Stem Cell Biology and Regenerative Medicine

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Dental Stem Cells

Humana Press

Stem Cell Biology and Regenerative Medicine

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Springer

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ISSN 2196-8985 ISSN 2196-8993 (electronic)
Stem Cell Biology and Regenerative Medicine
ISBN 978-3-319-28945-8 ISBN 978-3-319-28947-2 (eBook)
DOI 10.1007/978-3-319-28947-2

Library of Congress Control Number: 2016931885

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

Preface

Stem cells are a class of undifferentiated master cells that have robust self-renewal kinetic and differentiation potential into many specialized cell types in the body.

Stem cell research has been a field of great clinical interest with immense possibilities of using the stem cells to replace, restore, or enhance the biological function of damaged tissues and organs due to accidents, diseases, and/or developmental defects.

Recent studies have demonstrated that mesenchymal stem cells (MSCs) are found in various tissues in an adult organism. MSCs derived from teeth and supporting tissues, called dental stem cells (DSCs), have been mainly characterized into five different cell types including dental pulp stem cells (DPSCs), dental follicle stem cells (DFSCs), periodontal ligament stem cells (PDLSCs), stem cells from human exfoliated deciduous teeth (SHEDs), and stem cells from the apical papilla (SCAPs).

The knowledge of stem cell technology is moving extremely fast in both dental and medical fields. Advances in DSC characterization, standardization, and validation of stem cell therapies and applications have been leading to the development of novel therapeutic strategies.

Several investigators, especially those who have made significant contribution to the field of DSC research, have been invited to create this book. With the help of their intense and substantive efforts, this book reviews different aspects, challenges, and gaps of basic and applied dental stem cell research, cell-based therapies in regenerative medicine concentrating on the application and clinical use, and recent developments in cell programming and tissue engineering. This review will be useful to students, teachers, clinicians, and scientists, who are interested or working in the fields of biology and medical sciences related to dental stem cell therapy and related practices.

Istanbul, Turkey — Fikrettin Şahin

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Chapter 1
Dental and Craniofacial Tissue Stem Cells: Sources and Tissue Engineering Applications

Paul R. Cooper

Abbreviations

ADSCs	Adipose stromal/stem cells
BMP	Bone morphogenetic protein
BMMSCs	Bone marrow stromal cells
DFSCs	Dental follicle stem cells
DSCs	Dental stem cells
DPSCs	Dental pulp stem cells
EGF	Epidermal growth factor
DMP1	Dentin matrix protein 1
DSPP	Dentin sialophosphoprotein
ESC	Embryonic stem cell
FBS	Fetal bovine serum
ECM	Extracellular matrix
FGF	Fibroblast growth factor
GMP	Good manufacturing practice
GMSCs	Gingiva-derived MSCs
HERS	Hertwig's epithelial root sheath
HS	Human serum
IEE	Inner enamel epithelium
iPSC	Induced pluripotent stem cell
OEE	Outer enamel epithelium
OESCs	Oral epithelial progenitor/stem cells
PDL	Periodontal ligament

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F. Şahin et al. (eds.), *Dental Stem Cells*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-3-319-28947-2_1

PDLSCs	Periodontal ligament stem cells
PSCs	Periosteum-derived stem cells
SCAPs	Stem cells from apical papilla
SGSCs	Salivary gland-derived stem cells
SHEDs	Stem cells from human exfoliated deciduous teeth
Shh	Sonic hedgehog
SR	Stellate reticulum
TGF-β	Transforming growth factor-β
TGPCs	Tooth germ progenitor cells
TMJ	Temporomandibular joint
VEGF	Vascular endothelial growth factor

1.1 Introduction

Stem cells are present in many tissues throughout the body and at the different developmental stages of the organism. They are reported to reside in specific areas within each tissue, in a so called "stem cell niche". They are also frequently described as being located within close proximity to the vasculature, *i.e.* in a perivascular niche [1–3], and this anatomical localisation may facilitate their rapid mobilisation to sites of injury [4]. Stem cells have been characterised based on their abilities to self-renew, along with their multi-lineage differentiation capabilities which enable complex tissue regeneration [5]. They have varying degrees of potency ranging from totipotent, pluripotent, multipotent through to unipotent. Totipotent stem cells are derived from the zygote, and can form embryonic and extra-embryonic tissues, including the ability to generate the placenta [6]. Pluripotent stem cells include embryonic stem cells (ESCs), and are derived from the inner cell mass of the developing blastocyst. Notably, ESCs can differentiate into the three main germ layers of the organism including the endoderm, mesoderm and ectoderm. Postnatal/adult stem cells are regarded as being multipotent and include populations of hematopoietic and mesenchymal stem cells (MSCs). They are capable of differentiating toward several germ layer lineages giving rise to cell types which are necessary for natural organ and tissue turn-over and repair. In addition, along with these naturally present stem cell types, induced pluripotent stem cells (iPSCs) have been generated within laboratory settings by transcriptional reprogramming of somatic cells. Notably, sources of these somatic cells have included ones of oral and dental origin. iPSCs are reprogrammed to an embryonic-like state and hence are pluripotent and can differentiate into cells of all three germ layers [7, 8].

