Chapter 5

Tooth

Tissue-engineered odontogenesis

One important goal of dental research is the efficient regeneration of lost teeth [1, 2]. Tooth formation, or odontogenesis, is a complex process that has been characterized as a series of reciprocal epithelial-mesenchymal interactions, culminating in the differentiation of the interacting tissues [3-6]. Tissue engineering of tooth structures on biodegradable polymer scaffolds has been recently achieved [7]. The method involves dissociating porcine third molar tooth buds into single-cell suspensions and seeding them onto biodegradable polymers, but this regeneration process is not yet fully understood. To characterize the process in greater detail, we followed the regeneration of tissue-engineered teeth by histology and immunohistochemistry with specific markers of epithelial and mesenchymal differentiation as well as ameloblasts, odontoblasts, and cementoblasts. In the present study, we show that the development of these engineered teeth closely parallels that of natural odontogenesis.

Preparation of biodegradable polymer scaffolds

Three-dimensional scaffolds were prepared as described previously [8], with the following modifications: polyglycolic acid (PGA) fiber mesh (fiber diameter = 13 μm; density = 60 mg/ml) was packed into 96-well plates and sterilized in 75% ethanol. The scaffold dimensions were approximately 1 cm^3. Before seeding the cells, the scaffolds were collagen-coated overnight at 4°C (1 mg/ml type I collagen in 10 mM HCI), followed by three times washings in PBS and three times in DMEM.

Isolation and dissociation of porcine third molar tissue

Tooth bud cells were harvested and prepared as described previously [7]. The tooth buds were removed early in development at the early stage of crown formation from the mandibles of 6-month-old pigs, which were purchased from a local slaughterhouse and transported to the laboratory on ice. Briefly, third molar tooth buds were removed from the fresh mandibles with their inner and outer epithelial layers as well as the dental papilla and dental follicle intact (Fig. 25). After the calcified tissue was removed, all tooth bud tissue, including the dental follicles, was minced into <1 mm^3 pieces in Hanks Balanced Salt Solution (HBSS) and dissociated with 2 mg/ml collagenase and dispase for 50 minutes at 37°C, followed by gentle trituration. Digested tooth bud tissues were then strained through a nylon filter (70 μm pores), and the isolated single cells (1.0×10^7 cells) were seeded onto a PGA fiber mesh scaffold which had been precoated with type I collagen (1 mg/ml type I collagen in 10 mM HCl) for 3 hours at 4°C before placing them into the omentum in the host rats (Fig. 26).

The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (15 mg/kg), and the scaffolds seeded with cells were implanted into the omenta of the athymic

rats (n = 30) [9]. Samples developed for 2, 4, 6, 8, 10, 15, 20, and 25 weeks were dissected and immediately fixed in freshly prepared 4% paraformaldehyde in PBS at 4°C for 6-8 hours. After fixation, the tissues were demineralized for 4 h in 0.2N HCl and, after extensive washing in PBS, were dehydrated in an ethanol gradient, cleared in xylene, and embedded in paraffin. Tissue sections 6 µm thick were mounted on glass slides and stained with hematoxylin and eosin (H-E) .

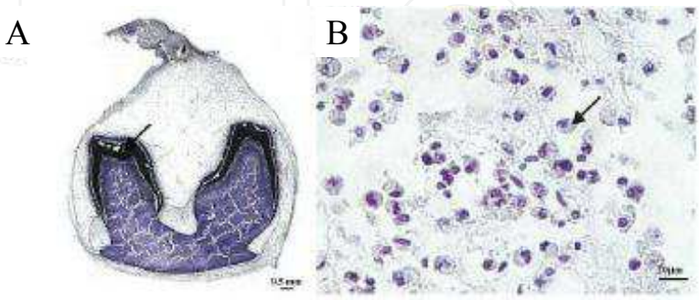

Fig. 25. A: A third molar tooth bud used in experiments stained with toluidine blue. The cusps of the molar are already calcified (black arrow). B: Single cells dissociated by straining through a nylon filter (70-µm pores) before seeding onto the PGA fiber mesh scaffold (black arrow) (From Honda et al. 2005. Reprinted with permission).

