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The apical region of developing tooth root constitutes a complex and maintains the ability to generate root and periodontium-like tissues

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Background and Objective: Mammalian tooth root development is a long-term process during which root elongates along the apical direction and is accompanied with the formation of periodontium. Considering the heterogeneous apical region of developing root as a functional entity, we observed and characterized the developing apical complex of rat molar root. The aim of the present study was to investigate the characteristics and developmental capability of developing apical complex *in situ* and *in vitro*.

Material and Methods: Histological analysis of rat developing apical complex was performed using hematoxylin and eosin staining and immunohistochemical staining. Cell counting, bromodeoxyuridine incorporation, flow cytometry assays and western blot analyses were performed to assess the proliferation potential of developing apical complex cells *in vitro*, and its mineralization potential was investigated by alkaline phosphatase activity, alizarin red staining and reverse transcription–polymerase chain reaction analysis. *In vivo* transplantation of both developing apical complex tissues and cells were used to characterize the differentiation capacity of developing apical complex cells. Dental pulp cells were used as a control in this study.

Results: Isolated developing apical complex maintained the developmental capability to form tooth root/periodontal complex ectopically. Developing apical complex cells exhibited relatively higher proliferation and mineralization potential compared with dental pulp cells in culture. When cultured developing apical complex cells were putatively depleted of Hertwig's epithelial root sheath cells, only osteodentin-like tissues and fibrous connective tissues formed in cell-scaffold explants.

Conclusion: The sustainable development ability of developing apical complex qualifies it as the growth center of tooth root and as a promising candidate source of cells for tooth root and periodontal regeneration.

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Mammalian tooth root development is a long-term process (1,2). By acting as a putative root growth center, the apical end of the developing root proliferates throughout the period of root development, promoting the elongation of root along the apical direction coincident with periodontium development (3). Clinically, when necrotic pulp in young permanent teeth is removed, viable root apical tissue can maintain subsequent root formation, implying the enormous independent developmental potential of apical root tissue *in situ* (4).

The development of tooth root and periodontium involves a complicated sequence of events and requires the participation of various types of cells (2,5). The elongating apical portion of developing root contains a bilayered epithelial sheath, termed Hertwig's epithelial root sheath, which subdivides the adjacent dental ectomesenchymal tissues into dental papilla and dental follicle (6–8). Despite the heterogeneity of this region, it exists as a single entity where the interaction and function of these components are essential for the establishment of a structurally intact root/periodontal complex. Therefore, the apical portion of developing root seems to be a developmentally viable complex comprising multiple, closely related cell types that are dependent on each other. The above clinical and developmental phenomena motivated us to characterize this developing structure and uncover its possible roles in regenerating tooth root and the supporting structure using tissue engineering techniques. We refer to this structure as developing apical complex of tooth root and regard it as a functional entity in root and periodontium development.

In the present study we characterized the developing apical complex of the rat molar root *in situ*, then evaluated the proliferation and differentiation features of cells from this region. Using an ectopic transplantation model, we demonstrated that both isolated tissues and cultured cells of the developing apical complex can independently generate tooth root-like and periodontium-like tissues *in vivo*, mimicking normal root/periodontal complex structures *in situ*.

Material and methods

Histological and immunohistochemical analysis *in situ*

Sprague–Dawley rats (21 d postnatal) were purchased from the Laboratory Animal Research Centre of the Fourth Military Medical University. All experimental animal use protocols were approved by the Animal Care Committee of the Fourth Military Medical University. Following decalcification with 10% EDTA, the mandibles were embedded in paraffin and 5-μm sections were prepared. Sections were then stained by hematoxylin and eosin. Immunohistochemical evaluation was performed using the streptavidin–biotin complex method according to the manufacturer's recommended protocols. Primary antibodies included mouse anti-STRO-1 (1:50 dilution; R&D Systems, Minneapolis, MN, USA), mouse anti-proliferating cell nuclear antigen (1:100 dilution; Dako Corporation, Copenhagen, Denmark) and rabbit anti-pan-cytokeratin (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell isolation and culture

Developing apical complex and dental pulp of the extracted mandibular first molar were dissociated along the apical foramen under stereomicroscopy. Dissociated developing apical complex and dental pulp were respectively digested at 37°C for 1 h in a mixture of dispase I and type I collagenase (Gibco BRL, Bethesda, MD, USA). Single-cell suspensions of developing apical complex and dental pulp were seeded at 1×10^5 cells/mL and cultured in the alpha-modification of Eagle's medium (Gibco BRL) supplemented with 15% fetal bovine serum. Through repeated differential trypsinization, as reported previously (9), purified epithelial-like cells and mesenchymal-like cells were obtained. Mesenchymal-like cells were cultured under the conditions described above and epithelial-like cells were cultured in keratinocyte serum-free medium (Gibco-BRL). We then prepared developing apical complex cells by contact coculture of third-passage epithelial-like and mesenchymal-like cells, and we prepared developing apical complex mesenchymal cells by culture of third-passage purified mesenchymal-like cells.

