Tissue Engineering and Regeneration in Dentistry

Current Strategies

EDITED BY

Rachel J. Waddington, PhD
School of Dentistry, Cardiff Institute of Tissue Engineering and Repair, Cardiff University, Cardiff, UK

Alastair J. Sloan, PhD
School of Dentistry, Cardiff Institute of Tissue Engineering and Repair, Cardiff University, Cardiff, UK

WILEY Blackwell

This edition first published 2017 © 2017 John Wiley & Sons, Ltd

Registered Office
John Wiley & Sons, Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

Editorial Offices
9600 Garsington Road, Oxford, OX4 2DQ, UK
The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK
1606 Golden Aspen Drive, Suites 103 and 104, Ames, Iowa 50010, USA

For details of our global editorial offices, for customer services and for information about how to apply for permission to reuse the copyright material in this book please see our website at www.wiley.com/wiley-blackwell.

Library of Congress Cataloging-in-Publication Data

Names: Waddington, Rachel (Rachel J.), editor. | Sloan, Alistair J., editor.
Title: Tissue engineering and regeneration in dentistry : current strategies / edited by Rachel J Waddington and Alistair J Sloan.
Description: Chichester, West Sussex, UK ; Ames, Iowa : John Wiley & Sons Inc., 2017. | Includes bibliographical references and index.
Identifiers: LCCN 2016022478 | ISBN 9781118741108 (pbk.) | ISBN 9781118741047 (Adobe PDF) | ISBN 9781118741078 (epub)
Subjects: | MESH: Tissue Engineering–methods | Dentistry–methods | Guided Tissue Regeneration–methods | Stomatognathic Diseases–therapy
Classification: LCC RK320.T47 | NLM WU 100 | DDC 617.6/340592–dc23
LC record available at https://lccn.loc.gov/2016022478

A catalogue record for this book is available from the British Library.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Cover image: © Andrew Brookes/Gettyimages

Set in 8.5/12pt Meridien by SPi Global, Pondicherry, India

1 2017

Contents

List of contributors

Wayne Nishio Ayre
School of Dentistry
Cardiff Institute of Tissue Engineering and Repair
Cardiff University
Cardiff, UK

P. Mark Bartold
Colgate Australian Dental Research Centre
Dental School
University of Adelaide
Adelaide, SA, Australia

Vanessa Chrepa
School of Dentistry
University of Texas Health Science Center
San Antonio, TX, USA

John Colombo
School of Dentistry
University of Utah
Salt Lake City, UT, USA

Lindsay C. Davies
Centre for Hematology and Regenerative Medicine
Karolinska Institutet
Stockholm, Sweden;
School of Dentistry
Cardiff University
Heath Park
Cardiff, UK

David de Silva Thompson
University College London
London, UK

Anibal Diogenes
School of Dentistry
University of Texas Health Science Center
San Antonio, TX, USA

Ikbale El Ayachi
Department of Bioscience Research
College of Dentistry
University of Tennessee Health Science Center
Memphis, TN, USA

Stan Gronthos
School of Medical Sciences
Faculty of Health Sciences
University of Adelaide
Adelaide, SA, Australia
South Australian Health and Medical Research Institute
Adelaide, SA, Australia

George T.-J. Huang
Department of Bioscience Research
College of Dentistry
University of Tennessee Health Science Center
Memphis, TN, USA

Dietmar W. Hutmacher
Institute of Health and Biomedical Innovation
Queensland University of Technology
Brisbane, Queensland, Australia

Saso Ivanovski
Menzies Health Institute Queensland
School of Dentistry and Oral Health
Griffith University
Gold Coast, Queensland, Australia

S. Quentin Jones
School of Dentistry
Cardiff Institute of Tissue Engineering and Repair
Cardiff University
Cardiff, UK

Katarina Le Blanc
Centre for Hematology and Regenerative Medicine
Karolinska Institutet
Stockholm, Sweden

Ryan Moseley
School of Dentistry
Cardiff Institute of Tissue Engineering and Repair
Cardiff University
Cardiff, UK

Roman Perez
Institute of Tissue Regeneration Engineering (ITREN)
Dankook University
Cheonan, Republic of Korea

Carlotta Peticone
University College London
London, UK

Jessica Roberts
North Wales Centre for Primary Care Research
Bangor University
Wrexham, UK

Nikita B. Ruparel
School of Dentistry
University of Texas Health Science Center
San Antonio, TX, USA

Alastair J. Sloan
School of Dentistry
Cardiff Institute of Tissue Engineering and Repair
Cardiff University
Cardiff, UK

Rachel J. Waddington
School of Dentistry
Cardiff Institute of Tissue Engineering and Repair
Cardiff University
Cardiff, UK

Ivan Wall
University College London
London, UK;
Institute of Tissue Regeneration Engineering (ITREN)
Dankook University
Cheonan, Republic of Korea

Xiao-Ying Zou
Department of Cariology, Endodontology and Operative Dentistry
Peking University School and Hospital of Stomatology
Beijing, China

Preface

Over the past twenty years there has been an explosion in published research studies characterising and investigating the behaviour of adult stem cells from the dental and oral tissues, and much excitement has been anticipated in their ability to regenerate a variety of connective tissues. Research in this field is rapidly expanding, facilitated by the many interdisciplinary collaborative opportunities for the repair of dental and craniofacial tissues. Their use has been championed for much wider translational opportunities, from large tissue volume regeneration of the musculoskeletal system to repair of ischaemic heart and liver tissue injury, replacement of misfunctioning cells such as pancreatic islet cells, and regeneration of neuronal networks and spinal cord injury. However, when entering the expansive literature, it is clear that many different experimental protocols have been utilised that examine heterogeneous stem cells, subpopulations, and clonally established cell lines where consideration of the environmental conditions are a critical for interpreting biological response. It is now very clear that adult stem cells represent a heterogeneous family of mesenchymal stem cells, where biological responses and translational applications are clearly going to be affected by the age and tissue source, with isolation and culture procedures affecting their peri-cellular and niche environment. In addition, the clinical use of such cells requires consideration of a number of practical limitations that need to be overcome, such as scale up and delivery. As the field of stem cell biology develops, characterisation of the cell populations is becoming ever more complex, although it should remain an important research element in assessing the therapeutic potential of stem cells. Indeed, exciting opportunities exist for reprogramming these cells, which may hold promise for expanding therapeutic potential. It is evident that much research in the area is needed to further our understanding.

In compiling this book, our aim was to highlight the varied breadth and considerations of the current research and the plethora of published literature to display key findings and current hypotheses. However, rather than simply produce a review of the current "state of the literature" we also aim to help active researchers in the field, both scientists and clinicians, through the provision of invaluable tools and methodologies utilised in undertaking research in this field, and to highlight important biological and practical considerations to facilitate successful migration of research from bench to clinic. As such, the chapters contained within this book not only provide a comprehensive overview of the published literature, but they highlight considerations that must be made for current data, indicate areas for development, and also provide clear protocols, methods, or "case studies" for aspects of research that can be used by other researchers in the field. With the help of leading experts in craniofacial and dental stem cell research and tissue engineering, we wanted to produce a textbook that becomes a valuable reference handbook and a practical guide that comes to be an invaluable lab text.

Professor Rachel J. Waddington
Professor Alastair J. Sloan

CHAPTER 1

Induced pluripotent stem cell technologies for tissue engineering

George T.-J. Huang[1], Ikbale El Ayachi[1], and Xiao-Ying Zou[2]

[1] *Department of Bioscience Research, College of Dentistry, University of Tennessee Health Science Center, Memphis, TN, USA*

[2] *Department of Cariology, Endodontology and Operative Dentistry, Peking University School and Hospital of Stomatology, Beijing, China*

Induced pluripotent stem cells (iPSCs) were first established by delivering the four factors c-Myc/Klf4/Oct4/Sox2 or Lin28/Nanog/Oct4/Sox2 into dermal fibroblasts via a viral vector-based approach (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). To avoid permanent integration of these introduced exogenous genes, plus the vector that carries them, significant efforts have been put into removing the transgenes and vectors from cells after they have been reprogrammed into iPSCs (Gonzalez et al., 2009; Kaji et al., 2009; Soldner et al., 2009; Woltjen et al., 2009; Yu et al., 2009a). Because of the reactivation of endogenous pluripotent genes that function to maintain the pluripotent state after reprogramming, these exogenous transgenes can be removed without affecting the reprogrammed status. In fact, removing these exogenous transgenes renders iPSCs more similar to human embryonic stem cells (hESCs) (Soldner et al., 2009). Besides using viral vector systems to reprogram cells, other methods that can completely circumvent the use of vectors have been utilised, including delivery of recombinant protein-based or synthetic mRNAs of the four factors to generate iPSCs (reviewed by Rao and Malik, 2012). There are many applications that iPSCs can contribute to; among others, this chapter focuses on (1) cell-based tissue regeneration and (2) generation of patient-specific iPSCs to study disease mechanisms.

With respect to the source of cells for human iPSC generation, various cell types are capable of converting into iPSCs, although dermal fibroblasts are most commonly used due to their relative ease of access and availability (Aasen et al., 2008; Giorgetti et al., 2009; Giorgetti et al., 2010; Li et al., 2009; Loh et al., 2009; Miyoshi et al., 2010a; Nakagawa et al., 2008; Park et al., 2008b; Sun et al., 2009; Takahashi et al., 2007; Yan et al., 2010). In general it is easier to reprogram more immature cells than more differentiated cells. From the perspective of clinical applications, cells that are not easily accessible, such as neural stem cells, are not a suitable cell source for iPSC generation. The oral cavity harbours a rich source of mesenchymal stem cells (MSCs), including those from various dental tissues, gingival/mucosal tissues, and alveolar bone (Huang et al., 2009; Morsczeck et al., 2013). Extracted teeth are considered biomedical waste and gingival/mucosal tissues are easily accessible and available. Oral MSCs are also relatively robust in respect to cell proliferation and population doubling (Huang et al., 2009); therefore, these cells may be one of the best sources for generating iPSCs.

While many aspects of iPSCs require investigation concerning their clinical safety, utilising iPSCs for cell therapy is anticipated to take place in the future. Studies focusing on guiding iPSCs to differentiate into various cell types for regeneration purposes have been rigorously undertaken. This chapter will overview current progress in this area, particularly emphasising neurogenesis. Additionally, utilising iPSCs as a tool for studying genetics and disease mechanisms will also be reviewed.