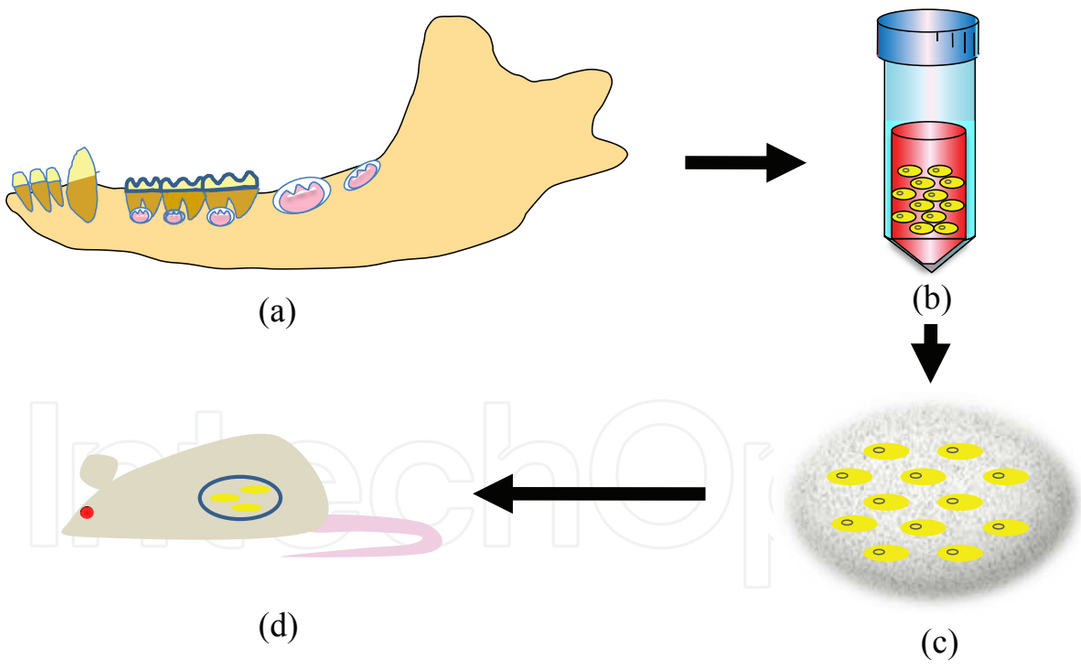

Fig. 26. Schematic diagram of the strategy used to produce the tissue-engineered tooth. Tooth-germ was derived from isolated cells seeded on a PGA fiber mesh scaffold. (a) Tissue was harvested from third molar tooth buds. (b) Isolate the tooth bud. (c) Isolated cells were seeded on PGA mesh. (d) Implantation in rat omentum (From Honda et al. 2005).

In vivo implantation and histology

Aggregates of epithelial cells were first observed 4-6 weeks after implantation (Fig. 27). These aggregates assumed three different shapes: a natural tooth germ-like shape, a circular shape, or a bilayer-bundle. Based on the structure of the stellate reticulum in the dental epithelium, the circular and bilayer-bundle aggregates could be clearly classified into two types: one with extensively developed stellate reticulum, and the other with negligible stellate reticulum. The epithelial cells in the circular aggregates differentiated into ameloblasts. The continuous bilayer bundles eventually formed the epithelial sheath, and dentin tissue was evident at the apex of these bundles. Finally, enamel-covered dentin and cementum-covered dentin formed, a process most likely mediated by epithelial-mesenchymal interaction. These results suggest that the development of these engineered teeth closely parallels that of natural odontogenesis derived from the immature epithelial and mesenchymal cells.

