takano-Yamamoto T, et al. Fully functional bioengineered tooth replacement as an organ replacement therapy. *Proc Natl Acad Sci U S A.* 2009;106:13475–80.
- Oshima M, Mizuno M, Imamura A, Ogawa M, Yasukawa M, Yamazaki H, et al. Functional tooth regeneration using a bio-engineered tooth unit as a mature organ replacement regenerative therapy. *PLoS One.* 2011;6:e21531.
- Jussila M, Thesleff I. Signaling networks regulating tooth organogenesis and regeneration, and the specification of dental mesenchymal and epithelial cell lineages. *Cold Spring Harb Perspect Biol.* 2012;4:a008425.
- Zhang YD, Chen Z, Song YQ, Liu C, Chen YP. Making a tooth: growth factors, transcription factors, and stem cells. *Cell Res.* 2005;15:301–16.
- Mina M, Kollar EJ. The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. *Arch Oral Biol.* 1987;32:123–7.
- Hu X, Lin C, Shen B, Ruan N, Guan Z, Chen Y, et al. Conserved odontogenic potential in embryonic dental tissues. *J Dent Res.* 2014;93:490–5.
- Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res.* 2009;88:792–806.
- Wang B, Li L, Du S, Liu C, Lin X, Chen Y, et al. Induction of human keratinocytes into enamel-secreting ameloblasts. *Dev Biol.* 2010;344:795–9.
- Angelova Volponi A, Kawasaki M, Sharpe PT. Adult human gingival epithelial cells as a source for whole-tooth bioengineering. *J Dent Res.* 2013;92:329–34.
- Cai J, Zhang Y, Liu P, Chen S, Wu X, Sun Y, et al. Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. *Cell Regen (Lond).* 2013;2:6.
- Byrne JA, Pedersen DA, Clepper LL, Nelson M, Sanger WG, Gokhale S, et al. Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature.* 2007;450:497–502.
- Kawasaki H, Suemori H, Mizuseki K, Watanabe K, Urano F, Ichinose H, et al. Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. *Proc Natl Acad Sci U S A.* 2002;99:1580–5.
- Thomson JA, Marshall VS, Trojanowski JQ. Neural differentiation of rhesus embryonic stem cells. *APMIS.* 1998;106:149–56.
- Huang AH, Snyder BR, Cheng PH, Chan AW. Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. *Stem Cells.* 2008;26:2654–63.
- Lu R, Zhang X, Huang D, Huang B, Gao N, Wang Z, et al. Conjunctival reconstruction with progenitor cell-derived autologous epidermal sheets in rhesus monkey. *PLoS One.* 2011;6:e25713.
- Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell.* 1993;73:713–24.

26. Coulombe PA, Kopan R, Fuchs E. Expression of keratin K14 in the epidermis and hair follicle: insights into complex programs of differentiation. *J Cell Biol.* 1989;109:2295–312.
27. Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis. *Cell.* 1995;80:83–93.
28. Waseem A, Dogan B, Tidman N, Alam Y, Purkis P, Jackson S, et al. Keratin 15 expression in stratified epithelia: downregulation in activated keratinocytes. *J Invest Dermatol.* 1999;112:362–9.
29. Senoo M, Pinto F, Crum CP, McKeon F. p63 Is essential for the proliferative potential of stem cells in stratified epithelia. *Cell.* 2007;129:523–36.
30. Lavker RM, Sun TT. Epidermal stem cells: properties, markers, and location. *Proc Natl Acad Sci U S A.* 2000;97:13473–5.
31. Alonso L, Fuchs E. Stem cells of the skin epithelium. *Proc Natl Acad Sci U S A.* 2003;100(Suppl 1):11830–5.
32. Breitkreutz D, Mirancea N, Nischt R. Basement membranes in skin: unique matrix structures with diverse functions? *Histochem Cell Biol.* 2009;132:1–10.
33. Bragulla HH, Homberger DG. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *J Anat.* 2009;214:516–59.
34. Nekulova M, Holcakova J, Coates P, Vojtesek B. The role of p63 in cancer, stem cells and cancer stem cells. *Cell Mol Biol Lett.* 2011;16:296–327.
35. Lu R, Ge J, Huang B, Huang DP, Gao N, Wei YT, et al. Induction of epidermal stem cells of rhesus monkey into human conjunctival epithelial cells: an in vitro experiment. *Zhonghua Yi Xue Za Zhi.* 2005;85:2554–8.
36. Kippenberger S, Bernd A, Loitsch S, Guschel M, Müller J, Bereiter-Hahn J, et al. Signaling of mechanical stretch in human keratinocytes via MAP kinases. *J Invest Dermatol.* 2000;114:408–12.
37. Levy L, Broad S, Diekmann D, Evans RD, Watt FM. beta1 integrins regulate keratinocyte adhesion and differentiation by distinct mechanisms. *Mol Biol Cell.* 2000;11:453–66.