Overview of iPSCs

iPSC derivation

While various approaches or conditions may lead to the derivation of pluripotent stem cells in mammals (Cowan et al., 2005; Gómez et al., 2006; Miyashita et al., 2002;

Tissue Engineering and Regeneration in Dentistry: Current Strategies, First Edition. Edited by Rachel J. Waddington and Alastair J. Sloan.

Oh et al., 2009; Thuan et al., 2010; Wilmut et al., 1997; Yu et al., 2006), attempts to generate human (h) ESCs by somatic cell nuclear transfer continues to be unsuccessful. Human triploid blastocysts have been generated and are capable of giving rise to ESCs (Noggle et al., 2011); however, triploid hESCs are an unlikely or favorable cell source for clinical applications. Cells that have potential clinical value are hESCs derived from the parthenogenetic approach (Revazova et al., 2007; Revazova et al., 2008). Nonetheless, such a technology is inconvenient and difficult to perform. Yamanaka and his team utilised a *Fbx15*$^{\beta geo/\beta geo}$ mouse model and found that by introducing 4 factors, c-Myc, Klf4, Oct4 and Sox2 were sufficient to reverse fibroblasts to ES-like cells, termed "induced pluripotent cells (iPSCs)" (Takahashi and Yamanaka, 2006). These mouse (m) iPSCs demonstrate the features resembling ES cells. These include similar morphology in cultures, growth rate, key pluripotent genes, global gene profiles, epigenetic profiles, and capability of embryoid body (EB) formation. In addition, differentiation into cells of all germ layers is observed in EBs *in vitro*, as well as formation of teratomas *in vivo* containing tissues of all germ layers, and above all, the formation of chimeras after iPSCs were injected into blastocysts in an animal system. Subsequently, Yamanaka's group further demonstrated that the same four factors c-Myc, Klf4, Oct4 and Sox2 were also effective in humans in reprogramming fibroblasts into iPSCs, exhibiting similar features mentioned above for miPSCs, except the formation of chimeras which cannot be tested for the human system (Takahashi et al., 2007). Thomson's group independently identified a core set of 4 genes, Oct4, Sox2, Nanog and Lin28 that were also able to reprogram human fibroblasts into iPSCs (Yu et al., 2007).

The successful rate of iPS generation is generally low; the highest was at 0.1% in a mouse system using embryonic fibroblasts as the cell source (Smith et al., 2009). With a single lentiviral vector expressing all four Yamanka's factors, Sommer et al. (2009) were able to demonstrate a reprogramming efficiency of 0.5% using mouse tail-tip fibroblasts. In human systems, adipose tissue stem cells can reach a successful reprogramming rate of 0.2% (Sun et al., 2009). In general, it is difficult to assess the absolute efficiency as different laboratories are using various vector systems and the viral activities can vary widely as well. Compared to other means of deriving human pluripotent stem cells, iPSCs appear to be the desired method for potential clinical utilisation.

Characteristics of iPSCs

One critically important hallmark of ESCs as pluripotent stem cells is the capability to form embryos and be born into live animals via a tetraploid-complementation procedure. Using a mouse system, such cell characteristics can be demonstrated and the generation of live pups by iPSCs, some of which lived to adulthood, has been demonstrated (Boland et al., 2009; Kang et al., 2009; Zhao et al., 2009). The successful rate of giving rise to tetraploid complementation by iPSCs is similar to that by ESCs; however, there are variables in iPSC lines. Some iPSC lines showed early termination of fetal development at the embryonic stage (Zhao et al., 2009). Generally, iPSCs are functionally similar if not identical to ESCs. One drawback is the variability among different iPSC clones. hiPSCs cannot be tested by such methodologies; therefore, characterisation at genetic and epigenetic levels should be carried out to establish the molecular basis of the reprogrammed hiPSC clones.

In the human system, the global gene-expression patterns and epigenetic profiles between iPS and ES cells were shown to be similar (Takahashi et al., 2007; Yu et al., 2007). Regarding the telomere regaining length in iPSCs, this was addressed in the reprogramming of cells from patients with Dyskeratosis congenita (DC), a disorder of telomere maintenance (Agarwal et al., 2010). Reprogramming can restore telomere elongation in DC cells despite genetic lesions affecting telomerase (Agarwal et al., 2010).

Examining the whole-genome profiles of DNA methylation at single-base resolution of hiPSC lines revealed that there is reprogramming variability, including somatic memory and aberrant reprogramming of DNA methylation (Lister et al., 2011). iPSCs are thought to harbor a residual DNA methylation signature related to their cell of origin, termed "epigenetic memory". This predisposes them toward differentiation along lineages related to that cell type and restricts differentiation to alternative cell fates (Kim et al., 2010; Polo et al., 2010). Epigenetic memory can also be correlated with a residual transcriptional profile in iPSCs that is related to the cell from which it was originally reprogrammed (Ghosh et al., 2010). Epigenetic analysis of the iPSC clones may be needed to provide a critical baseline for studying cellular changes occurring during the controlled *in vitro* differentiation concerning the utility of these cells for future therapies. There are also reprogramming-associated mutations that occur during or

after reprogramming. It is suggested that extensive genetic screening should become a standard procedure to ensure hiPSC safety before clinical use (Gore et al., 2011). Despite these caveats, efforts have been made to produce pure, stable, and good manufacturing practice (GMP)–grade hiPSCs potentially suited for clinical purposes (Durruthy-Durruthy et al., 2014).

While mutations may occur during reprogramming, whether hiPSCs cause tumors has yet to be fully investigated. Neural precursor cells derived from miPSCs have been shown to form teratomas after *in utero* transplantation into the brain of mouse embryos. This may be avoided by FACS (fluorescent activated cell sorting) depletion of the SSEA1-positive cell fraction prior to transplantation (Wernig et al., 2008a). Although there is concern of the genomic instability in pluripotent stem cells such as ESCs, it is not known whether genomic instability in hPSCs increases the likelihood of tumorigenesis. It has been proposed that high-resolution methods such as single nucleotide polymorphism genotyping be performed before any hPSCs are used for clinical transplantation (Peterson and Loring, 2014).

Feasible cell types for iPSC generation

Dermal fibroblasts have been the popular cell type of choice to generate iPSCs because they are ubiquitous and easily acquired in the skin. However, another source of cells, which is possibly more feasible and accessible, is the oral cavity. Fibroblasts from the oral mucosa can be reprogrammed into iPSCs, and acquiring a small amount of oral mucosa tissue leaves behind no scar (Miyoshi et al., 2010a), while it harbours a robust mesenchymal stem cell population (Morsczeck et al., 2013). MSCs in the jawbone can also be easily accessed. Acquiring alveolar bone in the jaw may be slightly more invasive; if needed, its acquisition protocol to isolate MSCs has been well established (Mason et al., 2014), and no report associates significant postoperative pain with this procedure. Blood cells are another easily obtainable cell type for iPSC generation; however, it requires subpopulation isolation and growth factor stimulation before reprogramming. This tedious process makes them makes them less attractive as a feasible cell source for reprogramming (Loh et al., 2009; Staerk et al., 2010; Su et al., 2013).

Discarded extra-embryonic tissues such as umbilical cord are a good cell source to generate iPSCs, as they are immature cells, highly suitable for such a purpose (Jiang et al., 2014; Song et al., 2014). Extracted teeth contain mesenchymal-like stem/progenitor cells including dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), and periodontal ligament stem cells (PDLSCs) that are also a good cell source to derive iPSCs (Tamaoki et al., 2010; Wada et al., 2011; Yan et al., 2010). These stem cells are normally from children or young adults—SHED are from children around ages 6–12; SCAP, DPSCs, and PDLSCs from third molars are from those ages 16–22. These age groups contain more immature stem cells suitable for generating iPSCs. As summarised in Figure 1.1, a number of cell sources may be used for transgene-/vector-free iPSC generation and their subsequent medical applications.

Applications for iPSCs in cell-based therapy

While adult stem cells are multipotent and some are near pluripotent, their acquisition is nonetheless often inconvenient, and they have a limited life span in cultures (Kim et al., 2007; Kolf et al., 2007). Partial reprogramming by directing fibroblasts into specific lineages appears to be a good option for cell-based therapy; however, the key issue is still the limitation of cell source and their life span *in vitro*. With respect to their capacity for tissue regeneration, the pluripotency of ESCs, which can generate all cell types, is unparalleled by adult stem cells. The main concern of using hESCs is their safety, as ESCs may form teratomas *in vivo* if they fail to differentiate. One report of two clinical cases and phase I/II studies of 18 patients using hESCs for restoring eyesight of patients showed no adverse effects after a median of 22 months of follow-ups (Schwartz et al., 2012; Schwartz et al., 2015). Teratoma formation normally occurs within 8 weeks, suggesting that these clinical cases are unlikely to develop any tumor formation in the future. Currently, there are a number of clinical trials mainly using hESC-derived retina pigmented epithelial cells for transplantation to treat retinal degenerative diseases, and none have shown development of tumors (Peterson and Loring, 2014). The clinical trials operated by Geron for treating spinal cord injury using hESCs have, unfortunately, been discontinued due to financial reasons. If proven that hESCs are clinically safe, it is possible that iPSCs are also safe, and the

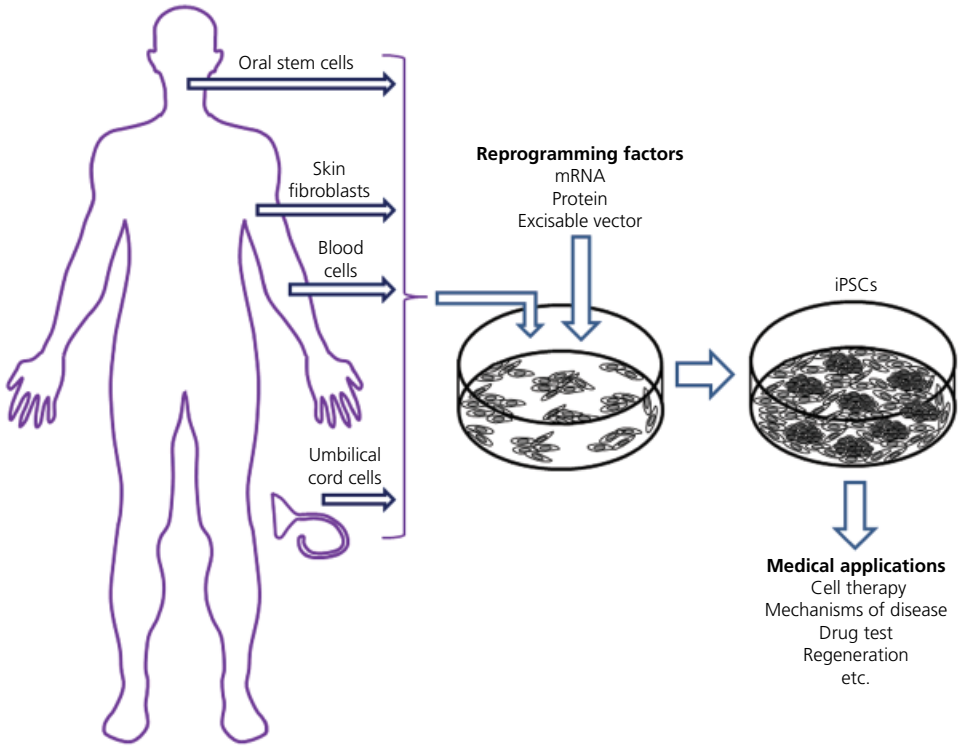


Figure 1.1 Feasible source of cells or stem cells for transgene-free iPSC generation and subsequent medical applications. Oral stem cells are the most accessible and easiest cells for the reprogramming process. Use of mRNA, protein, or an excisable vector approach allows generation of transgene-free iPSCs. Note: Although blood cells appear to be the easiest cell type to obtain, multiple steps are involved in their processing before they are ready for reprogramming, which is very inconvenient.

ongoing clinical trials with iPSCs will verify this possibility (Cyranoski, 2013). Recently it has been shown that transplantation of hiPSC-derived neural stem cells (NSCs) enhanced axonal sparing, regrowth and angiogenesis, prevented demyelination after spinal cord injury, and promoted functional recovery in the common marmoset animal model without tumor formation (Kobayashi et al., 2012).

Guiding hESCs towards differentiation into various tissue specific cells *in vitro* has been rigorously tested, and various protocols have been established. These protocols are being utilised for iPSC differentiation. Various differentiation pathways have been tested for guiding iPSCs into specific lineages representing all of the three germ layers (Efthymiou et al., 2014). Examples are ectodermal-related neural cells (Cai et al., 2010; Hu et al., 2010), mesodermal-related haematopoietic and endothelial cells (Choi et al., 2009; Feng et al., 2010) and skeletal muscle cells (Mizuno et al., 2010), and endodermal-related hepatocytes (Gallicano and Mishra, 2010; Si-Tayeb et al., 2010; Sullivan et al., 2010). Studies are being continuously undertaken to refine the differentiation protocols of guiding ESCs or iPSCs into specific cell types, and to understand the extent of variation in differentiation potential among different cell lines and clones that is related to the effects of cell origin or reprogramming methods. Most importantly, iPSC-derived differentiated cells must have equivalent functions to the naturally formed tissue cells (Efthymiou et al., 2014).

iPSCs for tissue engineering and regeneration

iPSCs for tissue regeneration in general

Cell-based therapy to regenerate tissues may be the best and the only option when defect size reaches a point where non-cell-based approaches cannot work. iPSCs,

similar to ESCs, undergo continuous self-renewal in cultures and may provide unlimited cell source for tissue regeneration (Efthymiou et al., 2014; Hirschi et al., 2014; Lengner, 2010). With regard to human systems, a number of different cell types may be differentiated from hiPSCs for regenerative medicine. The following listed are a few examples.

(a) Cardiac regeneration with iPSCs

hiPSCs can differentiate into functional cardiomyocytes (Germanguz et al., 2011; Seki et al., 2014; Zhang et al., 2009; Zwi et al., 2009). Successful differentiation of hiPSCs into cardiomyocytes was first reported in 2009 (Zhang et al., 2009). Electrophysiology studies indicated that iPSCs differentiate into nodal-, atrial-, and ventricular-like phenotypes and exhibit responsiveness to beta-adrenergic stimulation. Overall, cardiomyocytes obtained from iPSCs are functionally similar to ESC-derived cardiomyocytes (Zhang et al., 2009). Furthermore, iPSC-derived cardiomyocytes have been engrafted successfully into the hearts of experimental animals (Zwi-Dantsis et al., 2013) and used to improve cardiac function after ischemic cardiomyopathy in a porcine model (Kawamura et al., 2012).

(b) Skeletal tissue regeneration with iPSCs

Skeletal tissue engineering includes bone and cartilage regeneration. Osteogenic differentiation of iPSCs for bone tissue regeneration has been reported using scaffolds such as macro-channeled polycaprolactone scaffolds (Jin et al., 2013), polyethersulfone nanofibrous scaffolds (Ardeshirylajimi et al., 2013) and fibrin or hydroxyapatite/β-tricalcium phosphate (Park and Im, 2013).

Osteogenic differentiation of hiPSCs could be conducted with EB formation (Ardeshirylajimi et al., 2013; Park and Im, 2013), or without the EB formation step, by using osteogenic factors, ascorbic acid, β-glycerophoshate and dexamethasone (Jin et al., 2013). Based on *in vitro* studies, iPSCs seem to have the similar characteristics to hESCs in osteogenic differentiation (Ardeshirylajimi et al., 2013). *In vivo* bone formation by iPSCs was also demonstrated in rats (Park and Im, 2013) and in nude mice (Duan et al., 2011). Studies have also shown that hiPSCs combined with gels containing an enamel matrix-derived protein complex from the amelogenin family provide a valuable tool for periodontal tissue engineering by promoting the formation of new alveolar bone and cementum formation, with normal periodontal ligament between them (Duan et al., 2011).

Cartilage tissue engineering using differentiated and purified iPSCs has also been reported (Diekman et al., 2012). Robust chondrogenic differentiation of iPSCs using BMP-4 treatment in micromass culture was observed. These iPSC-derived chondrocyte-like cells were effective at promoting the integration of nascent tissue with the surrounding adult cartilage in an *in vitro* cartilage injury model (Diekman et al., 2012). Besides direct differentiation from iPSCs, osteoblasts (Villa-Diaz et al., 2012) and chondrocytes (Koyama et al., 2013) could also be derived from iPSCs via a selection of cells that can adapt to MSC growth conditions. MSCs could be derived from iPSCs through EB formation, with typical expression of MSC surface markers and the potential to differentiate into adipocytes, chondrocytes, and osteoblasts (Tang et al., 2014).

(c) Tooth regeneration with iPSCs

iPSCs have the capacity to differentiate into oral tissue cells including dental epithelial and mesenchymal cells. miPSCs cultured with dental epithelial cell line cells display an epithelial cell–like morphology expressing the ameloblast markers ameloblastin and enamelin (Arakaki et al., 2012). miPSCs can differentiate into neural crest–like cells (NCLCs) (Lee et al., 2007), and if cocultured with dental epithelium, they express dental mesenchymal cell markers (Otsu et al., 2012). If culturing NCLCs in the conditioned medium of mouse dental epithelium cultures, their differentiation into odontoblasts is enhanced (Otsu et al., 2012). Such findings have led to a proposed protocol for whole-tooth regeneration using iPSCs (Figure 1.2) (Otsu et al., 2014). Using a tooth germ reconstitution and transplantation model, miPSCs were able to participate in the regeneration of alveolar bone and pulp of the engineered tooth unit *in vivo* (Wen et al., 2012). Human iPSC-derived epithelial cells combined with mouse dental mesenchyme can give rise to tooth-like structures *in vivo* (Cai et al., 2013; Liu et al., 2014). These results suggest that iPSCs may be a useful cell source for tooth regeneration and tooth development studies.

Neural regeneration with iPSCs

Many neurological disorders await therapeutic strategies including cell-based therapies. A good example would be Parkinson's disease, a common chronic progressive neurodegenerative disorder characterised primarily by major loss of nigrostriatal dopaminergic neurons. In a proof-of-principle experiment using a mouse model, iPSCs were

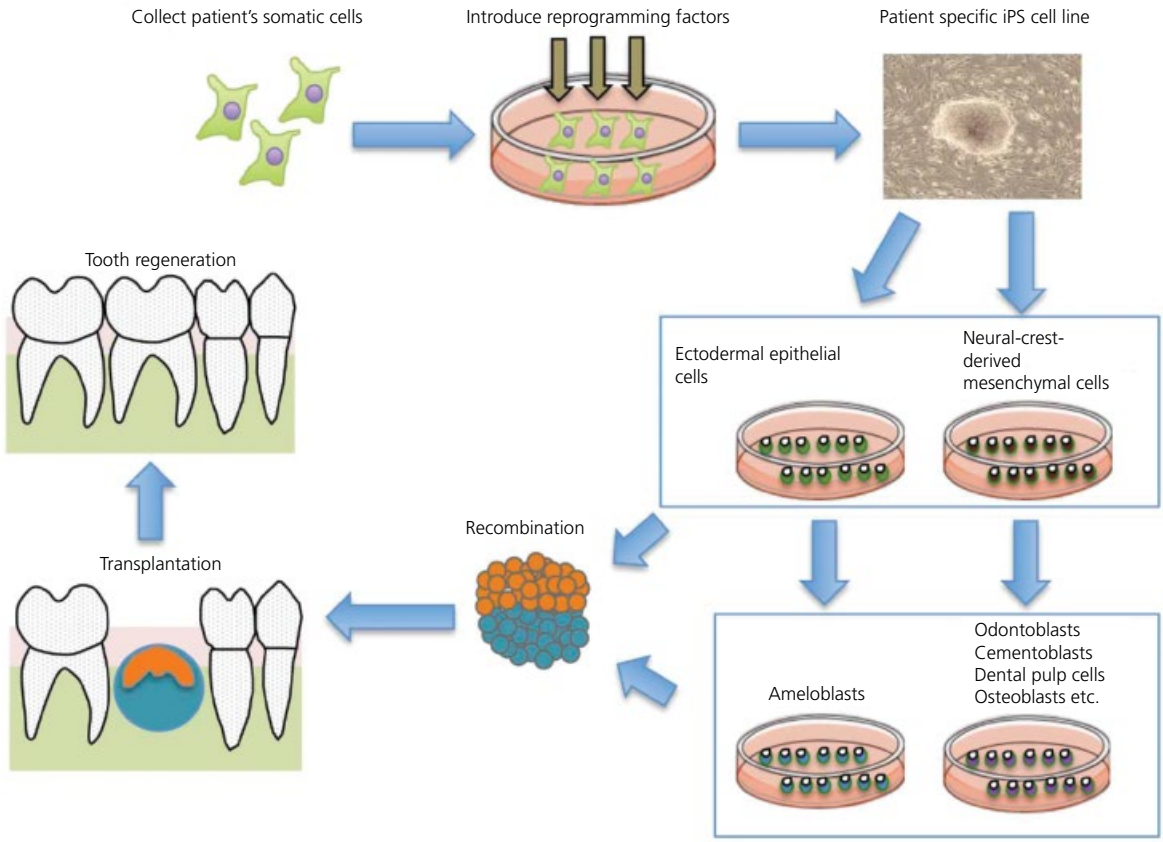


Figure 1.2 Schematic representation of a strategy for whole-tooth regeneration using iPSCs. The patient's somatic cells are harvested and reprogrammed into patient-specific iPSCs, which are then induced to form ectodermal epithelial cells and neural crest-derived mesenchymal cells. They may be further induced to form odontogenic cells *in vitro*. The two cell populations are combined by direct contact, mimicking the *in vivo* arrangement. Interaction of these cells leads to formation of an early-stage tooth germ. Once transplanted into the edentulous region, the recombinants develop into a functional tooth. (Adapted from Otsu et al., 2014. Used under CC-BY 3.0. http://creativecommons.org/licenses/by/3.0/.)

first guided to differentiate into neural precursor cells and transplanted into the fetal mouse brain. These iPSC-derived cells migrated into various brain regions and differentiated into glia and neurons, including glutamatergic, GABAergic, and catecholaminergic subtypes (Wernig et al., 2008a). These grafted neurons showed mature neuronal activity and were functionally integrated in the host brain. iPSC-derived dopamine neurons transplanted into a rat model of Parkinson's disease improved behavior (Wernig et al., 2008a; Wernig et al., 2008b). Furthermore, iPSCs reprogrammed from fibroblasts of Parkinson's disease patients can be guided to differentiate into dopaminergic neurons (Soldner et al., 2009). hiPSCs can be differentiated to form motor neurons with a similar efficiency as hESCs. The differentiation of iPSCs appeared to follow a normal developmental progression associated with motor neuron formation and possessed prototypical electrophysiological properties (Hu et al., 2010; Karumbayaram et al., 2009). A recent report using a mouse model showed that transplantation of neural precursor cells derived from transgene-/vector-free hiPSCs into the mouse brain that had suffered ischemic stroke injury enhanced functional recovery (Mohamad et al., 2013a).

Protocols of neural regeneration using iPSCs

In vitro differentiation of iPSCs to neural cells has been achieved using various approaches. There are three major methods (summarised in Table 1.1): (a) EB

Table 1.1 Neural differentiation protocols of ESCs/iPSCs.

Protocol	Culturing Method	Differentiation Strategy	Reference
EB formation and rosette isolation	EB formation in suspension and following adherent culture of EBs	Induction and isolation of neural rosettes without morphogens	(Zhang et al., 2001)
Dual-SMAD inhibition	Adherent single cell culture of dissociated iPSCs	Inhibition of BMP/Nodal signals	(Chambers et al., 2009; Morizane et al., 2011)
SFEBq	EB-like formation by reaggregation of dissociated iPSCs	Inhibition of WNT/BMP/Nodal signals	(Mariani et al., 2012b; Watanabe et al., 2005; Watanabe et al., 2007)

Note: EB = embryoid body; SFEBq = serum-free culture of EB-like aggregates, quick method.
Source: Adapted from Kim et al., 2014. Reproduced with permission.

formation and rosette isolation method, (b) dual-SMAD inhibition method, and (c) SFEB method (serum-free culture of EB-like aggregates) (Kim et al., 2014).

(a) EB formation and rosette isolation method

One popular and powerful approach to mobilising iPSC/ESC differentiation through to a neural lineage is the EB formation and rosette isolation method (Dimos et al., 2008; Wang et al., 2011; Zhang et al., 2001), or even using a rotary cell culture protocol to increase EB homogeneity (Mohamad et al., 2013b). Four types of colonies can develop at the rosette stage of iPSCs, namely, colonies with rosette structure, colonies with differentiated neurons, colonies with myofibroblasts, and a small number of undifferentiated colonies. The unique cellular arrangement of epithelial cells is reminiscent of cross-sections of the developing neural tube and is considered a hallmark of successful neural induction. These rosette colonies are positive for neural crest markers AP-2, nestin, and p75, and may be used for nerve regeneration (Wang et al., 2011). Timely treatment with particular morphogens such as Shh and Wnts or their agonists/antagonists, redirects the regional identity of these progenitor cells to either ventral or caudal fate, leading to many methods for generating different neuronal subtypes. iPSCs have been shown to differentiate into dopaminergic neurons (Kwon et al., 2014; Wernig et al., 2008b) and motor neurons (Dimos et al., 2008; Hu et al., 2010; Karumbayaram et al., 2009). The schematic representation of EB-mediated neurogenesis is depicted in Figure 1.3 with Protocol 1.2 describing steps for EB-mediated neuronogenesis to generate neuronlike cells *in vitro* (Hu et al., 2010; Zou et al., 2012).

(b) Dual-SMAD inhibition method

A representation of the dual-SMAD inhibition method is illustrated in Figure 1.4. Chambers et al. (2009) first reported this method using hESCs/hiPSCs. Here, the hESCs/iPSCs are dissociated to single cells and grown as adherent cultures for neuroectodermal cell differentiation. PAX6+ cells emerge and form neural rosettes in 11 days. Subsequent differentiation into neural cells that express PAX6, FOXG1 (BF1), and OTX2 indicate dorsal telecephalic identity (Chambers et al., 2009). With slight modification of this protocol, iPSCs are able to convert to dopaminergic neurons (Morizane et al., 2011). Adding the BMP signaling inhibitor dorsomorphin and a TGF-β/activin/nodal signaling inhibitor SB431542 into single cell cultures of iPSCs/ESCs was shown to promote highly efficient neural differentiation. This method is referred to as the dual-SMAD inhibition approach because each signaling pathway recruits SMAD proteins as intracellular signal transducers. The small molecule compounds dorsomorphin and SB431542 are stable and cost effective, and this method may provide a promising strategy for controlled production of neurons in regenerative medicine (Morizane et al., 2011; Wattanapanitch et al., 2014).

(c) Serum-free EB-like (SFEB) method

Watanabe et al. (2005) first reported the SFEB method using mESCs. Here, the ESC colonies are dissociated into single cells and allowed to grow in suspension. Approximately 90% of cells spontaneously form aggregates of defined size in cultures, and the Wnt inhibitor Dkk1 and nodal signaling antagonist LeftyA are present to guide cells toward neural differentiation (Watanabe et al., 2005). Cells can be further guided into subpopulations of neuronal lineage with different growth factors.

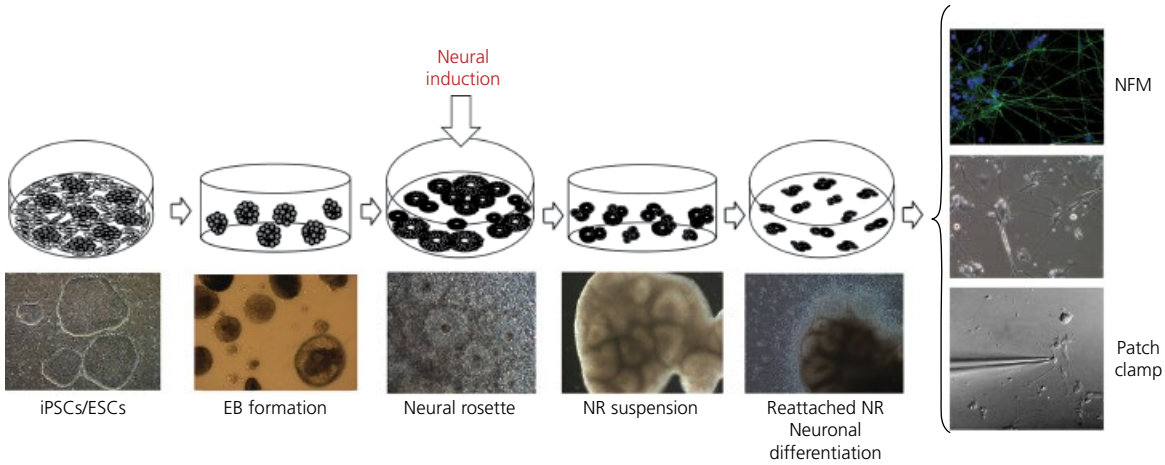


Figure 1.3 Embryoid body (EB)-mediated neurogenesis. iPSC/ESC colonies are lifted into suspension to form EBs, followed by growth in adherent culture in defined media containing N2 supplement and basic fibroblast growth factor (bFGF) and allowed to form neural rosettes (NR). Cells in the NRs express many neural stem cell markers such as Nestin, Musashi-1, and polysialylated-neuronal cell adhesion molecule. NRs are then detached and grown in suspension followed by reattachment onto a laminin-coated dish under neurogenic stimulation for further neural differentiation prior to functional assessment through patch clamp electrophysiology. (Patch clamp image courtesy of Dr. Kristen O'Connell, University of Tennessee Health Science Center. Reproduced with permission from K. O'Connell.)