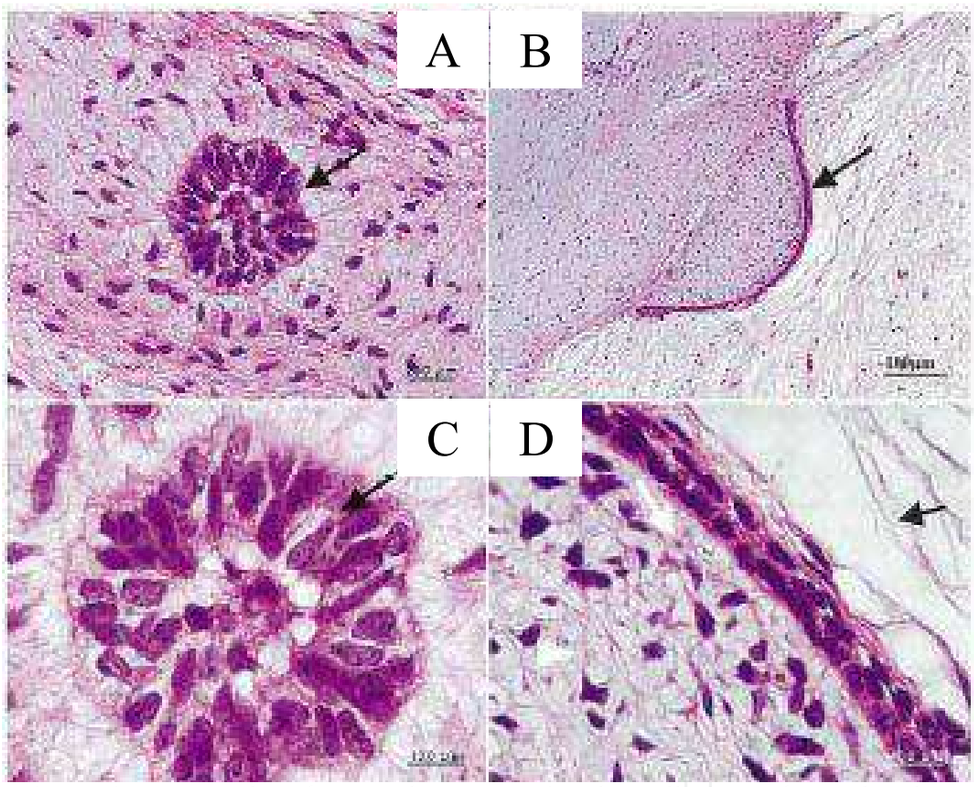

Fig. 27. A, B: At 6-8 weeks after implantation. A: The cells have formed either circular aggregates (black arrow). B: Sheets of a bilayer of cells (black arrow) which separate two very different densities of surrounding cells. C: At higher magnification, the cells in circular aggregates are low columnar in shape with nuclei in the center of the cytoplasm (black arrow). D: While the cells in the sheets clearly form a two-cell thick layer, which separates a low density of cells (black arrow) from a denser region of cells (white arrowhead), which is densest just adjacent to the bilayer (white arrow) (From Honda et al. 2005. Reprinted with permission).

Effects of shear stress

During tooth organogenesis, reciprocal interactions between epithelial-mesenchymal cells result in the cytodifferentiation of epithelial cells into ameloblasts and ectomesenchymal cells into odontoblasts [5, 11-13]. These differentiated cells produce the enamel and the dentin matrix. Amelogenesis is the process of enamel formation which occurs in three distinct stages: the presecretory stage, consisting of ameloblast cytodifferentiation; the secretory stage, with the bulk of enamel matrix formation; and the maturation stage, associated with matrix mineralization [14-16]. Dentinogenesis is the process of dentin formation, by which the dental papilla cells located at the epithelial-mesenchymal interface gradually differentiate into odontoblasts concomitant with ameloblast differentiation [17-20]. Recently, several *in vitro* and *in vivo* studies have demonstrated that dental pulp cells are also capable of differentiating into odontoblasts and producing a mineralizing matrix [21]. There is accumulating evidence that mechanical stress has a variety of effects on cell growth and differentiation [22, 23]. For example, such stress is known to facilitate the differentiation of osteoblasts [24] and chondrocytes [25]. In particular, shear stress induced by fluid flow facilitates the secretion of bone matrix protein [24, 26]. It is demonstrated that subjecting osteoblasts to fluid shear stress increases expression of genes including c-fos and cyclooxygenase-2 (COX-2) [27]. We hypothesized that appropriate shear stress is essential to facilitate the differentiation of odontogenic cells, which would facilitate the regeneration process. The purpose of this study was to investigate the effect of applying shear stress on the differentiation of odontogenic cells and histogenesis of odontogenic tissues. This study shows for the first time that shear stress facilitates tissue-engineered odontogenesis. Furthermore, using RT-PCR and Western blot technique, we provide evidence for the expression of teeth-related marker as to whether tooth cells facilitate cell differentiation by exposure to shear stress.

Shear stress exposure

In preliminary studies, we examined the effects of three different types of mechanical stress on the differentiation of the cells. Uniaxial stretch, ultrasonic wave, and shear stress generated by bi-directional fluid flow were given, and the levels of alkaline phosphatase (ALP) activity were evaluated as a marker for pulp cellular differentiation [28, 29]. Among the mechanical stresses examined, only shear stress succeeded in increasing ALP activity. From this result, the shear stress appeared to influence most clearly the cells on the ALP activity. Shear stress was generated by bi-directional fluid flow inside a tube, the velocity of which depended on the frequency of the agitating motion of the Shaker. After cells were seeded onto the scaffolds, cell-polymer constructs (CPCs) were first cultured for 2 hours under static conditions. Subsequently, CPCs were placed into 15-ml centrifuge tubes with DMEM containing 10% of fetal calf serum (FCS). To determine the more effective stress, we compared three distinct shear stresses by exposing seeding cells and evaluated the ALP activity. The used frequencies were between 10-20, 40-50, and 70-80 rpm at 37°C for 12 hours. Swing frequencies of constructs were 40, 90, and 120 rpm, respectively. Their mechanical load exerted on the CPCs was estimated at 0.7×10^{-4} N, 1.2×10^{-4} N, and 1.6×10^{-4} N, respectively. *In vitro* studies, the expression of both epithelial and mesenchymal odontogenic-related mRNAs was significantly enhanced by shear stress for 2 hours. Twelve hours after exposure to shear stress, the expression of amelogenin, bone sialoprotein and vimentin protein was significantly enhanced compared with that of control. After 7 days,