The dental and craniofacial tissues are known to be a rich source of MSCs which are relatively easily accessible for dentists. Stem cell populations which have been identified and characterised within these tissues include dental pulp stem cells (DPSCs) [9], stem cells from the apical papilla (SCAPs) [10–12], dental follicle

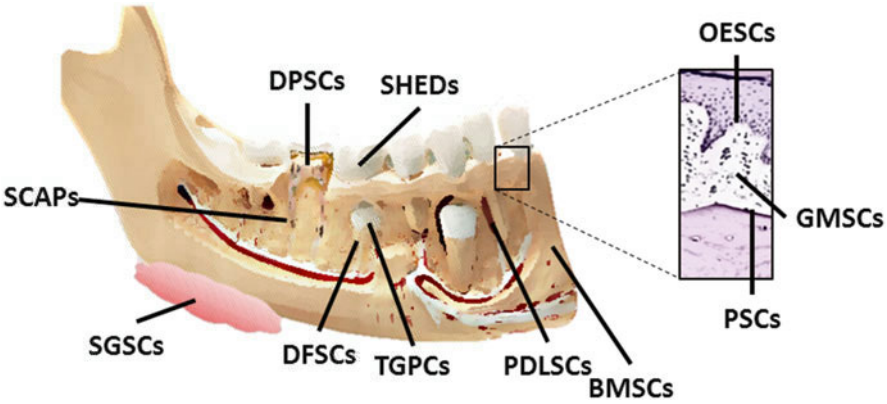

Fig. 1.1 The locations of developmental and postnatal stem cell populations in the dental and craniofacial region indicating sources for isolation from the mandible and teeth. The *insert* (*to the right*) shows the histology of the overlying masticatory mucosa (including oral epithelium, submucosa and bone tissue) and indicates the locations of the stem cell populations within it. Further details on all the stem cell populations shown are provided in the main text body. Abbreviations used are: BMMSCs—bone marrow-derived mesenchymal stem cells (MSCs) from mandible (also maxilla); DPSCs—dental pulp stem cells; SHEDs—stem cells from human exfoliated deciduous teeth; PDLSCs—periodontal ligament stem cells; DFSCs—dental follicle stem cells; TGPCs—tooth germ progenitor cells; SCAPs—stem cells from the apical papilla; OESCs—oral epithelial progenitor/stem cells; GMSCs—gingiva-derived MSCs; PSCs—periosteum-derived stem cells; SGSCs—salivary gland-derived stem cells

precursor cells (DFSCs) [13–16], periodontal ligament stem cells (PDLSCs) [17, 18], stem cells from human exfoliated deciduous teeth (SHEDs) [19] and tooth germ progenitor cells (TGPCs) [20]. Furthermore, the presence of other, perhaps as yet less well characterised stem cell types within the orofacial region have been reported including oral epithelial progenitor/stem cells (OESCs) [21], gingiva-derived MSCs (GMSCs) [22, 23], periosteum-derived stem cells (PSCs) [24] and salivary gland-derived stem cells (SGSCs) [25–27]. In addition, well characterised MSCs which are not exclusive to the oral and craniofacial tissues, include bone marrow-derived MSCs (BMMSCs) [28], which can be harvested from maxilla and mandibular bone, as well as adipose tissue-derived stem cells (ADSCs) [29]. These stem cell populations and their isolation and application will be discussed in greater detail in the following sections. Figure 1.1 pictorially shows the dental and craniofacial locations of these stem cell groups.

The oral and dental stem cell (DSC) populations are defined as MSCs according to the minimal criteria proposed by the International Society for Cellular Therapy (ISCT) in 2006 [30]. The criteria defining them, which are tissue independent, include their ability to adhere to standard tissue cultureware along with their expression profile of Cluster of Differentiation (CD) and other markers. According to the ISCT, MSCs should express CD105, CD73 and CD90 but lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR cell surface molecules.

More recently, the expression of other cell surface markers for human MSCs, including CD271 and MSC antigen-1, have been reported [31, 32]. The presence of (or lack of) combinations of these markers are not only used to define stem cell populations but are also used for their isolation, although across species, this may not be entirely reproducible. Further defining criteria from the ISCT state that MSCs must be capable of differentiating into osteogenic, adipogenic and chondrogenic lineages in vitro [33].

The harvesting of MSCs from postnatal dental, craniofacial and other tissues is not always straightforward and this can be hampered by these cells being present at relatively low frequencies within tissues, *i.e.* <1 % of the total cell population. The simplest approach for isolating postnatal MSCs utilises their ability to adhere to cultureware which was initially demonstrated for BMMSCs [34]. This approach has also been used for craniofacial and dental MSCs, and generates a heterogeneous population of cells which exhibit the MSC-like properties of clonogenicity and a high proliferative capacity [9, 19]. However, frequently reported in the literature is the increasing use of fluorescence-activated cell sorting (FACs) and magnetic activated cell sorting (MACs) approaches [35]. These methods enable the isolation of cells from dissociated tissue which are positive and/or negative for many of the defining markers previously described. For DPSC isolation, several studies have applied positive selection for a range of different markers including STRO-1, CD105, c-kit, CD34 and low-affinity nerve-growth-factor receptor (LNGFR) with negative selection for CD31 and CD146 [36–40]. These studies indicate that the dental pulp likely contains several different MSC populations/niches, and this is also probably true for other dental and craniofacial tissues. It should, however, be noted that selection of MSCs using STRO-1, CD146 and pericyte-associated antigen also supports the premise that perivascular niches exist in a variety of tissues throughout the body including those from the dental and craniofacial regions [9, 11, 19, 41].