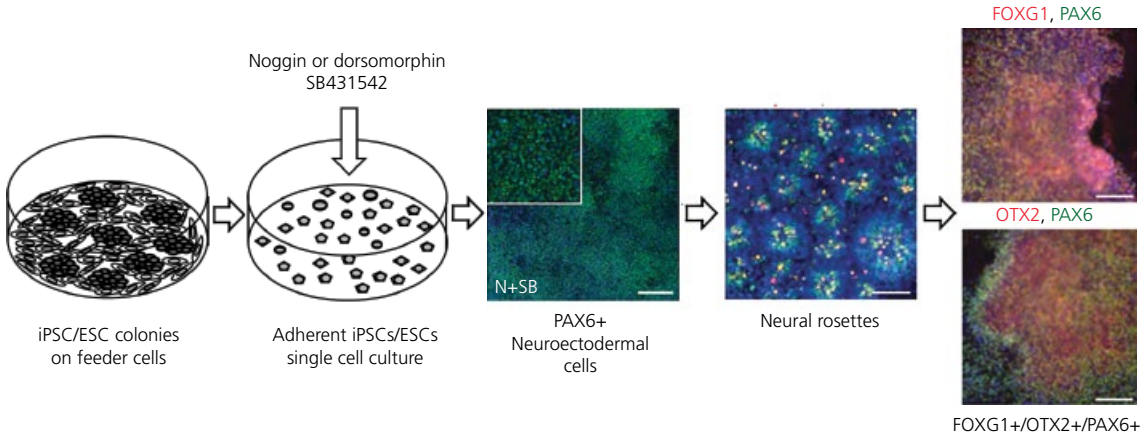


Figure 1.4 Dual-SMAD inhibition method for neural differentiation. Under serum-free conditions, adherent single cell-cultures of iPSCs/hESCs are treated with Noggin or dorsomorphin (BMP inhibitor) and SB431542 (Activin/Nodal inhibitor) to convert iPSCs/hESCs to largely PAX6-positive (green) neuroectodermal cells that subsequently form neural rosettes (Ki67, green phosphor-histone H3, red) following 11 days of differentiation. Neural cells generated express FOXG1 (red) and OTX2 (red), along with PAX6 (green). (Scheme based on Chambers et al., 2009. Reproduced with permission from Nature Publishing Group).

This group later tested this protocol on hESCs and had to use a ROCK inhibitor to increase survival of cells during culture (Watanabe et al., 2007). The modified protocol is depicted in Figure 1.5, showing that hESCs/hiPSCs are dissociated into single cells and allowed to form floating EB-like aggregates in the presence of Dkk1, LeftyA, and BMPRIA-Fc, followed by reattachment onto coated dishes/wells. Under further differentiation stimulation, different populations of neurons emerge in response to certain signals. For example, Shh treatment for ventralisation leads to an increased population of NKx2.1+ cells (basal region of

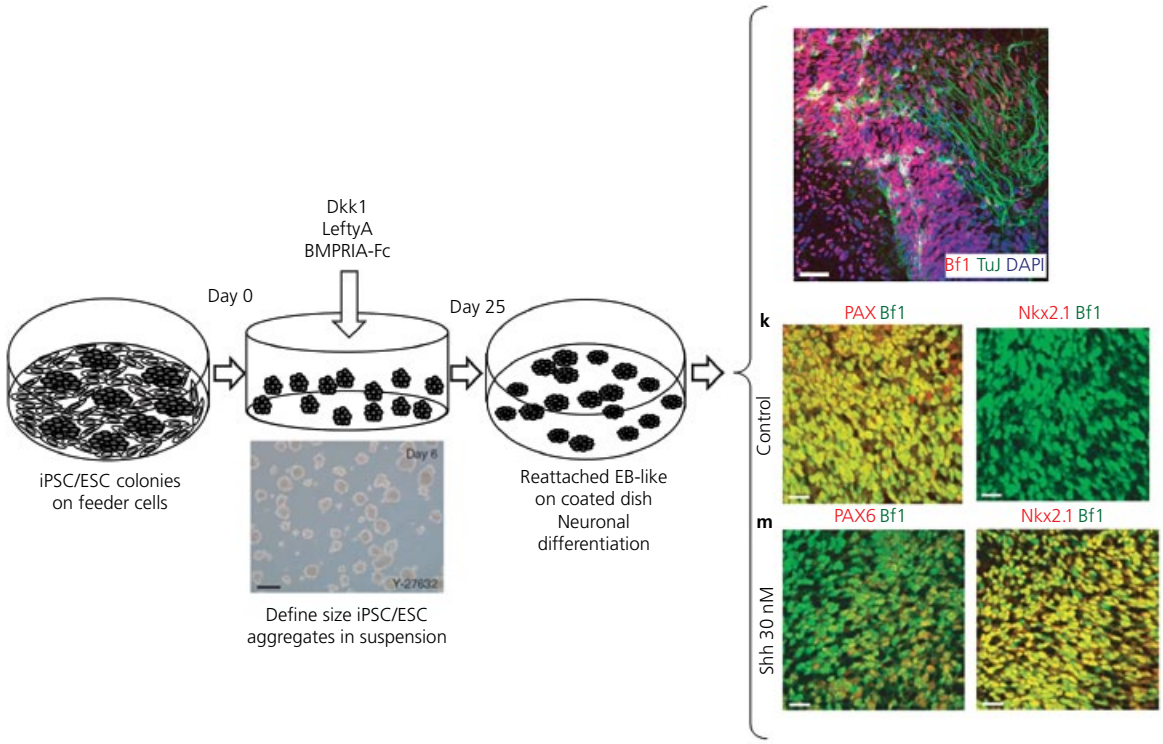


Figure 1.5 Serum-free EB-like (SFEB) method for neural differentiation. iPSC/ESC colonies are dissociated to single cells (2×10^5 cells/mL) and cultured in nonadherent dishes. Cell aggregates form spontaneously in the presence of a ROCK inhibitor. Dkk1 (Wnt inhibitor), LeftyA (Nodal signaling antagonist), and soluble BMPRIA-Fc (BMP-4 antagonist) are added to the culture from Day 0 to Day 24. The cell aggregates are then replated *en bloc* on dishes coated with poly-D-lysine, laminin, and fibronectin, and cultured until Day 35 in a neural differentiation medium (Neurobasal + B27 and glutamine). For ventralisation experiments, Shh is added. On Day 35, hESC-derived neural cells express Bf1 (32.9% ± 2.6%, far right image panel, top). The early embryonic telencephalon is subdivided into the pallial (Bf1+/PAX6+ cortical anlage) and basal (e.g., Nkx2.1+) regions. The majority of Bf1+ cells derived from Y-27632-treated hES cells coexpressed PAX6 (95.8 ± 0.7%), whereas Nkx2.1 was detected in only a few Bf1+ cells (1% or less) (far right middle image panel). Shh treatment (Days 15–35) decreased the PAX+ population (23.2% ± 5.3%) and increased the proportion of Nkx2.1+ cells among the Bf1+ cells (41.5% ± 14.5%) (far right bottom image panel). (Scheme based on Watanabe et al., 2007. Reproduced with permission from Nature Publishing Group.)

telencephalon) among BF1+ cells (Watanabe et al., 2007). Mariani et al. (2012a) adopted this protocol to use with hiPSCs, which were able to form multilayered structures expressing a gene profile typical of the embryonic telencephalon region.

iPSCs as disease study models

iPSCs can be generated from patients with specific diseases. If the generated iPSCs recapitulate the disease phenotype either *in vitro* or *in vivo*, these cells can be used to establish a patient iPSC library that can be used to study the disease mechanisms and novel drug development (Nishikawa et al., 2008; Park et al., 2008a). iPSCs generated from cells of patients with Hutchinson-Gilford progeria syndrome, caused by a single-point mutation in the lamin A (LMNA) gene, recapitulate the disease phenotype at the cellular and molecular level, providing an *in vitro* iPSC-based model to study the pathogenesis of this disease (Liu et al., 2011).

Genetic disease

Park et al. (2008a) established a disease iPSC library from patients with a variety of genetic diseases of Mendelian or complex inheritance. Examples include

adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Duchenne and Becker muscular dystrophy, Parkinson's disease, Huntington disease, juvenile-onset, type 1 diabetes mellitus, and Down syndrome/trisomy 21 (Park et al., 2008a). iPSCs generated from patients with single-gene disorders can not only be used to study disease mechanisms, but can also be used to correct the genetic defect *ex vivo* such that the correct cells may be transplanted back to the patient. In the case of Huntington disease, iPSCs from such patients that carry the mutant Huntingtin gene (mHTT) can be differentiated into NSCs or neural progenitor cells (NPCs). mHTT expression in NSC and NPCs can be silenced by using RNAi or antisense oligonucleotides. The corrected cells can then be transplanted into the brain of the patient to replenish the lost cell population (Chen et al., 2014).

For genetic diseases involving haematopoietic systems, Hanna et al. (2007) first showed that in a humanised sickle cell anemia mouse model, iPSCs can be generated from the diseased mouse fibroblasts and the mutation corrected *in vitro* followed by differentiating the corrected iPSCs into haematopoietic progenitors for transplantation and curing the disease (Hanna et al., 2007). Similarly, in a human model, fibroblasts from Fanconi anaemia patients after genetic correction can be reprogrammed into pluripotency to generate patient-specific iPSCs. Corrected Fanconi-anaemia-specific iPS cells can give rise to haematopoietic progenitors of the myeloid and erythroid lineages that are phenotypically normal (Raya et al., 2009).

iPSCs have also been established from patients with various neurological disorders, including Rett syndrome, Fragile X syndrome, Angelman syndrome, Timothy syndrome, familial Alzheimer's disease, and Parkinson's disease (Israel et al., 2012; Wang and Doering, 2012; Yagi et al., 2011; Soldner et al., 2009;). iPSCs have additionally been used as a tool to study X-linked genes with mutations that are either dominant or recessive. The situation is not clear-cut. For example, the X-linked neurodevelopmental disorder, Rett syndrome (RTT), has been studied using iPSCs from cells of patients with Rett syndrome. The disease affects girls due primarily to heterozygous mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2) (Cheung et al., 2012). X-chromosome inactivation (XCI) status of RTT-hiPSCs has been inconsistent with some reports showing that RTT-hiPSCs retain the inactive X-chromosome of the founder somatic cells, retaining their allele specific expression patterns. Conversely, other reports show reactivation of the inactive X-chromosome in RTT-hiPSCs derived from the founder somatic cells. Subsequently, random XCI ensues with RTT-hiPSCs undergoing differentiation, resulting in cellular mosaicism with cells either expressing MECP2-WT or MECP2-Mut transcripts (Cheung et al., 2012).

Cancer-iPSCs

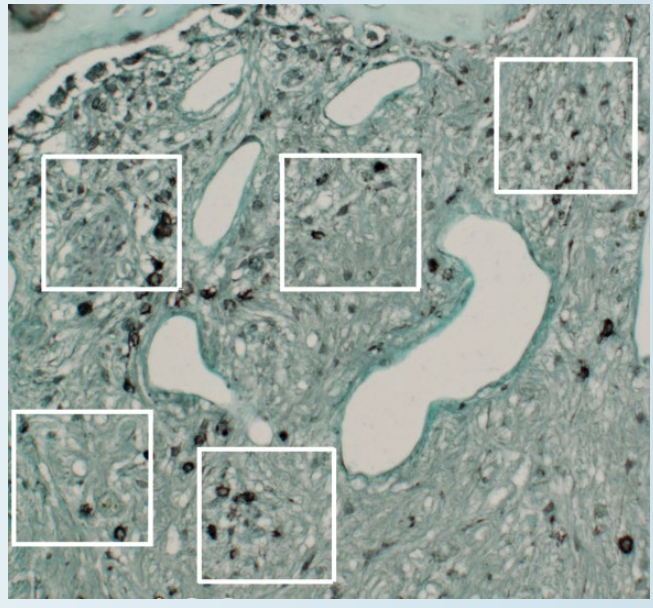
Reprogramming specific cancer cells into pluripotent state followed by differentiating into different lineages may help develop cancer vaccines, be applied in drug screening, or be used to understand the biological nature of cancer cells. For example, KBM7 cells derived from chronic myeloid leukemia have been reprogrammed into iPSCs. These cancer-iPSCs, in contrast to parental KBM7 cells, were completely resistant to the therapeutic drug Imatinib (Carette et al., 2010). Miyoshi and colleagues (2010b) reprogrammed cancer cells of endodermal origin including esophageal, stomach, colorectal, liver, pancreatic, and cholangiocellular cancer cells. The reprogrammed cancer-iPSCs express morphological patterns of ectoderm, mesoderm, and endoderm, which were not expressed in the parental cells (Miyoshi et al., 2010b). These cancer-iPSCs showed slow proliferation, were sensitised to differentiation-inducing treatment, and had reduced tumorigenesis in NOD/SCID mice. Additionally, the tumor-suppressor gene P16 (INK4A) was repressed in induced pluripotent cancer (iPC) cells while its expression increased in differentiated iPC cells. The findings suggest that the reactivation of tumor suppressor genes by reprogramming may play a role in increased chemosensitivity to 5-FU and the regression of cell proliferation and invasiveness under differentiation-inducing conditions (Miyoshi et al., 2010b). Since cancer cells can potentially be reprogrammed into pluripotency and be capable of differentiation into multiple cell lineages of all three germ layers, it has been speculated that converting cancer cells into highly immunogenic tumor antigen–presenting dendritic cells for cancer immunotherapy may be a distinct possibility (Lin and Chui, 2012).

Protocol 4.4 Immunocytochmistry for detection of cellular synthesis of osteogenic markers as a semiquantitiative measurement of bone healing.

1. Carefully dissect osseous tissue blocks for 3–5 time points relating to the presumed inflammatory stage (1–3 weeks) and the reparative stage (1–4 months).
2. If necessary, remove nonsectionable biomaterials, such as metallic implants. Take care not to damage adjacent biological tissue. Cut tissue blocks of 2–5 mm length and width.
3. Fix tissue blocks in 10% neutral buffered formalin for 24 h; demineralise in 10% formic acid for 48 h; dehydrate through 70%, 90%, and 100% alcohols and clear with xylene prior to embedding in paraffin wax; cut 5-μm sections with a microtome; mount onto poly-L-lysine coated glass slides (for additional adhesion) and dry overnight at 60°C.
4. For histological examination, deparaffinise with xylene for 10 min; rinse with industrial methylated spirit for 5 min; wash in tap water for 5 min; stain sections with haematoxylin and eosin for 5 min; mount glass coverslip using DPX glue; view using a light microscope at 40x magnification; obtain x300 dpi digital images (TIFF) using imaging software.
5. For immunocytochemical analysis, deparafinise and rehydrate sections as above; quench endogenous peroxidase activity by incubating sections in 3% H_2O_2 for 10 min.
6. Incubate sections with the appropriate 1°antibody, diluted in 1% fetal bovine serum (in Tris buffered saline) for 1 h (determine initial antibody dilution from manufacturer's recommendation, but optimise dilution factor to obtain an ideal staining level without suspician of nonspecific binding of the antibodies). As negative controls, substitute the 1° antibody with a nonimmunogenic IgG control antibody (Sigma Aldrich, UK) (used at the same dilution as the 1° antibody) and/or exclude the 1° antibody. If available, preincubate the 1° antibody with a blocking peptide used to generate the antibodies for 30 min prior to incubation with the section to block antibody epitope interaction and confirm specific antibody interation.
7. Visualise immunoreactivity using the Vectorstain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK) and the DAB peroxidase kit (Vector Laboratories UK) or equivalent; counterstain with 0.1% methyl green for 1 min, with excess stain removed by excessive rinsing with tap water. Soak sections in xylene for 5 min and mount for viewing by light microscopy at x20 magnification.
8. For detection of cell surface proteins and their ligands it may be necessary to retrieve the antigen by treatment with 24 μg/mL proteinase K for 10 min prior to quenching of endogenous peroxidase activity.

Semiquantitative image analysis:

9. Using image analysis package, a minimum of five random view areas of 50 μm^2 are randomly placed over the image (see enclosed figure).
10. Counts of positively staining cells within and on the borders of squares are recorded; cell counts are averaged from a minimum of three images from the same tissue block.
11. To increase statistical validity, cell counts should be averaged with other tissue blocks obtained from the same and experimental repeat sampling sources; note intensity of staining cannot be recorded since level of staining can vary between sections depending upon length of incubation with substrate.



Example of cellular osteo-pontin synthesis detected in healing bone stained black by immunocytochemistry.

techniques, the effect of new tissue repair therapies, and the effect of new bioactive agents (such as antimicrobial agents or anti-inflammatory agents). Many popular composite placement techniques, particularly in relation to operatively exposed dentine, are not evidence based and defy common sense. These are mainly "hang overs" from previous amalgam placement techniques. An important example of such confusion in contemporary clinical dentistry is the dilemma of "bonding" or "basing" composite restorations. Following a technique used with amalgam placement, many practitioners choose to place a "base" cement under a composite restoration to "protect" operatively exposed dentin at the expense of the mechanical properties of the completed restoration. However, a growing number of practitioners choose not to use a "base" cement and instead simply "bond" the composite in place without using a cement base. There is no real reliable evidence, biological or clinical, to support the merit of either technique. Therefore, patients are potentially exposed to risk. Clear elucidation of the biological effects of such materials via appropriate model systems is required for proper investigation. The opportunity also exists to explore the development and selection of materials, which can drive dental tissue repair via studying material/dentine interactions in appropriate dentine/pulp complex models. The ultimate aim of such an approach is to improve treatment outcomes for patients and retain tooth viability.

Models for dental tissue regeneration

In vivo, the mineralised dentine, the odontoblast cells, and the pulpal soft connective tissue are considered a cooperative functional complex. Various attempts have been made to culture odontoblasts and other cells of the pulp *in vitro* (Nakashima et al., 1991; Bègue-Kirn et al., 1992, 1994), but these and other previous attempts to culture odontoblasts *in vitro* have demonstrated the need to maintain direct contact between those cells and the dentine to preserve the cell's phenotypic morphology (Munksgaard et al., 1978; Heywood and Appleton, 1984). The importance of the dentine, especially the bioactive proteins contained within it, was clearly demonstrated by the successful culture of mouse dental papillae with a dentine matrix extract (Bègue-Kirn et al., 1992, 1994). Contact between the papillae and the dentine matrix extract led to differentiation of an odontoblast cell population from the papillae cells that were in contact with the dentine extract. These cells were also able to synthesise a new dentine matrix. In those culture systems, the papillae was embedded in a semisolid agar-based medium prior to being cultured at the liquid-gas interface, and it was this successful organ culture method that was modified to develop a culture model system for the mature dentine-pulp complex.

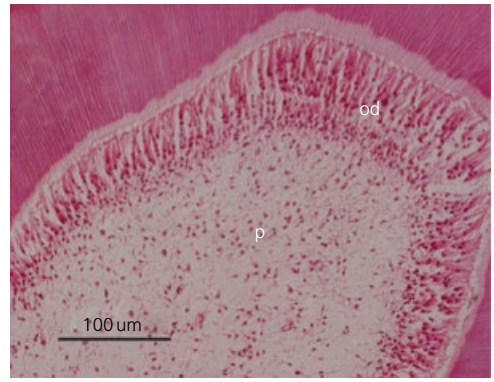


Figure 5.1 Histological appearance of a rodent tooth slice cultured for 14 days demonstrating maintenance of cell and tissue architecture and viability. Pulpal cells (p) and the odontoblast cell layer (od) remain viable during culture.

The culture of the dentine-pulp complex of 28-day-old male Wister rat incisor teeth (Sloan et al., 1998) when embedded in a semisolid agar-based medium and cultured in Trowel-type cultures at the liquid-gas interface allowed for culture of the tissue successfully for up to 14 days with maintenance of the tissue architecture of the entire tissue complex during culture period (Figure 5.1). An *in vitro* model of human dental tissue repair has also been developed, whereby thick tooth slices have been cultured in liquid media (Magloire et al., 1996; Melin et al., 2000). Such tissue organotypic culture models now facilitate the investigation of dentinogenesis and tissue repair mechanisms, as the odontoblasts can be examined within the normal environment of the dentine-pulp complex, but in the absence of the normal inflammatory processes that occur *in vivo*. This tooth slice culture system (Protocol 5.1) has been pivotal in understanding the bioactive nature of the dentine matrix and the role of TGF-β1, BMP-7, and other growth factors in directing reparative events in dental tissue repair (Sloan and Smith, 1999;

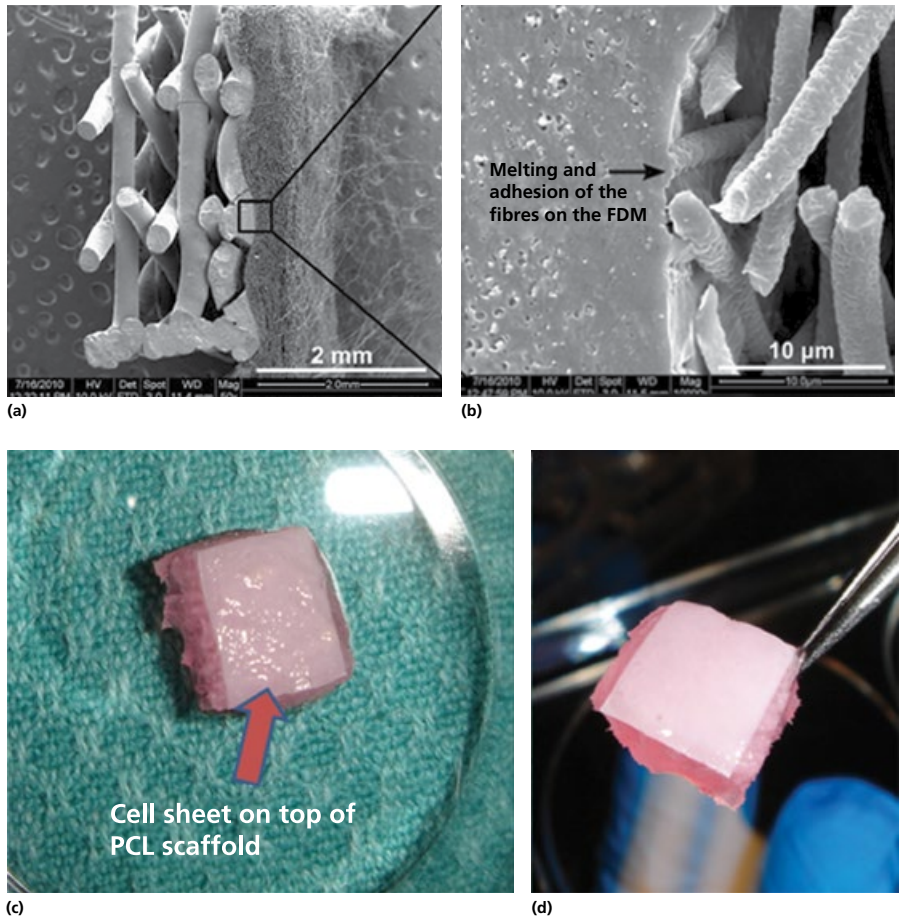


Figure 7.4 Biphasic scaffold (a) demonstrating excellent integration between periodontal ligament and bone compartments (b). Tissue-engineered construct with a periodontal ligament cell sheet placed on periodontal side of biphasic PCL scaffold (c), ready for insertion into periodontal defect (d).

Another approach has been to utilise PCL-HA (90:10 wt%) scaffolds, which were fabricated using three-dimensional printing in three phases: 100-µm microchannels in Phase A designed for cementum/dentine interface, 600-µm microchannels in Phase B designed for the PDL, and 300-µm microchannels in Phase C designed for alveolar bone (Lee et al., 2014). Recombinant human amelogenin, connective tissue growth factor, and bone morphogenetic protein-2 were delivered in Phases A, B, and C, respectively. Upon 4-week *in vitro* incubation with either dental pulp stem/progenitor cells, PDL stem/progenitor cells, or alveolar bone stem/progenitor cells, distinctive tissue phenotypes were formed in each compartment. The strategy used for the regeneration of multiphase periodontal tissues in this study involved the spatiotemporal delivery of multiple proteins. Using this method, it was shown that a single stem/progenitor cell population appeared to differentiate into putative cementum, PDL, and alveolar bone complex by using the scaffold's biophysical properties, combined with spatially released bioactive cues.

Tissue-engineered decellularised matrices and periodontal regeneration

The use of decellularised matrices as a biologic scaffold is gaining increasing attention in regenerative medicine. The rationale of using this approach is to produce three-dimensional scaffolds that mimic natural tissue's composition, microstructure, and biological and

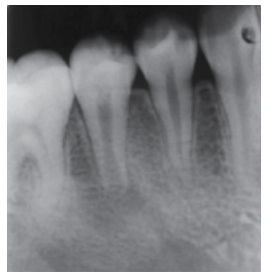
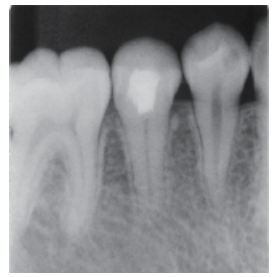
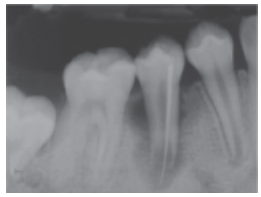
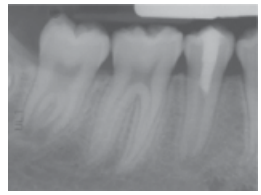
Case report	Pre-operative status	Post-operative status
Iwaya S. et al., 2001		 30 month recall
Banchs F and Trope M, 2004		 18 month recall

Figure 8.3 Two case reports showing successful clinical outcomes using REPs. Both cases reported immature permanent premolar teeth diagnosed with pulp necrosis. Large radiolucent areas are seen surrounding the apex and roots of both teeth, suggesting advanced apical periodontitis in the preoperative radiographs. Teeth were treated with revascularisation procedures, resulting in complete resolution of signs and symptoms of disease. Complete radiographic healing of apical periodontitis is seen in the postoperative radiographs. (Modified from Iwaya et al., 2001, and Banchs and Trope, 2004. Reproduced with permission from Wiley and Elsevier.)

Textbox 8.1 A recommended clinical protocol for regenerative endodontics.

First treatment visit for regenerative endodontics

1. Informed consent, including explanation of risks and alternative treatments or no treatment.
2. After ascertaining adequate local anesthesia, dental dam isolation is obtained.
3. The root canal systems are accessed and working length is determined (radiograph of a file loosely positioned at 1 mm from root end).
4. The root canal systems are slowly irrigated first with 1.5% NaOCl (20 mL/canal, 5 min) and then irrigated with saline (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end.
5. Canals are dried with paper points.
6. Calcium hydroxide, or DAP or TAP (0.1–1 mg/mL) is delivered to canal system.
7. Access is temporarily restored.

Final (second) treatment visit for regenerative endodontics

1. A clinical exam is first performed to ensure that there is no moderate to severe sensitivity to palpation and percussion. If such sensitivity is observed, or a sinus tract or swelling is noted, then the treatment provided at the first visit is repeated.
2. After ascertaining adequate local anesthesia with 3% mepivacaine (no epinephrine), dental dam isolation is obtained.
3. The root canal systems are accessed; the intracanal medicament is removed by irrigating with saline (20 mL) followed by 17% EDTA (10 mL/canal, 5 min).
4. The canals are dried with paper points.
5. Bleeding is induced by rotating a precurved K-file size #25 at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cementoenamel junction.
6. Once a blood clot has formed, a premeasured piece of Collaplug (Zimmer Dental Inc., Warsaw, IN) is carefully placed on top of the blood clot to serve as an internal matrix for the placement of approximately 3 mm of white MTA (Dentsply, Tulsa, OK) or Biodentine (Septodont, France).
7. A 3–4 mm layer of glass ionomer (e.g., Fuji IX, GC America, Alsip, IL) is flowed gently over the MTA.
8. A bonded reinforced composite resin restoration is placed over the glass ionomer.
9. The case needs to be followed up at 3 months, 6 months, and yearly after that for a total of 4 years.

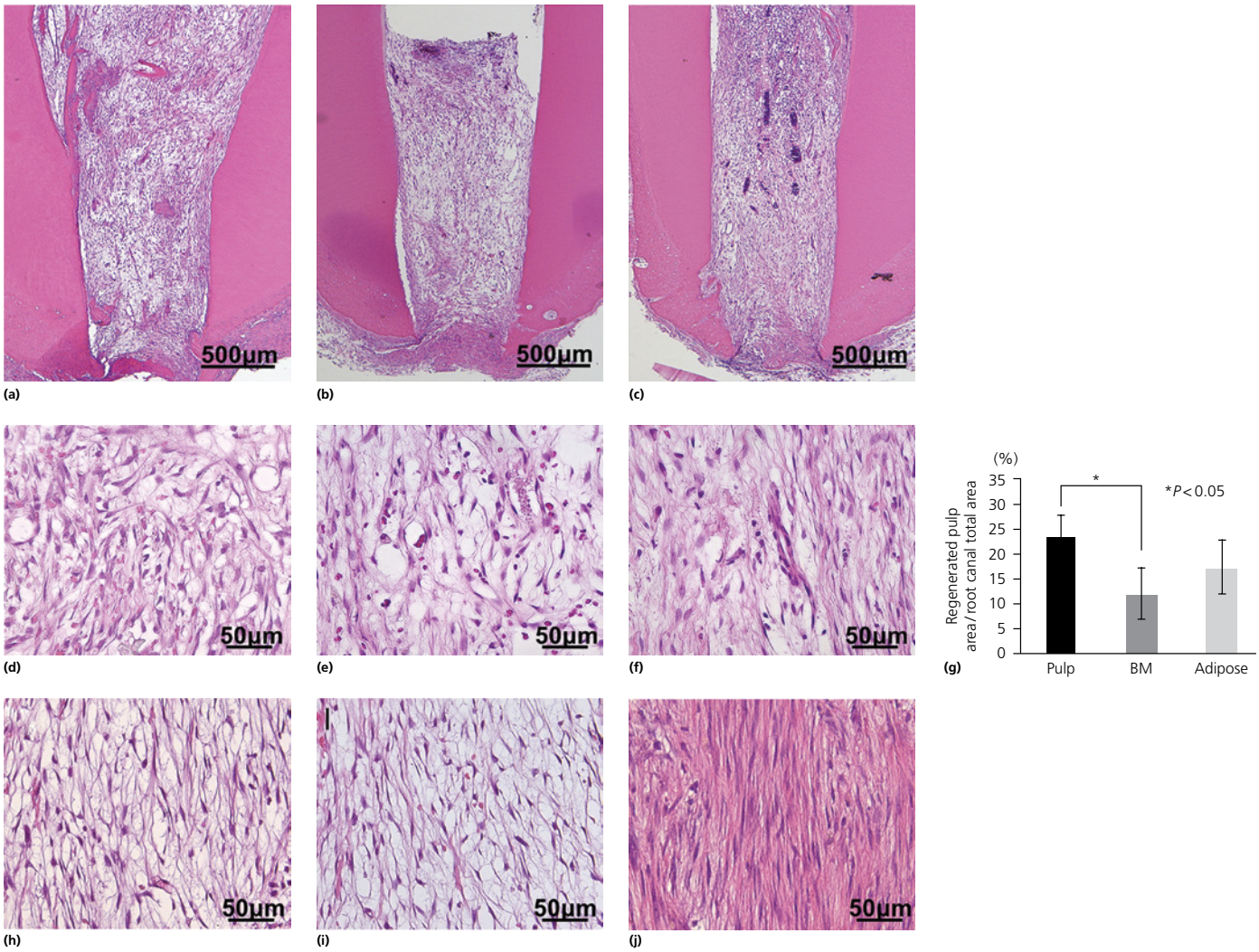


Figure 8.5 Complete regeneration of pulp tissue after autologous transplantation of CD31– SP cells with SDF-1 in the emptied root canal after pulpectomy in dogs. (a, d, and h) Pulp CD31– SP cell transplantation. (b, e, and i) Bone marrow CD31– SP cell transplantation. (c, f, and j) Adipose CD31– SP cell transplantation. (a–g) 14 days, (h–j) 28 days after transplantation. (j) Enhanced matrix formation. (g) Ratio of regenerated area to root canal area. Data are expressed as means ± SD. (From Nakashima and Iohara, 2014. Reproduced with permission from Elsevier.)