alkaline phosphatase activity exhibited a significant increase without any significant effect on cell proliferation *in vitro*. *In vivo*, enamel and dentin tissues formed after 15 weeks of *in vivo* implantation in constructs exposed to *in vitro* shear stress for 12 hours. Such was not the case in controls. We concluded that shear stress facilitates odontogenic cell differentiation *in vitro* as well as the process of tooth tissue engineering *in vivo*.

Collagen sponge as a 3-D scaffold

Tooth structure can be regenerated by seeding dissociated tooth cells onto PGA fiber mesh, although the success rate of tooth production is low. The present study was designed to compare the performance of collagen sponge with PGA fiber mesh as a 3-D scaffold for tooth-tissue engineering (Fig. 28). Porcine third molar teeth at the early stage of crown formation were enzymatically dissociated into single cells, and the heterogeneous cells were seeded onto collagen sponge or the PGA fiber mesh scaffolds. Scaffolds were then cultured to evaluate cell adhesion and ALP activity *in vitro*. An *in vivo* analysis was performed by implanting the constructs into the omentum of immunocompromised rats and evaluating tooth production up to 25 weeks (Fig. 29). After 24 hours, there were a significantly higher number of cells attached to the collagen sponge scaffold than the PGA fiber mesh scaffold. Similarly, the ALP activity was significantly higher for the collagen sponge scaffold was than the PGA fiber mesh scaffold after 7 days of culture. The area of calcified tissue formed in the collagen sponge scaffold was also larger than in the PGA fiber mesh scaffold (Fig. 30). The results from *in vivo* experiments show conclusively that a collagen sponge scaffold allows tooth production with a higher degree of success than PGA fiber mesh. Taken together, the results from this study show that collagen sponge scaffold is superior to the PGA fiber mesh scaffold for tooth-tissue engineering.

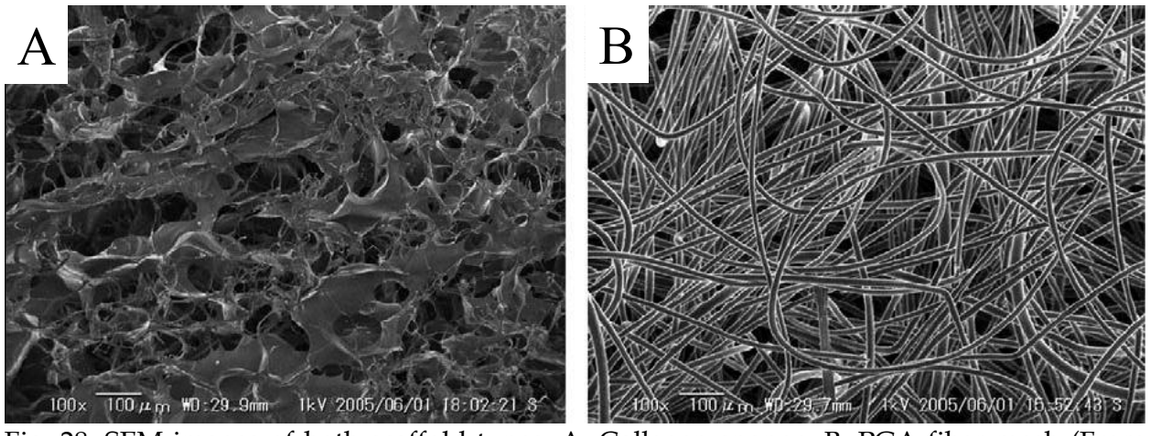

Fig. 28. SEM images of both scaffold types. A: Collagen sponge. B: PGA fiber mesh (From Sumita et al. 2006. Reprinted with permission).