Cell counting

Approximately 1×10^5 primary developing apical complex cells and dental pulp cells were respectively seeded in a six-well dish. Culture medium was changed every 3 d. After 1, 4, 7 and 10 d of culture, plates were washed three times with phosphate-buffered saline to remove unattached cells, and attached cells were collected by trypsinization. The number of cells was counted using a hemocytometer.

Detection of bromodeoxyuridine incorporation

Single-cell suspensions of developing apical complex and dental pulp were respectively seeded on coverslips at a density of 2×10^5 cells/mL. After serum starvation-induced cell cycle synchrony, 10 μM bromodeoxyuridine (Sigma-Aldrich, St Louis, MO, USA) was added 4 h prior to fixation with 4% polyoxymethylene. Bromodeoxyuridine incorporation in proliferating cells was revealed using immunostaining against anti-bromodeoxyuridine immunoglobulin G (1:100 dilution; Boster Biotechnology, Wuhan, China). The total cell number was counted in a rectangular frame under a phase-contrast microscope (Olympus Optical Co. Ltd., Tokyo, Japan) 10 cells for each coverslip (×200 magnification). The number of bromodeoxyuridine-positive cells in the same frame was counted. The percentage of bromodeoxyuridine-positive cells was calculated using a previously described method (10).

Flow cytometry

Single-cell suspensions of developing apical complex and dental pulp at day 7 of culture were treated as described

previously (11). Approximately 1×10^6 cells per sample were counted, and the number of cells in the G1, S and G2 phases of the cell cycle was determined.

Alkaline phosphatase activity

Alkaline phosphatase activity was determined on days 1, 3, 5, 7, 10 and 14, using an alkaline phosphatase assay kit (Zhongsheng Co., Beijing, China), as described previously (12). Single-cell suspensions of dental pulp and developing apical complex were respectively seeded at a density of 1×10^3 cells/well in 96-well plates. The cells were washed in phosphate-buffered saline and lysed with 0.05% Triton X-100 solution. The alkaline phosphatase activity of the lysates was determined using *p*-nitrophenol-phosphate as a substrate. The absorbance of each well was measured spectrophotometrically at 405 nm using a microplate reader.

Alizarin red S staining

The calcium deposition of developing apical complex and dental pulp cells was studied using 0.1% alizarin red S staining solution. In brief, when nodule formation was detected on day 14, the samples were fixed with 4% polyoxymethylene for 15 min, rinsed twice with phosphate-buffered saline and stained with 0.1% alizarin red solution at 37°C for 30 min.

Reverse transcription–polymerase chain reaction

Developing apical complex cells and dental pulp cells at 80% confluence were harvested after 7 d of culture. Total RNA isolation, first-strand complementary DNA synthesis and polymerase chain reaction (PCR) processes were performed as reported elsewhere (11). The following primer sequences were used: bone sialoprotein, sense, 5′-ACA GCT GAC GCG GGA AAG TTG-3′ and antisense, 5′-ACC TGC TCA TTT TCA TCC ACT TC-3′; osteopontin, sense, 5′-GCT GAA GCC TGA CCC ATC T-3′ and antisense, 5′-TCC CGT TGC TGT CCT GAT-3′; osteocalcin, sense, 5′-ATG AGG ACC CTC TCT CTG CTC-3′ and antisense, 5′-CTA AAC GGT GGT GCC ATA GAT-3′; alkaline phosphatase, sense, 5′-TTT GCT ACC TGC CTC ACT TCC G-3′ and antisense, 5′-GGC TGT GAC TAT GGG ACC CAG-3′; dentin sialophosphoprotein, sense, 5′-TGG TAC CCA AAC AAC ACA GG-3′ and antisense, 5′-CAT CAT CAC TTC CGT CAC TT-3′; dentin matrix protein 1, sense, 5′-CGG CTG GTG GTC TCT CTA AG-3′ and antisense, 5′-GTC CCT CTG GGC TAT CTT CC-3′; ameloblastin, sense, 5′-CAT CAG AGA ACC CAC CA-3′ and antisense, 5′-ATG GGA CTT CCT CTT TCT-3′; and β-actin, sense, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and antisense, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. The amplification process was performed for 27–30 cycles after an initial 45-s denaturation at 94°C, annealed for 30 s at 56–60°C and extended for 5 min at 74°C. Polymerase chain reaction products were separated by agarose-gel electrophoresis and revealed by staining with ethidium bromide. This experiment was repeated three times and β-actin was used as the positive control.

Western blot

Total proteins of developing apical complex cells and developing apical complex mesenchymal cells were extracted for analysis of the expression of pan-cytokeratin (1:1000 dilution) and ameloblastin (1:400 dilution; a gift of Dr Yu Tian, Fourth Military Medical University, Xi'an, China) using western blotting, as described previously (13). Primary developing apical complex cells and dental pulp cells were harvested after a 7-d induction, and were lysed on ice for 30 min in lysis buffer [10 mM Tris (pH 8.0), 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 5 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol]. Equal amounts of protein (25 μg) were loaded onto a sodium dodecyl sulfate-poly acrylamide gel. For western blotting, anti-cytokeratin, anti-ameloblastin and anti-β-actin were added for 3 h. The blots were then washed, and species-matched peroxidase-conjugated secondary antibody was added (1:2000 dilution). Labeled bands from washed blots were detected by enhanced chemiluminescence (Amersham Biosciences, Bucks., UK). The expression levels of β-actin in both groups were detected as an internal control.