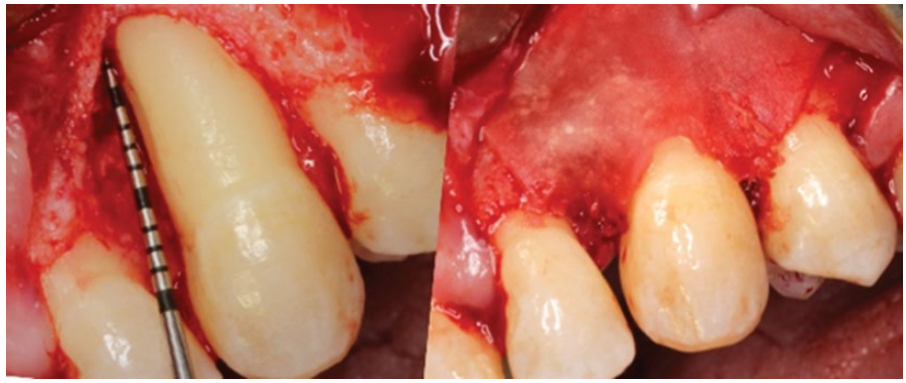


Figure 8.6 Intraoral clinical view following flap elevation showing significant bone loss due to periodontal disease. In this case, a resorbable collagen membrane in combination with a calcium phosphosilicate (bioglass) bone substitute was utilised for periodontal regeneration to enhance the long-term prognosis of this maxillary canine. (Clinical case courtesy of Dr. George Kotsakis, University of Washington, Seattle, WA.)

Numerous periodontal regeneration techniques have been developed and evaluated with overall successful clinical outcomes (Nabers and O'Leary, 1965; Anderegg et al., 1991; Blumenthal, 1993; Heijl et al., 1997; McClain and Schallhorn, 2000; Scheyer et al., 2002; Nevins et al., 2005). These treatment modalities may incorporate the use of bone grafting materials, barrier membranes, and growth factors/matrix proteins as well as their combination to achieve true regeneration of the periodontium. Guided tissue regeneration and osseous grafting are the most extensively evaluated techniques that also have histological documentation (Dragoo and Sullivan, 1973; Nyman et al., 1982; Bowers et al., 1989; Bowers et al., 1989; Bowers et al., 1989). Histological evaluation of the regeneration outcome is crucial to confirm the presence of truly regenerated tissue; however, ethical limitations often impede harvesting periodontal tissues from humans to provide evidence for true periodontal regeneration. The majority of the published evidence on periodontal regeneration deals with clinical outcomes such as clinical attachment level (CAL), pocket depth (PD) reduction, and bone fill (BF) as surrogate endpoints for treatment. Despite the lack of histological support, these criteria are clinically important and have been shown to be associated with tooth survival (Kao et al., 2014).

Historically, attempts for periodontal regeneration have been reported since the 1950s (Prichard, 1957). Autogenous grafts were initially investigated for regenerating bone in human periodontal defects (Schaffer, 1958; Nabers and O'Leary, 1965; Schallhorn, 1968; Dragoo and Sullivan, 1973). Schallhorn and coworkers were among the first to show clinical reattachment in periodontal bone defects after the implantation of autogenous iliac bone. Histological analysis in these defects revealed a true reattachment with osteogenesis, cementogenesis, and new PDL formation (Schallhorn, 1968; Dragoo and Sullivan, 1973). Subsequently, Bowers et al. (1989) performed a three-part human study and compared regeneration of intrabony defects in submerged or nonsubmerged environments with or without the use of decalcified freeze-dried bone allograft (DFDBA). Histological results showed that grafted areas had significantly greater periodontal regeneration than nongrafted areas (Bowers et al., 1989). Moreover, regeneration of the periodontal apparatus was far greater in submerged sites when compared with nonsubmerged sites, which indicated that secluded defects that were protected from the microbial challenge of the oral environment were advantageous in terms of regeneration (Bowers et al., 1989). Collectively, landmark human studies have provided substantial histological evidence confirming beyond any doubt that true periodontal regeneration is feasible under clinical conditions following appropriate surgical technique and careful selection of biomaterials (Cortellini and Bowers, 1995). The following section will focus on current periodontal regenerative techniques and biomaterials.

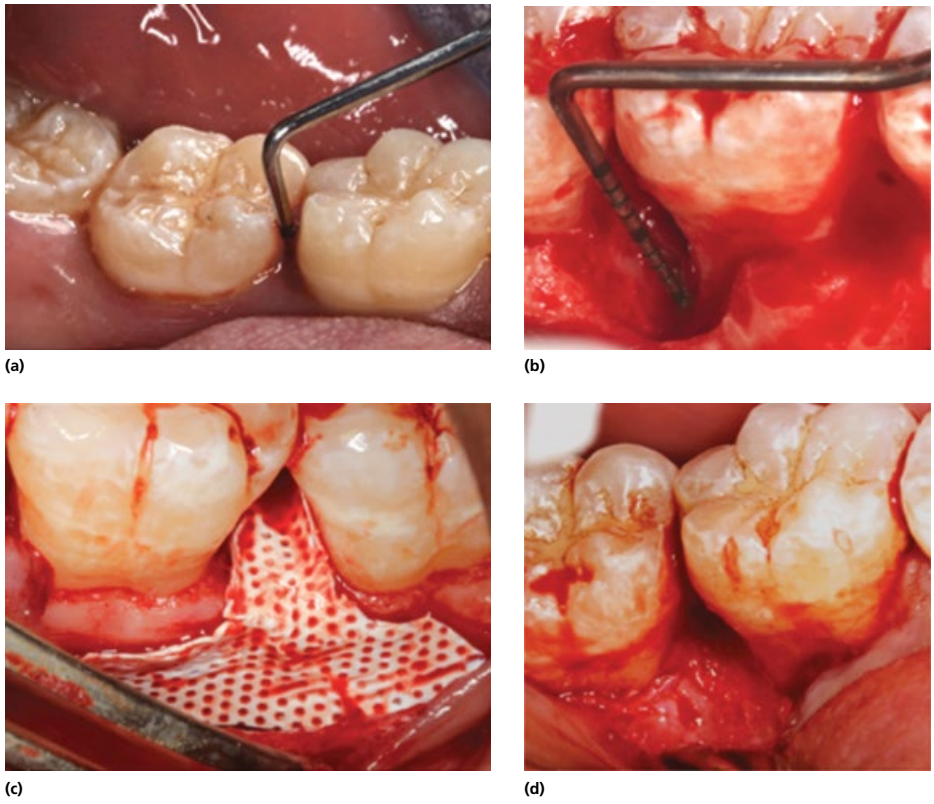


Figure 8.7 Series of intraoral clinical photographs showing (a) 12 mm probing depth in the distal region of the lower left 1st mandibular molar; (b) combination of a two-wall intrabony defect with a three-wall component at the base of the defect; (c) placement of a nonresorbable barrier membrane through the buccal aspect to provide space maintenance for regeneration in combination with a bone substitute; and (d) 6-month reentry surgical procedure. Note the newly formed tissue covering approximately 65% of the original defect volume. (Clinical case courtesy of University of Washington, Seattle, WA.)

their modulation by local environmental factors, will likely pave the road for the future generation of regenerative procedures.

References

ACCORINTE MDE, L., HOLLAND, R., REIS, A., BORTOLUZZI, M. C., MURATA, S. S., DEZAN, E. JR., SOUZA, V., & ALESSANDRO, L. D. 2008. Evaluation of mineral trioxide aggregate and calcium hydroxide cement as pulp-capping agents in human teeth. *J Endod*, 34, 1–6.

AICHELMANN-REIDY, M. E., & REYNOLDS, M. A. 2008. Predictability of clinical outcomes following regenerative therapy in intrabony defects. *J Periodontol*, 79, 387–93.

ALPAR, B., LEYHAUSEN, G., GUNAY, H., & GEURTSEN, G. 2000. Compatibility of resorbable and nonresorbable guided tissue regeneration membranes in cultures of primary human periodontal ligament fibroblasts and human osteoblast-like cells. *Clin Oral Investig*, 4, 219–25.

ALTHUMAIRY, R. I., TEIXEIRA, F. B., & DIOGENES, A. 2014. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. *J Endod*, 40, 521–5.

ANDEREGG, C. R., MARTIN, S. J., GRAY, J. L., MELLONIG, J. T., & GHER, M. E. 1991. Clinical evaluation of the use of decalcified freeze-dried bone allograft with guided tissue regeneration in the treatment of molar furcation invasions. *J Periodontol*, 62, 264–8.

ANDREASEN, J. O., BORUM, M. K., JACOBSEN, H. L., & ANDREASEN, F. M. 1995. Replantation of 400 avulsed permanent incisors. 2. Factors related to pulpal healing. *Endod Dent Traumatol*, 11, 59–68.

ATILLA, G. 1993. A rare find in Anatolia: a tooth implant (mid-sixth century B.C.). *J Oral Implantol*, 19, 54–7.

BANCHS, F., & TROPE, M. 2004. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? *J Endod*, 30, 196–200.