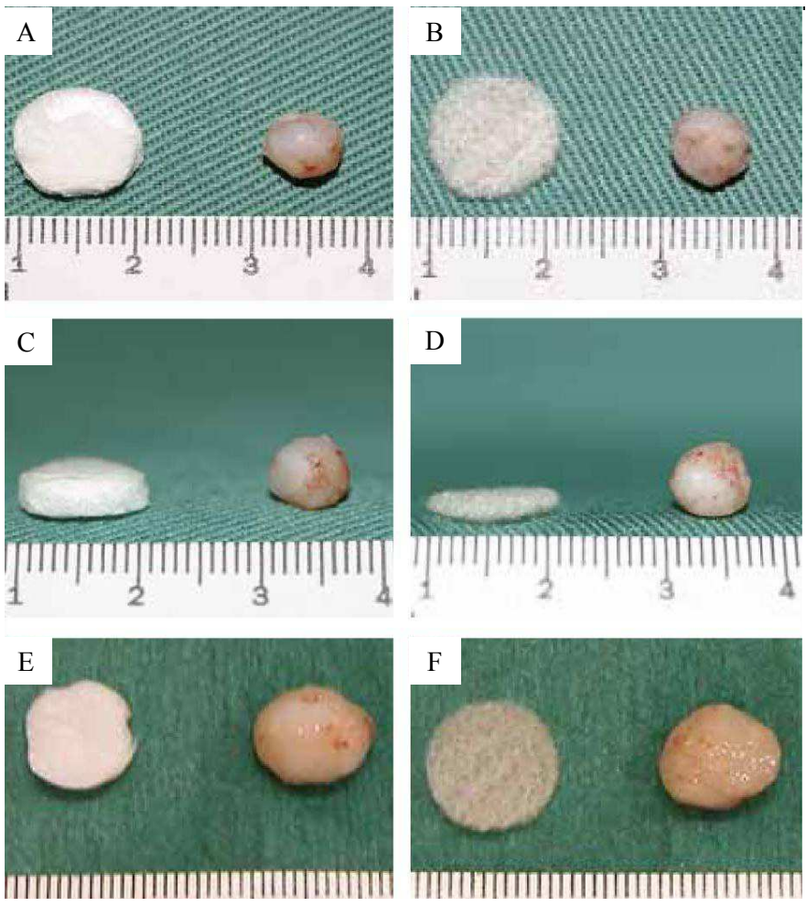

Fig. 29. Gross appearance of the original scaffold and implants after 8 and 20 weeks implantation. (A, C and E) show the collagen sponge scaffold before implantation and the implants obtained from the collagen sponge scaffold at 8 (A and C) and 20 weeks (E) after implantation. (B, D and F) show the PGA fiber mesh scaffold before implantation and the implants at 8 (B and D) and 20 weeks (F) after implantation. A: The original collagen sponge scaffold (left) and the implant at 8 weeks after implantation (right). The diameter of the original collagen sponge scaffold was approximately 11 mm, while the diameter of the implant was approximately 7 mm. B: The original PGA fiber mesh scaffold (left) and the implant at 8 weeks after implantation (right). The diameter of the original PGA fiber mesh scaffold was approximately 11 mm, while the diameter of the implant was approximately 7 mm. C: The perpendicular thickness of the original collagen sponge scaffold was approximately 2 mm (left), while the perpendicular thickness of the implant at 8 weeks was approximately 7 mm (right). D: The perpendicular thickness of the original PGA fiber mesh scaffold was approximately 1–2 mm (left), while the perpendicular thickness of the implants at 8 weeks was approximately 7 mm (right). E: The original collagen sponge scaffold (left) and the implant at 20 weeks after implantation. The diameters of the implant were approximately 11 mm by 11 mm by 10 mm (right). F: The original PGA fiber mesh scaffold (left) and the implant at 20 weeks after implantation. The diameters of the implants were approximately 11 mm by 11 mm by 10 mm (right) (From Sumita et al. 2006. Reprinted with permission).