Subrenal transplantation

Eight-week allogenic Sprague–Dawley rat hosts were used for subrenal transplantation. The procedures were performed as previously described (12,14). For tissue transplantation, isolated developing apical complex was implanted into the left side of the rat host. As a control, the dental pulp was implanted into the other side of the same host. For cell transplantation, approximately 5×10^6 third-passage developing apical complex cells or developing apical complex mesenchymal cells were mixed with 30 mg of ceramic bovine bone powder (Research and Development Center for Tissue Engineering, Fourth Military Medical University, Xi'an, China) for 2 h and then transplanted. The transplants were recovered at 4 wk post-transplantation, fixed with 4% paraformaldehyde and decalcified with buffered 10% EDTA; the sections were stained with hematoxylin and eosin.

Statistical analysis

The results of cell counting analysis, bromodeoxyuridine incorporation and alkaline phosphatase activity were analyzed for significance using the independent-samples *t*-test. Differences in values of the cell cycle between the two groups were analyzed using the chi-square test. *p*-Values of less than 0.01 were considered to be statistically significant. The SPSS 12.0 software package (SPSS Inc, Chicago, IL, USA) was used for the statistical tests. For histomorphological analyses of implantation, all slides were examined by a single masked and calibrated examiner.

Results

Characterization of developing apical complex *in situ* and ectopic developmental capability of developing apical complex

The tooth roots of 21-d postnatal rats reached approximately half the complete root length. Except for the apparent subdivision position of Hertwig's epithelial root sheath, no boundary of dental papilla and follicle was observed (Fig. 1A). Generally, the cells in developing apical complex were denser than those in the pulp, especially in the sites adjacent to Hertwig's epithelial root sheath (Fig. 1B, C). A greater number of STRO-1-positive and proliferating cell nuclear antigen-positive cells were detected in developing apical complex than in dental pulp (Fig. 1D, E). Hertwig's epithelial root sheath was immunopositive for cytokeratin (Fig. 1F), as reported previously (7).

A microphotograph of rat mandibular first molar is shown in Fig. 1H. At this stage, developing apical complex of 21-d postnatal rat mandibular first molar was a loose connective tissue and bulged out of the developing apical foramen with diameter of about 0.5 mm. Developing apical complex and dental pulp were dissociated along the apical margin of the tooth at the level of hard tissue deposition (Fig. 1H, I). Dissociated developing apical complex contains continuous cytokeratin-positive epithelial cells (Fig. 1J). To assess the developmental capacity of developing apical complex *ex situ*, we transplanted isolated developing apical complex into the renal capsules of rat host using dental pulp implants as a control. After 4 wk of incubation *in vivo*, histological examination of recovered implants revealed that a total of 18 out of 20 (90%) developing apical complex grafts formed typical root-like structures with supporting tissues (Fig. 1K and Fig. S1). Histologically, most explants were highly ordered with a well-formed dentin–pulp complex containing identifiable dentinal tubules and pulp-like tissue. A layer of unmineralized predentine was clearly visible between mineralized dentine and odontoblasts, while the newly formed cementum-like structure with fibrous tissue were deposited on the dentin surface. The highly ordered structure observed in developing apical complex explants resembled the profiles of the normal root–periodontal complex formed during development (Fig. 1A). By contrast, 20 dental pulp implants only formed irregular dentin–pulp complex-like structures with no distinct tubular structure (20/20, 100%) (Fig. 1L and Fig. S1).

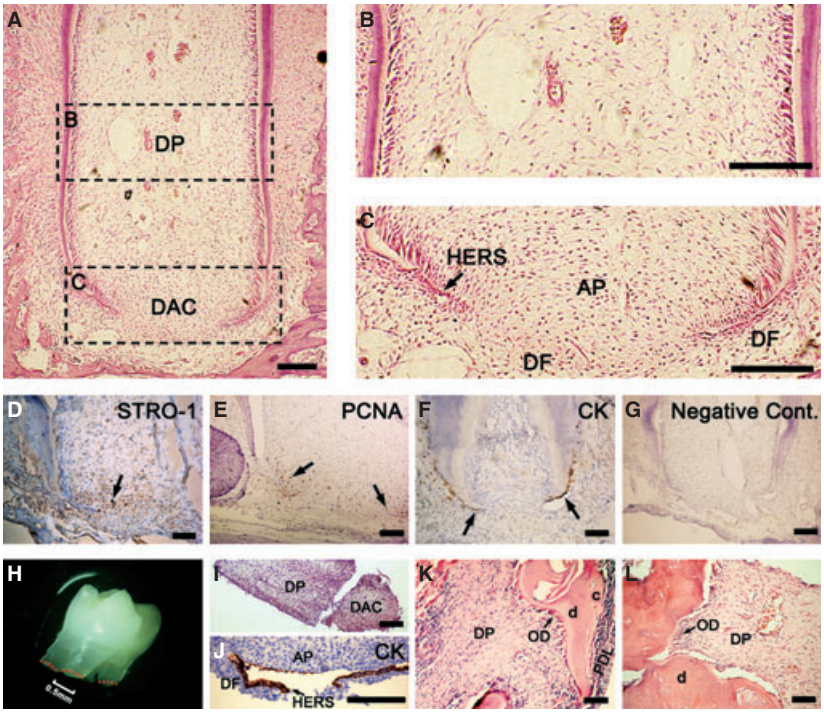

Fig. 1. Characterization of DAC *in situ* and ectopic development of isolated DAC. AP, apical papillae; c, cementum; CK, cytokeratin; d, dentin; DAC, developing apical complex; DF, dental follicle; DP, dental pulp; HERS, Hertwig's epithelial root sheath; OD, odontoblasts; PCNA, proliferating cell nuclear antigen; PDL, periodontal ligament.

Developing apical complex cells exhibit high proliferative activity *in vitro*

In normal culture medium, developing apical complex cells showed a fast proliferative stage during initial culture, with the total number of developing apical complex cells being significantly higher than the total number of dental pulp cells until day 10 (Fig. 2A). In addition, the level of bromodeoxyuridine uptake was fourfold higher in developing apical complex cells than in dental pulp cells (Fig. 2B). The percentage of cells in different phases of the cell cycle, as analyzed by flow cytometry (Fig. 2C), showed a higher percentage of developing apical complex cells in S (23.8%) and G2 (5.9%) phases, and a lower percentage in G1 phase (70.3%) compared with dental pulp cells ($x^2 = 20.4$, $p < 0.01$), whereas almost all dental pulp cells were found to be in G1 phase (Fig. 2D).

High mineralization potential of developing apical complex cells in culture

To determine the mineralization potential of developing apical complex cells *in vitro*, alkaline phosphatase activity and mineralized nodules were analyzed. Alkaline phosphatase activity was about fourfold higher in developing apical complex cells than in dental pulp cells on day 14 (Fig. 3A). Obvious alizarin red-positive nodules appeared in developing apical complex cells on day 14 (Fig. 3B, left panel), and the number increased remarkably with culture time. However, no alizarin

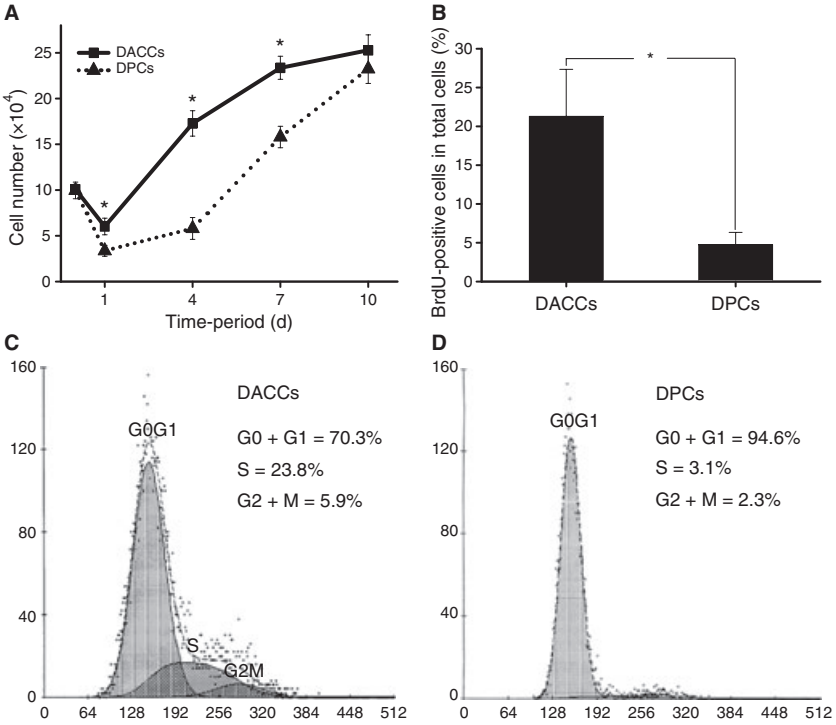

Fig. 2. Comparison of cell proliferation capability between DACCs and DPCs. DACCs, developing apical complex cells; DPCs, dental pulp cells.

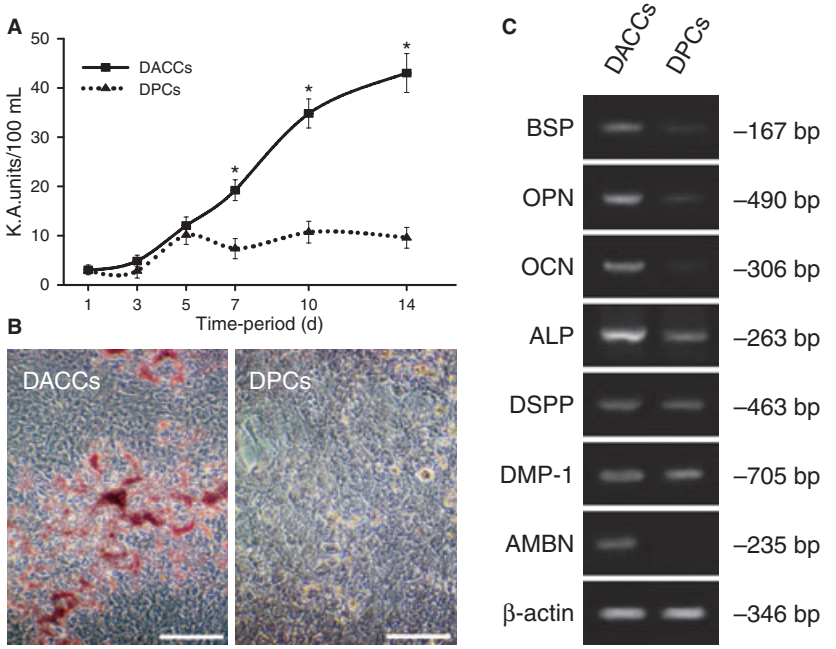

Fig. 3. Comparison of mineralization potential and mRNA expression between DACCs and DPCs. ALP, alkaline phosphatase; AMBN, ameloblastin; BSP, bone sialoprotein; DACCs, developing apical complex cells; DMP-1, dentin matrix protein1; DPCs, dental pulp cells; DSPP, dentin sialophosphoprotein; K.A. units; OCN, osteocalcin; OPN, osteopontin.

red-positive grains were detected in dental pulp cells on day 14 (Fig. 3B, right panel). Reverse transcription-PCR evaluation produced results similar to those described above. The developing apical complex cells, but not dental pulp cells, strongly expressed osteopontin, osteocalcin, bone sialoprotein and alkaline phosphatase (Fig. 3C), which have been reported previously as cemento/osteoblast-related markers (15–18). Both groups expressed the odontoblast-specific markers dentin sialophosphoprotein and dentin matrix protein 1. Only developing apical complex cells showed detectable expression of ameloblastin implicative of presence of dental epithelial cells, that is Hertwig's epithelial root sheath cells at the stage (19,20).

Effect of the presence or absence of epithelial components in developing apical complex cells on histological appearance of the explants

Similarly to cells of the primary passage, developing apical complex cells produced by contact coculture of third passage epithelial-like and mesenchymal-like developing apical complex cells appeared heterogeneous, containing both cobblestone-like epithelial cells and polygonal or spindle-shaped mesenchymal cells (Fig. 4B). Cells with an epithelial appearance were completely absent in third-passage purified mesenchymal-like developing apical complex mesenchymal cells (Fig. 4E). Cytokeratin and ameloblastin were confirmed to be expressed in cocultured developing apical complex cells and absent in developing apical complex mesenchymal cells (Fig. 4A). Both cell groups were recombined with ceramic bovine bone scaffolds and transplanted into the renal capsules of rat hosts. After 4 wk, nearly half of the developing apical complex cell grafts (13/25, 52%) generated root–periodontal complex-like tissue (Fig. 4C), which included dentin-like, cementum-like, periodontal ligament-like and bone-like tissues. Some explants did not form such intact structures but remained as dentin–pulp complex-like tissue (Fig. S2) and cementum-like deposits with fibrous bundle embedded in them, similarly to periodontal ligament *in situ* (5/25, 20%, Fig. 4D). However, in developing apical complex mesenchymal cells, abundant osteodentin-like tissue was observed and

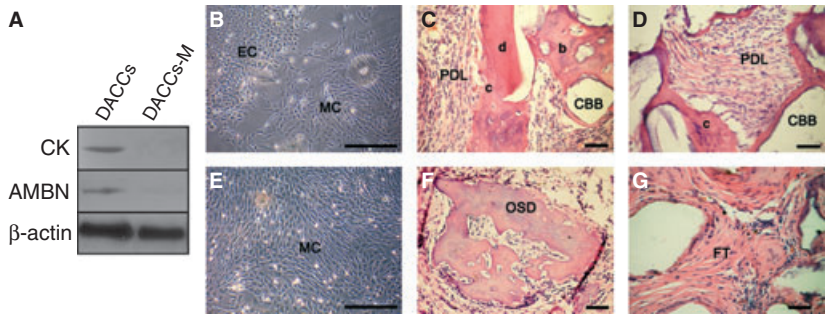

Fig. 4. Difference in the presence of epithelial component between DACCs and DACCs-M cells and in the morphological appearance of transplants *in vivo*. AMBN, ameloblastin; b, bone; c, cementum; CBB, ceramic bovine bone; CK, cytokeratin; d, dentin; DACCs, developing apical complex cells; DACCs-M, developing apical complex mesenchymal cells; EC, epithelial cells; FT, fibrous tissue; MC, mesenchymal cells; OSD, osteodentin; PDL, periodontal ligament.

fibrous tissue, but no cementum-like tissue, formed at the surface of osteoinductive ceramic bovine bone (25/25, 100%, Fig. 4F,G).

Discussion

The simultaneous elongation of tooth root and formation of periodontium is attributed to the continuous growth of apical tissues (21), which in this context was termed the developing apical complex. In the present study, we found that both the viable tissues and cultured cells of developing apical complex, when separated from the molar root of young rat, retained the capacity of independent development and generated a well-shaped tooth root-like structure with its supporting tissue, lending support to the concept that the developing apical complex may act as a development center and lead to the further development of root and periodontium.

In the present study, ectopic developing apical complex implants re-exhibited the correct morphogenesis of tooth root and periodontium, whereas only irregular dentin–pulp complex-like structures formed in the dental pulp group. Several factors may account for this discrepancy. First, the heterogeneous composition of developing apical complex enabled it to form a multilayered structure; this was supported by the observation that Hertwig's epithelial root sheath was located apically between the dental papilla and follicle, as indicated by the presence of cytokeratin-positive regions *in vivo* and *ex vivo* using immunostaining. Second, developing apical complex featured a more 'embryonic' profile than its neighboring adult dental pulp. This is typically inferred by the denser expression of STRO-1 and proliferating cell nuclear antigen in developing apical complex than in dental pulp, which indicated the presence of highly proliferative progenitor/stem cells in developing apical complex (22–24). Putative stem cells in the same apical area of humans and pigs were confirmed as SCAP (stem cells from apical papilla), which had a greater capacity for dentin regeneration than dental pulp stem cells (25). Our findings, of a denser expression of STRO-1 in a similar region of rats, coincided well with the expression profile of SCAP (26). Besides, we deemed that possible stem cell properties of cells within the apical dental follicle and Hertwig's epithelial root sheath may also facilitate the correct morphogenesis from developing apical complex to the root/periodontal complex. By using the cells of cap-stage tooth germs, Nakao *et al.* developed a successful method for bioengineering teeth, which produced an appropriately arranged periodontal structure. Hence, the speculation that the developing apical complex possesses more characteristics of primary tooth root primordium may well explain its developmental ability, even when dissociated and combined with particle-shaped scaffold (27). Lastly, developing apical complex is situated in a 'younger' environment in which more signals and elements favor the maintenance of undifferentiated state of cells within it. We observed that the developing apical complex featured a characteristic of cell condensation, especially of the ectomesenchymal cells adjacent to Hertwig's epithelial root sheath, which may be related to more extensive cell–cell and cell–matrix interactions. Findings that disclosed the recruitment of multiple signaling molecules (such as Bmp, Msx, Fgf, Shh and GDF) in this developing region may be indicative of specific epithelial–mesenchymal interactions, and signals regulating histomorphogenesis may be conserved within the developing apical complex during formation of the root and periodontium (28–31). In addition, the postulation that developing apical complex contains features younger than its neighbouring tissues was strongly supported by the persistence of an abundant vasculature network in this region during root elongation (32), which actually confers on developing apical complex the advantage of more nutrition and primitive cells. Together, developing apical complex featured a unique 'embryonic' characteristic, which not only contains stem/progenitor cells capable of differentiation into multiple tooth root-forming and periodontium-forming cells, but also provides a proper local environment leading to the establishment of an integral tooth-supporting structure.

As mentioned above, a unique population of extensively proliferative postnatal stem cells was previously identified in the apical papilla of developing tooth (25). In the present study we regarded developing apical complex (comprising apical papilla, dental follicle and Hertwig's epithelial root sheath) as an inseparable integrity and demonstrated a high proliferation tendency of cells from this entity. The population of proliferating cell nuclear antigen-positive cells in developing apical complex was distinctly denser than that in dental pulp tissue, in

accordance with proliferation markers, such as a higher rate of bromodeoxyuridine uptake (10) and an increased percentage of S-, G2- and M-phase cells (33) in culture. Because of a higher cellular alkaline phosphatase activity and a higher spontaneous occurrence frequency of calcified nodules (15), than of dental pulp cells, developing apical complex cells seem to be particularly prone to mineralize, an essential characteristic of the cells involved in tooth root and periodontium formation. The RT-PCR results further supported this by determining the intrinsic expression pattern related to cemento/osteo-associated differentiation. The propensity of developing apical complex cells to mineralize compared with dental pulp cells not only coincided with the difference of the physiological and functional property between developing apical complex and dental pulp, the latter remaining unmineralized to ensure that the integral pulp is a soft tissue, but also highlighted the potential utility of developing apical complex cells as a candidate for root and periodontium regeneration. All of these proliferation and differentiation profiles potentiated developing apical complex to function as a long-term active growth center of tooth root and periodontium and promised the regeneration of tooth-supporting structure using cells from developing apical complex.

Holding that expectation, we observed the formation of a typical tooth root- and periodontium-like structure in explants of early-passage cultured developing apical complex cells combined with ceramic bovine bone scaffold, known as bone-inductive material. This may well argue for maintenance of the development capability of developing apical complex cells, even after *ex vivo* expansion. Zhao *et al.* found that the delivery of cementoblasts with polymer sponges could promote mineralization in periodontal defects, whereas the delivery of dental follicle cells alone inhibited periodontal healing, which was inconsistent with the *in vitro* mineralization feature of dental follicle cells. The fact that the heterogenous developing apical complex cells generated periodontium-like mineralized tissues strongly emphasized the importance of the developmental microenvironment created by the interaction of multiple cells within the developing apical complex (34). The point especially worthy of note is that after we performed differential trypsinization to obtain relatively pure dental mesenchymal cells (developing apical complex mesenchymal cells), which included dental follicle and papilla cells and eliminated the contamination with Hertwig's epithelial root sheath cells, as shown by cell morphology and protein analysis, we only found osteodentin-like tissues and fibrous connective tissues in explants. This conceivably implied that the absence of Hertwig's epithelial root sheath would attenuate the ability of developing apical complex cells to re-exhibit histomorphogenesis, as observed in the intact developing apical complex cells–scaffold transplants ectopically. It has long been postulated that Hertwig's epithelial root sheath plays an important role in the development of root and periodontium (19,35). Most recently, Sonoyama *et al.* (36) successfully demonstrated that human Hertwig's epithelial root sheath cells could not only promote the differentiation of human periodontal ligament stem cells along the cemento/osteogenic lineage *in vitro*, but could also directly undergo epithelial–mesenchymal transition under specific induction and develop into cementum-forming cells. Therefore, our results confirmed again the importance of Hertwig's epithelial root sheath cells and, more significantly, supported our disclosure of functionality of developing apical complex as an integrated part.

In summary, our study demonstrated the sustainable developmental ability of the apical region of rat tooth root, which featured distinctive signatures for a structurally and functionally integral entity, proposed as developing apical complex. The unique characteristics of developing apical complex cells might delineate them as promising candidates for the regeneration of human tooth root and periodontium, as they can be obtained from discarded autologous developing wisdom teeth and cryopreserved. However, the specific mechanisms required for the fine-tuned regulation of the heterogeneous cell types and their extracellular environment in the developing apical complex need further investigation and elucidation.

Acknowledgements

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Supporting Information

The following supplementary material is available for this article:

Fig. S1. Histological view of DAC and DP implants at low-power field. (A) DAC grafts formed typical root like structures with supporting tissues which included dentin-pulp complex, cementum- and periodontal ligament-like tissue. (B) DP implants only formed irregular dentin-pulp complex like structures. C, cementum; D, dentin; DAC, developing apical complex; DP, dental pulp; OD, odontoblasts; PDL, periodontal ligament; R, renal tissue. Scale bar = 300 μm.

Fig. S2. Histologic analysis of DAC cells (DACCs) implants, 4 wk post-transplantation and stained with hematoxylin and eosin. DACCs implants showed a distinctive dentin-pulp complex structure including odontoblasts, predentin and dentin. The bone like tissue can also be found in DACCs implants. B, bone; CBB, ceramic bovine bone; D, dentin; DACCs, developing apical complex cells; OD, odontoblasts. Scale bar = 100 μm.

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References

1. MacNeil RL, Somerman MJ. Molecular factors regulating development and

regeneration of cementum. *J Periodontal Res* 1993;**28:**550–559.
2. Foster BL, Popowics TE, Fong HK, Somerman MJ. Advances in defining regulators of cementum development and periodontal regeneration. *Curr Top Dev Biol* 2007;**78:**47–126.
3. Margarita ZD. Regeneration of periodontal tissues: cementogenesis revisited. *Periodontol 2000* 2006;**41:**196–217.
4. Rafter M. Apexification: a review. *Dent Traumatol* 2005;**21:**1–8.
5. Wright T. The molecular control of and clinical variations in root formation. *Cells Tissues Organs* 2007;**186:**86–93.
6. Ten Cate AR. The role of epithelium in the development, structure and function of the tissues of tooth support. *Oral Dis* 1996;**2:**55–62.
7. Luan X, Ito Y, Diekwisch TG. Evolution and development of Hertwig's epithelial root sheath. *Dev Dyn* 2006;**235:**1167–1180.
8. Alatli I, Lundmark C, Hammarström L. The localization of epithelial root sheath cells during cementum formation in rat molars. *J Periodontal Res* 1996;**31:**433–440.
9. Jinga VV, Gafencu A, Antohe F *et al*. Establishment of a pure vascular endothelial cell line from human placenta. *Placenta* 2000;**21:**325–336.
10. Lossdörfer S, Götz W, Jäger A. Parathyroid hormone modifies human periodontal ligament cell proliferation and survival *in vitro*. *J Periodontal Res* 2006; **41:**519–526.
11. Yu J, Wang Y, Deng Z *et al*. Odontogenic capability: bone marrow stromal stem cells versus dental pulp stem cells. *Biol Cell* 2007;**99:**465–474.
12. Yu JH, Shi JN, Deng ZH *et al*. Cell pellets from dental papillae can reexhibit dental morphogenesis and dentinogenesis. *Biochem Biophys Res Commun* 2006;**346:**116–124.
13. Xing XH, Deng ZH, Yang FS, Watanabe S, Wen LY, Jin Y. Determination of genes involved in the early process of molar root development initiation in rat by modified subtractive hybridization. *Biochem Biophys Res Commun* 2007;**363:**994–1000.
14. Yu JH, Deng ZH, Shi JN *et al*. Differentiation of dental pulp stem cells into regular-shaped dentin-pulp complex induced by tooth germ cell conditioned medium. *Tissue Eng* 2006;**12:**3097–3105.
15. Arzate H, Alvarez MA, Narayanan AS. Cyclosporin A promotes mineralization by human cementoblastoma-derived cells in culture. *J Periodontal Res* 2005;**40:**218–224.
16. Rincon JC, Xiao Y, Young WG, Bartold PM. Production of osteopontin by cultured porcine epithelial cell rests of Malassez. *J Periodontal Res* 2005;**40:**417–426.
17. Pi SH, Lee SK, Hwang YS, Choi MG, Lee SK, Kim EC. Differential expression of periodontal ligament-specific markers and osteogenic differentiation in human papilloma virus 16-immortalized human gingival fibroblasts and periodontal ligament cells. *J Periodontal Res* 2007;**42:**104–113.
18. Shimonishi M, Hatakeyama J, Sasano Y, Takahashi N, Komatsu M, Kikuchi M. Mutual induction of noncollagenous bone proteins at the interface between epithelial cells and fibroblasts from human periodontal ligament. *J Periodontal Res* 2008;**43:**64–75.
19. Margarita ZD, Keiji O, Zhengyan S *et al*. Role of Hertwig's epithelial root sheath cells in tooth root development. *Dev Dyn* 2003;**228:**651–663.
20. Hasegawa N, Kawaguchi H, Ogawa T, Uchida T, Kurihara H. Immunohistochemical characteristics of epithelial cell rests of Malassez during cementum repair. *J Periodontal Res* 2003;**38:**51–56.
21. Thomas HF. Root formation. *Int J Dev Biol* 1995;**39:**231–237.
22. Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood* 1994; **84:** 4164–4173.
23. Chen SC, Marino V, Gronthos S, Bartold PM. Location of putative stem cells in human periodontal ligament. *J Periodontal Res* 2006;**41:**547–553.
24. Yoshimoto T, Yamamoto M, Kadomatsu H, Sakoda K, Yonamine Y, Izumi Y. Recombinant human growth/differentiation factor-5 (rhGDF-5) induced bone formation in murine calvariae. *J Periodontal Res* 2006;**41:**140–147.
25. Sonoyama W, Liu Y, Fang D *et al*. Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS ONE* 2006;**1:**e79.
26. Sonoyama W, Liu Y, Yamaza T *et al*. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod* 2008;**34:**166–171.
27. Nakao K, Morita R, Saji Y *et al*. The development of a bioengineered organ germ method. *Nat Methods* 2007;**4:** 227–230.
28. Yamashiro TM, Tummers M, Thesleff I. Expression of bone morphogenetic proteins and Msx genes during root formation. *J Dent Res* 2003;**82:**172–176.
29. Khan M, Seppala M, Zoupa M, Cobourne MT. Hedgehog pathway gene expression during early development of the molar tooth root in the mouse. *Gene Expr Patterns* 2007;**7:**239–243.
30. Madan AK, Kramer B. Immunolocalization of fibroblast growth factor-2 (FGF-2) in the developing root and supporting structures of the murine tooth. *J Mol Histol* 2005;**36:**171–178.
31. Gao J, Symons AL, Bartold PM. Expression of transforming growth factor-beta receptors types II and III within various cells in the rat periodontium. *J Periodontal Res* 1999;**34:**113–122.
32. Tsukada H, Ishikawa H, Nakamura S, Yoshida S. Developmental changes of the vasculature in the periodontal ligament of rat molars: a scanning electron microscopic study of microcorrosion casts. *J Periodontal Res* 2000;**35:**201–207.
33. Mitchison JM. Growth during the cell cycle. *Int Rev Cytol* 2003;**226:**165–258.
34. Zhao M, Jin QM, Berry JE, Nociti FH Jr, Giannobile WV, Somerman MJ. Cementoblast delivery for periodontal tissue engineering. *J Periodontol* 2004;**75:** 154–161.
35. Thomas HF, Kollar EJ. Differentiation of odontoblasts in grafted recombinants of murine epithelial root sheath and dental mesenchyme. *Arch Oral Biol* 1989; **34:**27–35.
36. Sonoyama W, Seo BM, Yamaza T, Shi S. Human Hertwig's epithelial root sheath cells play crucial roles in cementum formation. *J Dent Res* 2007;**86:**594–599.

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