Recent work has also built upon the cultureware adhesion approach initially reported for BMMSC isolation with studies now demonstrating that several MSC-types can be derived via selective adhesion to cultureware surfaces coated with extracellular matrix (ECM) derived molecules. This potentially biomimetic approach may be based on the in vitro recapitulation of the niche environment whereby MSCs in vivo are maintained in a quiescent state by the ECM until released and activated during tissue disease or trauma. This MSC selection technique has been shown to be successfully applied using ECM-derived proteins such as fibronectin, type I collagen, type II collagen, vitronectin, laminin and poly-L-lysine [42–45].

It is also notable that isolated cells may not always be of a pure population and may be somewhat heterogeneous in nature, subsequently representing various differentiation states. It remains unclear, and is under considerable debate, as to whether a pure population of cells is indeed needed for therapeutic application, as within tissues stem cells interact with a variety of other cell types to enable repair. Further confounding this issue is the fact that MSCs are derived from different donors, *e.g.* age range and sexes, and isolated cells may subsequently respond differently in vitro and in vivo [28]. Current research, therefore, aims to identify the

most appropriate isolation conditions which will enable predictable clinical application and outcomes.

Over the coming years within the dental field, stem cells combined with tissue engineering strategies are expected to provide novel therapeutic approaches to regenerate teeth or tooth component tissue and for repair of defects in periodontal tissues and alveolar bone. Specific oral tissues and organs which are already being targeted for regenerative medicine strategies include the salivary glands, tongue, craniofacial skeletal muscles, and component structures of the temporomandibular joint. The properties and characteristics of craniofacial and dentally relevant MSCs are subsequently discussed below as is dental tissue development, tissue engineering and clinical application progress.

1.2 Dental Tissue Development and Repair

In general, the development of many organs requires heterologous cell and tissue interactions. For tooth development these interactions occur between the ectodermally-derived enamel organ epithelium and cranial neural crest–derived ectomesenchyme. These epithelial-mesenchymal interactions also underpin the development and morphogenesis of many other human organs including hair, mammary gland and salivary glands. Significant work over recent years has shown that complex growth and transcription factor signalling are critical to coordinate these cellular events [46]. Gene and protein expression profiles are tightly regulated throughout all stages of tooth development, and the signalling networks generated are similar to those found in the development of other organs. Notably, it is these networks which are reactivated during many repair and regeneration events later on in life. Indeed, recent studies have now made significant in-roads into the characterisation of these intracellular signalling cascades essentially for coordinating tooth development [47].

The initiation stage of tooth development is characterized by the formation of the dental lamina and this occurs at around the fifth week of human gestation [10th embryonic day (ED 10) of mouse development]. During this stage, a variety of cellular and molecular events occur which determine tooth type, position and orientation within the developing jaws. Subsequently, the dental epithelium begins to proliferate to give rise to a narrow horseshoe-like ribbon of cells termed the dental lamina, and their morphology reflects the future position of the dental arches. Embryonic epithelial thickenings (ectodermal/dental placodes) of the dental lamina subsequently develop which are the first morphological indications of teeth and precede the local appearance of an ectodermal organ. Many growth factors and signalling molecules such as fibroblast growth factors (FGFs), Paired box's (PAXs), WNTs, sonic hedgehog (SHH), msh homeobox's (MSXs), distal-less homeobox's (DLXs) and bone morphogenetic proteins (BMPs) are the main regulators of this process which provide the relevant positional information for dental placode development [48, 49].

The dental epithelium continues to proliferate and begins to invaginate into the ectomesenchyme, and forms tooth buds with the dental placodes continuing to secrete potent signalling molecules [50–52]. Subsequently, at 20 locations in the human dental lamina, at around weeks 7–9 of human gestation and mouse (ED 11–11.5), the epithelial cells begin to proliferate and intrude into the mesenchyme to give rise to an early bud stage structure. The ectomesenchymal cells proliferate and accumulate around each epithelial bud, and the innermost cells of the epithelial develops a star-like morphology with the onset of synthesis and secretion of glycosaminoglycans. This structure becomes hydrated resulting in the cells becoming more widely distributed with this internal area of the tooth bud now containing the stellate reticulum and the intermediate layer. During the bud stage of tooth development, the odontogenic potential no longer resides with the epithelium but is driven by the ectomesenchyme [53].

The tooth bud becomes transformed into a cap-like structure by differential proliferation and infolding of the epithelium. The local mesenchymal cells begin to secrete a range of ECM molecules, such as tenascin and syndecan, which bind to, and increase the local concentrations of growth factors. The inductive signalling results in differential multiplication of the epithelial layer with concomitant transformation of the tooth bud into a pyramid-like structure with the dental lamina at its tip which marks the future site of the tooth crown. Evidence indicates that BMP4 is key to the mesenchymal signalling that induces transition from bud to cap stage due to its regulation of several key transcription factors. Subsequently, an epithelial mass, the enamel knot, within the central base of this structure develops, and this reportedly acts as a transient organizer of the morphogenetic signalling for adjacent cells via its expression of FGFs. The enamel knot is removed via apoptosis at the end of the cap stage and is entirely lost by the time of the bell stage [54–56]. The epithelium expands and folds inside the core of the bud in an anterior to posterior manner and the whole structure begins to resemble an upturned cap. The inner enamel epithelium (IEE) is found internally within the cap while the outer structure is covered by the outer enamel epithelium (OEE). Between the IEE and OEE sheets are vacuolised cells of the stellate reticulum and an intermediate cell layer which is referred to as the enamel or dental organ. The condensed mesenchymal tissue within the IEE and between the cervical loop (outer rim of the entire structure) is the dental papilla which develops into the future dental pulp tissue. The condensed mesenchyme surrounding the dental papilla and dental organ is the dental follicle which gives rise to the cementoblasts, osteoblasts and fibroblasts of the periodontal ligament [57].