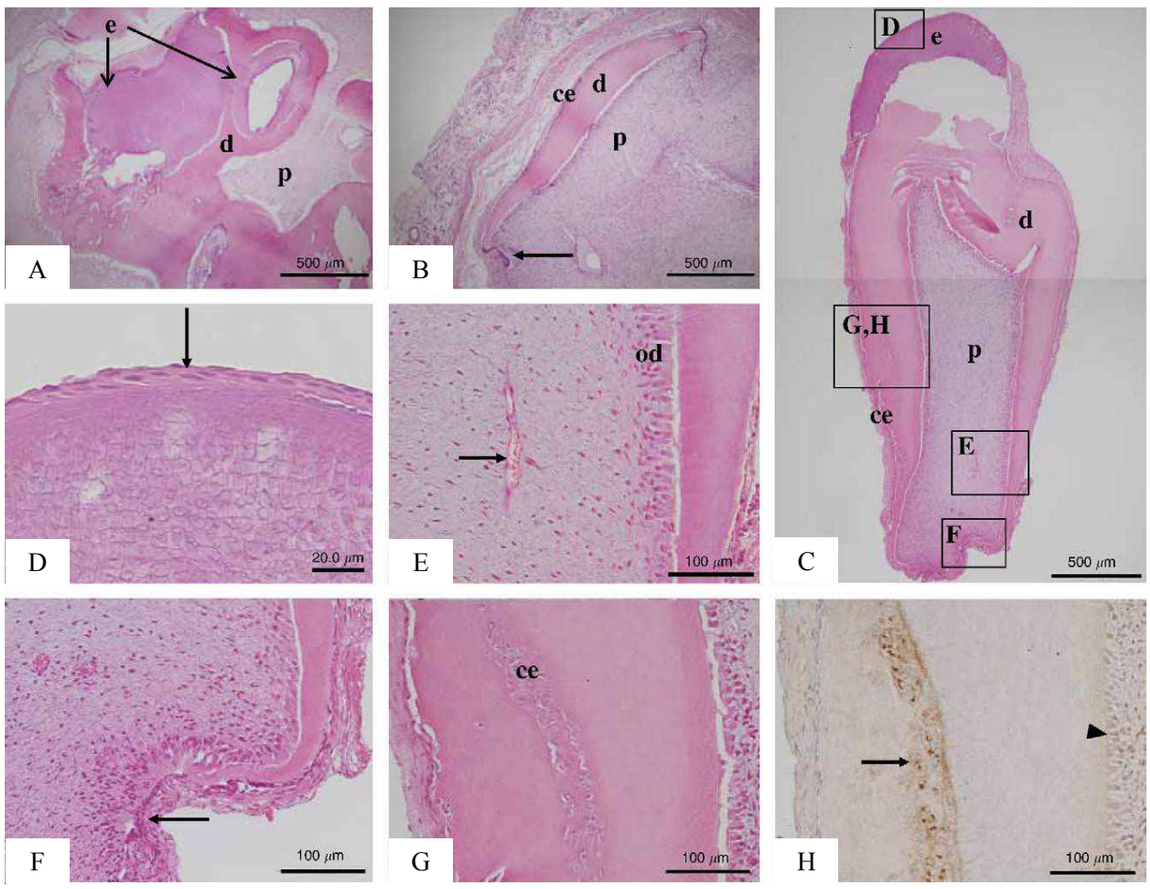

Fig. 30. Histological and immunohistochemical analyses of TE-teeth at 25 weeks after implantation. (A–G) show H-E staining and panel (H) shows immunohistochemical staining for BSP. A: The circular-shaped TE-teeth obtained from the collagen sponge scaffold revealed the enamel (e) and dentin (d). B: The stick-shaped TE-teeth obtained from the collagen sponge scaffold revealed the dentin (d), cementum-like tissue (ce), pulp (p) and HERS (black arrow). C: A typical tooth with normal morphology was observed in the implant obtained from the collagen sponge scaffold. TE-teeth revealed (e), (d), (p) and (ce). (D–H) are higher magnifications of (C) as indicated by the squares. D: The reduced enamel epithelium cells (black arrow) were recognized on the surface of the enamel. E: The odontoblasts (od) and blood vessels (black arrow) were identified in the pulp. F: Bilayers of epithelial cells were similar to Hertwig's epithelial root sheath (black arrow). G: Cellular cementum-like tissue (ce) was observed on the surface of the root dentin. H: BSP expression was located in cellular cementum-like tissue (black arrow) and odontoblasts (black arrowhead) (From Sumita et al. 2006. Reprinted with permission).

Sequential seeding

Progress is being made toward regenerating teeth by seeding dissociated postnatal odontogenic cells onto scaffolds and implanting them *in vivo*, but tooth morphology remains difficult to control. In this study, we aimed to facilitate tooth regeneration using a novel technique to sequentially seed epithelial cells and mesenchymal cells so that they developed

appropriate interactions in the scaffold. Dental epithelium and mesenchyme from porcine third molar teeth were enzymatically separated and dissociated into single cells. Mesenchymal cells were seeded onto the surface of the scaffold and epithelial cells were then plated on top so that the two cell types were in direct contact. The cell-scaffold constructs were evaluated *in vitro* and also implanted into immunocompromised rats for *in vivo* analysis (Fig. 31). Control groups included constructs where direct contact between the two cell types was prevented. In scaffolds, seed using the novel technique, ALP activity, was significantly greater than controls, the tooth morphology *in vivo* was developed similar to that of a natural tooth, and only one tooth structure formed in each scaffold. These results suggest that the novel cell-seeding technique could be useful for regulating the morphology of regenerated teeth.

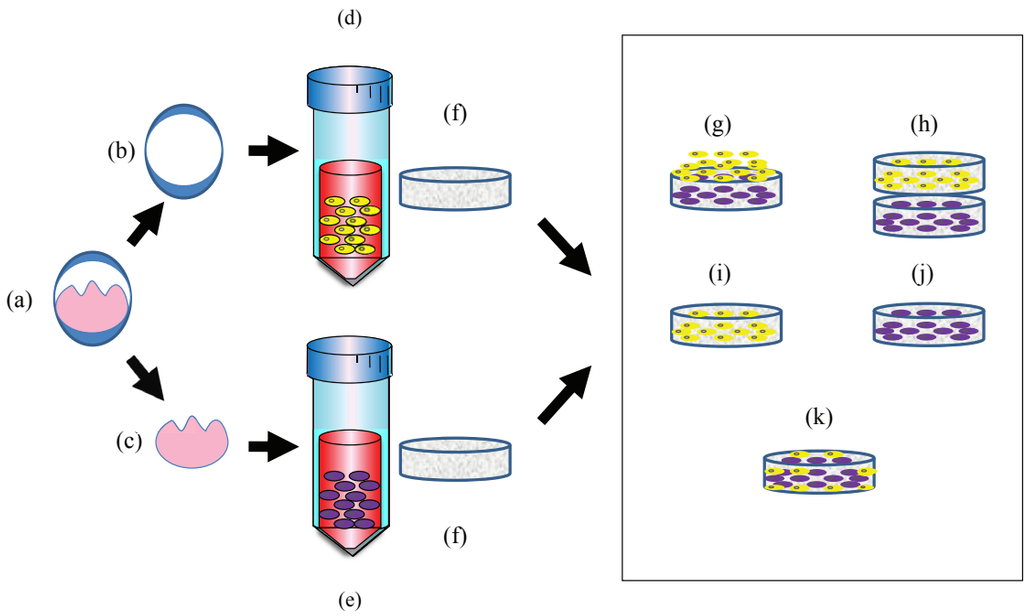

Fig. 31. Experimental design for the study.(a) Tooth germ. (b) Epithelium. (c) Mesenchyme. (d) Epithelial cell suspension. (e) Mesenchymal cell suspension. (f) Collagen sponge. (g) Group I, mesenchymal cells were plated onto the scaffold, and epithelial cells were then plated on top of the mesenchymal cells; (h) Group II, mesenchymal cells were plated onto a scaffold in the lower compartment, and epithelial cells onto a scaffold in the upper compartment of a dish divided by a microporous membrane; (i) Group III, epithelial cells alone were seeded into the scaffold; and (j) Group IV, mesenchymal cells alone were seeded into the scaffold. For the *in vivo* study, the groups were similar but with an additional (k) Group V, in which both epithelial cells and mesenchymal cells were plated randomly into each scaffold (From Honda et al. 2007. Reprinted with permission).

References

1. Earthman JC, Sheets CG, Paquette JM, Kaminishi RM, Nordland WP, Keim RG, et al. Tissue engineering in dentistry. Clin Plast Surg. 30: 621,2003
2. Chai Y, Slavkin HC. Prospects for tooth regeneration in the 21st century: a perspective. Microsc Res Tech. 60: 469,2003