Cup position and height are tooth- and species-specific; therefore, correct spacing and size are accurately regulated in multicuspid teeth via primary and secondary enamel knots. Indeed, secondary knot formation marks the onset of the bell stage of tooth development and the IEE continues infolding according to the organising signals that they express. The IEE subsequently displaces the stellate reticulum, and the structure acquires the form of a bell. At this point, the dental mesenchyme does not appear to be undergoing cell proliferation, and the enamel organ is separated from the dental papilla, with the tooth cusps starting to form and the crown height

increasing. Crown morphogenesis and cytodifferentiation occur during the bell stage with the cells differentiating in situ to give the crown its final shape [58–61]. Subsequently, the mesenchymal cells bordering the dental papilla are attached to the basement membrane of the IEE, and they take on a polarised columnar form and differentiate into the odontoblasts which secrete the predentine. Immediately following the deposition of the predentine the basement membrane breaks down and subsequent signalling leads to cells of the IEE, which are in contact with the predentine, differentiating into polarised columnar ameloblasts which begin their synthesis of enamel. Mineralization occurs and converts the predentine to dentine, and further secretion of predentine results in the odontoblasts receding from the dentino-enamel junction. The odontoblasts leave cellular processes within dentinal tubules as they traverse towards the pulp core. The two hard tissues of the tooth matrix, the enamel and dentine, are characterised by their apposition of hydroxyapatite crystal. Notably, the basal cells of the intermediate layer support the process of enamel formation and following tooth eruption transform into the junctional epithelium. The dental lamina disintegrates, and the pulp and enamel organ are encased in a condensed mesenchyme, which constitutes the dental follicle which ultimately gives rise to cementoblasts, osteoblasts and fibroblasts [62, 63].

A multitude of genes have been identified as being active during tooth development and morphogenesis which indicate the complexity of the process. Our increased understanding of these molecular and cellular events is necessary to underpin the development of future stem cell-based therapies for bio-tooth engineering.

1.2.1 Dentinogenesis

Whilst primary dentinogenesis occurs at a rate of ~4 μm/day during tooth development, namely secondary dentinogenesis continues to occur at ~0.4 μm/day following tooth root formation throughout the life of the tooth. Tertiary dentinogenesis refers to the process of repair and regeneration in the dentine–pulp complex which represents a natural wound healing response. Following relatively mild dental injury, such as during early stage dental caries, primary odontoblasts are reactivated to secrete a reactionary dentine which is tubular and continuous with the primary and secondary dentin. However, in response to injury of a greater intensity, *e.g.* a rapidly progressing carious lesion, the primary odontoblasts die beneath the lesion. Subsequently, if conditions are appropriately conducive, *e.g.* caries is arrested; the stem/progenitor cells within the pulp are signalled to home to the site of injury and differentiate into odontoblast-like cells. These cells deposit a tertiary reparative dentine matrix resulting clinically in dentine bridge formation walling off the dental injury. Clearly, the relative complexity of these two tertiary dentinogenic processes differ with reactionary dentinogenesis somewhat more simply requiring only the up-regulation of existing odontoblast activity, whereas reparative dentinogenesis involves recruitment, differentiation, as well as up-regulation of dentine synthetic

and secretory activity. It is understood that tertiary dentine deposition rates somewhat recapitulate those of development and are also reported to be ~4 μm/day. Notably, tertiary dentinogenic events are understood to be signalled by released bioactive molecules similar to those present during tooth development which were initially sequestrated within the dentine during its formation [64–66]. Indeed, an array of molecules are bound within dentine and are known to be released from their inactive state by carious bacterial acids and restorative materials, such as calcium hydroxide, and are known to stimulate dentine bridge formation. At the stem cell level, released dentine matrix components may stimulate cell proliferation and expansion, recruitment to the site of injury, differentiation into odontoblast-like cells and the up-regulation of synthetic and secretory activity. Indeed, prime candidate signalling molecules for stimulating these events come from the BMP and transforming growth factor (TGF)-β superfamilies with TGF-β1 alone being shown to stimulate many of these processes in vitro and in animal models. However, it is likely that synergistic signalling due to many of the bioactive molecules released from the dentine ECM are potent regulators of DSC repair processes in vivo (reviewed in [67, 68]). Notably, however, while it is generally assumed regenerative processes utilises tissue resident cell sources, a mouse parabiosis model has recently demonstrated that progenitor cells can be derived externally to the pulp [69]. The source and properties of stem cells involved in repair and regenerative responses are discussed in Section 1.3.

1.3 Stem Cell Populations

1.3.1 BMMSCs

Originally in 1970, Friedenstein et al. [34] reported the isolation of adherent colony forming cells from bone marrow, and demonstrated their ability to differentiate toward various mesenchymal tissue lineages. In 1999, Pittenger et al. [70] characterized human BMMSCs from the iliac crest, and showed that they could be expanded in culture, and were able to differentiate down osteogenic, adipogenic and chondrogenic lineages. More recent work has gone on to demonstrate BMMSCs also have the capacity to differentiate into non-typical mesenchymal lineages such as ones involved in neurological repair [71]. Perhaps predictably BMMSCs most robustly form bone in vitro and in vivo, indicating their utility in bone regenerative therapy which is frequently exploited clinically in oral and dental procedures. While BMMSCs are generally isolated from bone marrow aspirates derived from the iliac crest during a relatively invasive and painful surgery, they can also be isolated from the maxilla and mandible. These orofacially-derived BMMSCs, derived from cranial neural crest cells, are subsequently likely more applicable for dental treatments although their safe expansion in numbers is required prior to use in therapeutic procedures [72–74].

1.3.2 Adipose Tissue-Derived Stem Cells (ADSCs)

ADSCs can be relatively abundantly harvested via lipectomy or from lipoaspirates from many sites within the adult human body including craniofacial regions. Notably, their harvest generally results in low donor-site morbidity, and the tissue isolated is regarded as clinical waste as liposuction is routinely performed during cosmetic surgery, *e.g.* cheek and chin reshaping. While intrinsically ADSCs exhibit some differences compared with BMMSCs, ADSCs appear to exhibit good mineralised tissue lineage responses, and therefore have potential for use in bone and tooth tissue repair including applications in osseointegration [29]. For dental structures, ADSC transplantation has been used to regenerate pulp tissue and whole teeth containing dentine, with periodontal ligament and alveolar bone attachments in animal models [75–78]. Further work characterising the application of ADSCs for bone, tooth and periodontal tissue regeneration should result in the development of robust protocols which utilise waste fat tissue for clinical application.

1.3.3 Dental Tissue Stem Cells

1.3.3.1 Postnatal Dental Tissue-Derived Stem Cells

A clonogenic and highly proliferative DPSC population exhibiting phenotypic characteristics similar to those of BMMSCs were initially isolated by enzymatic disaggregation of adult dental pulp. Only a few years later, SHEDs were isolated, which were also shown to exhibit the stem cell properties of self-renewal and multi-lineage differentiation potential. In animal studies, DPSCs and SHEDs have demonstrated the ability to generate a mature dentine–pulp-like structure. Further studies using SHEDs have shown that they can induce bone-like matrix formation which may relate to processes that occur in deciduous tooth roots, whereby resorption occurs concurrently with new bone formation. Notably, DPSCs and SHEDs have significant clinical application potential for autologous regenerative treatment approaches as both can be derived from what is regarded as clinical waste tissue. Indeed, DPSCs can be obtained from teeth extracted for orthodontic reasons, whilst SHEDs are harvestable from primary teeth which are naturally exfoliated (reviewed in [79]). Interestingly, up until recently, it was believed that due to the reciprocal interactions which occur between the embryonic oral epithelium and neural crest-derived mesenchyme during tooth morphogenesis, the stem cells from the tooth were derived from a neural crest origin. However, Kaukua et al. [80] recently demonstrated that a significant population of MSCs involved in development, self-renewal and repair of teeth are derived from peripheral nerve-associated glia. While this study was performed in a murine incisor model system, which may limit its relevance to humans, it does, however, indicate our continued need to better understand both tooth development and regeneration events.

The periodontal ligament provides another source of postnatal MSCs in the form of PDLSCs which can also be isolated from extracted waste teeth. Perhaps not surprisingly due to their localisation, PDLSCs have been demonstrated to be able to regenerate several periodontal tissues including cementum, periodontal ligament and alveolar bone in animal studies. However, recent work has indicated that the local derivation of the PDLSCs may significantly influence their differentiation capabilities as PDLSCs from the alveolar bone surface exhibited superior alveolar bone regeneration properties compared with PDLSCs from the root surface [17, 18, 81].

1.3.3.2 Stem Cells Derived from Developing Dental Tissue

Within the developing dental tissues of the dental follicle, including the dental mesenchyme and apical papilla, MSC-like cell populations have been identified. The dental follicle, also termed the dental sac, contains the developing tooth and within it, DFSCs with the ability to regenerate several periodontal tissue types are found [13–16]. At the late bell stage of tooth development, stem cells derived from the dental mesenchyme of the third molar tooth germ have also been identified and these are termed as TGPCs [82]. These isolated MSC-like cells demonstrated a high proliferative capacity along with the requisite capability to differentiate in vitro into the three germ layer lineages. SCAPs [11, 12] have also been identified in developing tooth roots. In comparison with DPSCs, SCAPs have demonstrated increased proliferation rates and enhanced regenerative capabilities for dentine-pulp complex tissue in animal model studies. Furthermore, as these cells exhibit a developmentally immature phenotype and can be isolated from the clinical waste postnatal or adult tissue of extracted wisdom teeth, they could provide a valuable source of autologous stem cells for future regenerative therapies.

1.3.3.3 Oral Mucosal and Periosteum-Derived Stem Cells

The oral mucosa comprises stratified squamous epithelium composed of oral keratinocytes and an underlying connective tissue. The connective tissue consists of a well vascularised lamina propria and a submucosa which can contain minor salivary glands, adipose tissue, neuronal structures and lymphatics. Within the oral mucosa two different types of human postnatal stem cells have been identified; OESCs and GMSCs [21–23]. OESCs are reportedly relatively small oral keratinocytes (<40 mm in diameter) and while being unipotent, they can regenerate oral mucosal tissue ex vivo which may have clinical utility for intra-oral grafts.

GMSCs are reported in the gingival lamina propria which attaches directly to the periosteum of the underlying bone [21]. In addition, a neural crest stem cell-like population has also been isolated from the adult human gingival lamina propria which are termed oral mucosa stem cells (OMSCs) [22]. The relative clinical ease by which relatively high numbers of both GMSCs and OMSCs could be isolated makes these cells promising candidates for use in future clinical therapies.

The periosteum of bone comprises two distinct layers; the outer layer which contains mainly fibroblasts and elastic fibres, while the inner layer contains MSCs along with other progenitor cell populations. Periosteum-derived cells may have preferential application for bone regeneration and subsequently may have application in craniofacial therapies [83–85]. Indeed, locally derived periosteum cells may have particular application for bone repair in procedures such as periosteal flap surgery in conjunction with implant placement along with use in large defect repair procedures [86–88].

1.3.3.4 Salivary Gland-Derived Stem Cells

Salivary glands develop from the endoderm and when mature comprise of acinar and ductal epithelial cells with exocrine function. While the existence of salivary gland stem cells have been proposed following in vivo studies, stem cells that give rise to the entirety of the epithelial cell types present within the gland have yet to be identified [25, 27]. MSC-like cells from human salivary glands have, however, been reported based on their expression of embryonic and postnatal stem cell markers along with their ability to differentiate toward adipogenic, osteogenic and chondrogenic lineages [89–91]. Stem cells isolated from this tissue may have particular application for use in the rescue of dysfunctional gland activity in particular in head and neck irradiated cancer patients who exhibit salivary gland dysfunction [92].

1.3.3.5 Induced Pluripotent Stem Cells (iPSCs)

The possibility of reprogramming somatic cells to an early embryonic development stage by introducing the four transcriptional factors, Oct3/4, Sox2, Klf4 and c-Myc, was initially reported by Takahashi and Yamanaka [93]. Originally, normal mouse adult skin fibroblasts were used and the resultant reprogrammed cells were termed as iPSCs. A year later, this work was replicated using human skin cells which subsequently indicated the potential to generate patient-specific cells with ESC-like characteristics [7, 94]. Indeed, animal studies have demonstrated iPSCs can generate all the tissues and organs of the body. Notably, it has been shown that iPSCs can be derived from many cell types derived from oral and dental tissues which can be relatively easily harvested by dentists. Interestingly, many of these cells have exhibited relative high reprogramming efficiencies which may be explicable as oral and dental MSCs already express relatively high levels of endogenous multipotent transcription factors [82, 95–99]. In the future, the use of oral and dental waste tissue may, therefore, provide an ideal cell source for use in iPSC technology in particular for the regeneration of autologous craniofacial soft and hard tissue structures. Indeed, recent work utilising iPSCs in a mouse model using enamel matrix derived molecules demonstrated increased periodontal tissue regeneration, while in vitro work has demonstrated iPSC application for biotooth-engineering of ameloblast- and odontoblast-like cells [100–102].

Notably, there remain drawbacks with the use of iPSC technology. Much is still to be learned as to how to optimise their generation and reprogramming efficiency as well as in controlling their differentiate fate. A major concern also lies with the risk of tumour formation by iPSCs following clinical implantation. Such a concern arises due to the use of the c-Myc oncogene as a reprogramming factor along with the use of the retroviral insertion system for gene transfer. Recent research, however, may have resolved these issues by using alternative genes for reprogramming along with the application of small reprogramming molecules. Indeed, the use of non-viral components such as proteins, microRNAs, synthetic mRNAs and episomal plasmids is being pioneered. A further clinical concern also arises due to delivery of residual undifferentiated iPSCs remaining amongst the differentiated target cell population. These cells may proliferate uncontrollably and generate teratomas at the site of implantation. To overcome this issue the use of selective ablation approaches to remove teratomas via suicide genes and chemotherapy, as well as the use of antibody-based cell sorting approaches to remove teratoma-forming cells, are being developed [7, 103–113].

1.4 Scaffolds and Morphogens for Stem Cell Tissue Engineering

1.4.1 Scaffolds

For dental and oral tissue engineering strategies, along with stem cells, suitable biomimetic scaffolds and appropriate morphogens/growth factors are required [114]. Clinically, for periodontal tissue repair, material-based guided tissue regeneration (GTR) approaches have been developed. Subsequently, biocompatible or bioinert scaffolds are used to enable connective tissue and bone regeneration from local tissue MSC populations [115–118]. Alveolar bone augmentation approaches, such as guided bone regeneration (GBR), utilise bioactive materials, such as calcium phosphate (CaP)-based biomaterials and collagen-based grafts. While these materials are bioactive and osteoconductive, they are not osteoinductive; hence, scaffolds are being developed, which incorporate bone formation promoting growth factors [119–122].

Fibrous silk protein (fibroin) biomaterial scaffolds are also being developed for their use in tooth and bone repair. These scaffolds can be generated and harvested from silkworms and spiders, and can exhibit properties of controllable porosity, surface roughness and stiffness. They can be further functionally modified to mimic the natural ECM environment to facilitate stem cell recolonization, differentiation and tissue regeneration for therapeutic applications [123–125].

Recent studies have demonstrated the utility of hydrogel scaffolds for tooth tissue engineering applications and their promise is likely based on them exhibiting similar biomechanical properties to pulp tissue. The seeding of pulp derived cells on

collagen scaffolds with subsequent animal implantation has demonstrated the formation of dental tissue structures [126, 127]. Furthermore, DPSCs encapsulated in collagen hydrogels have been shown to differentiate and deposit a mineralised ECM in the presence of natural tissue morphogens [40, 128]. Others have generated pulp-like tissue in vivo following the seeding of SHEDs and human endothelial cells on biodegradable poly-L-lactic acid hydrogel scaffolds [129]. A peptide-amphiphile hydrogel scaffold containing bioactive osteogenic supplements has also been shown to promote differentiation of encapsulated SHED and DPSCs [130]. While challenges still remain, the development of the most appropriate scaffolds which optimise stem cell responses for clinical application is progressing at a rapid rate.

1.4.2 Role of Growth Factors and Morphogens for Tissue Regeneration

Our understanding of the molecules involved in signalling tissue development and repair will underpin the generation of novel naturally inspired clinical therapies. Current knowledge of this molecular signalling is advancing with the tooth's hard and soft tissue ECM being shown to provide both biochemical and biomechanical regulatory cues. Indeed, comparable with repair processes in other tissues, the regulation of dental tissue regeneration involves signalling derived from its ECM with inherent growth factors known to coordinate recruitment, proliferation and differentiation of MSC populations [65, 68, 131, 132].

In the periodontal tissues, the application of platelet rich plasma (PRP) enables the delivery of a cocktail of potent growth factors and morphogens. Indeed currently, there is significant interest in the use of PRP in combination with bone grafts and/or stem cells to enable more predictable periodontal regeneration [133]. Enamel matrix derivatives (EMDs), obtained from porcine tooth buds, also contain a complex cocktail of growth factors and can also stimulate periodontal tissue regenerative events. Indeed both PRP and EMD are morphogenically complex and have been shown to include BMP-2, platelet derived growth factor (PDGF)-BB and FGF-2, amongst others [134–136]. These molecules likely act synergistically on MSCs. However, the action of individual growth factors has been exploited with BMP-2 being used in absorbable collagen sponge scaffolds to induce bone formation for sinus and alveolar ridge augmentation therapies. Both PDGF-BB and FGF-2 in combination with CaP or hydrogel scaffolds have also shown some clinically efficacious potential based on their ability to stimulate vascular responses which underpin many MSC-based repair mechanisms [137–145].

The indirect application of MSCs due to their release of growth factor with paracrine effects has also recently been highlighted. These secretomes contain a multitude of bioactive molecules such as insulin-like growth factor (IGF)-1 and vascular endothelial growth factor (VEGF) which promote many tissue repair mechanisms [146]. Notably, DPSC secretomes exhibit significant neurogenic repair

activity as well as being able to immunomodulate T-cell, B-cell, natural killer cell, and dendritic cell function [147, 148]. Further work is still required to better characterise the active components of the secretomes to determine optimal concentrations for targeted tissue repair and regeneration application.

1.5 Stem Cell Applications for Dental and Craniofacial Tissue Regeneration

The use of stem cells for regenerative medicine/dentistry is progressing and currently, the use of adult/postnatal stem cells exhibits the most realistic clinical opportunity. Regeneration of bone and periodontal tissues using MSCs has received considerable attention with several studies already reporting clinical application. Clearly, stem cells used in dental tissue engineering should be; (i) relatively easily isolated, (ii) straightforward to deliver in a reproducible and clinically simple procedure, (iii) clear of any patient safety issues, and (iv) ultimately differentiate into and regenerate the target tissue or organ.

BMMSCs and ADSCs, in particular those derived from the orofacial region, may provide an appropriate source for craniofacial tissue repair. Other dental and craniofacial tissue-derived MSCs may be more appropriate for regenerating dental mesenchyme-derived hard and soft tissues, including those of the dentine, pulp and supporting periodontal tissues. The application of MSCs for complete repair of complex oral organs, such as teeth and salivary glands, which also require cells to differentiate down epithelial lineages may however be challenging. Pluripotent embryonic stem cells may, therefore, have utility in these cases; however, medical and ethical issues associate with their application and the use of iPSCs still require further technical and safety advancements before they can be applied. For all stem cell sources, their downstream processing following isolation still remains an issue for the clinician who would also require onsite specialist equipment and expertise to enable their purification and expansion.

1.5.1 Tooth and Tooth Component Tissue Regeneration

Ultimately, it is aimed that a lost tooth will be replaced by a fully functional bioengineered one; however, current studies indicate that tooth component tissue, such as root and crown dentine are more realistically clinically achievable. Recent work using animal models has shown that complex root/periodontal structures can be regenerated using PDLSCs and SCAPs in conjunction with hydroxyapatite scaffolds [11, 149]. The structures regenerated provided suitable abutments for prosthetic devices enabling the support of an artificial crown with dental functionality. Clearly, future work in this area may enable development of the underpinning technology necessary for human application.

The regeneration of an entire tooth structure is now appearing feasible in the future based on animal studies utilising several different MSC sources. For tooth bioengineering, the generation of embryonic tooth primordia has been commonly used. Initial studies have transplanted pelleted dissociated porcine tooth buds in the omentum of athymic rats which resulted in the generation of complex tooth structures which comprised a pulp chamber, dentine, putative Hertwig's Epithelial Root Sheath (HERS) and an enamel organ. Transplantation of dissociated rat and mouse tooth buds have also resulted in the development of similar tooth structures. Notably, as is described previously, tooth development requires the reciprocal interactions between embryonic oral epithelial cells and neural-crest derived mesenchyme. Subsequently, recent work has attempted to determine if mouse-derived ESCs, neural stem cells and BMMSCs can appropriately respond to mouse embryonic oral epithelium derived cells. Data indicated that odontogenic differentiation was most apparent in explants containing BMMSCs although other cell types demonstrated some potentiality [100, 102, 150–154]. Work conducted by Volponi et al. [155] has demonstrated tooth tissue regeneration following transplantation of human adult gingival epithelial cells combined with mouse embryonic tooth mesenchyme cells in kidney capsules. The tooth structures generated at six weeks of transplantation contained vascularized pulp-like tissue and signs of root development including the presence of ameloblast-like cells and epithelial rests of Malassez.

Significantly, a murine model has recently demonstrated that, following the transplantation into the alveolar bone of a bioengineered tooth germ, reconstituted in vitro in a collagen hydrogel scaffold using epithelial and mesenchymal progenitor/stem cells, a functioning tooth was formed. Notably, the in vitro step used recapitulated the developmental events necessary for complex tooth tissue generation and the subsequent bioengineered tooth, which when erupted and occluded, exhibited appropriate mineralised tissue properties [151]. Furthermore, the pulpal tissue was appropriately innervated and relevantly serviced by a blood supply. The generation of fully functional tooth units in animal models which have utilised MSC and iPSC sources support the concept that bioengineered structures may one day be routinely generated for patients. Clearly, significant work is still required to bring this to fruition and to provide clinically relevant alternatives for patients who require dental implants.

1.5.2 Regeneration of Other Complex Craniofacial Tissues and Organs

The regeneration of salivary gland function is important in particular for head and neck oncology patients who have undergone surgery and/or radiotherapy. Recent mouse model studies using ADSCs, BMMSCs and primitive salivary gland stem cells have shown that this may one day be clinical feasible. [89, 92, 156, 157]. The temporomandibular joint (TMJ) disc or condyle can become damaged due to disease such as arthritis or through trauma. MSCs in conjunction with hydrogels and ultrasound approaches have been used successfully to reconstruct condylar defects

in animal model systems [158–160]. The regeneration of tongue tissue is also important to many patients. Several animal model systems using MSCs and relevant scaffold systems has now shown tongue tissue repair is possible [161–163]. Overall studies are now showing that for many complex tissue and organ systems within the oro-craniofacial region, bioengineering approaches may one day become a clinical reality for patient treatment.

1.6 Stem Cell Storage and Processing

While growing evidence demonstrates that dental and oral tissues provide a rich source of MSCs, their use in regenerative therapies may be limited due to the requirement to isolate tissue at the time of need, *e.g.* tooth extraction. The banking of DSCs or tissues obtained from deciduous and wisdom teeth may, therefore, provide a practical approach for future stem-cell-based regenerative therapies. Recently, in several countries worldwide stem cell and tissue banks in the dental field have been developed, *e.g.*, Advanced Center for Tissue Engineering Ltd., Tokyo, Japan (http://www.acte-group.com/); Teeth Bank Co., Ltd., Hiroshima, Japan (http://www.teethbank.jp/); Store-A-ToothTM, Lexington, USA (http://www.store-a-tooth.com/); BioEDEN, Austin, USA (http://www.bioeden.com/) and Stemade Biotech Pvt. Ltd., Mumbai, India (http://www.stemade.com/) (reviewed in [164]). These banking approaches routinely utilise cryopreservation which aims to enable the long term storage of viable stem cells from tissues such as the PDL, pulp, apical papilla and whole tooth tissue. Subsequently, it is envisaged that the stem cells will be retrieved in the future from this cryopreservation and applied in autologous regenerative therapies for the patient. Much work, however, is still needed to determine the utility of these biobanks, their longevity, and value for money and the MSC processing procedures required.

Currently, it is not entirely clear as to how long term cryopreservation affects MSC viability and phenotype [165]. Therefore, alternative storage approaches are being developed which may be beneficial. Indeed, recent studies have shown that MSCs encapsulated in hydrogels may provide a means to decrease archiving costs while maintaining MSC phenotype and properties. Furthermore, the potential of tissue engineered product vitrification has also been investigated with studies using bone constructs consisting of a hydroxyapatite scaffold-cell complexes demonstrating higher cell survival rates compared with conventional freezing approaches [166, 167]. Further studies of these emerging biobanking approaches are clearly needed.

MSC handling and ex vivo expansion will be required for clinical application due to the relatively low number of stem cells, <0.1 % of all cell types, present within tissues. To achieve this, Good manufacturing practice (GMP)-compliant environments have been developed and are reported to generate clinical-grade MSCs from several tissue-types, *e.g.* adipose and bone marrow [168]. Currently, there are minimal published reports evident on GMP-handling and processing for dental MSC-types. It is proposed that standard GMP procedures should be more routinely applied