3. Thesleff I. Interactions between the extracellular matrix and the cell surface determine tooth morphogenesis and the cellular differentiation of the dental mesenchyme. Ontogenez. 20: 341,1989
4. Thesleff I, Vaahtokari A, Kettunen P, Aberg T. Epithelial-mesenchymal signaling during tooth development. Connect Tissue Res. 32: 9,1995
5. Jernvall J, Thesleff I. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev. 92: 19,2000
6. McCollum M, Sharpe PT. Evolution and development of teeth. J Anat. 199: 153,2001
7. 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. 81: 695,2002
8. Mikos AG, Bao Y, Cima LG, Ingber DE, Vacanti JP, Langer R. Preparation of poly (glycolic acid) bonded fiber structures for cell attachment and transplantation. J Biomed Mater Res. 27: 183,1993
9. Choi RS, Vacanti JP. Preliminary studies of tissue-engineered intestine using isolated epithelial organoid units on tubular synthetic biodegradable scaffolds. Transplant Proc. 29: 848,1997
10. Bancroft JD, Stevens A. Theory and practice of histological techniques. 3rd ed. Edinburgh ; New York: Churchill Livingstone;1990
11. Thesleff I. Tooth morphogenesis. Adv Dent Res. 9: 12,1995
12. Maas R, Bei M. The genetic control of early tooth development. Crit Rev Oral Biol Med. 8: 4,1997
13. Thesleff I, Sharpe P. Signalling networks regulating dental development. Mech Dev. 67: 111,1997
14. Zeichner-David M, Diekwisch T, Fincham A, Lau E, MacDougall M, Moradian-Oldak J, Control of ameloblast differentiation. Int J Dev Biol. 39: 69,1995
15. Smith CE, Nanci A. Overview of morphological changes in enamel organ cells associated with major events in amelogenesis. Int J Dev Biol. 39: 153,1995
16. Robinson C, Brookes SJ, Shore RC, Kirkham J. The developing enamel matrix: nature and function. Eur J Oral Sci. 106 Suppl 1: 282,1998
17. Linde A, Goldberg M. Dentinogenesis. Crit Rev Oral Biol Med. 4: 679,1993
18. Butler WT. Dentin matrix proteins and dentinogenesis. Connect Tissue Res. 33: 59,1995
19. Sasaki T, Garant PR. Structure and organization of odontoblasts. Anat Rec. 245: 235,1996
20. Smith AJ, Cassidy N, Perry H, Begue-Kirn C, Ruch JV, Lesot H. Reactionary dentinogenesis. Int J Dev Biol. 39: 273,1995
21. Couble ML, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M, Magloire H. Odontoblast differentiation of human dental pulp cells in explant cultures. Calcif Tissue Int. 66: 129,2000
22. Banes AJ, Link GW Jr., Gilbert JW, Tran Son Tay R, Monbureau O. Culturing cells in a mechanically active environment. Am Biotechnol Lab. 8: 12,1990
23. Wang N, Butler JP, Ingber DE. Mechanotransduction across the cell surface and through the cytoskeleton. Science. 260: 1124,1993
24. Kapur S, Baylink DJ, Lau KH. Fluid flow shear stress stimulates human osteoblast proliferation and differentiation through multiple interacting and competing signal transduction pathways. Bone. 32: 241,2003

25. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-term intermittent shear deformation improves the quality of cartilaginous tissue formed *in vitro*. J Orthop Res. 21: 590,2003
26. Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE, et al. Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain. Am J Physiol. 273: C810,1997
27. Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG, Pilbeam CC. Fluid flow induction of cyclo-oxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. J Bone Miner Res. 17: 266,2002
28. Pavasant P, Yongchaitrakul T, Pattamapun K, Arksornnukit M. The synergistic effect of TGF-beta and 1,25-dihydroxyvitamin D3 on SPARC synthesis and alkaline phosphatase activity in human pulp fibroblasts. Arch Oral Biol. 48: 717,2003
29. Alliot-Licht B, Bluteau G, Magne D, Lopez-Cazaux S, Lieubeau B, Daculsi G, et al. Dexamethasone stimulates differentiation of odontoblast-like cells in human dental pulp cultures. Cell Tissue Res. 321: 391,2005
(Honda MJ, Sumita Y, Kagami H, Shinohara Y, Tonomura A, Ohara T, Tsuchiya S, Sagara H, Ueda M)

Applied Tissue Engineering

Edited by

ISBN 978-953-307-689-8
Hard cover, 76 pages
Publisher InTech
Published online 08, June, 2011
Published in print edition June, 2011

Tissue engineering, which aims at regenerating new tissues, as well as substituting lost organs by making use of autogenic or allogenic cells in combination with biomaterials, is an emerging biomedical engineering field. There are several driving forces that presently make tissue engineering very challenging and important: 1) the limitations in biological functions of current artificial tissues and organs made from man-made materials alone, 2) the shortage of donor tissue and organs for organs transplantation, 3) recent remarkable advances in regeneration mechanisms made by molecular biologists, as well as 4) achievements in modern biotechnology for large-scale tissue culture and growth factor production.

This book was edited by collecting all the achievement performed in the laboratory of oral and maxillofacial surgery and it brings together the specific experiences of the scientific community in these experiences of our scientific community in this field as well as the clinical experiences of the most renowned experts in the fields from all over Nagoya University. The editors are especially proud of bringing together the leading biologists and material scientists together with dentist, plastic surgeons, cardiovascular surgery and doctors of all specialties from all department of the medical school of Nagoya University. Taken together, this unique collection of world-wide expert achievement and experiences represents the current spectrum of possibilities in tissue engineered substitution.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen