Stem Cell Biology and Regenerative Medicine

Marco Tatullo *Editor*

MSCs and Innovative Biomaterials in Dentistry

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Preface

Future Directions of Modern Dentistry: Dental-derived Stem Cells, Autologous Biomatrices and Injectable Biomaterials

Dentistry is going through a period of profound change. The old conception of the dentist collides with the modern view of dental science. The future challenges of modern dentistry are focused on regenerative medicine, with a particular interest on stem cells and biomaterials. Nowadays, it is very common to hear about stem cells and regenerative medicine; however, they are fairly recent concepts, therefore still not well understood. Moreover, they often create in the inexperienced reader several highly suggestive but unrealistic ideas, such as that stem cells are a kind of magical remedy that defies the laws of physiology and biology. Instead, it is important to have a correct point of view of regenerative medicine, particularly in the emerging field of regenerative dentistry.

Stem cell research is a growing part of biomedical research aimed at regenerating damaged or lost tissues and organs.

Although the ability to self-regenerate shown by some tissues, such as the liver, was already reported in 460 B.C. by Aeschylus in the *Promethéus desmótes*, the definition "Stammzelle", the German translation of "stem cells" was reported in the scientific literature by Ernst Haeckel in 1868.

Maximow is commonly reported to be the first scientist of the modern age to discuss "stem cells" in a lecture held at a special meeting of the Berlin Hematological Society on 1 June 1909; however, the term "stem cell" has been used in earlier publications (Ramalho-Santos M, Willenbring H. On the origin of the term "stem cell". *Cell Stem Cell*. 2007 Jun 7;1(1):35-8).

Starting from 1932, the query "stem cell" on PubMed, the most used scientific search engine worldwide accessing primarily the MEDLINE database of references and abstracts on life sciences and biomedical topics, shows over 169,000 scientific contributions on this topic. The impressive number of articles is rapidly growing with a particular boost over the recent years, proving the great interest of the scientific community on this subject.

Recently, dental tissues have also been reported as an accessible source of mesenchymal stem cells, introducing the innovative and engaging topic of the dental-derived stem cell research (Mao JJ, Prockop DJ. Stem cells in the face: Tooth regeneration and beyond. *Cell stem cell*. 2012;11:291-301).

The pioneer of the study of dental-derived stem cells (DDSCs) is Stan Gronthos, who first described in 2000 the stem cells in dental pulp, defining them as dental pulp stem cells (DPSCs) (Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97:13625-13630).

After the isolation of DPSCs, other dental and periodontal tissues have been found to be rich in stem cells, such as human exfoliated deciduous teeth (SHED), periodontal ligament (PDLSCs), apical papilla (SCAP) and dental follicle (DFSCs) (Tatullo M, Marrelli M, Paduano F. The regenerative medicine in oral and maxillofacial surgery: the most important innovations in the clinical application of mesenchymal stem cells. *Int J Med Sci*. 2015 Jan 1;12(1):72-7).

Basic research has been highly stimulated by these discoveries in the field of dental-derived stem cells research. One of the most authoritative experts on DDSCs is Gianpaolo Papaccio, who has contributed high-quality studies, supported by his great ability to communicate the results of basic research to the entire scientific community, focusing on the potential of these findings on clinical applications in dentistry and maxillofacial surgery (Papaccio G, Laino G. First International Meeting on "Stem Cell Applications in the Craniofacial Region". *J Cell Physiol*. 2006 Sep;208(3):473-5).

Following the example of Papaccio and colleagues, the interaction between dentists, maxillofacial surgeons and biologists has developed new professional profiles, opening a new section of modern dentistry, namely, regenerative dentistry.

As a result of this interest in the field of oral tissue regeneration, several research groups have carried out numerous experiments to exploit the intraoral tissues as a source of stem cells, characterized by an easy surgical access and minimal morbidity of the donor site. In fact, following the discovery of DPSCs, in the scientific literature significant studies have been reported about the ability of these cells to differentiate towards the main cell phenotypes. These studies have undoubtedly confirmed the excellent quality of such cells in the field of regenerative dentistry, but they also stimulated the research towards alternative sources.

In 2013, Marco Tatullo, Massimo Marrelli and Francesco Paduano published a scientific article describing a newly discovered source of stem cells: the human periapical dental cysts; this discovery came from a clinical observation, concerning the heightened capability of odontogenic cysts to recur in the same location, if not eradicated completely (Marrelli M, Paduano F, Tatullo M. Cells isolated from human periapical cysts express mesenchymal stem cell-like properties. *Int J Biol Sci*. 2013 Nov 16;9(10):1070-8). Following rigorous research, Tatullo et al. isolated the human periapical cyst-mesenchymal stem cells (hPCy-MSCs). These cells possess high proliferative potential, self-renewal ability and the potential to differentiate into osteoblast-like, adipocyte-like cells in vitro; furthermore, in a recent paper

hPCy-MSCs also showed the ability to differentiate into neural/glial cells for cell-based therapies aimed at treating neurologic diseases (Marrelli M, Paduano F, Tatullo M. Human periapical cyst-mesenchymal stem cells differentiate into neuronal cells. *J Dent Res*. 2015 Jun;94(6):843-52. doi: 10.1177/0022034515570316).

After a couple of years, in 2015, in a multicenter study carried out by Giorgio Mori, Lorenzo Lo Muzio, Maria Grano and other collaborators, MSCs were isolated in the dental bud. Dental Bud Stem Cells (DBSCs) were described as a promising source for bone regeneration of stomatognathic as well as other systems (Di Benedetto A, Brunetti G, Posa F, Ballini A, Grassi FR, Colaianni G, Colucci S, Rossi E, Cavalcanti-Adam EA, Lo Muzio L, Grano M, Mori G. Osteogenic differentiation of mesenchymal stem cells from dental bud: Role of integrins and cadherins. *Stem Cell Res*. 2015 Nov;15(3):618-28). Several academic groups have performed extensive research on MSCs from dental tissues, such as the group of Roberto F. Grassi and colleagues (Ballini A, De Frenza G, Cantore S, Papa F, Grano M, Mastrangelo F, Tetè S, Grassi FR. In vitro stem cell cultures from human dental pulp and periodontal ligament: new prospects in dentistry. *Int J Immunopathol Pharmacol*. 2007 Jan-Mar;20(1):9-16. Review). The group of Enrico Gherlone and Filiberto Mastrangelo performed several studies on tissue regeneration, by using MSCs, with a translational approach, thus creating interest in readers even less close to cell biology (Mastrangelo F, Quaresima R, Grilli A, Tettamanti L, Vinci R, Sammartino G, Tetè S, Gherlone E. A comparison of bovine bone and hydroxyapatite scaffolds during initial bone regeneration: an in vitro evaluation. *Implant Dent*. 2013 Dec;22(6):613-22). The group of Rajiv Saini and Andrea Ballini worked on bone regeneration improved by DPSCs under low-level laser irradiation. The low-level laser therapy (LLLT) has also been demonstrated to be a useful aid in bone regeneration, because of its activity on MSCs, as described by the Indo-Italian group reported above (Ballini A, Mastrangelo F, Gastaldi G, Tettamanti L, Bukvic N, Cantore S, Cocco T, Saini R, Desiate A, Gherlone E, Scacco S. Osteogenic differentiation and gene expression of dental pulp stem cells under low-level laser irradiation: a good promise for tissue engineering. *J Biol Regul Homeost Agents*. 2015 Oct-Dec;29(4):813-22).

The cell manipulation and the production of large quantities of MSCs have always constituted one of the major limitations in the use of this technology on the patient. In this light, regenerative medicine has developed, on a parallel track, a great interest in endogenous growth factors capable of promoting tissue regeneration and improving the quality of healing. The platelet concentrates have played a very interesting role since the Robert Marx studies on PRP (Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? *Implant Dent*. 2001;10(4):225-8). The PRP had many advantages, but also some disadvantages, such as the need to manipulate the blood with anticoagulants. This technical necessity has made it difficult to use in dental surgeries. The breakthrough in the use of platelet concentrates was the discovery of "platelet-rich fibrin" (PRF). Joseph Choukroun and colleagues first described this innovative platelet concentrate. PRF showed the ability to trap both platelets and growth factors released by them, in a complex three-dimensional network; such high presence of fibrin allowed PRF to act as an autologous biocompatible matrix, able to

treat some soft tissues injures and to improve the grafting of bone defects. Currently Choukroun PRF is a widely used aid in regenerative dentistry, along with the latest generation of biomaterials (Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, Gogly B. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2006 Mar;101(3):e37-44).

Studies on DDSCs have stimulated the search for the ideal biomaterials to use as cell scaffold.

Antonio Apicella and his research lab carried out several researches on biomaterials used in dentistry. These studies have developed composite biomaterials, capable of simulating the bone structure, while still maintaining great biocompatibility; furthermore, the interaction between engineers of materials and dentists has increased *in silico* analysis about the biomechanical behavior of dental prostheses, as demonstrated by studies with finite element models (Schiraldi C, D'Agostino A, Oliva A, Flamma F, De Rosa A, Apicella A, Aversa R, De Rosa M. Development of hybrid materials based on hydroxyethylmethacrylate as supports for improving cell adhesion and proliferation. *Biomaterials*. 2004 Aug;25(17):3645-53).

The main limitation to the use of scaffolds was their morphology, often incompatible with the bone defects with complex shape.

The recent literature has reported several studies on biomaterials adaptable to anatomical sites with complex morphology. The latest studies on hydrogels have deeply modified the clinical approach to the use of biomaterials, overcoming the limit of the shape of the surgical receiving site. In this context, an interesting innovation is represented by the development of new strategies for scaffold release; in fact, this action has always been flawed by the operator-dependent variability; the National Research Council group headed by Luigi Ambrosio has developed an innovative approach to the release of the scaffold in the surgical site, thanks to the "injectable scaffold" concept. This method allows you to have more control in the filling of bone defects during regenerative surgery; in addition, it recreates the complex morphology of the bone defect with high precision (Raucci MG, Alvarez-Perez M, Giugliano D, Zeppetelli S, Ambrosio L. Properties of carbon nanotube-dispersed Sr-hydroxyapatite injectable material for bone defects. *Regen Biomater*. 2016 Mar;3(1):13-23).

The future of dentistry is a challenge that must be accepted today. The dentist can no longer be a figure with skills limited to clinical or surgical branches related to the oral district. Future directions of modern dentistry are leading towards dental-derived stem cells, autologous biomatrices and injectable biomaterials, as widely described in this book by the most authoritative researchers who first understood the importance of enhancing the dental science. The take-home message that I would like to leave to the reader is that the future of today is the past of tomorrow; therefore we must always be receptive to innovations and curious with respect to basic research.

Crotone, Italy Marco Tatullo

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Chapter 1
Dental Pulp Stem Cells: What's New?

Agnieszka Arthur, Songtao Shi, and Stan Gronthos

1.1 Tooth Development and Injury

Understanding the developmental processes required during tooth formation is an essential step to regenerate or repair a tooth-like structure; or tissues associated with teeth, such as pulp, dentin, cementum, periodontal ligament (PDL) or even alveolar bone. Predominantly two cell types, the oral epithelial cells and the cranial neural crest derived ectomesenchymal cells give rise to the tooth during development [1, 2]. Precise temporal and spatial expression of inductive signals (such as bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), Sonic Hedgehog (SH), and Wnt molecules) is required for the epithelial–mesenchymal interactions for tooth formation [3–5]. The oral epithelium gives rise to ameloblasts which form the outer layer of enamel. The ectomesenchymal cells that contain the dental pulp stem cells (DPSC) give rise to the remaining tooth structures. Odontoblasts synthesis an extracellular matrix (ECM) comprised predominantly of collagen type I and other matrix proteins to form the template for mineralised dentin. The cellular processes of odontoblasts remain within the dentin tubules, while the cell bodies reside in the pulp tissue [6, 7]. The central pulp chamber is formed by the ectomesenchymal cells comprising fibrous tissue, while neural and vascular networks invade the chamber

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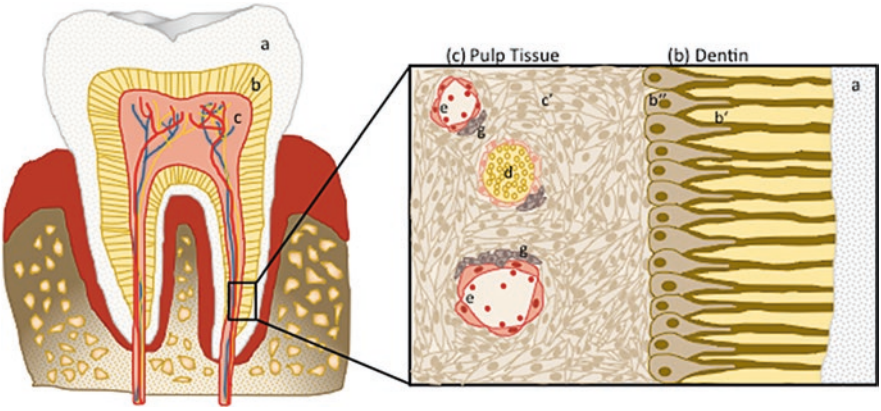

Fig. 1.1 The structure and components of a mature human tooth maintained in the alveolar bone. Interactions between epithelial and ectomesenchymal cells give rise to the tooth during development. The (**a**) enamel is formed from ameloblasts that are epithelial derived. The ectomesenchymal cells give rise to odontoblasts that synthesis the (**b**) mineralised dentin. The cellular processes of the odontoblasts lay perpendicular within the (**b′**) dentin tubules, while the (**b″**) cell bodies reside within the (**c**) pulp chamber. The ectomesenchymal cells give rise to the fibrous tissue within the (**c′**) pulp chamber, while the (**d**) neural and (**e**) vascular networks invade from the (**f**) apical foramen. The dental pulp stem cells (DPSC) reside in (**g**) perivascular niches within the pulp

from the apical foramen. The tooth itself is stabilised within the alveolar bone socket by a fibrocellular stratum of PDL, which interacts with specialised connective tissue fibers (Sharpey's fibers) that extend between cementum the alveolar bone socket [8] (Fig. 1.1).

Teeth are rigid structures that are relatively durable and hard, however bacterial infection, mechanical or chemical insults leading to attrition, congenital defects or cancers can cause inflammation and subsequently pain. Conventional root canal treatment for the remove of the damaged tissue followed by the use of synthetic implants or compounds ensues for the repair or restoration of damaged dental tissue. Alternatively pulp capping is also used to protect any remaining living pulp; however compounds currently used in clinical practice can often lead to inflammation and necrosis of the pulp tissue [9]. These restorative procedures have been used because damaged enamel cannot be regenerated, as the ameloblasts that form the enamel during development subsequently undergo apoptosis. Recently it was shown that mouse perivascular stem cells appear to assist in protecting the pulp from the external environment by producing a mineralised dentin-like material [10]. Importantly, the underlying dentin and dental pulp demonstrate limited self-repair in humans [11]. However, it was the identification DPSC in 2000 [12] that has resulted in the expansion of cellular-therapy based reparative approaches for endodontic regenerative medicine.

1.2 The Identification of Dental Pulp Stem Cells: How Far We Have Come

DPSC and stem cells from human third molars or exfoliated deciduous teeth [13] are mesenchymal stem-like cell populations. They are derived from disaggregated pulp tissue with the ability to generate clonogenic adherent cell clusters called colony forming units-fibroblastic (CFU-F) as described for bone marrow derived MSC [12, 14–16]. These DPSC when cultured ex vivo have the capacity to form mineralized deposits [12, 15], physiologically similar to hydroxyapatite crystal globular formation found in native dentine. Furthermore, using the xenogeneic transplantation system ex vivo expanded SHED and DPSC transplanted with a hydroxyapatite/tricalcium phosphate (HA/TCP) carrier developed vascularized fibrous pulp tissue, a well-defined layer of odontoblast-like cells, mineralized dentin-like material, consistent with the structure of dentin in human teeth; with the processes extending into tubular structures [12, 15]. Furthermore, the odontoblast-like cells and the pulp were of donor origin [12, 15, 17, 18]. These seminal studies clearly establish the regeneration capacity of DPSC into differential tissue, maintaining epigenetic memory of their tissue of origin.

Elegant studies using the mouse incisor model reported that stem cells within pulp originate from periarterial cells and are maintained by the neurovascular bundle niche [19]. Transgenic reporter mouse models and lineage tracing studies clearly showed that the release of sonic hedgehog (shh) from the trigeminal nerves that infiltrate the tooth active Gli1. Gli1 is expressed by the quiescent stem cells that line the arterioles; these quiescent stem cells have been shown to regulate odontogenesis. Furthermore, the study demonstrated that the $Gli1^+$ cells that surround the neurovascular bundle niche are the most primitive population of MSC important for homeostasis and repair and can give rise to all MSC populations in culture. Interestingly, these stem cells did not express the normal MSC markers such as CD146, CD105 and Sca1. It was the $NG2^+$ pericyte cells, a subpopulation of MSC-like population derived from the Gli1+ cells, that where required for repair but not homeostasis [19]. Interestingly, a third population of multipotent Schwann cells was identified of neural crest origin, derived from peripheral nerve-associated glia. The study indicated that these multipotent Schwann cells were able to yield dental pulp cells and odontoblasts during tooth development, maturation and regeneration [20].

Notably, other MSC-like populations have also been identified in human teeth. These include apical pulp derived cells (APDC), isolated from immature apices [21] ; stem cells from the apical papilla (SCAP), isolated from the apical papilla of permanent immature teeth [22] ; and PDL stem cells (PDLSC) [23]. While this book chapter will focus on the use of DPSC for endodontic regeneration, certainly other cell types, including bone marrow derived MSC, PDLSC, adipose derived stem cells are also being investigated for their endodontic regenerative capacity [24].

Importantly, DPSC lack cell surface expression of various immune helper antigens and exhibit the capacity to modulate immune/inflammatory responses, analogous to that described for other MSC-like populations [25–28]. Therefore, DPSC

have the potential to be used as an off the shelf allogeneic preparation for bioengineering applications, in contrast to autologous stem cells; where appropriate storage and production of allogeneic DPSC can be maintained on a clinical scale allowing for better quality control processes and lower costs of manufacturing [29].

1.3 The Use of DPSC in Regenerative Medicine

The grand challenge in endodontic regenerative medicine is the generation or reconstruction of a functional dentine/pulp complex in the form of a living functional root (reviewed by [30, 31]). The primary focus in this chapter is to highlight current approaches being developed for the bioengineering and repair of dental pulp regeneration and tooth reconstruction, including alveolar bone reconstruction to improve dentition. A number of strategies are employing multi-disciplinary approaches combining different models and harnessing our knowledge of DPSC, biomaterials and growth factors for directed tissue engineering.

Early work in the bioengineering field demonstrated that it was possible to regenerate a tooth-like structure from a suspension of single cells derived from the tooth bud of either pig or rat origin [32]. These organoid structures consisted of a pulp-like chamber, dentin, odontoblasts, cementoblasts, and enamel, demonstrating for the first time the presence of both epithelial and ectomesenchymal cell types. Others have further expanded on this concept by generating a three-dimensional (3D) bioengineered tooth germ that recapitulated a functional tooth when transplanted into a tooth cavity within the alveolar bone in an adult mouse [33]. Importantly, the bioengineered teeth retained the correct tooth structure for mastication and were responsive to noxious stimuli, demonstrating neural and vascular infiltration. Other investigations have examined DPSC in vitro, using 3D models of epithelial invagination into the mesenchyme [34] and matrigel DPSC-spheroid systems, to explore the molecular basis of cell survival, cavitation and organogenesis [35]. Taken together, these advances have paved the way for developing novel bioengineered organ replacements as potential regenerative therapies for endodontic applications.

1.3.1 Characteristics of Scaffolds Required for Endodontic Applications

One of the main technological challenges being addressed by bioengineers involves understanding the interactions between DPSC and 3D scaffolds, where the surfaces need to mimic the physiologically in vivo environment in order to allow for correct cell-cell and cell-ECM crosstalk, attachment, growth, proliferation and differentiation. Given the large body of work in this field a summary of the different properties required for the regeneration of the dentine/pulp complex and tooth structures have been outlined in Fig. 1.2.

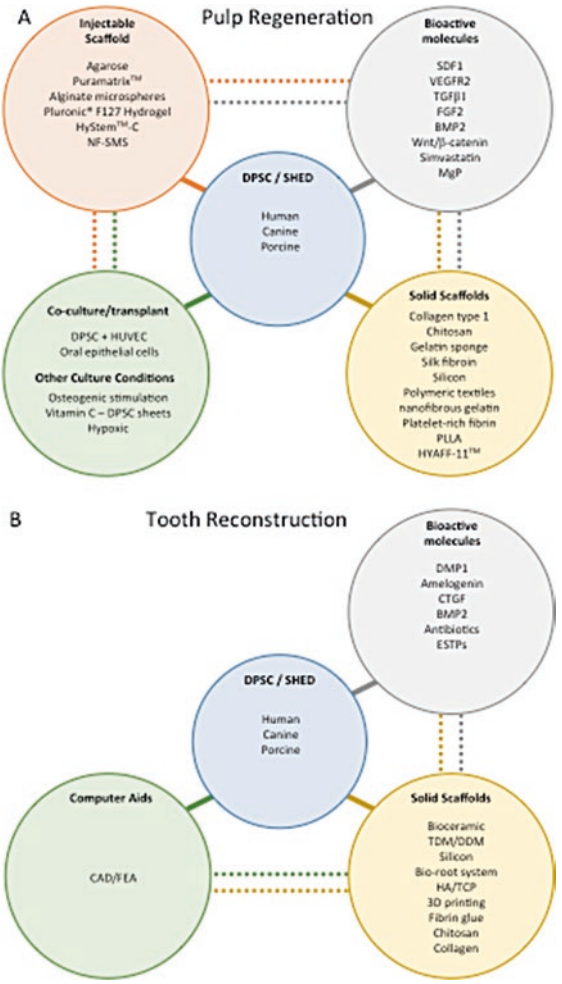

Fig. 1.2 Schematic representation of the bioengineering approaches to recapitulate a pulp-like structure or tooth reconstruction. The schematic represents a summary of the individual (*solid lines*) or multifaceted (*dashed lines*) approaches employed to regenerate (**a**) the pulp tissue or (**b**) tooth like structure using DPSC from various animals in proof-of-principle studies.(**a**) Dental pulp regeneration has encompassed the use of numerous biomaterials to generate an injectable or solid scaffold for the appropriate delivery of DPSC. The scaffolds or DPSC may have been loaded with specific bioactive molecules; co-cultured or pre-conditioned prior to transplantation. (**b**) Bioengineering approached for tooth reconstruction require greater structural rigidity that mimics the architecture of the tooth, as such the solid scaffolds and bioactive molecules vary to those utilised in pulp regeneration. Additionally, computer aids have been utilised to assist in the process (*NF-SMS* nanofibrous spongy microspheres, *SDF-1* stromal derived factor 1, *VEGFR2* vascular endothelial growth factor receptor 2, *BMP2* bone morphogenic protein 2, *CTGF* connective tissue growth factor, *MgP* magnesium phosphate, *PLLA* poly-L-lactic, acid, *DMP1* dentin matrix acidic phosphoprotein 1, *ESTPs* ethylenediaminetetraacetic acid-soluble tooth proteins, *TDM* treated dentin matrix, *DDM* demineralised dentin matrix, *HA/TCP* hydroxyapatite/tricalcium phosphate, *CAD/FEA* computer aided design (CAD) and finite element analysis (FEA)

Important aspects being considered in the fabrication or choice of scaffold is the physical structure of the target tissue such as surface topography and the biocompatibility, including the nature of the polymer, moulding ability and swelling, porosity, cationic nature and degradation rate [36–38]. The porosity or pore size of scaffolds is also an important factor for the formation of the correct 3D structures, while providing the right surface topography for cell attachment and transfer of nutrients.

Collart-Dutilleul and colleagues have demonstrated that porous silicon scaffolds are a promising nanostructured biomaterial for tissue engineering. It ideally mimics ECM environment properties, to support cell attachment, development and migration in vitro [39, 40]. The composition (nanopourous or mesoporous) and treatment of the silicon influence its function [41]. Poly-L-lactic acid (PLLA), which has previously been shown to produce a de novo pulp like structure [42] is ideally used at 150–425 μM pore size [43]. Chitosan, obtained from shellfish, is being used more in current scaffold constructs due to its non-toxic natural polymer structure, where it has been successfully used for regenerative purposes in other tissue systems [44]. Alginate, is another natural polymer that is degradable, which also appears to maintain DPSC viability and promote osteogenesis in vitro [45]. Furthermore, graphene oxide-based substrates have previously been shown to promote osteogenesis in addition to enriching physical and mechanical properties of biomaterials. It has also been demonstrated that these substrates are that not cytotoxic, maintain cell viability with increased proliferation, attachment and expression of genes expressed during mineral formation [46].

Multifaceted or hybrid approaches are favoured tissue engineering options combining the appropriate scaffold with ECM components, growth factors or chemical signals required for dental formation. One novel alternative biocompatible scaffold, is the FDA approved resorbable hydrophobic fabrics. The textile fibre is knitted from polyglycolic acid (PGA) braided multifilaments or polydioxanone (PDO) monofilaments, respectively, providing different roughness to the surface on which the cells attach [36]. The scaffold was also functionalised with either chitosan or peptide arginine-glycine-aspartic acid (RGD).This preliminary study demonstrated that functionalised PGA multifilament scaffolds were conducive to DPSC survival, cell adhesion and viability, although, in vivo biocompatibility analysis is still required [36]. Others have tried decellularised bone ECM in conjunction with a hydrogel scaffold to provide the stimulatory environmental cues for osteo/odontogenic differentiation in addition to growth factors (FGFb and EGF) to enhance osteogenic differentiation [47].With the same concept in mind, demineralised dentin matrix (DDM) has also been investigated. This organic material has been shown to release key osteogenic growth factors such as BMP2, bFGF and TGF-β1 stimulating cell proliferation, migration and osteogenic differentiation of porcine DPSC in vitro. Interestingly, implants of porcine DPSC with HA/TCP carrier demonstrated greater osteo-conductive ability than with DDM. However, greater DSPP gene and protein levels were identified in DDM implants, suggesting that the DDM environment provided cues for a dentin-specific phenotype [48]. These observations underscore the importance of directing the right kind of tissue architecture during the regenerative process.

The potential of a biomimetic hybrid scaffold with the controlled release of chemical components is another concept currently being investigated. This study combined a nanofibrous gelatin with magnesium and phosphate (NF-gelatin/MgP) to demonstrate enhanced proliferation, differentiation and biomineralisation of hDPSC both in vitro and in vivo ectopic transplant studies [49]. Bakopoulou and colleagues also utilised multi-faceted approaches for the regeneration of dentin tissue in vitro, investigating DPSC, dual scaffolds and morphogens [50].The dual scaffold composed primarily of a zinc-doped, Mg-based bioceramic scaffold, was amalgamated with human treated dentin matrices (hTDMs).The hTDM constructs were comprised of the crown of impacted third molars from health donors and a source for dentinogenic-related growth and morphogenetic factors. DPSC were then spotted inside this hybrid scaffold in the presence or absence of growth factors DMP1 and BMP2. This multifaceted scaffold supported long-term DPSC attachment and viability. While the controlled release of elements including Mg^{2+}, Ca^{2+}, Si^{4+} and Zn^{2+}, was non-toxic to the DPSC and the release of DMP1 and BMP2 enriched hydroxyapatite formation [50].

Other investigators have used hybrid scaffolds utilizing of silicon. Mineral trioxide aggregate (MTA), is a calcium silicate based cement used in a number of endodontic applications including regenerative applications. Calcium silicone based materials developed for endodontic applications show good cell biocompatibility. Si ions released from calcium silicate based material are essential to the formation and calcification of mineralised tissues and promotes angiogenesis in dental pulp cells [51]. It has now been shown that MTA enhances DPSC proliferation, which correlates with Wnt/β-catenin signalling [52]. The Si released from polycaprolactone (PCL)/submicron bioactive glass (smBG) hybrid scaffold has been developed for pulp and dentin tissue regeneration, in a proof-of-principle in vitro study [53]. Furthermore, the preparation/treatment of the damaged tissue allowing for favourable regeneration is also an aspect to consider. Galler and colleagues have shown that the treatment of dentin discs with EDTA prior to seeding with DPSC enhances cell attachment, migration and mineralisation capacity [54].

1.4 Bioengineering Approaches for Pulp Regeneration

One of the main issues faced in endodontics is infection arising from either microbial, chemical, thermal or mechanical insult. Infection can result in irreversible pulp disease caused by pulp necrosis and disrupted dentin formation; subsequently resulting in an enlarged pulp chamber or an opened apical foramen. Currently the function of the damaged dental pulp cannot be restored, and as such root canal treatment becomes the only viable option, resulting in permanent devitalization of the tooth. This can result in the loss of structural integrity of the remaining tooth and potential re-infection. Therefore, the development of novel regenerative therapies for the repair or regeneration of the pulp tissue is essential. However, the challenges faced when attempting to regenerate a pulp structure include the differentiation into

functional odontoblasts, their capacity to form dentin on established dentin, the correct ECM composition allowing vascularisation and nerve innervation of the pulp tissue and potential reinfection.

Endeavours thus far have utilised scaffolds, bioactive molecules, and stem/progenitor cells individually and in combination with some success. One study demonstrated that the release of bioactive molecules (heparin, sucrose, VEGF, TGF-B1 and FGF2) from a hydrogel scaffold and dentin conditioning influenced cell fate determination [55]. Other reports in recent years have opted for a versatile injectable scaffold that is biodegradable when addressing dental pulp regeneration or even a scaffold-free system (Fig. 1.2 and Table 1.1). These scaffolds range from self-assembling hydrogels, gelatine, to commercially available Puramatrix™,Pluronic®F127 Hydrogel and HyStem™-C [55–65].

The eradication of any infectious material within the damaged tissue has been addressed with the use of calcium hydroxide or antibiotic pastes. However, adverse effects on the endogenous dental pulp cells [66, 67] and PDL fibroblasts [68] have been documented with antibiotic pastes. Therefore, there needs to be a balance between optimal and efficient antibiotic administration, while sustaining the physiological function of the stem cells [69–71]. With the expansion of the endodontic regenerative field and use of scaffolds for delivery of stem cell populations, it has been proposed that perhaps the slow release of antibiotics from a scaffold-based system would reduce cell toxicity and inhibitory effect on DPSC proliferation [72]. An alternative approach is an injectable scaffold, such as the Pluronic®F-127 hydrogel, an FDA approved delivery system that is non-toxic, while supporting osteogenic and adipogenic differentiation in vitro [57].

Another pressing issue in pulp regeneration is the maintenance of an adequate blood supply following transplantation; to sustain nutrient transfer and thus survival of the implant. As the invasion of endogenous blood vessels is slow, the implant relies on the diffusion of oxygen from surrounding capillaries. Therefore a number of studies have focused on the pre-vascularisation or enhanced vascularisation during transplantation to improved functional integration. One study addressed this issue by developing a system whereby endothelial (HUVEC) and DPSC are co-transplanted with or without a scaffold [73]. Their strategy was to develop a scaffold-free system that consisted of co-culturing endothelial human HUVEC with human DPSC to provide a physiologically relevant system for dental pulp regeneration. The in vitro studies established that DPSC:HUVEC co-cultures produced large amounts of mineral in odonto/osteogenic assays, which correlated with ALP expression when compared to DPSC only samples. Furthermore, the addition of DPSC with HUVEC cells stabilized the vessel-like structures generated in matrigel assays [73]. In subsequent studies, the researchers fabricated scaffold-free microtissue spheroids of DPSC that were pre-vascularised by HUVEC [60]. These microtissues were transplanted into empty tooth-root slices that were subcutaneously transplanted into immunocompromised mice. The study reported evidence of a vascularised pulp-like structure that consisted of odontoblast-like cells lining the dentin, with cellular processes extending into dentinal tubules following 4 weeks post transplantation [59]. The interaction between DPSC and HUVEC also attenuated

Table 1.1 Summary of current approaches used for endodontic regeneration

Scaffold	Experimental design	Cells/bioactive molecules	Duration	Functional outcome	Ref
Puramatrix™	sc explant–toot root	hDPSC: HUVECs	4 weeks	Establishes a microenvrironment conducive to vascularisation and generation of pulp-like tissue	[58]
Puramatrix™ or rhCollagen I	sc explant–tooth root	hSHED-GFP labelled	35 days	Vascularisation and dentin formation evident in the root canals	[62]
Silk fibroin	(1) sc explant, (2) pulp injury canine	hDPSC/SDF-1α	(1) 8 weeks, (2) 3 months	hDSPC and SDF-1α embedded silk fibroin scaffolds promote (1) regeneration of pulp and (2) revascularisation of pulp accompanied by autophagy	[77]
Gelatin sponge	Pulpectomy–canines	canine DPSC/Simvastatin	10 weeks	Simvastatin treated cDPSC enhances both pulp and dentin formation following injury	[65]
Hyaluronan-based (HYAFF-11™)	Calvarila defect implant in rat	hDPSC	1 month	Evidence of blood vessel and collagen matrix formation and osteogenic differentiation within the implanted scaffold	[61]
Self-assembling hydrogel	sc explant–dental cylinders	hDPSC/VEGF, TGF-β1, FGF2	6 weeks	Pre-treatment of dentin cylinders influences response of DPSC in transplant	[55]
Nanofibrous spongy microspheres(NF-SMS)	(1) sc explant, (2) pulpectomy in rat	hDPSC/hypoxic conditions	4 weeks	Hypoxic-primed hDPSC delivered in NF-SMS support the regeneration of a vascularised dental pulp structure in both explants and in situ, integrating with native dentin	[64]
Nanofibrous gelatin/magnesium phosphate Hybrid scaffold	sc explant–tooth slice	hDPSC/prior osteogenic stimulation	5 weeks	The biomimetic hybrid scaffold enhances DPSC proliferation, differentiation and biomineralisation.	[49]

(continued)

Table 1.1 (continued)

Scaffold	Experimental design	Cells/bioactive molecules	Duration	Functional outcome	Ref
Nanofibrous gelatin—differing stiffness	sc explant	hDPSC	4 weeks	Modulating the stiffness of a scaffold influences differentiation; and assist in the recapitulation of a dentin-pulp complex	[88]
Poly-L-lactic acid (PLLA)	sc explant–tooth slice	hDPSC and SHED/rhVEGF, rhWnt1, B-catenin silencing	4 weeks	VEGF signalling activates Wnt/β-catenin signalling mediating vasculogenic fate of DPSC	[76]
Platelet-rich fibrin (PRF) granules	(1) sc explant, (2) pulpectomy–canine	canine DPSC sheets/Vitamin C	8 weeks	PRF promotes cDPSC regeneration of dentin and a vascularised pulp-like tissue	[74]
Demineralised dentin matrix (DDM)	sc explant	porcine DPSC	12 weeks	sDPSC in DDM generates a dentin-specific bone phenotype	[48]
3D printed multiphase scaffolds	sc explant	hDPSC/amelogenin, CTGF and BMP2	6 weeks	Multiphase 3D scaffolds containing bioactive molecules and DPSC can form a dentin/cementum/PDL/alveolar bone complex	[87]
Fibrin glue	Implantation under the renal capsule	Apical bud epithelium: hDPSC/ESTPs	4 weeks	Human ESTPs consist of dentogenic factors that support dentin formation	[86]
Bio-root system	Implant, socket of jaw bone—miniature porcine	PDLSC sheet with DPSC in HA/TCP–Bio-root system/Vitamin C	6 months	Functional tooth restoration via a bio-root system in conjunction with allogeneic MSC can be achieved	[91]
Bio-root system	Tooth loss model—incisors miniature porcine	PDLSC sheet with DPSC in HA/TCP–Bio-root system/Vitamin C	6 months	The bio-root system as a tooth replacement alternative, with the formation of root and dentin-like structures	[92]

Summarised in the table is the scaffold investigated, the experimental design (explant or in vivo); the use of bioactive molecules, duration and brief explanation of the outcomes (*sc* subcutaneous implant of material using immunocompromised mouse strain, *CAD* computer-aided design, *FEA* finite element analysis)

ECM deposition; which enhanced the stabilisation of the microenvironment [59]. These microtissues were constructed with agarose, however, more recent studies have utilised an injectable scaffold (PuraMatrix™) [58, 62]. PuraMatrix consists of the amino acid sequence R-A-D-A in an aqueous solution that self assembles/polymerises instantly into nanofibers upon exposure to physiological concentrations of salts, thus providing a biodegradable scaffold [56, 58].This is ideal for pulp regeneration to access the pulp though the apex of the root. A proof-of-principle study highlighted that human DPSC survived and proliferated when cultured with Puramatrix™, where DPSC also expressed putative odontogenic genes following 21 days culture in a 3D tooth slice model [56]. In a similar study, SHED were injected into the root canal of a human tooth delivered with either PuraMatrix™ or a human recombinant collagen type I matrix [62].This study demonstrated that the engineered pulp within the root canals resembled endogenous pulp, although the matrix was less dense. However, the vascularity and cellularity between engineered and endogenous pulp was similar.GFP labelled and tetracycline staining also confirmed that newly formed dentin was derived from the transplanted SHED cells [62]. Collectively, these findings provide promising results for future pulp regeneration.

Dissanayaka and colleagues have taken this research one step further with their co-culture studies, addressing not only odontogenic differentiation by DPSC, but also enhanced neovascularisation by the endothelial counterpart [58]. Co-transplantation of hDPSC:HUVEC in the PureMatrix™ hydrogel identified that DPSC could instigate the vascular network formed by HUVECs through the release of VEGF within 4 weeks post transplantation. Conversely, the co-culture to hDPSC and HUVEC enhanced the ECM composition, vascularisation and mineralisation within the transplant than the transplants consisting of DPSC alone [58]. Alternatively, a canine study performed orthotopic transplantation of a novel canine DPSC sheet and plate-rich fibrin (PRF) granules complex into the root canal in a canine pulpectomy model. Eight weeks post transplantation a dentin-pulp like complex had formed, which was vascularised by the transplanted DPSC as evidenced by positive BrdU staining [74]. This study suggests that the slow release of a number of growth factors, including VEGF from the PRF granules contributed to the increase in vascularisation in the regenerated pulp complex.

The underlying signalling mechanisms responsible for DPSC differentiation into vascularized endothelial cells is of particular interest [64, 75, 76]. The use an antagonist inhibitor of VEGF-A and shRNA knockdown of VEGF2A in DPSC in both in vitro and in vivo transplant experiments clearly demonstrated that the vascular differentiation of DPSC was VEGFR2 dependent [75]. The signalling mechanism by which VEGFR2 induces vasculogenic differentiation of DPSC and SHED was mediated by Wnt/β-catenin signalling [76]. Studies by Nor et al. utilised both in vitro and in vivo tooth slice/PLLA scaffold transplant study to investigate vasculogenic differentiation potential of DSPC or SHED. These findings elegantly showed that VEGFR2 induces the vasculogenic fate of DPSC and SHED via activation of the canonical Wnt/β-catenin signalling pathway, with the use of growth factors, inhibitors and gene silencing [76].

Pre-treatment of DPSC under hypoxic conditions has also been shown to instigate the expression of VEGF and subsequently enhance vascularisation following transplantation [64]. This study utilised an injectable nanofibrous spongy microsphere system (NF-SMS) for the delivery of hDPSC that were pre-cultured in a normoxic or hypoxic bio-reactor and subsequently injected into pulpectomized rabbit molars and implanted into immune compromised mice or injected into a rat pulpectomy model. Both experiments resulted in enhanced pulp regeneration and vascularisation when compared to DPSC alone, scaffold alone or DPSC with scaffold under normoxic conditions [64]. While the origin of the vascularised tissue was not determined in this study, the observations highlight the regenerative efficiency of priming cells prior to transplantation. Interestingly, Yang and colleagues demonstrate that both the transplant and host contribute to blood vessel formation in the regenerated pulp tissue [77]. Furthermore, the study also demonstrated the importance of SDF-1 for DPSC migration and pulp regeneration. Other studies have shown that DPSC express high levels of the chemokine CXCL12 or stromal derived factor 1 [78], which is a known potent mitogen for vascular tube formation and endothelial cell migration [79]. Collectively, these findings demonstrate the importance of understanding the physiological properties of the tissue being regenerated and harnessing our understanding of the basic cell biology in the regenerative process.

The majority of in vivo transplant studies have demonstrated the ability to regenerate the appropriate dentin-pulp-like complex using a range of scaffolds. However, the percentage of cell survival and the level of stem cell maintenance, following implantation remains to be determined. There is considerable conjecture on this topic, where transplanted cells could not be detected after 6 weeks post-transplantation in the mandible [80]. However, another study was able to harvest DPSC following ectopic transplantation, and show that these cells retained their MSC-like properties in vitro. They were still able to undergo colony formation and osteogenic differentiation, although they were somewhat hampered compared to freshly isolated DPSC. These DPSC, which contributed to the regeneration of a pulp-like structure, were isolated 60 days post subcutaneous transplantation into immunocompromised mice [81]. These findings are in accord with seminal studies demonstrated the self-renewal capacity of human DPSC using a serial ectopic xenogenic transplantation model [17]. Human DPSC were isolated by fluorescence activated cell sorting from primary transplants, expanded ex vivo then re-transplanted into immunocompromised mice. These secondary transplants were also able to generate a dentine-pulp-like structure similar to the primary transplants, comprised of odontoblasts and organised collagen fibers of human origin [17]. Collectively, these observations suggest that while some of the transplanted cells contribute to regeneration of a pulp-like structure, the stemness of a minor fraction of transplanted cells is also retained. Furthermore, the scaffold in which the DPSC reside may also contribute to their survival, regenerative potential and no doubt self-renewing capacity. However, a number of studies have commented on the regenerative tissues formed in scaffold only controls, with the potential of endogenous cells contributing to the regenerated tissue [74].

Studies conducted by the Nakashima et al. have established a good manufacturing practice protocol for isolating DPSC subsets based on their migratory response to G-CSF (labelled MDPSC) in a canine model [82, 83].They reported that there were few differences between young and aged MDPSC, with respect to their proliferation, migration, anti-apoptotic, angiogenic and neuronal properties, with similar regenerative potential in an ectopic tooth transplantation model [82]. However regeneration of the pulpectomised tissue was reported to be less in aged dogs [82] than in the young dogs [84]. These observations imply that perhaps the regeneration potential is also dependent of the age of the host. Interestingly, these studies showed that transplanted MDPSC did not directly contribute to the tissue regeneration, but rather were thought to have released trophic factors that inhibited apoptosis and stimulated migration and proliferation of host stem cells [83, 84]. These findings suggest that the host environment influences the regenerative process [82].The discrepancies in DPSC survival and contribution to the regenerated tissue proposes that the environmental factors, such as the scaffold/biomaterial used for DPSC delivery and the location of transplant need to be taken into account when assessing cell survival and involvement in tissue regeneration.

1.5 Bioengineering Approaches for Tooth Reconstruction

The approach to dentine/whole tooth regeneration is distinctly different to that of pulp regeneration, and as such, alternative strategies and materials vital for the process are required (Fig. 1.2). Furthermore, it is also important to keep in mind the composition of the mineral that is produced by the stem cell population used in the dentin regenerative process [85].

Preliminary studies assessing the feasibility of whole tooth regeneration have employed mass spectrometry to identify soluble proteins expressed within the human adult tooth, denoted as ethylenediaminetetraacetic acid-soluble tooth proteins (ESTPs) [86]. In vitro studies demonstrated that the ESTPs selectively enhanced odontogenesis of cultured DPSC but not bone marrow or adipose derived MSC-like cells. In vivo xenografts were also performed, composed of human DPSC and murine apical bud tissue treated with ESTP, encapsulated in fibrin glue prior to being implanted under the renal capsule of immunocompromised mice. The constructs demonstrated enhanced formation of dentin structures 4 weeks post-transplantation. Similar experiments utilized mouse embryonic tooth-forming primordia treated with ESTPs, encapsulated in fibrin glue and implanted under the renal capsule of immunocompromised mice. These experiments showed the ESTP promoted the formation of teeth that were morphologically similar to normal teeth, demonstrated the utility of using a dual-pronged approach for endodontic tissue engineering [86].

Another approach to tooth reconstruction is using 3D printing to generate a scaffold that encompasses the three distinct structures required for a stable tooth structure (phase A. Cementum/dentin interface, B. PDL, C. Alveolar bone [87]. This

multiphase scaffold also consisted of the release of specific bioactive molecules within each phase that was specific to the formation of the appropriate tissue structure (amelogenin, CTGF and BMP2, respectively) [87]. While this was a proof-of-principle study using both in vitro and in vivo models, these findings provide new insight into potential endodontic regenerative strategies.

The construction of the scaffold has also been considered as a potential requirement for appropriate pulp-dentin regeneration. Qu and colleagues demonstrated that the stiffness of the scaffold influences the differentiation/mineralization capacity of DPSC whereby high stiffness of a 3D nanofibrous gelatin (NF-gelatin) scaffold resulted in bio-mineralization following osteogenic induction in vitro, while low stiffness promoted a soft pulp-like tissue. As such the researchers created a scaffold consisting of both high and low stiffness called S-scaffold with the successful regeneration of a pulp-dentin like complex following ectopic implantation into nude mice [88].

Another critical component to tooth regeneration is the formation of a stable and integrative root. Previous studies have reported that autologous dental stem cells were able to bioengineer tooth roots (bio-root) [89, 90]. However, the use of autologous dental stem cell preparations is dependent on various factors including patient age, oral health status, availability of viable tooth samples. Research efforts are now moving towards the use of allogeneic DPSC as a more readily available source of stem cells, where bio-root development has previously been reported to be viable using allogeneic PDSC and DPSC combined in a HA/TCP scaffold [91]. In a comparative study using miniature pigs, it was shown that bio-root regenerated teeth displayed similar tooth composition and functional ability to dental implants in vivo [92]. Tooth root restoration, has also been investigated using computer-aided design (CAD) modelling and finite element analysis (FEA).This study utilized a swine model to demonstrate that dental follicle cells seeded onto a treated dentin matrix (TDM) scaffold improved tooth root restoration, with sustained masticatory function for 3 months [93]. Furthermore, resin based materials are now been considered as an alternatives to ceramic based scaffolds for restorative dentistry. Indeed, four resin based biomaterials have been assessed to have no adverse effects on DPSC viability, morphology, the cytoskeleton of the cells or the production of the main ECM components [94]. However, the utility of these compounds in whole tooth regeneration remains to be determined.

1.6 Concluding Remarks and Future Directions

This chapter highlights the various approaches that have been undertaken in endodontic regenerative medicine, ranging from pulp, dentin, tooth root and total tooth regeneration. These investigations have assessed natural and synthetic biomaterials to determine suitable scaffold systems to maintain and enhance DPSC viability, attachment, migration and differentiation into the required structures to sustain dental repair or regeneration. Bioactive molecules including growth factors/mitogens,

matrix molecules, ions and transcription factors that are involved for tooth development are also being assessed as mediators of tissue regeneration. Furthermore, the field is moving towards the use of "off the shelf" allogeneic DPSC preparations for clinical scale manufacturing, due to their accessibility, immunomodulatory properties and lack of cell surface expression of immune helper antigens.

It is clear that a considerable amount of work has been conducted over the last two decades with regards to endodontic regenerative medicine. There is a greater appreciation that a multifaceted approach to dental tissue bioengineering is essential, whether by combining scaffolds with bioactive molecules and DPSC, and/or in conjunction with multiple cell types.

Future developments could employ the use of induced pluripotent stem cells (iPSC) derived from dental pulp [95–97], PDL [98], gingival [99] and other accessible tissues to be used for mineral [100], PDL [24] and potentially dentin-pulp regeneration. Importantly, it has been demonstrated that iPSC-derived MSC-like cells [101] exhibit similar multi-differentiation and immune-modulatory properties akin to primary MSC-like populations [102], which is clinically useful to generate an allogeneic source of cells for regenerative therapy. However, the safety and efficacy of using iPSC-MSC in a clinical setting due to issues concerning the efficiency of specific lineage differentiation and tumour formation requires rigorous assessment in vitro and in vivo [97, 103].

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Chapter 2
Platelet Rich Fibrin "PRF" and Regenerative Medicine: 'The Low-Speed Concept'

Joseph Choukroun, Alexandre Amir Aalam, and Richard J. Miron

2.1 Introduction

The multidisciplinary field of tissue engineering has tackled a wide variety of medical challenges over the years with the aim to predictably repair, regenerate or restore damaged and diseased tissues [1–4]. Defects frequently encountered are commonly produced by a variety of underlying conditions caused by congenital abnormalities, injury, disease and/or the effects of aging [1–4]. Many strategies have since been adapted to regenerate these tissues. One of (if not the) key component during the regenerative phases during wound healing is the absolute necessary for ingrowth of a vascular blood source capable of supporting and contributing to cellular function and the future development and maintenance of nutrients across this newly created blood supply [5]. Although normal biomaterial and tissue engineered scaffolds are typically avascular by nature, over 15 years ago a series of proposed motifs introduced blood concentrates as a regenerative modality in order to improve the vascular network to obtain successfully regenerated soft or hard tissues where lack of a blood supply was often at the forefront of the defect [5].

Wound healing is a complex biological process that includes the active participation of numerous cell types, a matrix consisting of extracellular matrix as well as soluble factors capable of facilitating regeneration. By nature, these are normal healing events that take place in response to normal tissue injury involving a cascade

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of complex, orderly and elaborate events [6]. Numerous studies have already demonstrated that the delivery of multiple growth factors in a well-controlled manner can enhance bone formation [7–9]. Generally, the events related to wound-healing are divided into four overlapping phases including hemostasis, inflammation, proliferation and remodeling [7–9]. One of the key players during these phases have been platelets, cells that have been shown to be important regulators of hemostasis through vascular and fibrin clot formation [6]. Ongoing studies over the past decades have revealed platelets are the responsible cell-type for the activation and release of important biomolecules including platelet-specific proteins and growth factors including platelet-derived growth factor (PDGF), coagulation factors, adhesion molecules, cytokines/chemokines and angiogenic factors capable of stimulating the proliferation and activation of cells involved in wound healing including fibroblasts, neutrophils, macrophages and mesenchymal stem cells (MSCs) [10]. For these reasons, it was proposed in the 1990s that platelet concentrates could be utilized and centrifuged to reach supra-physiological doses to achieve wound healing and tissue regeneration by facilitating angiogenesis. While numerous studies have previously demonstrated that the delivery of multiple growth factors can enhance new tissue formation, it has since been shown that more importantly blood vessel formation is tightly coupled with tissue regeneration, and that the ideal scenario for tissue regrowth is to deliver a multitude of growth factors designed to induce angiogenesis and tissue regeneration simultaneously in order to produce a vascularized remodelled/regenerated tissue fully vascularized and able to sustain itself long-term. Leading to the science behind platelet concentrates, a group of research begun to investigate platelet concentrates for tissue wound healing and regeneration in medicine beginning in the 1990s.

2.1.1 Brief History of Platelet Concentrates

Although recently the use of platelet concentrates have gained tremendous momentum as a regenerative autologous source of growth factors utilized in various field of medicine (especially due to the more recent development of platelet rich fibrin (PRF)), it is important to note that their utilization spans over two decades in surgery [11]. It was originally proposed that leading to their preparation, a belief that concentrated platelets derived from autologous sources could be collected in plasma solutions later to be utilized in surgical sites could potentially release supra-physiological doses of growth factors capable of promoting local healing [12, 13]. Further work in the 1990s led to the popular working name ‘platelet rich plasma’ (PRP) which was introduced in the 1990s in dental medicine [14–16]. Since the goal of PRP was to collect the largest and highest quantities of growth factors from platelets, PRP was fabricated with a protocol lasting over 30 min of centrifugation cycles and requiring the use of anticoagulants to prevent clotting. The final composition of PRP contains over 95% platelets, known cells responsible for the active

secretion of growth factors involved in initiating wound healing of various cell types including osteoblasts, epithelial cells and connective tissue cells [14, 17].

Following a few years of use with PRP, several limitations were observed. Since the technique and the preparation required the additional use of bovine thrombin or $CaCl_2$ in addition to coagulation factors, it was found that these drastically reduced the healing process during the regenerative phase. Furthermore, the entire protocol was technique sensitive with several separation phases lasting sometimes upwards of 1 h making it inefficient for everyday medical purposes. Since PRP is liquid in nature, it was originally required as an agent to be combined with various other biomaterials, most notably bone grafting materials. Interestingly, very recent data from our laboratories has shown that growth factor release with PRP is released very early in the delivery phase whereas a preference would be to deliver growth factors over an extended period of time during the entire regenerative phase as opposed to a quick short burst [18–20]. All these limitations have led to the emergence of a second generation of platelet concentrates which takes advantage of the fact that without anti-coagulants, a fibrin matrix that incorporates the full set of growth factors trapped within its matrix and slowly released over time could be achieved [21]. Furthermore, PRF (which was later renamed leukocyte PRF or L-PRF) contains white blood cells, which have been shown to be key contributors to wound healing later described in this chapter.

2.1.2 From PRP to PRF

Due to the reported limitations of PRP mainly derived from anti-coagulant incorporation, further research led by Dr. Joseph Choukroun in the early 2000s was focused at developing a second-generation platelet concentrate without utilizing anti-coagulation factors [22]. As such, a platelet concentrate lacking coagulation factors could be harvested from the upper layer of centrifugation tubes following single centrifugation cycles of 12 min at 2700 rpm (750 g). This formulation was termed platelet rich fibrin (PRF) owing to the fact it contained a fibrin matrix following centrifugation [23–26]. PRF (leukocyte-PRF or L-PRF) additionally contains white blood cells (WBCs) within the fibrin matrix; necessary cells involved in the wound healing process by improving defense immunity and secreting a large quantity of growth factors (Fig. 2.1) [27–32]. It's interesting to note that since WBCs are a combination of neutrophils and macrophages, they are always one of the first cell-types found in wounded infection sites as well as the first cell types in contact with biomaterials and thus play a major role in phagocytizing debris, microbes and necrotic tissue as well as directing the future regeneration of these tissues through release of cytokines and growth factors. As depicted in Fig. 2.2, macrophages areone of the three key cells found in PRF derived from the myeloid lineage (WBCs) and secrete a wide range of growth factors including transforming growth factor beta (TGF-beta), PDGF and vascular endothelial growth factor (VEGF) (Fig. 2.1). These cells, in combination with neutrophils and platelets, are

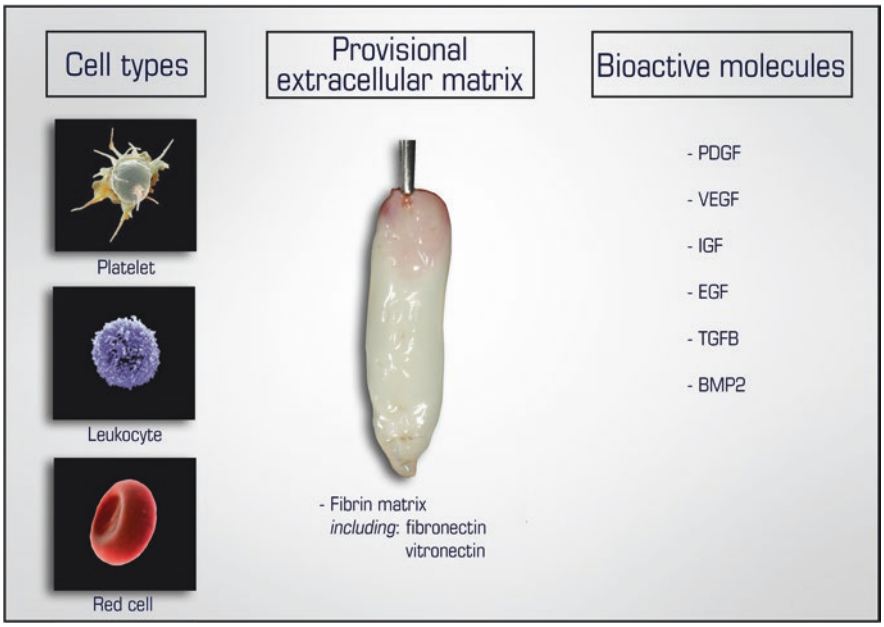

Fig. 2.1 Natural components of PRF include (1) cell types (platelets, leukocytes and red blood cells), (2) a provisional extracellular matrix three-dimensional scaffold fabricated from autologous fibrin (including fibronectin and vitronectin) as well as (3) a wide array of over 100 bioactive molecules including most notably PDGF, VEGF, IGF, EGF, TGF-beta and BMP2 (reprinted with permission from Miron et al. 2016)

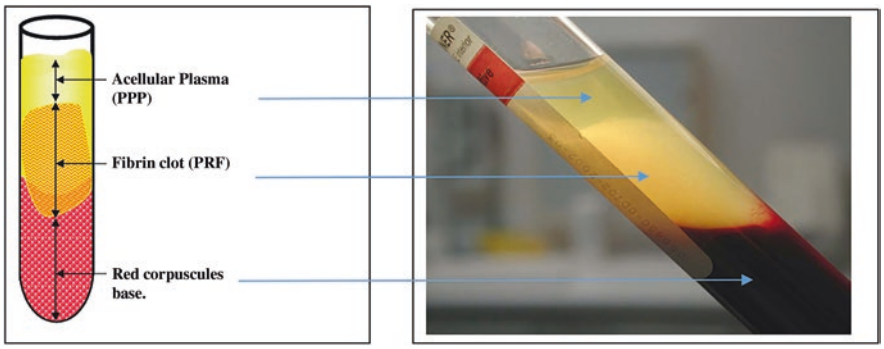

Fig. 2.2 Fibrin clot in the tube after centrifugation

the main players in tissue wound healing and together (as opposed to solely with platelets in PRP) are able to further enhance new blood vessel formation (angiogenesis) which subsequently leads to new bone and tissue formation [23–26, 29]. To date, numerous studies have investigated the regenerative potential of PRF in various medical situations. With respect to tissue engineering, it has long been

proposed that in order to maximize the regenerative potential of various bioactive scaffolds, three components are essential to improve tissue repair including (1) a three-dimensional matrix capable of supporting tissue ingrowth, (2) locally harvested cells capable of influencing tissue growth and (3) bioactive growth factors capable of enhancing cell recruitment and differentiation within the biomaterial surface. With respect to PRF, all three of these properties are met whereby (1) fibrin serves as the scaffold surface material, (2) cells including leukocytes, macrophages, neutrophils and platelets attract and recruit future regenerative cells to the defect sites and (3) fibrin serves as a reservoir of growth factors that may be released over time from 10 to 14 days. Below we summarize these three components in sections and explain the rational of each.

1. Major Cell Types in PRF

A. Platelets

Platelets are one of the cornerstone cells found in PRF and the cells that were first collected in previous versions of platelet concentrates including PRP. Interestingly, in PRF, platelets are theoretically trapped massively within the fibrin network and their three-dimensional mesh allowing their slow and gradual release and associated growth factors over time [20]. Recent research has shown that blood alone is enough to drastically improve wound angiogenesis and tissue regeneration [33].

Platelets are constantly being formed in the bone marrow from megakaryocytes. They are discoidal and anuclear structures by nature and their lifespan is typically in the range of 8–10 days. Their cytoplasm contains many granules whose contents are secreted at the time of activation. Alpha-granules contain many proteins, both platelet specific (such as b-thromboglobulin) and non-platelet specific (fibronectin, thrombospondin, fibrinogen, and other factors of coagulation, growth promoters, fibrinolysis inhibitors, immunoglobulins, etc.) that have been shown to possess many functions during wound healing [34, 35]. Moreover, the platelet membrane is a phospholipid double layer into which receptors for many molecules are inserted (collagen, thrombin, etc.) and act to improve wound healing. Activation is fundamental to initiate and support haemostasis because of aggregation on the injured site and interactions with various coagulation mechanisms [34, 35].

B. Leukocytes

Leukocytes are the other major cell type found in PRF playing a prominent role in wound healing. Interestingly, the major difference between PRF (which has since been renamed L-PRF specifically due to its high leukocyte content) apart from the fact anti-coagulants are not utilized in PRF, is the fact that both PRP and PRGF (first generation platelet concentrates) either do not or contain very low numbers of leukocytes. The literature dealing with platelet concentrates often ignores the impact of leucocytes on tissue wound healing. Several studies have already pointed out the key role of leucocytes, both for their anti-infectious action and immune regulation [36–38]. Apart from their anti-infectious effect, leucocytes produce large amounts

of VEGF and PDGF amongst other growth factors. Additional VEGF, which stems from leucocytes, might be crucially important for the promotion of angiogenesis. The amount of white cells in PRF has been determined at around 50% and newer formulations of PRF have further shown ways to collect a higher number of leukocytes.

Interestingly, studies from basic sciences have revealed the potent and large impact of leukocytes on tissue regeneration [30, 32]. They additionally release growth factors and play a large role in immune defense, but also serve as key regulators controlling the ability for biomaterials to adapt to new environments. For instance, studies conducted following extraction of third molars has shown that a tenfold decrease in third molar osteomyelitis infections was detected simply by placing PRF scaffolds into extraction sockets [39]. Furthermore, in a separate study, patients receiving PRF report having less pain and requiring less analgesics when compared to control, most notably due to the defense of these immune cells preventing infection, promoting wound closure and naturally reducing swelling and pain felt by these patients [40].

Recent research has further shown that macrophages (derived from the white blood cell lineage with leukocytes) are the necessary driving force for new bone formation [41–45]. It has been shown that in certain in vitro culture conditions with osteoblasts, removal of macrophages led to a 23-fold decrease in osteoblast mineralization, drastically and convincingly demonstrating the pronounced impact of macrophages and WBCs in bone biology [43]. Furthermore, it has been shown that monocytes and macrophages are one of, if not the most important cell type during biomaterial integration into host tissues [46]. Therefore, the influence of leukocytes derived from PRF matrixes should not be under-estimated as numerous basic and animal studies have recently pointed to their vast importance in wound healing and tissue regeneration and long-term integration.

2. Platelet Rich Fibrin–PRF: A Natural Fibrin Matrix and Its Biological Properties

While PRF was first developed in France by Choukroun et al. in 2001 [22]. The lack of an anticoagulant made it so that the fibrin clot begins to form during the centrifugation process and when centrifugation tubes are removed, a fibrin clot can be observed as depicted in Fig. 2.2. Naturally this technology requires a centrifuge and a collection system present within the office since anti-coagulants are not utilized, clotting forms rapidly. Therefore, centrifugation must take place within seconds after blood harvesting. The original PRF protocol was first established with a very simple protocol: A blood sample is taken without anticoagulant in 10-mL tubes which is immediately centrifuged at 750 g for 12 min. The absence of anticoagulant implies the activation in a few minutes of most platelets of the blood sample in contact with the tube walls and the release of the coagulation cascades. Fibrinogen is initially concentrated in the upper layer of the tube, before the circulating thrombin transforms it into fibrin. A fibrin clot is then obtained in the middle of the tube, just between the red corpuscles at the bottom of the tube and the acellular plasma at the top (PPP) (Fig. 2.2).

As previously mentioned, the success of this technique entirely depends on the speed of blood collection and its subsequent transfer to the centrifuge. Indeed, without anticoagulants, the blood samples start to coagulate almost immediately upon contact with the tube glass, and it takes a minimum of a few minutes of centrifugation to concentrate fibrinogen in the middle and upper part of the tube. Quick handling is the only way to obtain a clinically usable PRF matrix. If the duration required to collect blood and launch centrifugation is overly long, failure will occur. By driving out the fluids trapped in the fibrin matrix, practitioners will obtain very resistant autologous fibrin membranes.

2.2 What Is Fibrin?

Fibrin is the activated form of a plasmatic molecule called fibrinogen. This soluble fibrillary molecule is massively present both in plasma and in the platelet alpha-granules and plays a determining role in platelet aggregation during haemostasis. It is transformed into what resembles a biological glue capable of consolidating the initial platelet cluster, thus constituting a protective wall during coagulation. In fact, fibrinogen is the final substrate of all coagulation reactions. Being a soluble protein, fibrinogen is transformed into an insoluble fibrin by thrombin while the polymerized fibrin gel constitutes the first healing matrix of the injured site [47]. Studies from basic science have also pointed to the fact that fibrin alone (fabricated from various sources) is able to act as a provisional matrix allowing cell invasion and tissue regeneration [48–50].

3. Cytokines

Cytokines and growth factors have been observed released in high number from platelet alpha granules after clotting. They are active through specific cell receptors and play a predominant role in wound healing. One interesting finding that was recently discovered later described in this chapter is the effect of centrifugation times and speeds on growth factor release from PRF clots, most likely as a result in a higher number of leukocytes and more loosely dense PRF clot allowing better growth factor release from the PRF matrix over time. Below we describe the most commonly reported growth factors found in PRF.

– **TGFb-1:** Transforming growth factor b (TGFb) is a vast superfamily of more than 30 members known as fibrosis agents [51, 52]. The reference molecule from the TGFb superfamily is TGFb-1. In vitro research has demonstrated its effects are extremely variable according to the amount applied, the matrix environment and cell type in which applied. For example, it has been shown that it could stimulate the proliferation of osteoblasts just as easily as it could cause their inhibition [53]. Although its effects in terms of proliferation are highly variable, for the great majority of cell types, it constitutes the most powerful fibrosis agent among all cytokines and the growth factor commonly released from autogenous

bone during tissue repair and remodeling [52]. In other words, it induces a massive synthesis of matrix molecules such as collagen1 and fibronectin, whether by osteoblasts or fibroblasts. Thus, although its regulation mechanisms are particularly complex, TGFb-1 can be considered as an inflammation regulator through its capacity to induce fibrous cicatrisation.

- **PDGF:** PDGFs (platelet-derived growth factors) are essential regulators for the migration, proliferation, and survival of mesenchymal cell lineages. According to the distribution of their specific receptors, they are able to induce stimulation in these cells. This position of regulation node plays a fundamental role during the embryonic development and all tissue remodelling mechanisms. For this reason, PDGFs play a critical role in the mechanisms of physiologic healing and have been commercially available in a recombinant source (rhPDGF-BB) and FDA approved for the regeneration of various defects in medicine and dentistry. Interestingly, PDGF is naturally produced and accumulated in high quantities in PRF clots and are considered one of the important released molecules over time from PRF.
- **VEGF:** Vascular endothelial growth factor was previously isolated as the most potent growth factor leading to angiogenesis of tissues [54]. It has potent effects on tissue remodelling and the incorporation of VEGF alone into various bone biomaterials have demonstrated increases in new bone formation, thereby pointing to the fast and potent effects of VEGF [54].
- **The IGF axis**: Insulin-like growth factors (IGFs) I and II are positive regulators of proliferation and differentiation for most cell types, which act as cell-protective agents [55]. Although these cytokines are cell proliferative mediators, they also constitute the major axis of programmed cell death (apoptosis) regulation, by inducing survival signals protecting cells from many apoptotic stimuli. Moreover, even though IGFs are released during platelet degranulation, they are initially massively present in blood circulation [55].

The combination of these three properties including (1) host cells, (2) a three-dimensional fibrin matrix and (3) cytokine and growth factor release from PRF membranes acts to synergistically lead to a fast and potent increase in tissue regeneration.

2.3 Introducing the Low-Speed Concept

It is now more known that the most important factor for stimulation is not the amount of growth factors released but the maintenance of a low and constant gradient of growth factor delivery to the milieu. As the use of PRF has seen a continuous and study increase in regenerative medicine, there was great interest to determine if the clinical situations could be improved by optimizing centrifugation protocols to alter the PRF matrix. This hypothesis was derived from the fact that cells within the original PRF matrix were surprisingly found gathered at the bottom of the PRF

matrix [56]. Therefore it was found that centrifugation speeds (which naturally push cells towards the bottom of centrifugation tubes whereas the PRF is collected from the top one third) would benefit from slower speeds (g-force) to prevent from driving the cells downwards. This hypothesis was confirmed by a classical study by Ghanaati and co-workers whom showed that by decreasing centrifugation speeds from 2700 rpm (750 g) to 1300 rpm (200 g), a more optimal formulation of PRF could be created with a higher number of leukocytes more evenly distributed throughout the PRF matrix [56]. This new formulation of PRF was given the working name Advanced-PRF or A-PRF and is deemed natural evolution from over 13 years of research from the original L-PRF. It is now recognized that evidently the leukocytes were being pushed out of the fibrin clots unnecessarily down to the bottom of centrifugation tubes. More recently, it has further been shown in a recent study published in the Journal of Periodontology (August 2016) that both centrifugation speed and time could be reduced to further enhance growth factor release and cell performance from A-PRF.

One of the primary proposed reasons for a slower release of growth factors over time is the ability for the fibrin matrix to hold proteins within its fibrin network as well contain cells capable of further releasing growth factors into their surrounding micro-environment [57–61]. Therefore, if centrifugation protocols are optimized to contain more cells (most notably leukocytes), then evidently they will subsequently release more growth factors over a 10 day period as well as contribute to tissue defence, and biomaterial integration all factors necessary to further enhance tissue regeneration.

Another interesting observation has been that since centrifugation speeds have been drastically decreased from the first version of L-PRF, it was observed also that a liquid version of PRF could be obtained with even lower centrifugation speeds. This new formulation was given the working name 'Injectable-PRF' or I-PRF due to its hypothesized ability to be injected into defects or be combined with other biomaterials such as bone grafts or barrier membranes (in a similar fashion to PRP however without use of anti-coagulants) further improving tissue regeneration. While ongoing research is continuously underway, this new formulation of I-PRF has been shown to contain an increase in leukocytes and mesenchymal progenitor cells have also been detected utilizing lower centrifugation speeds which have been decreased from 2700 to 700 RPM (750 g to 60 g) for only 3 min. Below we summarize the effects of these two new formulations of 'smart' blood concentrates on leukcocyte number and VEGF growth factor quantity (Fig. 2.3).

1. Advanced Platelet Rich Fibrin: A-PRF

Considerable evidence has been accumulating demonstrating the pronounced and marked impact of white cells on vascularization and bone formation [36]. Furthermore, granulocytes have been shown to play an additional role on vascularization and improve the function of monocytes whom have been described by Soltan et al. to be the so-called "super cells for bone regeneration" [62]. Both cells are found in higher concentrations in A-PRF. Our understanding of the role of g-force on the loss of white cells during the spin cycle guided for new protocols to reduce

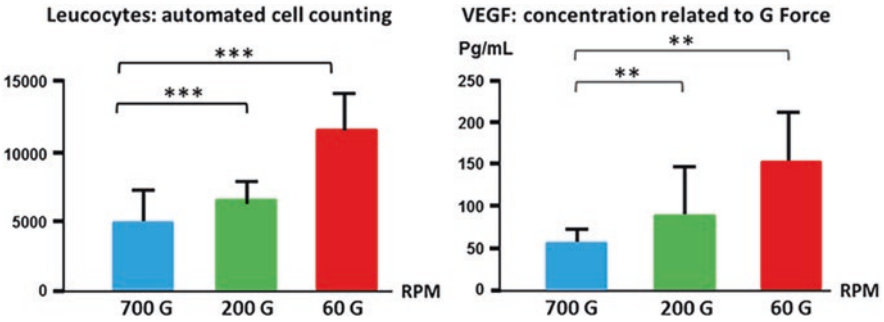

Fig. 2.3 Higher number of leucocytes and VEGF found in PRF centrifuged at lower g-forces. Figures adapted from (**Choukroun J.** et al. *Injectable Platelet Rich Fibrin: A smart blood concentrate achieved by the low speed concept.* **J.Cell Communication Signaling** *in revision*)

the rpm to maintain a higher amount of white cells in the fibrin matrix. Furthermore, the introduction of a special glass tube that induced a more rapid clotting allowed a marked reduction in centrifugation time from 12 to 14 min down to 8 min, further reducing the lost number of leukocytes from high centrifugations speeds and times. This new fibrin clot is richer in white blood cells (Fig. 2.3), with a fibrin matrix that is less dense allowing the invasion and penetration of incoming cells to repopulate the matrix in an ongoing more rapid process [56]. The newer formulation of PRF (A-PRF+) has been shown to increase growth factor release of TGF-beta1, PDGF-AA, PDGF-AB, PDGF-BB, VEGF, IGF and EGF (Fig. 2.4). Furthermore, it has subsequently been shown that gingival fibroblasts in contact with A-PRF produce higher collagen levels and a significantly higher cell migration towards A-PRF+ was observed when compared to either PRP or L-PRF (Fig. 2.4).

2. Injectable Platelet Rich Fibrin: i-PRF

With the same concept of non-additive platelet derivatives, i-PRF was developed to fulfil the goal of acting as a regenerative agent that could be delivered in liquid formulation by drawing blood rapidly in a specific centrifugation tube at a very low speed of 700 rpm (60 g) for an even shorter centrifugation time (3 min). Here the objective was to centrifuge without anti-coagulants nor additives, yet maintain the ability to separate two layers as depicted in Fig. 2.5. This new formulation can be utilized for a variety of procedures including mixing with bone grafts to form a stable fibrin bone graft for improved handling after a short period of time (1–2 min) which improves graft stability (as can be envisioned during sinus lifting procedures with bone grafting materials to improve graft stability by avoiding the migration of granules into the maxillary cavity). Subsequently, I-PRF alone can be used for a variety of procedures when utilized alone including knee injections for the management of osteoarthritis, temporo-mandibular joint disorders as well as various procedures in facial aesthetics to improve collagen synthesis naturally. The principle for I-PRF remains the same—it contains a larger proportion of leukocytes and blood plasma proteins due to the ‘low-speed concept’; known inducers of vascularization and thus speed the rate at which wound healing can take place.

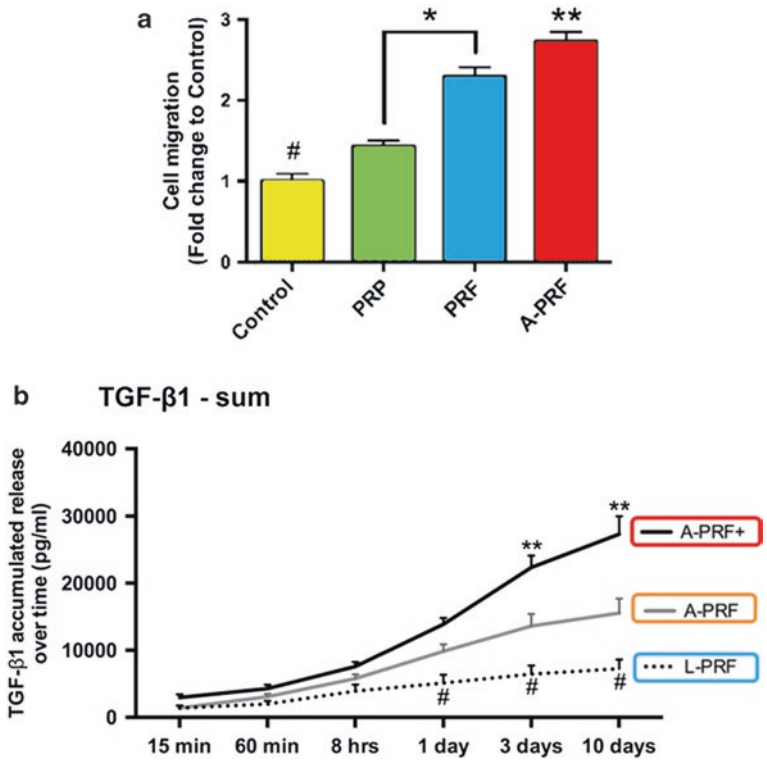

Fig. 2.4 Higher number of gingival fibroblast cell migration from A-PRF when compared to PRF and PRP as well as higher growth factor released from the slow speed concept. (**a**) The cell migration assay shows a higher gingival fibroblasts migration from A-PRF when compared to PRF and PRP, (**b**) it is observable a higher growth factor (TGF-beta1) release when the slow speed concept is performed. Adapted with permission from Kobayashi et al. [2016]: *Optimized Platelet Rich Fibrin with the Low Speed Concept: Growth Factor Release, Biocompatibility and Cellular Response*. Accepted for publication in **Journal of Periodontology** (not yet online)

2.4 Clinical Use of PRF and Indications

The clinical uses of PRF have exploded across many fields of medicine and dentistry over the past 15 years since its original development. Most notably, PRF has had a major impact in soft tissue regeneration as well as various indications in dentistry where PRF can be utilized as a fast and easy procedure to aid in the regeneration of various common bone and soft tissue defects often encountered in daily clinical practice.

Our group recently performed two extensive systematic review articles to elucidate the effects of PRF on (1) soft tissue wound healing and (2) its use in dentistry.

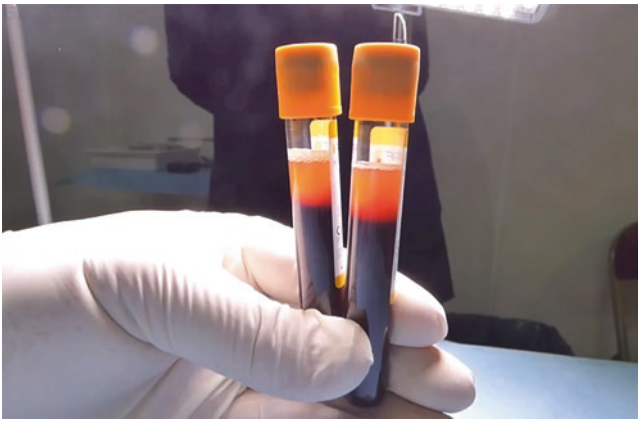

Fig. 2.5 The newer formulation of I-PRF is a liquid formulation of PRF found in the top 1 mL layer of centrifugation tubes following a 700 rpm spin for 3 min. This liquid can be collected in a syringe and re-injected into defect sites or mixed with biomaterials to improve their bioactive properties

In total 164 articles were screened for soft tissue wound healing and publications were divided into (1) in vitro, (2) in vivo and (3) clinical studies. In summary, it was found that 86% of all included articles found a significant increase in tissue wound healing and regeneration when PRF was used when compared to their respective controls. Most notably however, the use of PRF has remarkably now been utilized in over 20 different clinical procedures in medicine and dentistry; 7 of which coming from the oral and maxillofacial region. In the dental field, the most commonly utilized use of PRF is for the treatment of extraction sockets [39, 63–65], gingival recessions [66–68] and palatal wound closure [69–71] with PRF being additionally utilized for the repair of potentially malignant lesions [72], regeneration of periodontal defects [73], hyperplastic gingival tissues [74] and in conjunction with periodontally accelerated osteogenic orthodontics [75]. In general medicine, the use of PRF has been successfully utilized for hard-to-heal leg ulcers including diabetic foot ulcers, venous leg ulcers and chronic leg ulcers [76–80]. Furthermore, PRF has been utilized for the management of hand ulcers [81], facial soft tissue defects [82], laparoscopic cholecystectomy [83], in plastic surgery for the treatment of deep nasolabial folds, volume-depleted midface regions, facial defects, superficial rhytids and acne scars [84], induction of dermal collagenesis [85], vaginal prolapse repair [86], urethracutaneous fistula repair [87, 88], during lipostructure surgical procedures [89], chronic rotator cuff tears [90] and acute traumatic ear drum perforations [91]. Thus, there is evidently growing use of PRF for the treatment of various medical procedures due to its ability to (1) speed revascularization of defect tissues and (2) to serve as a three-dimensional fibrin matrix capable of further enhancing wound healing.

Furthermore, a second systematic review focused only on the regenerative potential of PRF in dentistry found that of roughly 200 articles that were investigated

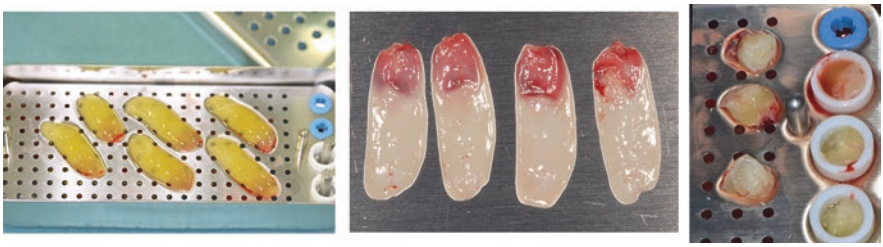

Fig. 2.6 PRF clots formed to either make membranes or PRF plugs

(only clinical studies), the most commonly utilized uses of PRF were shown to be for (1) guided bone regeneration procedures and extraction socket healing, (2) sinus lift procedures, (3) for the treatment of gingival recessions and (4) for intrabony and furcation defect regeneration. Of all the known clinical applications of PRF, it is known that PRF accelerates tissue cicatrisation due to enhanced neovascularization and ability to defend against an infectious environment found in the oral cavity.

When it comes to soft tissue management and maturation utilizing PRF, three key elements have been encountered. PRF is able to simultaneously support the development of angiogenesis, immunity and epithelial coverage. Fibrin has been shown to act as the natural scaffold guiding angiogenesis which consists of the formation of new blood vessels inside the wound. Thus, the requirement of an extracellular matrix scaffold that allows the migration, division, and phenotypic change of endothelial cells has been clearly demonstrated leading to faster angiogenesis. Furthermore, the angiogenic property of PRF may further be partially explained by the high number of trapped cytokines found within the fibrin mesh. Here a variety of cytokines and ECM proteins have been found within PRF providing structural and functional support for the cells and tissues involved in the regeneration process consisting of several molecules including collagen, proteoglycans, heparin sulfate, chondroitin sulfate, hyaluronic acid, elastin, fibronectin, and laminin. A few plasma-derived proteins such as fibrin, thrombospondin, and fibronectin have also been reported as provisional ECM.

Regarding the clinic use of PRF in daily dental practice, PRF scaffolds may be utilized as both a tissue matrix/scaffold (provisional ECM) with the ability to simultaneously release growth factors over a 10 day period. The clots are prepared in a PRF metallic box which allows the slight compression of their clots into membranes or plugs to be later utilized as depicted in Fig. 2.6.

1. Socket Preservation

The most often utilized application for PRF in dental practice has been in the management of extraction sockets [39, 64, 92, 93]. After extraction, the socket may be filled with PRF plugs as depicted in Fig. 2.7 by utilizing the philosophy “as much as you can” into the extraction socket. Since PRF is a natural matrix including various wound healing cell-types, it provides the ability to increase and speed tissue regeneration. This technique furthermore does not necessitate the use of having to

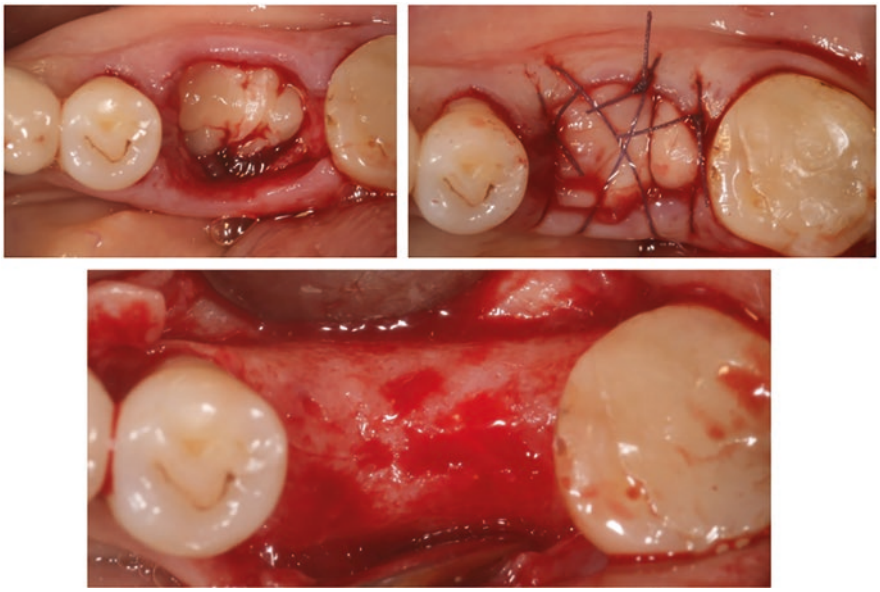

Fig. 2.7 PRF plugs that have been utilized to fill an extraction socket followed by appropriate suture for PRF stability. After a 3 month healing period, new bone formation taking place prior to implant bed preparation

use another barrier membrane or other biomaterials to cover the flap as the PRF scaffolds may be left exposed. Sutures are simply used for stabilization purposed of the PRF matrix within the socket. Primary closure is not necessary as the material in the socket is fully natural. Over time, the fibrin matrix is transformed into new tissue: bone in the socket and soft tissue at the surface. The healing of the site is completed after 3 months. Further advantages of using PRF for socket preservation is the fact that reports have shown that PRF reduces osteomyelitits infections in third molar extraction sites approximately tenfold and decreases the amount of pain and analgesics taken as reported by patients [39, 64, 92, 93].

2. Sinus Lift

The principle for the use of PRF for sinus lifting is quite the same as for socket preservation, it acts as a provisional matrix of ECM proteins which provide quick vascularization due to its simultaneous incorporation of autologous growth factors. Here, PRF can be utilized alone or mixed with a bone grafting material. In such combination cases, PRF membranes may be cut into small fragments with scissors and mixed with a bone grafting material. However, as in the sockets, PRF is often utilized alone and many reports now point to the fact that PRF can act as a sole grafting material when utilized (1) during sinus lifting procedures with simultaneous implant placement and (2) preferably in narrow sinus [94–96]. Furthermore, PRF may be utilized for the repair of Shneiderian membranes, or to close the maxillary window during lateral sinus lifting procedures (Fig. 2.8).

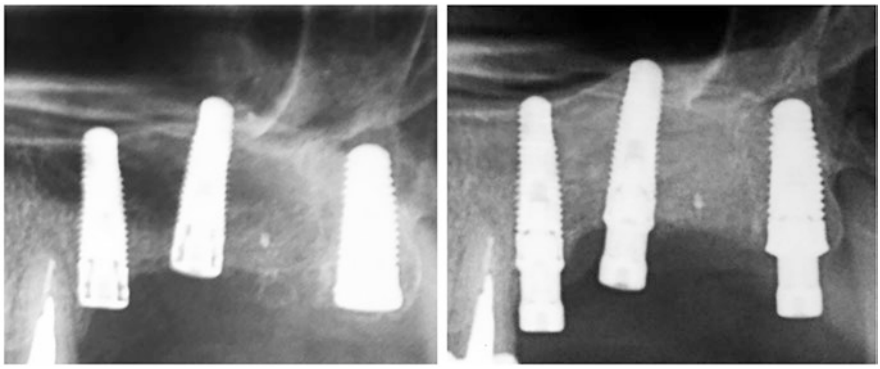

Fig. 2.8 Implant placement into the sinus in combination with PRF. Notice the new bone formation taking place around the apical portion of implants after a 6 month healing period

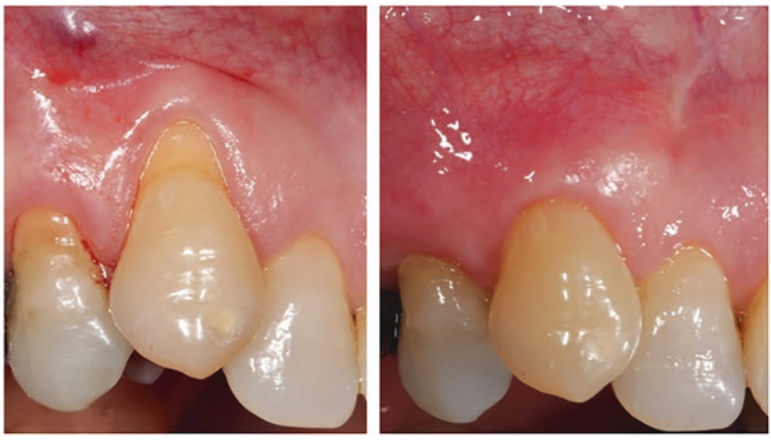

Fig. 2.9 Gingival recession of upper canine treated with PRF alone. Notice the excellent wound healing properties of PRF following a 6 month healing period with revascularization of the underlying soft tissues

3. Soft Tissue Management: Gingival Recession Regeneration

The treatment of gingival recessions with PRF has also been a highly utilized regenerative procedure used by many periodontists. Over ten clinical studies have now shown that in Miller Class I and II defects, PRF can be utilized as a sole grafting material often carrying the ability to replace connective tissue grafts harvested from the palatal sites [67, 97–108]. Therefore, PRF may be used an alternative graft material for treating multiple adjacent recessions of the gingiva without a requirement of a second surgical site thereby reducing patient morbidity. In such procedures, it has commonly been reported that although PRF has the ability to significantly improve root coverage to similar levels as CTG, one remaining limitation is it does not necessarily improve the thickness of keratinized tissue. Therefore, in clinical situations

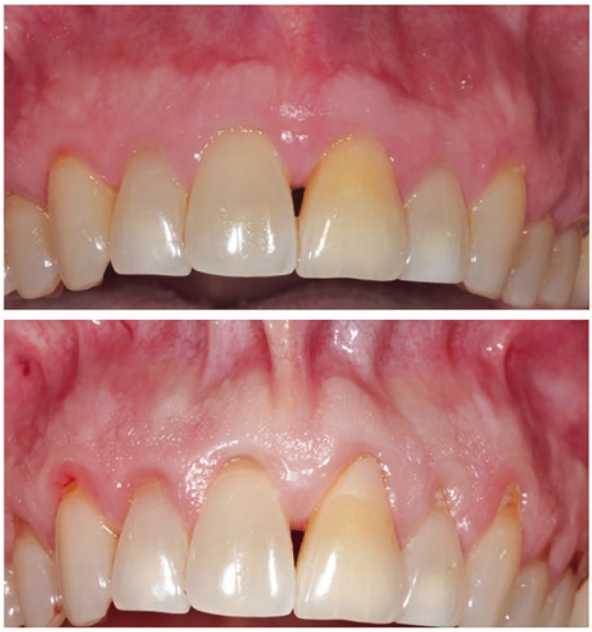

Fig. 2.10 Multiple gingival recession of upper 8 maxillary teeth treated with PRF alone. Notice the excellent root coverage of all teeth treated with PRF following a 6 month healing period with great keratinized tissue

where keratinized tissue is lacking, PRF may then be combined with a CTG in order to improve tissue thickness while simultaneously improving tissue revascularization and regeneration (Figs. 2.9 and 2.10).

4. Intrabony Defect Regeneration with PRF

Another area of research receiving much attention in recent years has been regarding the use of platelet concentrates for periodontal regeneration of intrabony and furcation [59, 60, 73, 109–116]. As such, PRF alone or combined with bone grafts has also been utilized in a number of clinical studies showing improved results when compared to controls alone. Recent evidence suggests that PRF alone can be utilized for intrabony defect as successfully as various leading bone grafting materials including demineralized freeze-dried bone allografts (DFDBA) [117]. Furthermore, PRF has been shown in three studies to significantly improves the regeneration of Class II furcation defects [118–120].

2.5 Conclusion

The use of PRF in regenerative medicine has now seen a huge increase in its use across many fields of medicine due to its ease of use and low-associated costs while providing a completely autologous source of growth factor delivery. Furthermore, recent advancements in our understanding of the regenerative potential of PRF has

further allowed modifications to the centrifugation speeds and times (A-PRF) to further enhance its regenerative potential and bring to clinical practice a liquid formulation that is injectable during use (I-PRF).

After more than 15 years of research and more than 450 publications available in Medline, there continues to be growing evidence and support for its use. Future strategies are continuously being developed to further improve the clinical outcomes following regenerative procedures utilizing platelet concentrates.

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Chapter 3
MSCs and Innovative Injectable Biomaterials in Dentistry

Ines Fasolino, Maria Grazia Raucci, and Luigi Ambrosio

3.1 Introduction

The dentistry health is critical to ensure life quality. Oral cavity defects often raise risk of several disorders including heart diseases [1]. As life expectancy increases the requirement for new bone substitute for tooth is growing very rapidly in the last decade. As a result, there is a great request of biomaterials with detailed properties such as anti-inflammatory, antibacterial and regenerative properties [2]. Currently people with a greater loss of alveolar bone has a risk 6.6 times higher of suffering from heart attack and stroke compared to people who have a healthy mouth. This correlation is more significant in younger people and may be more direct because mouth microorganisms are able to spread easily to the heart. The disorders caused by mouth microorganisms concern especially heart valve defects (such as mitral valve prolapse) because the germs are located directly on the valve, turning a trivial infection of the mouth in a much more serious disease such as endocarditis [3]. Another important direct binding between the heart and the mouth is the pain. The toothache is considered one of the most severe pain. It is well known that all particularly strong pain stimuli can cause a narrowing of the blood vessels. This reduces the normal blood supply to the heart. Vasoconstriction leads to increased blood pressure and may increase the risk of heart damage. It is necessary to prevent infections and dental problems that can cause intense pain, especially in the presence of risk factors for cardiovascular disease. Tooth loss is caused by periodontitis (i.e., a severe inflammation of the periodontium), advanced carious lesions, age-related alternations, or cancer [4]. Hence, the therapy of oral (traumatic and degenerative) diseases which lead to tooth loss including alveolar resorption is crucial. Oral disorders include periodontal disease that is an infectious, complex, multifactorial,

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chronic inflammatory disease of supporting periodontal tissues. Periodontal chronic inflammation not only damages the bone morphology but also leads to the reduction in bone height [5]. Different issues are associated to chronic periodontal disease: loss of attachment due to destruction of periodontal ligament, loss of adjacent supporting bone, a period of rapid destruction localized. In the case of deep intrabony defects the regeneration is difficult to attain because anatomy impedes the accessibility and obstructs the integration of the grafted material into the physiological architecture [6]. The oral surgery is yet considered the first approach to treat tooth degenerative diseases. In recent years, considerable attention has been given to regenerative medicine and tissue engineering in order to replace oral tissues. In this context, the main challenge in tissue engineering is to introduce biomaterial-based techniques which stimulate stem cell response in terms of oral tissue regeneration. Repair of dental pulp and periodontium is considered an enormous clinical challenge since human teeth have a very limited capacity to regenerate [7]. Teeth regeneration needs a big knowledge of the cellular and molecular events linked to odontogenesis. It is well known that mesenchymal cells give rise to the dental pulp, the dentin-secreting odontoblasts, and the periodontal ligament cells that anchor the tooth to the surrounding alveolar bone. As a result, the dental pulp is capable to generate a connective tissue that conveys vascularization and innervation and hosts stem cells, as well as the dentin [4]. Root growth, cementum matrix deposition, and periodontium formation occur simultaneously to dental pulp innervation [8]. Dental pulp integrity is crucial because it provides trophic support, sensation, and defense against the various pathogens; in fact, devitalized teeth are subject to severe complications that cause tooth fragility and fracture [9]. Hence, the maintenance of dental pulp vitality has a prominent role in endodontic clinics (Fig. 3.1).

Current regenerative therapies in dentistry involve biomaterials and implants with still questionable efficacy and durability [10]. Moreover, these treatments do not preserve the appropriate physiological function of the tooth organ. For this reason, there is an increasing need for new techniques based on biomaterial enabling a balance between new dental tissue formation and unaltered physiological functions of the tooth organ [11]. The endodontic surgery plays a key role in the treatment of traumatic or degenerative diseases that lead to a tissue loss and utilizes techniques that have been improved over time.

Since 1990s, numerous materials for supporting cell attachment, growth, and differentiation, as well as novel stem cell sources and bioactive molecules are identified and tested in order to improve tissue regeneration after lesions due to trauma and/or diseases. In this context, scaffolds in regenerative dentistry can repair dental tissue damaged by inflammation and/or trauma. Inflammation often causes pulp necrosis thus promoting the death of odontoblasts and tooth fracture. The tooth structure is hard to regenerate for the presence of dentin. In fact the dentin is a substance produced only by odontoblasts and consequently dentin-like tissue can be released only by odontoblast-like cells. The researchers developed new experimental models for dentin-like tissue regeneration through the combination of three key elements for tissue regeneration, namely, stem cells, bioactive molecules (e.g., growth factors),

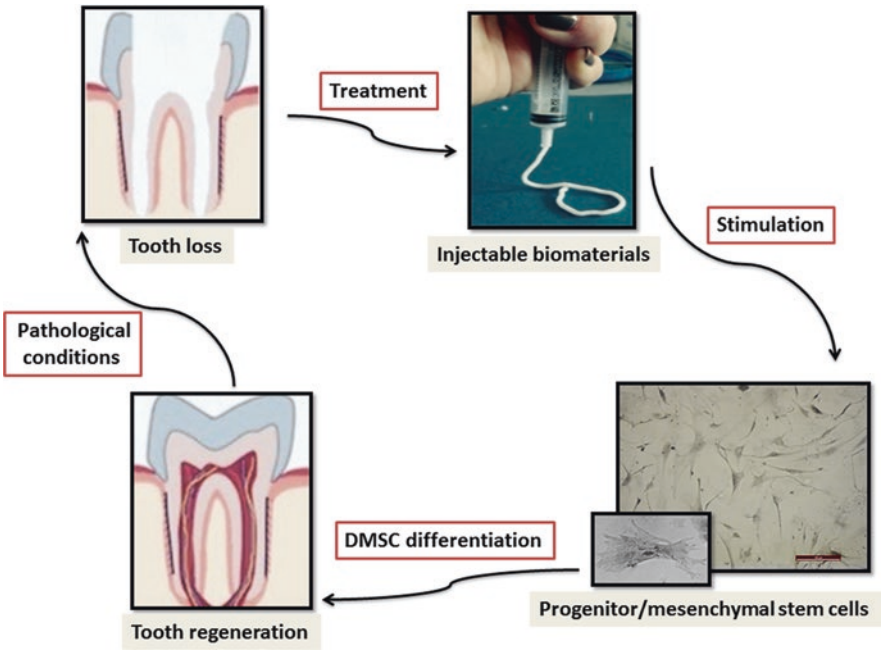

Fig. 3.1 Overview of chapter—injectable materials to stimulate tooth regeneration

and scaffolds [12]. Scaffolds mimicking extracellular-matrix endow mechanical support, promote biological response and regulate bioactive molecule effects [13].

A wide variety of polymer scaffolds—both synthetic (e.g., poly[lactic] acid) and natural (e.g., collagen), ranging from macroporous structures obtained through salt leaching/solvent casting and gas foaming, to nanofibrous scaffolds processed via electrospinning, self-assembly, and phase-separation—have been realized for regeneration of the pulp-dentin complex [14–16]. In regenerative medicine, medical devices are usually realized on the basis of a particular approach that utilizes specific bioactive, biodegradable synthetic or natural scaffolds combined with cells and/or biological molecules, to replace damaged tissue site. In medical research over the past 50 years, different biomaterials in order to replace tissue function, have been identified. Starting from 1950s, there was a predominant use of metal implants and associated devices with a good effectiveness on local tissues. Throughout the 1970s and 1980s, there was a wide use of polymers and synthetic materials for enhancing cell biological responses. Recently, there has been an increasing interest in the design of both natural and degradable scaffolds. These scaffolds are gaining more functions over the time. They are becoming: in three dimensions, structurally more acceptable, able to totally regenerate tissue [17].

At first tissue engineering proposed the use of platelet concentrates, which favored and accelerated the post-surgical with a lot of benefits for patients. These platelet concentrates have been enriched with growth factors that promote tissue

regeneration. Many authors have emphasized the advantages of the use of growth factors in tissue repair processes. The first studies were published on the use of growth factors (GFs) contained in platelet gel, called Platelet-Rich Plasma (PRP), which required a complex and expensive protocol for its production [18, 19]. The evolution of the PRP was the PRGF (Plasma Rich in Growth Factors) containing a higher concentration of growth factors. Moreover, the PRGF has produced by using a procedure relatively faster. Marrelli et al. have shown that the filling with PRF of a large osteolytic cavity promoted complete bone reformation [20]. Tatullo et al. have demonstrated the osteoinductive potential of PRF related to neoangiogenic ability and concentration of GFs that promoted the totipotent cell migration and activation of pre-osteoblastic cells present in the surgical site [21]. In fact, PRF when used as a membrane or as a grafting material promotes cell events such as osteoblast proliferation leading to mineralized tissue formation [22]. The latest discoveries related to the use of scaffolds and/or stem cells in regenerative endodontics have been focused on injectable materials synthesis because these materials, besides inducing cell response in terms of proliferation, adhesion and differentiation, are capable of controlling growth factor delivery and angiogenesis more effectively than other materials. Gelatin produced by the partial hydrolysis of collagen plays a pivotal role as biomaterial for tissue regeneration due to its useful properties such as biodegradability, biocompatibility and anti-immunogenicity [12]. Recent findings showed that also alginate and/or chitosan (natural polymers) are useful to achieve injectable biomaterial based scaffold for clinical applications aimed to regenerate teeth including dentinal-wall-thickening, root maturation, and, in the same cases, the formation of reparative cementum-like tissue [23, 24].

3.2 Mesenchymal Stem Cells: Tools for Tissue Regeneration in Dentistry

Many research studies have been performed on MSC capability of generating several tissue types including oral tissue. It was widely reported that MSC isolated from bone marrow in combination with scaffolds and growth factors promote bone repair in several *in vivo* and *in vitro* experimental models [25]. These studies demonstrated that MSC residing in the oral cavity represent a source for formation of new connective tissues such as dentin, cementum and periodontal ligament [26]. Nowadays the frontier of regenerative medicine is represented by the individuation of the ideal scaffold that enhances MSC residing response in terms of cell growth, spreading, adhesion and differentiation. Phenotypically, MSCs express the CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146 and STRO-1 surface antigens, and they do not express CD45 (leukocyte marker), CD34 (the primitive hematopoietic progenitor and endothelial cell marker), CD14 and CD11 (the monocyte and macrophage markers), CD79 and CD19 (the B cell markers), or HLA class II. Investigations on MSC from oral origin began in 2000 and oral tissues appear

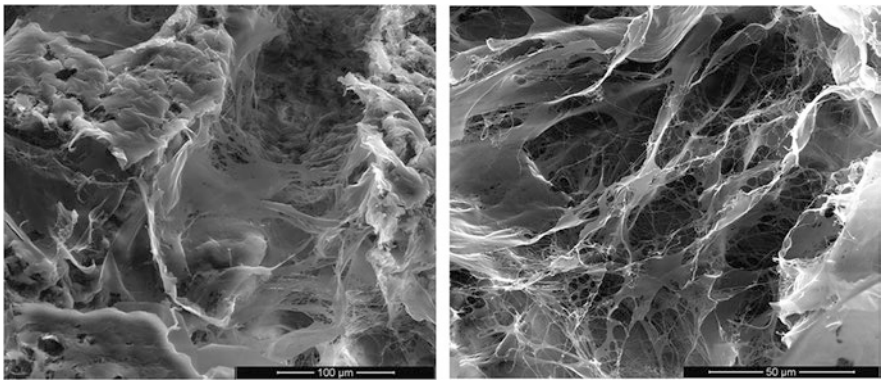

Fig. 3.2 Cell-material interactions—hMSCs after 21 days on scaffold biomaterials for tooth regeneration

simply available for dentists and a rich source for mesenchymal stem cells [27]. Most recent approaches aimed to tissue regeneration are performed by using MSCs taken from sites that are even more accessible and rich in stem cells: the oral cavity represent an important source of MSCs due to its easily accessibility to the surgeon. In oral cavity tissue regeneration exists naturally thanks to the ability of stem cells to renew themselves indefinitely and differentiate into multiple more specialized cell phenotypes. However, these regenerative mechanisms decrease with age and cells lose the capacity to repair damaged tissues [28].

The regenerative medicine introduced the combination of biomaterials, growth factors and stem cells for avoiding the lack of "self-renewal" in damaged tissue [29]. Recently, different materials with optimal physical and mechanical features have been identified. These biomaterial-based scaffolds used in tissue engineering approaches, have been produced using natural or synthetic polymers that are biocompatible and biodegradable. Scaffold properties are crucial for enhancing MSC biological response (Fig. 3.2). Furthermore, the stem cells for regenerative medicine should comply with the following features: they should be in abundant number, they should be able to differentiate in multiple cell lineages, they can be isolated by minimally invasive procedure, produced according to GMP (Good manufacture Practice) and transplanted safely [30, 31].

In the last decade, three main types of stem cells useful for tissue repair were identified: (1) the embryonic stem cells derived from embryos (ES); (2) the adult stem cells that are derived from adult tissue; and (3) the induced pluripotent stem (iPS) cells that have been produced artificially via genetic manipulation of the somatic cells [32]. ES and iPS cells are pluripotent stem cells because they can differentiate into all types of cells from all three germinal layers. By contrast, adult stem cells are multipotent because they can only differentiate into a restricted number of cell types. It is well known that each tissue consists of a specific area named "stem cell niche" containing adult stem cells. The first time MSCs were isolated from bone marrow by Friedenstein et al. in 1974 [33]. Currently, MSCs can be isolated from

different tissues such as peripheral blood, umbilical cord blood, amniotic membrane, adult connective, adipose and dental tissues [34]. Mesenchymal stem cells (MSCs) represent an advantageous therapeutic option for dental defects in presence of specific biomaterials that can manipulate the fate of stem cells leading to high quality tissue regeneration [35]. Nowadays, in bone tissue engineering, encapsulating the cells within hydrogel biomaterials is the major challenge because stem cell encapsulation in hydrogels prevents also the host pro-inflammatory response. Besides controlling the fate of stem cells, the biomaterials play a key role in regulating MSC physiological functions such as survival and host immune system control [36].

It is well known that pro-inflammatory mediators such as TNF-α (tumor necrosis factor alpha) and IFN-γ (Interferon gamma) induced down-regulation of osteogenesis thus inhibiting MSC-mediated bone regeneration [37]. Hence, by using encapsulating hydrogel biomaterials is possible to protect MSCs from the host immune cell/cytokine insult and regulate the crosstalk between immune cells and MSCs. For this purpose several preclinical immunocompromised animal models have been carried out for testing different types of scaffolds and stem cell sources in association with growth factors [12].

Most studies [38, 39] are focus on modification of the scaffold to enhance odontogenic differentiation and biomineralization. At present the effect of matrix stiffness on MSC fate in terms of odontogenic differentiation is still largely unclear. However, a study of Engler et al. showed that the elasticity of the matrix influences the differentiation of MSCs into osteoblast-like-phenotype in an ascending manner, with the stiffest matrices supporting MSC differentiation to osteoblasts [40]. Recently, MSC-like cells exhibited a tumorigenic potential but they might lose carcinogenic activities, implanting them safer into humans [41]. For this purpose, in a recent research the generation of iPSCs by combining primary human gingival fibroblasts and episomal plasmid vectors has been assessed. Such iPSCs could represent a promising source of stem cells in order to evaluate SC potential for future clinical applications.

Numerous investigations for evaluating the *in vivo* application of MSCs isolated from the oral cavity were carried out on animal models. MSCs isolated from the gingiva showed self-renewal and multipotent differentiation capacity similar to that of MSCs [42]. Moreover, MSCs isolated from the salivary glands could generate the salivary gland duct cells as well as mucin and amylase producing acinar cells *in vitro* [43]. In addition, MSCs isolated from peri-osteum are able to differentiate into bone tissue cells [44]. Unlike bone marrow that is a not easily accessible tissue, the orofacial tissues are the most accessible stem cell sources. MSCs can be isolated also from periapical cysts (hPCy-MSCs) thus overcoming surgical methods or tooth or pulp extraction [45]. MSCs obtained from the periapical cysts can be simply expanded and represent a promising source of adult stem cells in dentistry for oral tissue regeneration.

Hence, stem cell-based therapies are very promising long-term alterative in dentistry since they could restore dental tissues keeping structural integrity and physiological functions of teeth. In vivo studies confirmed the successful of stem cell-based therapies in dentistry not only in animal models but also in humans. Stem cells could be used for several applications in dentistry such as reestablishment of

dental pulp vitality and new dentin formation. The use of stem cell-based strategies has started to be applied in endodontic clinics. The main goal after tooth loss would be the regeneration of an entire tooth by using stem cell-based approaches. The distinction of various dental stem cell populations as well as their behavior after transplantation in ectopic sites keys a pivotal role in applying these novel approaches. Moreover, the innervation and vascularization control stem cell niche homeostasis, thus influencing stem cell fate and behavior [46]. Despite the limitations related to the translation of stem cell-based approaches into the clinics, these emerging strategies represent the future of dentistry that will benefit millions of patients worldwide. Due to the limitations of cell injection therapy, the investigation of biological mechanisms underlying tissue regeneration is of primary importance. In oral regenerative medicine the most likely candidate for such therapies remains the human oral mucosa-/gingiva-derived MSCs due to their immunomodulatory and anti-inflammatory properties. In fact, MSCs can modulate the intensity of immune response by inducing T-cell apoptosis, which have a great therapeutic potential in terms of antinflammatory effect when utilizing biomaterials for tissue engineering applications [28]. In order to generate a new oral tissue MSCs will be isolated, expanded in culture and finally seeded within or onto a natural or synthetic scaffold that can reproduces the shape of the newly forming tissue and then the newly formed "organoid" can be transplanted into the patient. Another opportunity is to directly implant acellular scaffolds into the oral defect thus the body cells can populate the scaffold to form the new tissue in situ. In this context, many authors have highlighted a relevant synergistic role of biological molecules for cell-based therapies in order to achieve properly functioning dental tissue regeneration.

3.3 Injectable Scaffolds in Dentistry: State of Art

3.3.1 Injectable Polymeric Scaffolds

No single implantable scaffold involved in the functional regeneration of the pulp-dentin complex exists. Tissue-engineering-based strategies for regenerative endodontics include very promising injectable-based scaffold. Injectable biomaterials allow the incorporation and the release of therapeutic agents, such as antimicrobial and anti-inflammatory drugs thus promotingoral cavity disinfection, as well as bioactive molecules that can trigger stem cell differentiation to aid in regeneration of the pulp-dentin complex. More recently, injectable electrospun-based scaffolds [47] have also shown an excellent structural stability over time, with better chances for overcoming the adaptation issue associated with initial testing of macroporous scaffolds [14, 15]. Notably, the use of injectable hydrogel polymers shows advantages compared to the use of non-injectable scaffolds because of their capability of intracanal delivery, which allows stem cell niche formation [16, 48]. Moreover, drugs such as antibiotics may also be incorporated into injectable hydrogel polymers, thus

treating oral cavity infections. In addition, growth factors may be encapsulated into hydrogels laded to the neovascularization and regeneration of tissues relevant to the dentin-pulp complex [49, 50]. Recently several evidences on potential clinical impact of a very promising hydrogel-based nanofibrous scaffold named Puramatrix™ have been reported. Puramatrix™, is a hydrogel bioactivated through a peptide that, upon interaction with physiological conditions, polymerizes and forms a biodegradable nanofiber hydrogel scaffold [16]. This mechanism favors clinical application that requires not only a biocompatible matrix, but also that can be rapidly formed. It was shown that Puramatrix™ supports dental pulp stem cell survival and proliferation *in vitro* [48]. The commercially available peptide hydrogel scaffold PuraMatrix™, a synthetic matrix comprising a repeated polymer of four amino acids (R-A-D-A) and water, supported the development of a capillary network when the HUVEC are co-cultured with DPSCs. Furthermore, several reports have demonstrated that the HUVECs had an inducing effect on mineralization by the DPSCs due to a direct cell–cell contact of HUVECs with osteoblasts. In vivo studies confirmed that the transplantation of PuraMatrix™ allows the partial regeneration of pulp-like tissue within the root canals. PuraMatrix™ hydrogel, through a pre-vascularization process, can enhance vascularization within a cell construct, because the regeneration of full-length pulps is inhibited when only the apical region is available for vascular connection. Hence, injectable systems like PuraMatrix™ is particularly attractive for clinical translation of dental pulp regeneration, because it can be easily realized with growth factors or drugs and cells by simple mixing. Moreover, PuraMatrix™ can conform to the variable shape of the pulp chamber, following injection [51]. In the design of the scaffold for dental pulp tissue engineering, to overcome the disadvantages associated to the use of natural biopolymer gels (collagen, Matrigel, PuraMatrix, and hyaluronic acid), which do not tune the mechanical properties independently from matrix composition and architecture, semisynthetic hydrogels have been realized. For example, PEG-fibrinogen (PF) based scaffold is able to retain mechanical properties by the addition of cross-linker that controls the hydrogel cross-linking degree, while maintaining a constant fibrinogen backbone.

These mechanical properties of PEG-fibrinogen confer to the structure biofunctional features that influence adhesion, proliferation and differentiation of dental stem cells and progenitors. Collectively, the injectable PF hydrogels are cytocompatible and determine an increase of odontogenic differentiation but lesser extent of proliferation. Notably, the injectable PF hydrogels are able to upregulate Col I gene expression, one of the most important components of extracellular matrix (ECM) of the demineralized dentin. These PF properties suggest that hydrogels as scaffolds can support the formation of new tubular dentin and pulp tissue complex for dental pulp regeneration [52].

Subperiosteal tunnelling injection is a method that allows bone regeneration in a minimally invasive manner. However, because of the poor plasticity of most of the injectable bone substitute materials used for this protocol the technique has not been used widely. To overcome this problem in a recent study authors have been developed an injectable, sol-gel reversible thermosensitive alginate hydrogel. The flowable material obtained by using sol-gel transformation was injected *in vivo* through

a syringe needle into tissue and at body temperature, in situ the biomaterial turned into a gel form and was stable on the bone surface. Alginate based hydrogel showed a degradation time of 28 days matching osteogenesis and retains RhBMP-2 through an electrostatic interaction thus providing sustained rhBMP-2 release. BMP-2 in presence of this alginate based-hydrogel stored its bioactivity, increased the ALP activity of hBMSCs until day 15 and promoted mineralization processes. Also marker of mature osteoblasts such as osteopontin and osteocalcin were induced in presence of alginate hydrogel and BMP-2 [53].

In recent studies, it is reported that also scaffolds made of chitosan form a dentine-pulp complex *in vivo* [24] in presence of stem cells and hydroxyapatite (HA).

In a specific study, porous chitosan/collagen scaffolds were manufactured by using a freeze-drying process, and then were loaded with the plasmid vector encoding human bone morphogenetic protein-7 (BMP-7) gene. These scaffolds *in vitro* and *in vivo* enhanced dental stem cell response in terms of oral tissue regeneration. In particular, chitosan/collagen-based scaffolds enhanced DPSCs differentiation toward an odontoblast-like phenotype *in vitro* and *in vivo*. Moreover chitosan/collagen-loaded with the plasmid vector encoding human bone morphogenetic protein-7 (BMP-7) gene showed good properties as substrate for gene delivery [54].

3.3.2 Injectable Calcium Phosphate Scaffolds

Since 1982 calcium phosphate cements (CSCs) have been investigated extensively as injectable bone replacement biomaterials due to their successful properties. In fact, CSCs possess a chemical composition similar to the mineral component of bone, a proven biocompatibility, osteoconductive capabilities and fast setting times (<5 min). Moreover, CPCs showed higher solubility than apatite and resorb more rapidly. Thus, CPCs have attracted considerable attention in recent years for orthopedic and cranio-maxillofacial applications [55].

In this context, some authors have proposed the regeneration of the periodontium using the enamel matrix (EMD) derivative in combination with injectable bone cements. By combining EMD and CaP is possible to obtain a synergistic effect, stimulating both soft periodontal tissue healing and bone regeneration. This model is cost-effective and especially easy to apply in patients [56]. In order to obtain fast resorption of the grafts, the CaP cement was tuned with a low molecular PLGA. In this device, CaP appeared to act much like a “membrane” in supplying wound stabilization. Besides as wound stabilizer, CaP is the major determining factor of cementum formation and bone regeneration due to its osteoconductive properties. Because the use of an injectable calcium phosphate cement accelerates bone formation, the combination with EMD is a promising curative strategy for bone tissue regeneration in the periodontium [56].

Another experimental study in dogs demonstrated for the first time that the use of an injectable bone substitute, composed of a calcium phosphate ceramic and a

polymeric carrier, favors bone regeneration around dental implants immediately placed into fresh extractions sockets [57].

After calcium phosphate-based ceramics such as hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP) and the HA/β-TCP association that replaced bone autografts thanks their chemical composition closely related to that of bone mineral, a ready-to-use injectable bone substitute (IBS) based on an association of BCP granules with a cellulosic hydrogel has been developed [58]. This IBS has been ranked among innovative biomaterials with osteoconductive properties in tooth bone regeneration. The effectiveness of IBS is comparable to that of conventional implants placed after a 3-month healing period thus encouraging its use in clinics. Furthermore, IBS confirmed its osteoconductive potential because the newly formed bone contains the same Ca and P values as in basal bone. Thus, IBS may satisfy immediate implantation requirements. Hence, the advantages of an injectable bone substitute (IBS) appear to be clear because these composite biomaterials are able to promote bone regeneration immediately placed after tooth extraction [59]. For this reason, injectable composite biomaterials are becoming of primary importance for clinical applications such as socket filling and pre-implant reconstruction. Novel cell aggregate-loaded macroporous scaffolds combining the osteoinductive properties of titanium dioxide (TiO_2) with hydroxyapatite-gelatin nanocomposites (HA-GEL) for regeneration of craniofacial defects were also approached. An in vivo study showed the applicability of these macroporous (TiO_2)-enriched HA-GEL scaffolds because they were able to promote osteointegration and newly formed bone tissue production in a craniofacial defect model [60].

3.3.3 Injectable Polymeric Scaffolds for Dentin Reconstitution

The most difficult challenge in tooth regeneration is to reconstitute dentin tissue. Dentin problems involve the entire adult population and about 60–70% of the pediatric population because of the prevalence of dental caries [61]. In the tooth, the role of dentin is crucial because dentin provides strong mechanical support and protection to delicate dental pulp tissue. When dentin is damaged loses its structural integrity, the pulp is exposed and may be affected by periodontitis, and other infections [62]. Current dental treatments to cure dentin disorders include pulp capping and root canal therapy [63]. However, these treatments cause several side effects such as tooth discoloration, increased brittleness, and tooth loss [64]. Therefore, novel alternative dentin repair therapies are highly required. Dentin is hard to regenerate because dentin matrix is only secreted by odontoblasts, a terminal differentiated cell type. This cell population is present in a limited number and is complicated to isolate. Tissue engineering suggests for dentin regeneration the use of stem cells that can differentiate under odontogenic stimuli. For this purpose, porous scaffolds have been explored as a biomimetic odontogenic microenvironment to guide stem cell differentiation in odontoblastic-like phenotype cell lines. New approaches to replace damaged dentin include dental pulp stem cells (DPSCs), stem cells from the apical

part of the papilla (SCAPs), and stem cells from human exfoliated deciduous teeth (SHED) in presence of a favorable microenvironment consists of a beneficial scaffolding for the cell attachment, proliferation and differentiation. To facilitate biological response in terms of cell seeding, adhesion and differentiation, scaffolds have to possess specific features such as high porosity and a high interconnection of pores thus scaffold can better mimic ECM [65]. Natural biomaterials such as gelatin, collagen, chitosan, and hyaluronic acid have been investigated for oral tissue regeneration but they present disadvantages due to their physical properties such as a poor mechanical behavior and uncontrolled degradation kinetics. To overcome the drawbacks of natural biomaterials, synthetic polymers with tailored degradation rates and high processability are increasingly introduced in tissue engineering. Hence, three-dimensional (3D) macroporous and nanofibrous PLLA scaffolds with a high porosity and well-interconnected pores have been realized for enhancing hDPSCs odontogenic differentiation [66]. Injectable formulations are preferable for dentin defects due to the small defect size and irregular defect shape. To this end, the clinical translation of stem-cells in presence of injectable scaffolds for dental pulp regeneration has been approached. The authors demonstrated that stem cells from exfoliated deciduous teeth (SHED) mixed with Puramatrix™ (peptide hydrogel) after 7 days, or when mixed with recombinant human Collagen (rhCollagen) type I after 14 days and injected into the root canals of human premolars can generate a functional dental pulp. After subcutaneous implantation in immuno-deficient mice self-assembling peptide hydrogel (Puramatrix™) and rhCollagen type I induced pulp-like tissues formation that consist of odontoblasts capable of generating new tubular dentin throughout the root canals. Surprisingly, newly formed tissue showed similar cellularity and vascularization of control human dental pulps. Moreover, the new-engineered pulp was capable of generating new dentin. The self-assembling peptide hydrogel (Puramatrix™) and rhCollagen type-I scaffold without surrounding tooth structure was not able to promote odontoblastic differentiation because is necessary dentin-derived signaling molecules [67, 68]. Interestingly, scaffolds increased expression of dentin sialophosphoprotein (DSPP) that is the first marker of odontoblastic differentiation. DSPP overexpression predicted mineralization processes [69]. Furthermore, the physical properties of the scaffold directly contributed to dental pulp tissue regeneration. Dentin stimulation plays a key role in dental pulp regeneration because dentin contains functional pro-angiogenic factors and chemotactic factors that induce blood vessels generation [70].

For dental tissue engineering, an injectable scaffold is more effective than an implantable 3D bulk scaffold because dental defects are often small and have irregular shapes. Porous microspheres are proposed as injectable cell carriers for tissue repair [71]. In fact, in a study novel injectable microspheres (NF-SMS) made of biodegradable and biocompatible poly (L-lactic acid)-block-poly (L-lysine) copolymers were tested as a cell carrier to regenerate dentin [65]. The biomimetic nanofibrous feature and the porous structure of the NF-SMS significantly improved hDPSC biological response in terms of cell attachment, proliferation and odontogenic differentiation. The diameter of NF-SMS pores is around 10–20 μm in order to facilitate the cell infiltration into the internal space. The high interconnection of pores enhanced

cell-cell interaction thus promoting the activation of several differentiation pathways. Consequently cell-cell interactions, DSPP expression and odontoblast maturation were observed. Notably, NF-SMS increased not only DSPP expression but also the levels of other important osteogenic markers such as ALP, an early marker of osteogenic differentiation, that regulates organic and inorganic phosphate metabolism [71]. The expression of OCN an important late marker of mineralization during odontogenic differentiation was induced by NF-SMS. Several research studies reported the effect of various scaffolds, such as gelatin, collagen sponge, porous ceramics or fibrous titanium meshes, on hDPSCs in order to form a connective tissue than a dentin-like tissue [65], but in presence of NF-SMS the largest newly formed tissue volume was obtained. In conclusion, the injectable NF-SMS seems to create a microenvironment useful for hDPSC proliferation, odontogenic differentiation, and dentin tissue regeneration. Hence, NF-SMS showed features useful for clinical applications as an injectable cell carrier with high potential for dentin repair [71].

3.4 The Sol-Gel Approach to Prepare Calcium Phosphate Injectable Biomaterials

Sol-gel method has recently attracted much attention because is capable of improving chemical homogeneity of the resulting HA compared to conventional methods such as solid state reactions, wet precipitation, and hydrothermal synthesis. In fact, the sol-gel approach improves the conditions for the synthesis of HA thus providing a much better structural integrity compared to the defects related to plasma spraying method [72]. Moreover, the lower temperature, used during the process, allows the inclusion of thermolabile drugs and bioactive molecules (i.e. growth factors, peptides, dendrimer, antibiotics) in the variously shaped materials [72]. Furthermore, hybrid organic-inorganic materials may be formed through sol-gel method by using three different approaches. The first one is based on the dissolution of organic molecules in a liquid sol-gel [72]. The second one consists of the impregnation of a porous gel in the organic solution. In the third approach, the inorganic precursor either already has an organic group or reactions occur in a liquid solution to form chemical bonds in the hybrid gel. The sol-gel process consists of four steps: (1) the evolution of inorganic networks, (2) formation of colloidal suspension (sol), (3) the gelation of the sol to form a network in a continuous liquid phase (gel) and (4) the "aging" step (the sol-gel derived material expulses the liquid phase). Variously porous materials may be formed by sol-gel technique and the pore size depends on such factors as time and temperature of the hydrolysis and the kind of catalyst used. The sol-gel method is useful for the synthesis of hydroxyapatite (HA)-based injectable materials due to the possibility to obtain nanoparticles that are able to rapidly improve the stability at the artificial/natural bone interface [72]. Hydroxyapatite has long been among the most studied biomaterials for medical applications due to both its high biocompatibility and for being the main constituent of the mineral part of

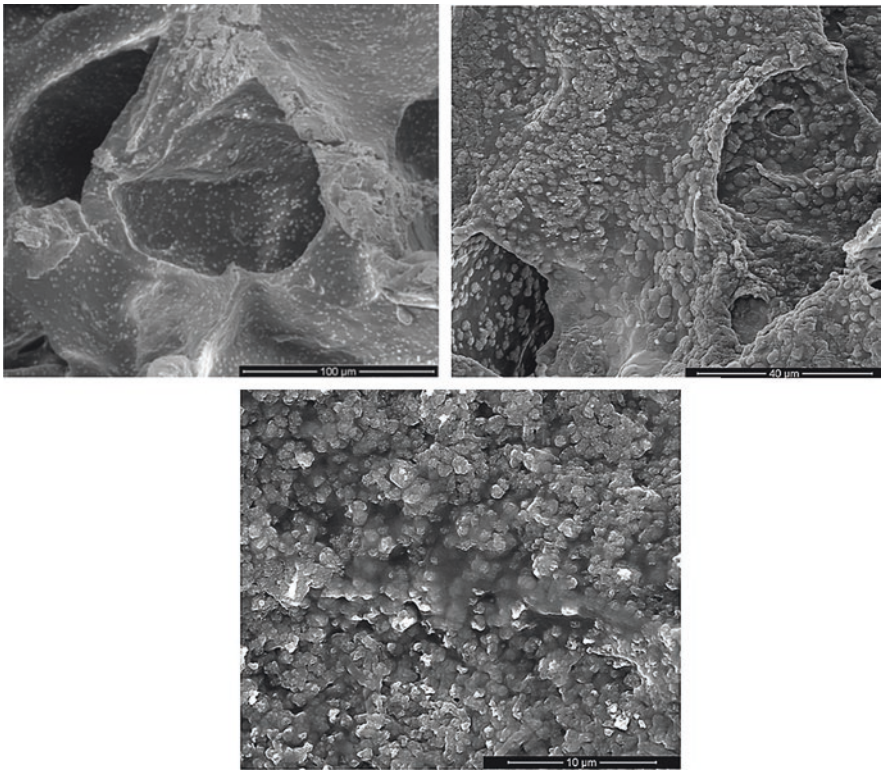

Fig. 3.3 Distribution of hydroxyapatite in the polymeric matrix by using sol-gel technique—hybrid materials

bone and teeth [73]. To overcome the limitations related to the preparation of HA by using sol-gel process such as the possible hydrolysis of phosphates, the high cost of the raw materials, a strict pH control, the vigorous agitation and a long time for, is possible to use a non-alkoxide based sol-gel approach where the calcium and phosphate precursors are calcium nitrate tetrahydrate and phosphorous pentoxide, respectively [72]. Organic-inorganic composite materials such as PCL/HA can be synthetized by sol-gel method. Sol-gel process allows mixing at molecular-level calcium and phosphorous precursors with the polymer chains in order to obtain composites having enhanced dispersion and exhibiting good interaction between the inorganic phase and the polymer matrix. A homogeneous distribution of nanoscale hydroxyapatite particles in the polymeric matrix by using sol-gel technique was observed (Fig. 3.3). This homogeneous distribution of nanoscale hydroxyapatite particles enhanced the bioactivity and the ability in bone repair of composites. In fact, these materials were able to increase osteoblast adhesion, proliferation and to inhibit osteoclast functions [72]. In addition, metals coated with nanoscale hydroxyapatite particles induced new bone formation compared to conventional apatite. Innovative injectable composite materials based on hydroxyapatite containing

strontium (Sr-HA) and carbon nanotubes (CNTs) as a reinforcing component for the treatment remodeling compromised bone have been developed. Besides the conventional processes to produce HA-CNT composite materials, innovative techniques such as sol-gel have been approached to obtain an increasing of the bone mineral density and a decreasing of bone resorption by strontium intake [2]. It is well known that Strontium (Sr) plays a key role both in the stimulation of bone formation and in the reduction in bone resorption. Moreover, Sr is able to enhance the bioactivity and biocompatibility of biomaterials. Conventional processes to produce HA-CNT composite materials are based on physicochemical blending methods including ball milling [74] and mixing in solvent [75]. Initially, the sol-gel method was used in the preparation of silicate from tetraethylorthosilicate (TEOS, $Si(OC_2H_5)_4$), which is mixed with water and a mutual solvent, to form a homogeneous solution. Recently, new reagents are appeared, so novel inorganic oxides and hybrid organic-inorganic materials can be synthesized using this methodology. Furthermore, the sol-gel technology provides the opportunity of working at lower temperature during the synthesis thus preventing mechanical degradation of substrates and/or of thermolable drugs and growth factors. Therefore, the literature reported that the sol-gel process leads to a high-quality HA coating after heat treatment at lower temperatures. The synthesis of HA requires a correct molar ratio of 1.67 between Ca and P in the final product. A number of combinations between calcium and phosphorus precursors were employed for sol-gel HA synthesis. However, calcium phosphate (CaP) materials show limited compressive strength and their uses are limited to non-stress-bearing applications exactly as maxillofacial surgery, or the repair of craniofacial defects and dental fillings [2]. On this basis, recent research studies are aimed to investigate the synthesis of an injectable composite material based on hydroxyapatite containing strontium (Sr-HA) and CNTs as a reinforcing component (Fig. 3.4). CNTs as a reinforcing component showed no acute toxicity and a good effect on the attachment and spreading of osteoblast cells [76]. Nayak et al. [77] have also shown that surface roughness of CNT thin films may show effects on proteins adsorption on the material surface thus improving biological response in terms of proliferation and differentiation of hMSCs into bone lineage. Moreover, a recent study [78] reported that MWCNT (multiwalled carbon nanotubes) has beneficial effects on inhibition of osteoclastic bone resorption *in vivo* and through the suppression of essential transcription factors involved in osteoclastogenesis *in vitro*. The injectable strontium-modified CaP gels reinforced with CNT material are able to induce osteogenic marker expression such as the phosphatase activity (ALP) that is one of the most widely used markers for osteogenic differentiation and is considered a necessary prerequisite for the onset on mineralization [2]. Furthermore, the expression of some bone-related molecules such as OPN and OCN was promoted in presence of the injectable strontium-modified CaP gels reinforced with CNTs thus confirming the ability of these biomaterials to support MSC differentiation toward the osteoblast-like phenotype [79]. These results suggest potential applications in regenerative endodontics of injectable hydrogels that can be dispersed inside a closed, small space, such as the root canal system. Injectable biomaterials can be involved also in angiogenic processes because they promote cell-cell and cell-extracellular matrix

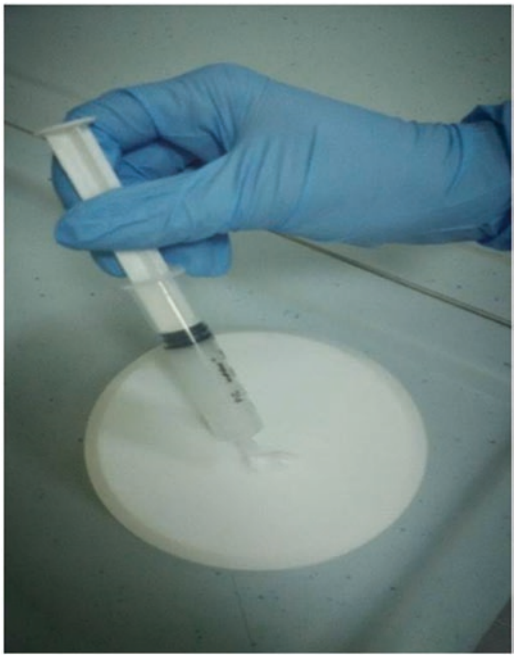

Fig. 3.4 Injectable Sr-modified calcium phosphates

cross-talk. This function plays a key role in pulp regeneration because these injectable scaffolds may create an interaction between DPSCs (Dental Pulp Stem Cells) and HUVECs (Human Umbilical Vein Endothelial Cells) thus remodeling of capillary-like structures. However, it is difficult to fabricate a stable vascular network *in vitro* because ECs (Endothelial Cells) require specific environment elements, such as a specific pH range, signaling molecules, and growth factors, for their survival, proliferation, migration, and vascular morphogenesis.

3.5 Conclusions

The main challenge of the biomedical sciences is to regenerate all tissue types starting from an initial stem cell line by using innovative scaffolds. This goal is opening the door to new stem cells based therapies for tissue regeneration. New therapies based on combination of scaffold and stem cells could ameliorate the expectation of quality of life in more than two billion of patients undergone to a regenerative surgery. In dentistry, the aim is to simply replace damaged or degenerated tissues with MSCs from dental and oral sources. Hence, the use of injectable biomaterials is particularly attractive for dental pulp and bone tissue engineering, as they can be easily formulated with growth factors, drugs and cells by simple mixing. In conclusion, the tunable of injectable biomaterials makes them appropriate for induction of odontogenic differentiation and mineralization of human dental MSCs.

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Chapter 4
Innovative Biomaterials in Bone Tissue Engineering and Regenerative Medicine

Antonio Apicella, Davide Apicella, Jamaluddin Syed, and Raffaella Aversa

4.1 Introduction

Innovation means creativity to new products, equipment or consumer services, increasing the value returned from invested capital. Today, manufacturers are aware that innovation and creativity are key factors in unravelling potential for development and growth even in the areas of biomedical applications.

Our philosophy is exploring novel area of innovation through a *systemic approach* in search of suitable solutions to be exploited for industrial applications, using new technological developments based on specific software tools of Artificial Intelligence for an integrated design process testing their fitness (integrated design method).

This evolutionary design underlines the concept of approaching research for new material and technologies development, and design advance, by an adaptive parallel evolution driven by the complex nature of self-sustainable systems.

In particular, our attention has been captured by the fact that each year an ever-increasing number of researchers from diverse disciplines enter the field of nano and advanced new materials. A growing extent of novel ideas and exciting new opportunities appearing on the international scene for nanostructured material are becoming feasible.

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Biomimetics, *biomechanics* and *tissue engineering* are three multidisciplinary fields that we have considered to attain the objective of increasing the reliability of new prosthetic implants.

The intersection of these disciplines resides where much of the innovation exists: nanotechnology, biology and structural design define a wide research area carrying great potential for true innovations such as smart and multifunctional nanostructured materials, 3D metal printing industrial processes, structural analysis and biomimetic prosthetic systems.

Starting from this natural evolutionary approach we argued that, when in search of innovations in complex systems such are those in the biomedical field, it is important to balance two conflicting objectives: Exploiting worthy existing solutions and exploring a new research space:

Mimicking mechanism of the natural evolution, where a biological population evolves over generations to adapt to an environment by selection, crossover and mutation, we should evaluate fitness of an objective function value of a design candidate and of design variables, during the creation process itself [1].

Since biological testing and mathematical methods could be closely related, the combination of *in vitro* and *in vivo* experiments with *in silico* computer simulations is a promising approach that has been followed by our group [2–9].

Our innovative approach combines biomimetics and biomechanics studies with the development of new hybrid nanostructured materials for osteoblast and stem cells cultures used in regenerative medicine. These new class of materials could provide to the a microenvironment, which is bio-mechanically coherent and nutrient conducive.

The new ceramo-polymeric hybrid nanocomposites have been parallely investigated using biomimetic finite element analysis (FEA), computed tomography anatomic characterization and reconstruction, and computer assisted design of tissue engineering scaffolds.

Our multidisciplinary methodological approach considers biomimetics, biomechanics, and tissue engineering as investigation fields that should be strongly correlated in order to design biomechanically active bone tissue scaffolds. The biomimetic and biomechanical approach will be followed in preparing the experimental procedures for in vivo scaffold ossification and mineralization experimental tests.

The *mechanical* signals imposed by physical activities drive the growth of the bone, as well its maintenance and ossification. The mathematical modelling of biotissue growth is correlated to local geometrical characteristics and strain distribution. Finite element analyses has been used to link the local skeletal morphology and bone tissue mechanics to endochondral ossification patterns, using multi-phase continuum representations of in vivo animal experimentations with traditional and innovative protocols [2–8, 10, 11]. These new protocols used new biologically tecto-structured hybrid materials. By considering the natural evolution of the bone, which is a hybrid biological material composed by an inorganic reinforcing nanocrystalline hydroxyapatite and structural component immersed in a connective

organic component (collagen), both organic and inorganic phases are integrated at a nano-scale level determining its nanostructure features and mechanical properties [12–14].

The understanding of the relationships existing between structure and properties is needed for the correct selection of the base materials and their further development. Biomechanics and Biomimetics could then foster the potentials of new and advanced materials and technologies.

With the today and still growing improvement of Artificial Intelligence, the "*in silico*" human *synthetic* calculation ability shortens the decisional time for the evaluation of the robustness of a design idea [2, 4, 6, 8, 9, 15, 16].

The present chapter evaluates the advances of use of hybrid materials for bone tissue repair, as well as the chemical procedures that allow controlling the material nanostructure, by covering the following scientific areas:

- Biomechanics: study of human bone by biofidel modelling,
- Biomimetics: utilization of nanotechnologies in medicine to develop nature inspired materials
- Bio-mechanically active scaffolds able to favour osteo-integration that are composed by porous structural nanocomposite and hybrid matrices

4.1.1 Advances in Biofidelity

Pioneering investigations on mathematical biomechanics and morphological modelling of mandible [2, 4–8, 10, 11, 15, 17] and tooth [3, 9, 18, 19] have confirmed suggest that this method could be successfully applied to bone tissue engineering.

Recent technological progresses in nano-materials sciences have designated *biomimetics* and *tissue engineering* as emerging fields that could lead to development of new restorative systems [2, 4, 15, 20, 21] restore the biomechanical, structural, functional, and aesthetic integrity of the tissue engineered bone.

Biomimetics, which investigates such features, becomes the natural connection between biology and engineering enabling the improvement of biological criteria and models for the production of bio-inspired materials and fully engineer prosthetic systems. However, the understanding of the basic mechanical and adaptive properties of bone is critical in designing new *biomimetic* prostheses with minimal biomechanical and biological invasiveness.

Since antiquity, parts of our body have been replaced by artificial prostheses. The materials used were selected to avoid any adverse response when in contact with human body tissues and fluids.

The choice criteria of a biomaterial were related to its specific biocompatibility and functionality with the organ or bone to replace. However, it is only in the past two decades that studies of these interfacial effects have been upgraded by using thin nanomeric coatings and surface modifications [22–27].

4.1.2 New Classes of Biomaterials

There are numerous modes in which living tissues react to the implants synthetic materials but they are usually confined to their responses at the interface.

Three terms describe the behaviour of a biomaterial as defined by Hutmacher [28], Jones and Clare [29], and Hoppe et al. [30], which are associated to the tissue responses:

- *Bioinert* such is the Alumina for dental application
- *Bioresorbable* such is the tri-calcium phosphate
- *Bioactive*, such is the hydroxyapatite used as coating on metal implants or such are the bio-glasses

Moreover, further improvement can be achieved if the biomechanical function on cell growth and specialization is considered. Nano structured bio-ceramics have been demonstrated to be biomechanically interactive materials, facilitating the bone tissue natural tendency to heal. These materials promote tissues regeneration and restoration of physiological functions [4, 5, 8, 31–34].

4.1.3 New Perspectives for Tissue Engineering

Bone implants are needed to operate for longer period without failure or surgical revision [35–39]. The development of association of material and prosthetic systems with high durability and biocompatibility is then essential [20, 40–44].

The evolutionary design path for combined material and product development is shown in Fig. 4.1. This path groups several researches and industrial activities such are those for the new biomimetic hybrid ceramo-polymeric material [4, 5, 8, 34, 45].

Exploitation of potentialities of the new additive metal manufacturing technologies in new production [4], and highly non-linear biofidel modelling where *in silico*, *in vitro* and *in vivo* tests have been coupled together [2, 15].

The scaffolding material and the prostheses replacing bone should possess comparable stiffness (which is the combination of material elastic modulus and prosthesis shape) matching that of bone area where it is implanted.

The current implant materials have higher stiffness than bone and they alter the bone stress physiological distribution preventing the stress transfer to adjacent bone, leading to stress shielding and bone reabsorption and implant loosening [19, 21, 46, 47].

A customized material owing the proper combination of high strength and stiffness that matches that of the bone has a great potential for biomechanical integrated implantation with higher service period [2, 4, 5, 8].

Moreover, other research fields that can benefit of this biomechanical integration is the stem cells seeding, differentiation and growth in 3-dimensional ceramic scaffolds. This new strategy is finalized to implant healthy cells directly in the damaged area of the bone [48–51].

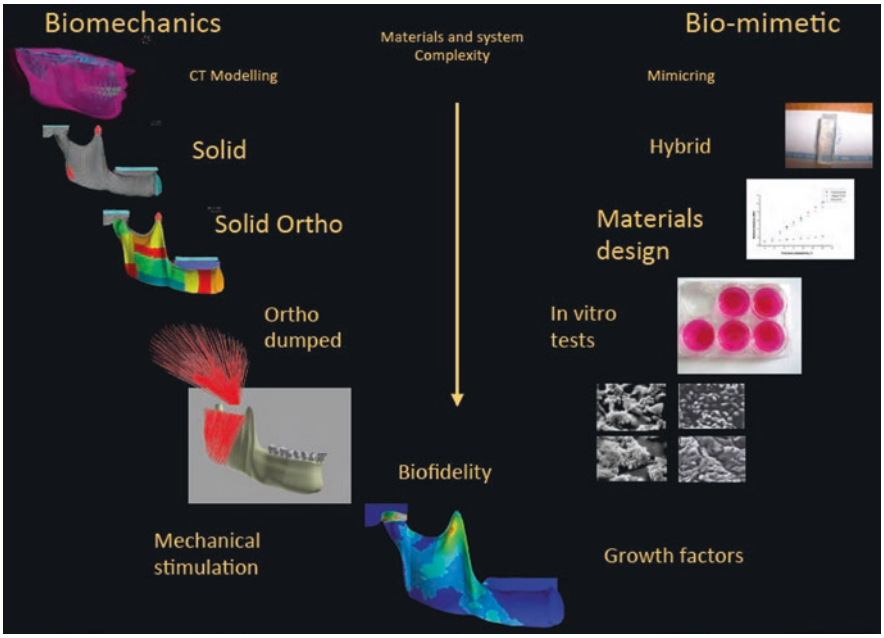

Fig. 4.1 Cooperative research development in the evolutionary design process in the field of biomedical prostheses

Our group is developing clinical practicable and productive strategies combining the traditional bio-ceramics implant with the already assessed knowledge of stem cells growth and differentiation. Namely, stem-cells cultured in bio-ceramic nanocomposites could be adopted for an extensive bone repair with high probability of complete functional recovery and integration of the new bone in hybrid scaffolds.

4.1.4 Biomimetics

Natural tough hybrid materials, such are sea urchin tooth, nacre or bone itself, retain their properties at nanoscale level. The organic phase acts at nano-scale level as a highly energy-dissipating plastic network that inhibits fracture propagation (high resiliency).

The adaptive characteristic of the bone is acting at micro scale and is due to coupling between bone formation and bone reabsorption mechanisms.

This process accounts for the dynamic equilibrium bone-forming activity between osteocytes reabsorption by osteoclasts and renewed generations of osteoblasts from precursors. Coupling can be then considered a complex dynamic remodelling mechanism involving the interactions of different types of cells and biochemical and mechanical controlling stimuli. Remodelling of the bone occurs at

many sites “asynchronously throughout the skeleton” [52], involving that mechanical stimulations at specific physiological strain levels are locally active. Osteoblasts mature under specific biochemical and mechanical stimulations, transforming in osteocytes that mineralize the bone. If osteoclasts activity is not supported by the correct physiological mechanical stimulation, as can occur after prosthesization, bone reabsorption may be observed.

4.1.5 Bioengineering and Bioactive Scaffolds

An ideal bone scaffolding material should provide a sufficiently rigid but resilient structure to momentarily replace the damaged bone function [34, 53–55]. Based on the bone regeneration criteria, we developed new bio-active-biomaterials that are designed to favour the bone tissue formation by fostering osteoblast proliferation and stem cells differentiation [34, 45].

The use of materials with nanostructure similar to that of natural bone tissue is one of the most promising options in bone healing [4, 19, 56].

New hybrid highly-bioactive amorphous fumed silica nano-composites mimicking the mechanical behaviour of the bone have been tested as a potential candidate scaffolding material.

The presence of fumed silica nano-particles improved the self-organizing properties of the polymeric network since it increases the amount of hydrogen bonding between polymer hydroxyl groups and the oxygen of the silica nanoparticles [7].

Internal strong hydrogen bonding increases the nano-filled composites stiffness while remaining transparent and exhibiting good nanoparticle distribution and final mechanical strength [4–8, 10, 11, 34]. Its high stiffness overwhelms the major problems for the application of hydrogels in bone tissue engineering.

Early studies have confirmed that these hybrid nano-composites attain the needed biomimetic and osteoconductive properties to become bio-mechanically active scaffolding materials [4–6, 8, 9, 34].

As previously mentioned, modeling and remodeling in healthy conditions cooperate to model the correct functional shape of bones. In an implanted bone, conversely, with a rigid metallic prosthesis, even if functionality is restored, the biomechanical equilibrium of stresses and strains distributions are significantly changed [9, 15, 19].

Loads cause bone deformations generating mechanical signals that Osteoblast cells can feel and respond to.

Bone modeling and remodeling are driven and controlled by these threshold signals [12–14, 57, 58]. Remodeling processes repair the injury by removing and replacing the damaged tissues with new bone. Moreover, excessive or insufficient loading alters such remodeling process [57]. Early studies by Wolff [58] stated that the bone mechanical response could induce modifications of its architecture. Frost [12] found mathematical relationships describing the modification of the bone

tissue under specific loading that quantitatively accounted for bone deformations [14]. Remodeling processes repair the bone by replacing the damaged tissues with new structured bone.

4.1.6 *Biofidelity Models and FEM Analysis*

The physiological mechanisms of a healthy bone dynamic growth may be described as an iterative process among biology and engineering. The knowledge that we can obtain by reverse engineering a biological system could give a feedback into biology, permitting a more certain and complete understanding of the possible routes for further developments in medical applied engineering.

Newly combined diagnostic and engineering tools, such are those maxillo-facial districts NMR or CT segmentation and solid CAD reconstruction have been utilised in our research (i.e. Materialize Mimics and 3Matics) that can detail the anatomy of hard and soft textures in an extremely precise way.

The integration of biological knowledge and clinical possibilities is thus essential. A more reliable and biofidel model begins with the biomechanical modelling of a bones, ligament, and alveolar bone, using Finite Element Analysis in order to gain insight into the biological response to changing biomechanical circumstances.

Due to the fact that experimental and numerical methods could be closely interlaced, combination of *in vitro* and *in vivo* experiments with *in silico* computer simulations is a promising methodological approach (Fig. 4.2).

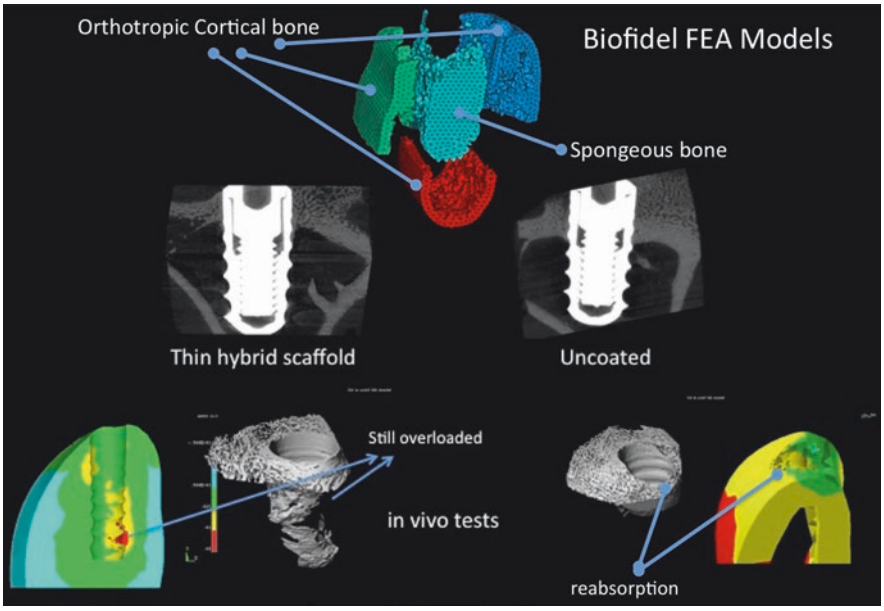

Fig. 4.2 *In silico* and *in vivo* validation for Osteoconduction of Titanium implants coated with a nanostructured hybrid osteoactive (*left side*) and without (*right*)

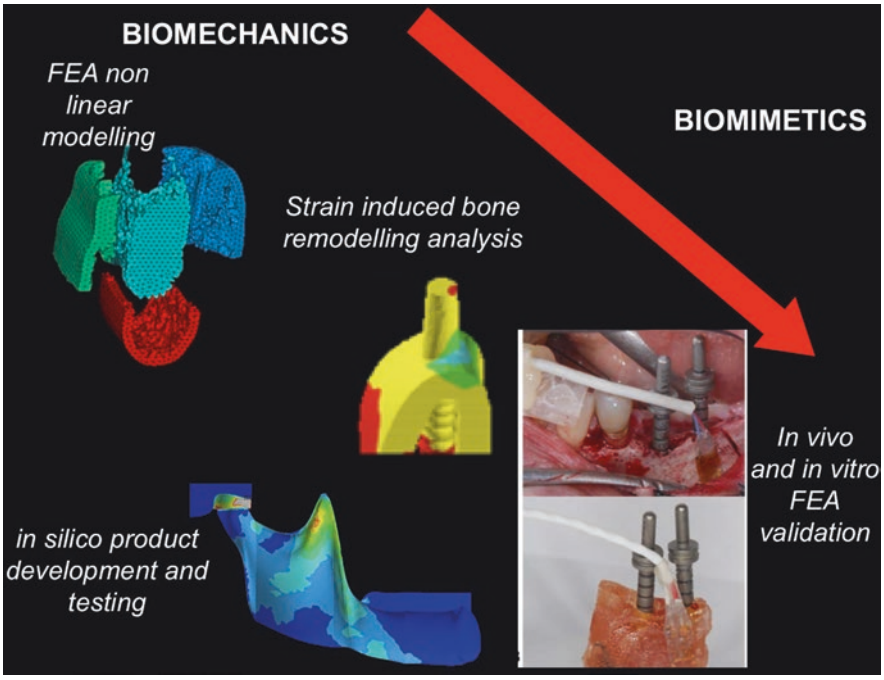

Fig. 4.3 Biomechanics, Biomimetic and Biofidelity cooperative path: An "*in silico*" tool for new prostheses design

However, several other stimulating aspects in the development of the mathematical model and its realization are still to be explored.

The concurrent interaction of the several mechanical variables influencing the scaffolding and prosthetic systems bio-efficiency should be further investigated by means of parallel biological testing on tissue engineering involving stem cells and simulation by finite element mathematical modelling.

Specific mathematical softwares evaluate the distribution of stresses and strains as a response to changing loading conditions [2, 4, 5, 8]. The mathematical modelling by Finite element analysis has been validated by in vivo or in vitro tests, confirming it as an useful tool in defining optimum restorative design and material choice criteria (Fig. 4.3).

4.1.7 Biomimetic/Biomechanical Approach

First, in our Biomechanical and Biomimetic approach we have carried out parallel physiological, mechanical and physical characterization of the hydrophilic hybrid material in presence of aqueous environments that simulated human physiological fluids.

The 20% of extracellular water is distributed in extra vascular sector (such as Lymph and interstitial fluid while 8% resides in vascular sectors (blood). Water molecules freely diffuse between these compartments in response to concentrations variations, maintaining the hydro-balance between these compartments (osmosis). Similarly, osmotic and diffusive exchanges have been described to occur in polymeric materials [59, 60].

Penetrant molecules and dissolved species diffusion and absorption govern the osmotic tension build-up in polymeric materials [59]. The hydrophilic hybrid material developed by our group is strongly affected by the presence of physiological solutions. Fig. 4.4 shows the significant swelling occurring to our materials when exposed to an aqueous medium [4–8, 10, 11].

The sample is initially in the glassy state but it progressively swells when it is immersed in distilled water or even in water based physiological solutions. This phenomenon is due to the water molecules diffusion and sorption that finally reach equilibrium in the swollen and rubbery state (right bottom in Fig. 4.4).

A clear front, which separates the unaffected glassy core and the surrounding swollen outer shell, develops and progressively advances through ha glassy core (left bottom in Fig. 4.4).

This sorption behaviour has been deeply described in literature [60, 61] and it is named Case II sorption to differentiate it from the ordinary Fickian diffusion.

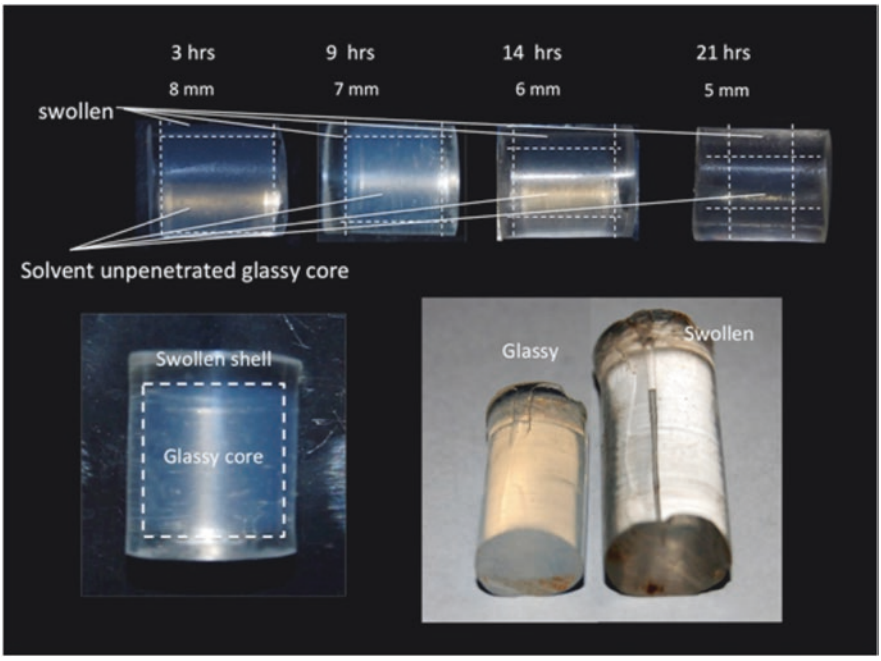

Fig. 4.4 Swelling behaviour of our hybrid hydrophilic nanocomposite in water

4.2 Mechanical Characterization in Glassy and Rubber Gel State

The dry and saturated in hypotonic and isotonic Hybrid pHEMA nanocomposites with compositions ranging from 5 to 25% by volume of nanosilica were isothermally shear tested in a Dynamic Mechanical Analyser operating at 10 Hz and 37 °C.

The Shear moduli of the dry samples range from 0.8 to 9 GPa. When plasticized by the physiological solutions, the elastic shear modulus drops to values ranging from 0.01 to 0.1 GPa, which are characteristic of the rubbery swollen state.

The less concentrated hypotonic solution induces a higher level of plasticization with still lower moduli. Aqueous isotonic and hypotonic saline (0.5 and 0.15 M NaCl) solutions were chosen to simulate potential physiological extracellular conditions in different patients since the percentage of water in extracellular fluid may differ for sex and body fat percentage (i.e. females, which are characterized by a higher percentage of fat than men, may average up to 5% less water than men of the same age).

The samples have shown an essentially elastic behaviour since the viscous component was negligible for all compositions. The shear modulus of pHEMA-Nanosilica composites does not follow classical Halpin and Kardos [62] equation for particulate composites and a linear dependency on increasing content of nanosilica has been observed, instead.

This behaviour confirmed the hybrid nature of our nanosilica pHEMA composites.

The mechanical shear moduli of the dry and swollen samples have been then interpolated by linear fitting (full lines in Fig. 4.5).

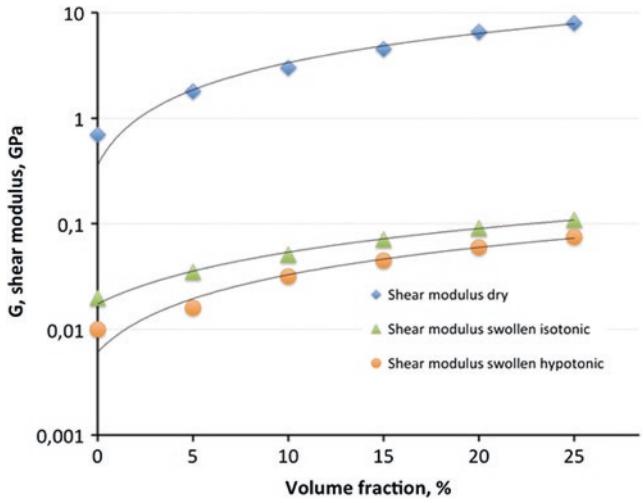

Fig. 4.5 Shear moduli of dry and swollen (rubbery) of our hybrid hydrophilic nanocomposites equilibrated in hypotonic and isotonic aqueous solutions (full lines represent the linear fittings)

Our hybrid system is a good candidate fulfilling the mechanical requirements needed for bone replacement, since its properties are:

- G = 2–4 GPa when in the glassy and dry state, which is comparable to the rigidity of the bone (useful during the early stage of surgical operations when a high mechanical strength of the scaffold is preferable)
- G = 10–40 MPa when swollen (which is comparable to cartilage and ligament elasticity that turns useful during the scaffold osteo-integration stages)

4.3 Sorption Kinetics and Swelling Tests

Water uptakes in the initially dry samples of composition of 5% of nanofiller have been described by Aversa and Apicella [59] and Aversa et al. [6]. Swelling and weight uptakes kinetics were plotted as a function of the square root of time (Fig. 4.5).

The advancement of the swelling fronts in the limiting Case II anomalous sorption [61, 63] for our hydrophilic samples was monitored as a function of time. The thickness of the un-swollen residual glassy core (Fig. 4.7) and the overall thickness % increases along the Z axis and % lengths variations along the two orthogonal axes (X and Y) in the plan of the sample slab are reported in Fig. 4.7.

Equilibrium sorption and swelling at 37 °C was reached in 100 h.

The hybrid nanocomposite in isotonic water solution picks up 42–45% of its dry weight and reducing its shear modulus to 15–25 MPa (as measured in DMA tests of Fig. 4.5).

A measure of the swelling kinetic is given by the rate of reduction of the glassy core thickness and of the increase of the overall swollen thickness. The swelling front advanced at constant rate according to the limiting relaxation controlled anomalous sorption mechanism indicated as “Case II sorption” [63]. The initial swelling rate is faster, about 0.13 mm/h, for the hypotonic solution and slower in the isotonic solution, about 0.10 mm/h.

As swelling proceeds water molecules diffusive resistance develops in the outer swollen layer reducing the swelling rate [61]. This generates a concentration gradient in the material that, even when swelling fronts meet, lets the sample weight uptakes to further steadily increase from about 25% to final equilibrium values, 45 and 42% (right axis in Fig. 4.6) in hypotonic and isotonic solutions, respectively.

This further water sorption increases the level of dimensional swelling along the X and Y axes of the sample slab (Fig. 4.7).

The swelling curve reaches a first plateau of 12% along the Z-axis (through the thickness), in correspondence of the glassy core exhaustions and then reprises to increase to a final value of 18%.

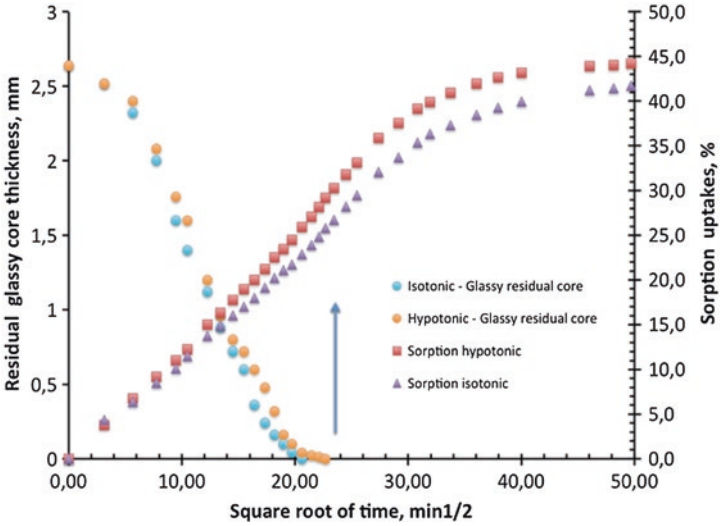

Fig. 4.6 Swelling and sorption kinetic as a function of the square root of the time in the 5% by volume hybrid nanocomposite in physiological 0.05 M (hypotonic) and 0.15 M (isotonic) NaCl solution

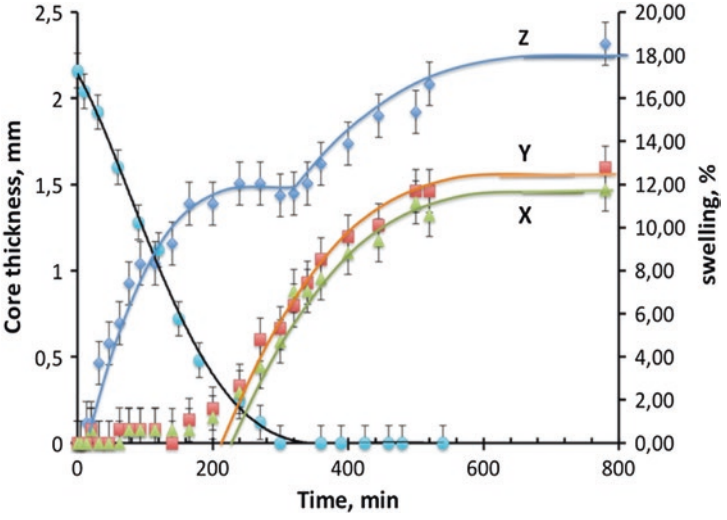

Fig. 4.7 Per cent swelling (right axis) and glassy core thickness reduction (left axis) for the 5% by volume fumed silica hybrid nanocomposite in physiological 0.15 M (isotonic) NaCl solution

4.4 Discussion

Due to this peculiar swelling behaviour, our hybrid materials can act as biocompatible and mechanically bioactive scaffolds.

A mechanical stimulation given by the stress state generated by the scaffold swelling once implanted in the bone could be finalized to favour adaptive directionally organized OB growths and, eventually, stem cell stimulation for differentiation.

The possibility of using mechanically bio-compatible hybrid hydrogels as scaffolding materials that improve biomimetics reproducing cartilage and ligaments biomechanical functions [2, 3, 5, 17, 18, 21]. The adaptive characteristics of the bone tissue could benefit of biomechanically compatible and bioactive scaffold biomaterials when coupled with new designed odontostomatological prostheses. New modified Titanium dental implants have been developed with hybrid ceramo-polymeric swelling inserts. The swelling insert stabilizes the implant in the bone while creating a bio-mechanically active interface for bone growth stimulation.

The level of stress and strain in the bone can be modulated by to fit the physiological state. In vivo tests have confirmed the improved capability of such implants to promoting early osseointegration [45].

The use of newly developed combined diagnostic and engineering tools, such as those utilised in our research (i.e. maxillo-facial district NMR or CT segmentation and solid CAD reconstruction) can detail the anatomy of hard and soft textures in an extremely precise way with smallest standard deviations. The integration of biological knowledge and clinical possibilities is thus essential. A more reliable and biofidel model begins with the biomechanical modelling of a bones, ligament, and alveolar bone, using Finite Element Analysis in order to gain insight into the biological response to changing biomechanical circumstances.

The bone ingrowth [25] and implant apposition, defined as the percentage of osteo-integrated implant length for the bio-mimetically coated with our hybrid material and uncoated implants in the six months in vivo test show a significant improvement of about 100% increase in the first two months and of the 30% after 6 months [45].

Micro-CT bone reconstruction of the bone ingrowth around the implant for in vivo tests on rabbit femur was validated by the use of FEA calculated physiological strain distributions in Titanium modified implants with a swollen scaffolding interface with the bone. The adaptive characteristics of the bone avoid bone reabsorption in the perimplantar hybrid scaffold areas. This behaviour has been predicted by the FEM analysis that have shown correspondence between strain areas s below the physiological lower limits for healthy growth and areas of bone reabsorption observed in Micro CT of rigid implant interface (right side of Fig. 4.1). The colour strain maps around in the bone surrounding the implant confirmed the critical role of the bioactive Ti-Bone interface and the experimentally observed bone growth validated these expectations. The bone proliferation and growth is favoured and accelerated by the presence of the hybrid nanostructured interface.

Research in progress has also shown that the mechanical stimulation could have a significant effect on the differentiation and development of mesenchymal tissues.

4.4.1 *Osseointegration Mechanisms to Account for in the Biofidel Models*

The osseointegration of the implants is essential for the attainment of prosthetic rehabilitations. The accomplishment and the maintenance of a stable functional anchilosis has shown to follow morpho-structural features, that are related to:

- Absence at the interface of idoneous tissues by the direct contact between bone and implant;
- The existence of primary bone in contact with the surface of the biomaterial;
- The deposition at the Titanium implant surface of a layer external to the primary bone layer of lamellar secondary;
- The overall perimplantar osseous density compared to the normal bone architecture;
- Growth of medullar spaces, which exhaust the tissue metabolic requirement in the area that is less involved in the dissipation of the load;
- The condensation of compact bone related to the strain distribution patterns determined by the specific implant shape;
- The organization of a strong trabecular structure that is radially departing from the compact perimplantar bone;
- The presence of a osseous crestal wall in the subepitelial connective allowing sulcular epithelium formation and junctional trophism.

The mature mineralised matrix has been described in dental and orthopaedic clinical studies to increase the mechanical stability in the early osseointegration phase (primary stability). In the case of use of our hybrid scaffolding interface, high levels of fluids are absorbed from the liquid external environment due its hydrophilic nature, leading to significant swelling and volume increase of the initially glassy hybrid material (Fig. 4.8) promoting early primary stability.

The biomimetic and bio-mechanically active scaffold is, therefore, accomplishing two biomechanical functions, the first is strictly related to the stabilization of the prosthesis after implantation (the prosthesis can be early loaded few hours after implantation), while the second function is associated to the mechanical bone growth stimulus exerted on the area surrounding the implant.

The volumetric expansion of the scaffolds is then effective in *improving the primary stability* of the implants, confirming the high bio-active performance of the tested nanocomposite material (Fig. 4.8 right side and Fig. 4.9).

According to Frost [13], who quantified the observations of Wolff [58], above (>3000 με) and below (<50 με) critical strain levels, bone growth is impaired (Fig. 4.10). In the mild range of strains, healthy bone growth and regeneration is favoured.

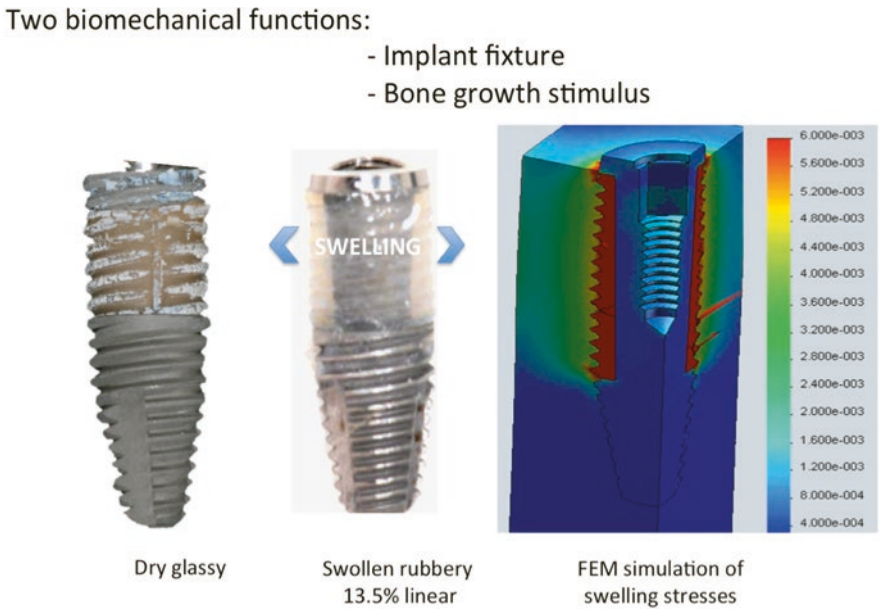

Fig. 4.8 Mechanisms of primary stability and osteoinduction improvements in Hybrid swellable scaffold modified Titanium implant. Glassy dry scaffold (*left*)

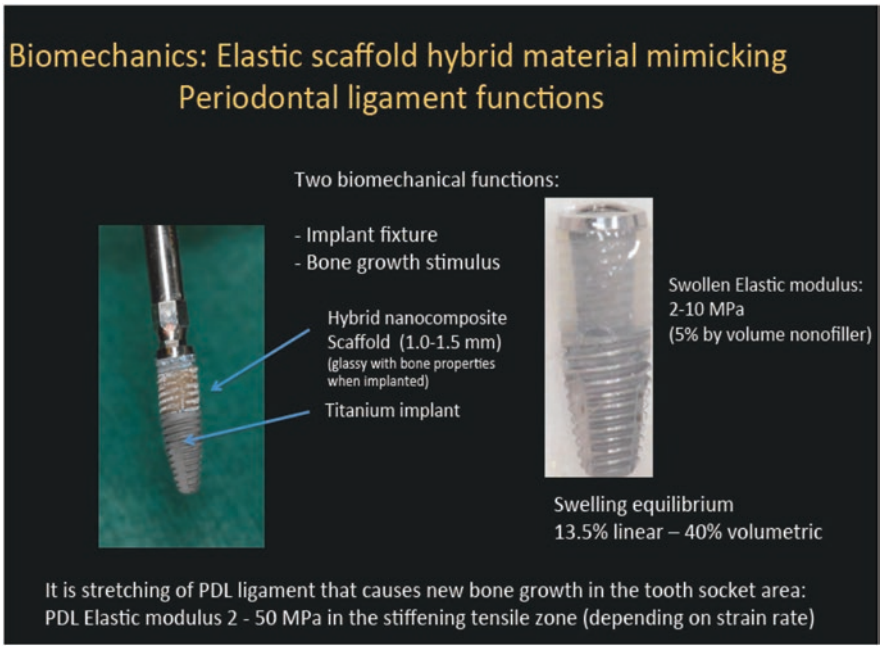

Fig. 4.9 Biomechanical functions of the new ceramo-polymeric hybrid nano-composites application in the development a new concept Titanium biomimetic dental implant [4]

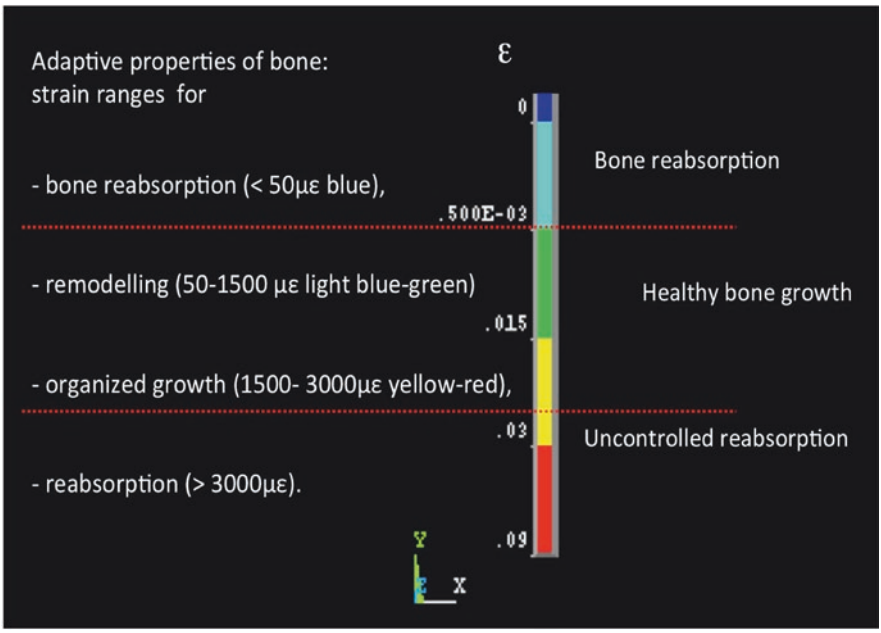

Fig. 4.10 Frost [13] adaptive window of bone physiology: Structural adaptations to mechanical usage

In fact, in order to maintain the stability of implants under load, it is of major importance for the bone-forming osteoblast to promote extra-cellular matrix in the vicinity of the implant.

The presence of the swellable scaffold contiguous the upper Ti core of the implant increases its removal torque after implantation when the system is in presence of organic fluids.

The removal torque measured at different times after implantation, in fact, increased of more than 100% at 24. Moreover, even just after 1 h, the removal torque already raised from 43 to 62 N (about 25% improvement). It has been described in a previous paper [5] that the retention improvement was directly following the swelling kinetic of the hybrid material scaffold.

This increase of the implant stability is due to the strong compressive strains generated in the swollen rubbery hybrid scaffold as it can be inferred from the coloured strains map reported in the right side of Fig. 4.3 (red colour of the hybrid insert of the implant). The implant is then constrained in its socket by the external bone, which then increases the retention and stability of the implant. The application of a higher removal torque for explanting is therefore needed at increasing swelling levels, as indeed it has been experimentally measured.

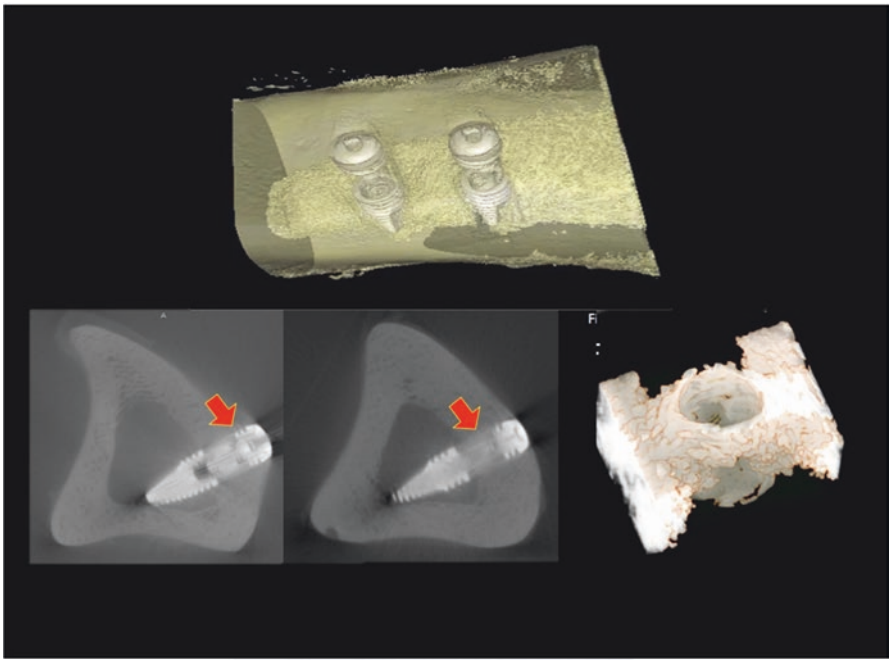

Fig. 4.11 The Bone to Implant Contact (BIC) and the relative bone density have shown similar characteristics at cortical (**a**) and medullar levels (**b**), Bone near to the implants shows similar characteristics (c) [45]

Moreover, the constraining bone is subjected to expansion strains as indicated by the Von Mises strains coloured map reported in the right hand side of Fig. 4.8. The colour scale utilized for this map is the same of that reported in Fig. 4.10, namely, the adaptive window of bone physiology where healthy bone growth and induction corresponds to the colours yellow and green and blue and red to bone reabsorption.

The surrounding bone is subjected to a healthy bone physiological deformation for a distance equivalent to the implant diameter. In this toroid volume surrounding the implant, then, it would be expected an osteoinductive effect and more rapid implant osteointegration (Fig. 4.11).

Micro Computer Tomography has confirmed these expectations. The Bone Implant Contact (BIC) and the relative bone density have shown similar characteristics at cortical (a) and medullar levels (b) indicating a good implant osteointegration with the original bone.

The newly formed bone near to the implants surprising shows characteristics similar to the previous one (c), indicating that a biomechanichally stimulating effect of the swollen hybrid scaffolding material.

4.5 Conclusion

The validation of the clinical efficiency and the estimate the long-term reliability of prosthetic restorative systems need the appropriate understanding of the physical variables that influence the biomechanical behavior of the material for biomedical advanced applications.

The tool of the Finite Element Analysis (FEA) is allowing biomaterials researchers to attain comprehensive evaluation of the biologic and mechanical behaviors of advanced restorative systems, even in the case of not homogeneous systems.

If validated by proper experimental procedures, the FEA turns useful in the optimization of the restorative design criteria and in the choice of the materials to be used. Moreover, this method allows the estimate of the location of fractures under given loading circumstances ([4–8, 10, 11]; Mullender and Huiskes, 1995).

New fabrication processes based on additive manufacturing technologies and studies on biomechanics and biomimetics [2, 15, 20] could enable the set-up of new design criteria for human prostheses. The Authors have taken up these studies to gathering the unexploited potential of such advanced materials and design technologies by developing biofidel Finite Element models able to correctly mimic the femur biomechanical behaviour (Fig. 4.1).

As an example of the theoretical approach presented here, the Authors refer to a design driven innovation obtained through an evolutionary design combining previous research results of experimental activities carried out on prostheses and new fabrication processes based on EBM (Electron Beam Melting) additive technologies of Titanium powders and related advanced academic studies on biomechanics and biomimetics of implanted bones. These activities have been taken up by the Authors in order to harvesting the hitherto untapped potential of such advanced materials and manufacturing technologies for the evolutionary design and fabrication of customized innovative “biomimetic prosthetic systems” that better integrate with the physiological biomechanics of the bones where they are implanted.

It is necessary to develop new technologies in biomaterials field, in order to obtain scaffolds and bone substitutes that could have a fundamental role in bone regeneration. It is requested to bone scaffolds to show particular intrinsic characteristics in order to work as a real bone substitute that satisfies biological, mechanical and geometrical constrains. Such features comprise:

- Biological requirements - the computed scaffolds must enable cell adhesion and homogeneous distribution, growth of regenerative tissue, and assist the passage of nutrients and chemical signals. This achievement has been attained by controlling the porosity of the scaffold;
- Mechanical requirement - the estimated scaffolds must preserve the mechanical and toughness properties that allow osteoblasts colonies to experience physiological and bioactive controlled deformations. This has been achieved by properly modifying the hybrid ceramo-polymeric compositional ratio (in our case, 10% by volume of amorphous nano-silica).

Combined clinical observation of traditional implant behaviour has been used to validate the biofidelity of the FEM models, while comparison between *in vitro* and computer aided simulation of osteoblast colony growth allowed us to explore many novel ideas in modelling, design and fabrication of new nanostructured scaffolds with enhanced functionality and improved interaction with cells. This turns particularly useful in designing and directly manufacturing complex bone tissue scaffolds.

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Chapter 5
Targeting MSCs for Hard Tissue Regeneration

Giorgio Mori, Adriana Di Benedetto, Francesca Posa, and Lorenzo Lo Muzio

Bone and cartilage injuries deriving from trauma, tumors, inflammation diseases, as well as natural aging, cause debilitation among affected individuals and represent a challenge for medicine. In particular, bone is subjected to frequent age and disease-related degeneration with mass decrease: the osteoporosis. Moreover tumors, trauma and chronic inflammation can determine localized bone loss. On the other hands, a major cause of disability in middle-aged and older people is represented by joint pain. Thus cartilage degeneration due to primary osteoarthritis, trauma and injuries resulting from sport activities are all possible causes of this kind of pain [1]. As the cartilage is a tissue with a little self-regenerative capacity, any alteration of its integrity might be carried on for years and eventually lead to further degeneration [2].

Frequently severe joint pain appears to be debilitating and this complication has motivated the research for scientists and surgeons to find a way to repair or regenerate lost cartilage. Given that it is a tissue with very distinctive properties, little success has been obtained until now.

Differently from bone, which has a consistent number of osteoblast precursors in the periosteum and bone marrow, cartilage has a matrix with no vascularization, with a consequent reduction of the tissue ability to recruit endogenous chondroprogenitor cells for healing; it is a flexible elastic tissue, compression resistant, it is able to distribute the loads to which it is subjected. It makes difficult the cartilage replacement with a tissue or any other designed device [3, 4].

Both in bone and in cartilage lesions it is important to distinguish between repair and regeneration. While a wound repair, which is mainly an inflammatory process with the subsequent recruitment of cells able to modulate the reparative processes occurs easily [5], the regeneration of the injured tissue consisting in an architectural and functional recovery hardly develop especially in cartilage. This process is

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thought to be due to the activation of resident stem cells or to the proliferation of quiescent cells with the restitution ad integrum of the tissues. In this case Mesenchymal Stem Cells (MSCs) are necessary [6, 7].

The current clinical practice for hard tissues defects repair employs autologous and allogeneic grafts. These strategies provide a scaffold support and a source of cells for the new tissue formation, but both approaches are often associated with post surgery complications as donor-site morbidity, pain, non-union fractures and infections [8, 9]. Therefore, the reconstruction of bone and cartilage defects is a challenge for regenerative medicine because of inadequate current treatments. To this purpose alternative approaches are desirable to address other possible strategies for hard tissues regeneration. Tissue engineering, relying on cell biology and material chemistry knowledge, aims to achieve the development of tridimensional substrates that can substitute bone implants. This approach can be optimized with the use of stem cells in particular Mesenchymal Stem Cells (MSCs), based on their direct ability to generate progenitors able to differentiate in chondroblasts and osteoblasts. *In vitro* differentiation of MSCs, represents the initial step to achieve the tissue regeneration, but to repair critical size defects, the cells must be seed on tridimensional scaffolds in order to recreate the structure and functionality of the lost tissues. Furthermore the integration of the new bone and cartilage with the resident tissue is needed, together with the appropriate degradation of the scaffold at the end of the healing process [10]. MSCs are stem cells of mesodermal origin and are responsible for the differentiation of properly defined connective tissues: the specialized ones, muscle tissues and also some epithelial tissues. MSCs, obviously forming the embryo and fetal mesenchyme, are present in many adult organism sites. Thus MSCs have the role to develop diverse differentiated tissues during embryogenesis and allow tissue renewal and repair during all life. MSCs residence in adults has been found in several locations and is still under investigation but bone marrow has been identified as the main residence site, here they are also defined as bone marrow stromal cells. Many other sites such as dental tissues, adipose tissue and peripheral blood has been demonstrated to contain a population of MSCs [11]. The research in the field of tissue regeneration has reached several goals in early clinical trials of therapies based on living cells. However, methods to regenerate bone and cartilage using living autologous cells are still under investigation. Thus, autologous bone still remains the gold standard for the repair of damaged bone tissues. Cartilage regeneration is still a step behind due to the lower chondrocyte proliferation and to the minor availability of convenient harvesting sites. Both bone and cartilage regeneration due to the morphology of the tissues is based on cell integration with opportune scaffolds. The choice of the most appropriate scaffold would bring optimal biological, chemical and geometrical characteristic to generate the more efficacious microenvironment to support the new bone formation. As first, the geometry of the scaffolds must be designed to ensure the correct transport of gas, nutrients and metabolites to the cells, but providing on the same time the adequate load support during the bone reconstruction therapy [12]. An adequate internal porosity in 3D scaffolds will better mimic the natural structure and the mechanical

properties of bone tissue [13]. Furthermore, the material composition and surface properties of the scaffolds will help MSCs adhesion and migration, thus favoring the osteogenic and chondrogenic differentiation. Since mineral bone matrix is naturally composed of calcium in hydroxyapatite (HA) crystals, to obtain bone regeneration, bioceramics able to incorporate hydroxyapatite or tricalcium phosphates (TCP), have been fabricated to mimic the tissue composition and stimulate the osteogenic differentiation of MSCs [14, 15]. These type of bioceramics have been also shown to facilitate the integration of the scaffold following *in vivo* implantation [16]. Synthetic polymers as polycaprolactone and polyethylene glycol have been used to generate scaffolds with the recent technology of 3D printing. These materials allow incorporating peptides containing the cell-binding sequence arginine-glycine-aspartate (RGD) or load regulatory molecules (BMP-2) to be gradually released, thus enhancing osteogenic differentiation of MSCs and promoting mineralization [17]. Several *in vitro* investigations have demonstrated that MSCs of different origin and osteoprogenitor cells were able to produce mineral matrix, when seeded on 3 D scaffolds [18]. Interestingly, composite of ECM protein Collagen and HA, or TCP, have been recently used to produce scaffolds and have shown to trigger and accelerate osteogenic differentiation and support *in vitro* bone formation from MSCs [19, 20] .

However the efficacy of a scaffold in generating *in vitro* bone formation could not be accompanied by the same properties *in vivo*, thus, to translate the technology of engineered scaffolds into clinical applications, *in vivo* approaches through animal models are necessary.

Different scaffold materials for cartilage engineering have been tested, they can be natural derivatives, synthetic polymers or a mix; they can present different forms: solid in fibers, powder, mesh, sheets, semi-solid gel, hydrogel, or glue form [21].

Several types of materials have been used for scaffolds manufacture. Some may contain proteins such as collagen, fibrin or gelatin, others may be made of carbohydrates (hyaluronic acid, alginate, agarose, polyglycolic acid (PGA) or poly-lactic-acid (PLA) and chitosan).

Chondrocytes have been seeded on biological matrices such as hyaluronic acid [22, 23] or collagen membranes [24]. These latter are the most widely used and have been studied for the first time in 1984 [25]. Collagen, as recognized by enzymes, can be modeled and degraded over time [26], it can induce transplanted cells to produce more Collagen [27]. Scaffolds constituted of commercial type I/III collagen membranes have been introduced [28].

Among the commercial products currently used for cartilage regeneration, there are two poly (lactic acid) (PLA) scaffold-based systems in particular: Bioseed ®-C and TRUFIT CB™ [29].

Bioseed ®-C from BioTissue Technologies is a scaffold of PGA/PLA and polydioxanone (PDO) on which autologous chondrocytes are cultivated. This is a method similar to the Autologous Chondrocyte Implantation (ACI); more than 3000 patients have been treated with it since 2002 and it is available in Europe [30].

TRUFIT CB™ from Smith & Nephew, differently from Bioseed ®-C, is a system similar to the Osteoarticular Transfer System (OATS), that is a replacement of

damaged cartilage with cartilage taken from a site which is subject to a lower weight. This scaffold is composed of a poly-(D-L-lactide-coglycolide) (PDLGA) and calcium sulfate bi-layer [31].

There is a great variety of products for cartilage engineering, many of which are in the clinical study phase. Most of these scaffolds requires the use of passaged chondrocytes, only in few cases it is possible to co-culture primary chondrocytes and less differentiated cells such as stem cells (in particular MSCs).

Primary chondrocytes can be enzymatically isolated digesting biopsies from less loaded regions of the knee. In order to obtain an adequate number of cells, chondrocytes need to be passaged one to four times, but it is important to maintain them at a low passage to avoid their dedifferentiation [32].

During chondrocytes differentiation there is a morphology change, the cells become round fibroblast-like at the same time specific cartilage matrix production is reduced. These changes are accompanied by a down regulation of chondrogenic genes and an up-regulation of fibroblast or mesenchymal genes [33, 34]. All these changes occur, generally, in the first passages. The passage number refers to the number of times in which the cells are passed in monolayers in culture and it appears to have little effect on the cells ability of new matrix synthesis. Generally it is preferable to use cells passaged less than 4 times [35].

Chondrocytes can be cultured in bi-dimensional cultures in the presence or not of serum, antibiotics and antimycotics. Advantages and disadvantages related to the use or not of these supplements are still under investigation since there are many conflicting data. Some growth factors, such as FGF-2, EGF and TGF-β1, appear to be indispensable to increase cell proliferation and enhance chondrogenic phenotype [36].

A particularly attractive strategy for improving the regenerative cartilage processes, compared to the use of differentiated cells such as chondrocytes, is represented by progenitor cells, in particular MSCs, with a chondrogenic differentiation potential [37, 38].

To obtain a correct hard tissues regeneration, MSCs have to be harvested, collected and isolated with opportune techniques. MSCs have been discovered by Friedenstein et al. who described a population of nucleated adherent cells from bone marrow that were fibroblast-like and capable to differentiate into fibroblast, osteoblast, chondrocyte and adipocyte [39].

In general, MSCs represent a small fraction of cells in the bone marrow, thus the frequency of MSCs in human bone marrow has been estimated to be in the order of 0.001–0.01% of total nucleated cells [38].

A higher percentage of MSCs can be isolated from dental pulp, the tissue harvested from a single tooth and following the opportune treatment can generate a large number of colonies considering the small volume of the tissue [40].

The identity of these cells as MSCs has been confirmed by their ability to differentiate also into neural-like cells, adipocytes, odontoblasts, and myoblast cells confirming their multipotency [41].

Such important features have led to define these cells as Dental Pulp Stem Cells (DPSCs).

These cells are located in the soft tissue within the tooth, in a niche of the pulp chamber mixed with the others cells normally present in the connective tissues and surrounded by the odontoblasts. The resident cells are fibroblasts, macrophages and granulocytes.

DPSCs can undergo to proliferation, allowing their self-renewal but also to differentiation process forming new odontoblasts and repairing the dentin damaged by bacterial action during caries [42].

DPSCs can be easily isolated from permanent and deciduous teeth; DPSCs are multipotent and have an high proliferation rate and the ability to undergo both osteogenic and chondrogenic differentiation.

Thus they have the potential to be used in the treatment of bone and cartilage injury and trauma having osteogenic and chondrogenic features and high proliferation rate [43–45]. MSCs, capable to differentiate into osteoblasts and chondrocytes, can be isolated not only from dental pulp but also from other dental tissues such as periodontal ligament, apical papilla and alveolar bone; anyway the dental bud which is the precursor of the tooth, or part of it as the dental follicle, are getting high interest as MSCs source [46]. The dental bud of the wisdom tooth can be harvested in teenagers and represents a productive source of MSCs. This undifferentiated organ contains an high number of stem cells, most of them expressing mesenchymal makers and, opportunely treated, can express excellent osteogenic features, representing an excellent model for bone regeneration (Fig. 5.1) [47]. Cartilage regeneration with MSCs is still a step behind bone regeneration. It is known that MSCs biologic processes are controlled by WNT/Beta-catenin pathway, which promotes osteoblastic differentiation during MSCs growth in culture. Therefore in normal conditions MSCs decrease the chondrogenic potential. Thus the inhibition of WNT signaling together with FGF2 administration during differentiation can promote chondrogenesis [48]. Also gene therapy showed to be effective in increasing chondrocyte differentiation from MSCs [49]. Chondrocyte obtained from MSCs differentiated on

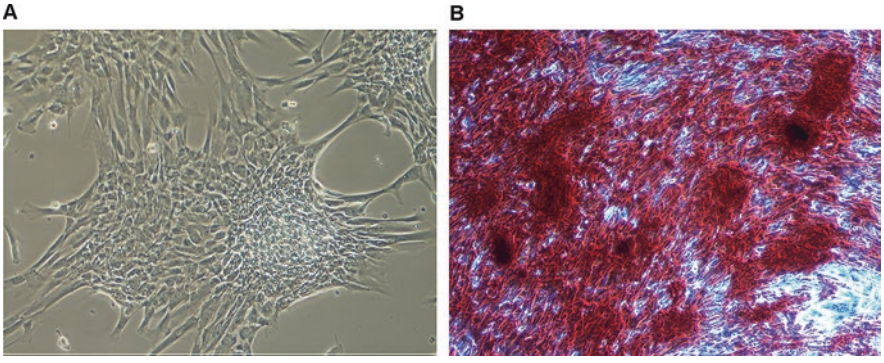

Fig. 5.1 (**A**) DBSCs isolated from dental bud and cultured *in vitro*. The cells were able to adhere to the plastic and after 48–72 h acquired the typical colony-forming unit appearance. (**B**) DBSCs cultivated in presence of mineralizing medium for 21 days and stained for Alizarin Red. Dense nodules stained in red are visible (40× magnification)

scaffolds of resorbable composite of natural hyaluronan matrix and synthetic polyglycolic acid showed to be more successful in repairing microfractures and large chondral defects in humans [50].

5.1 *In vivo* Models of Bone Tissue Repair and Regeneration

5.1.1 Ectopic Bone Formation

Ectopic bone formation represents the ossification of tissues in not usual places. This event is pathologic and has a clinical relevance, but has been used as a model to test *in vivo* experimental bone formation starting from cells and scaffolds. Subcutaneous implantation is the most used because surgically easier and is applied especially in rodents for three valid reasons: they are less expensive, have soft skin and can be readily immunocompromised for xenograft-based experiments. Basically, skin incisions are made on the dorsum of the animals under aseptic conditions and subcutaneous pockets are created where implants are placed. The most common implanted cell types are MSCs from bone marrow (BMMSCs), but experimental models have been also developed with DPSCs, Periodontal Ligament Stem Cells (PDLSCs), cord blood MSCs and osteoprogenitor cell lines [51]. Very few studies reported ectopic bone formation from direct transplantation of free-scaffold cells [52], few more revealed in some cases the ability of free-cell scaffolds to induce bone formation, but mostly in presence of BMP-2-loaded composite materials [53, 54]. Whereas the majority of the studies showed the best results when the cells were delivered with the help of different types of supports, in most cases the cells, after culture expansion, were seeded on biomaterials and predifferentiated *in vitro* with osteogenic factors before implantation [51, 55]. The use of immunodeficient animals, especially rats and mice, allow the implantation of allogenic or xenogenic cells with no adverse effects; the use of human cells, in particular, may have more important clinical implications. The subcutaneous experimental model is accomplished with small size rodents; the advantage of using small size animals consists in an easier accessibility for the scientists, allowing evaluation of bone regeneration and repair in relatively short time and with a high number of samples (implants) coming from the same animal. Furthermore in this model is examined the bone formation deriving from cells not naturally present in the subcutaneous, thus when xenogenic stem cells are implanted, it is confident enough to establish that the new formed tissue is of exogenous origin and not from native derivation. This latter aspect is especially important when implants with human stem cells are tested and has important clinical relevancies; however, it should be noted that this model does not take into account the influence of bone microenvironment and mechanical loading, thus restricting its availment to a first phase of *in vivo* evaluation of a tissue regeneration system. Indeed in order to translate the acquired experimental knowledge into real clinical cases it is necessary to develop more specific bone-defect models.

5.1.2 Craniofacial Models

5.1.2.1 Mandibolar Bone Defect Model

The choice of suitable animal model to test depends on which type of bone defects are object of the clinical demand and need to be simulated in animals. The craniofacial bones are often subjected to surgery due to tumors, traumas or congenital malformations. For example, parts of mandible can be removed for oncologic causes, thus the reconstruction of this bone represents a big challenge. To this purpose mandible defect models have been generated in rat, dog, goat and monkey to study bone regeneration from stem cells with and without the presence of scaffolds. These models include different size of defects ranging from 1 mm in mice, to 35 mm in sheep and are defined critical size defects (CSD), which is a defect size that will not undergo normal repair during the lifetime of the animal [56–58]. To repair such critical size defects, tridimensional scaffolds are necessary in order to recreate the structure and functionality of the lost bone tissue, furthermore the integration of the new bone with the resident tissue is needed, together with the appropriate degradation of the scaffold at the end of the healing process. The most common scaffold materials used in mandibular defect models that meet these characteristics are *Bioceramics* with incorporated (HA) (TCP). BMSCs seeded on these type of scaffolds have shown to have good bone repair potentials in jaw defects [57, 59–61] followed by dental tissues derived MSCs [55, 62]. Interestingly, also injected ASCs (Adipose-derived stem cells) ameliorated the healing time and promoted bone formation [63, 64].

5.1.2.2 Alveolar Bone Defect Models

Periodontitis is a disease of the tooth-supporting (periodontal) tissues characterized by inflammation and bone loss [47] impacting on health and life quality. Focusing on repair of alveolar bone defects caused by periodontal destruction would be a significant clinical goal. Periodontal defects have been surgically generated in animals to set *in vivo* tissue engineering of periodontium. The majority of the studies were conducted in large animals as dogs and minipigs using autologous cells for the regeneration, while nude rodents were employed for xenogenic cell implants. The data present in the literature indicate that autologous and allogenic MSCs of dental origin, mostly dental pulp and periodontal ligament, as well as bone marrow-derived have the ability to differentiate and regenerate the periodontium tissues, including bone and cementum, with well-oriented ligament fibers [56, 65].

5.1.2.3 Calvarial Bone Defects Models

The calvarial model is particularly suitable for evaluating the regeneration ability of high complex implant or new composite materials, because of poor vascularization of the bones and low presence of bone marrow [66]. This model has been most

commonly developed in rodents as rats and rabbits, but also in mice, pigs, sheep and goats. It is widely utilized because the bones shape allows to induce consistent and reproducible bone defects, easy to surgery, does not require fixation during the healing, and is easy to analyze with histological and radiological techniques after the healing period. Cranial defects generated in nude mice, were used to evaluate bone regeneration capacity of scaffolds made of TCP and granular deproteinized bovine bone, alone or in association with hDPSCs, indicating that addition of cells to scaffolds ameliorated the bone regeneration process [67]. Similar results were obtained in a rat model carried out with hDPSCs combined with a HA/TCP scaffold [68]. A recent study has used a calvarial defect model in rabbit to test a composite ceramic of TCP and HA in combination with rhBMP-2 and autologous MSCs, demonstrating that a certain composition of TCP and HA in synergy with rhBMP-2 and MSCs enhanced new bone formation as well as the resorption rate of the scaffold [69]. The limit of this experimental model is the absence of loading weight sites, thus in some applications others bones as mandibles, or long bones femur or tibia, may be preferred.

5.2 *In vivo* Models of Cartilage Repair and Regeneration

MSCs cells exhibiting trophic and immunomodulatory activities [70], could positively influence fate and activity of the unaffected cells surrounding the cartilage at the site where the damage is.

Notwithstanding the great interest in referring to MSCs, little data are available on studies with large-animal models.

As previously said for biomaterials used with chondrocytes in cartilage regeneration, MSCs can be delivered in cartilage defects through hyaluronic acid/hyaluronan, which is a glycosaminoglycan particularly abundant in the cartilage ECM, or collagen-based biomaterials [71, 72].

Kasemkijwattana C et al. have tried autologous implantation of BMMSCs, after their expansion on Collagen scaffolds, showing the advantages of this procedure compared to the convectional ACI [73].

However a problem to be solved is represented by the integration difficulty of different biomaterials with the neighboring cartilage to reach a continuity between the neo-synthesized tissue and the native one and long-term healing effects [74]. In this respect, promising results have been obtained recently in a rabbit model, using MSCs sheet incorporated in a bi-layer of Poly-lactic-glycolic acid (PLGA)/MCSs [75].

MSCs can be used also for transplantation in the damaged site after their chondrogenic differentiation *in vitro*. The optimal protocol for the differentiation of MSCs into chondrocytes is still under investigation since the use of the protocols available today has allowed to obtain hypertrophic cells that, when transplanted in Severe Combined ImmunoDeficiency (SCID) mice, led to the formation of an unstable cartilage [76].

An alternative is the use of chondrocytes and MSCs co-cultures. Studies carried out in this direction have led to phenotypically stable tissue constructs containing a

high amount of proteoglycans and Collagen Type II. This could in the future allow to reduce the use of chondrocytes for *in vivo* implantation [77].

Although the methods based on scaffolds are nowadays widespread, further studies are needed to find the ideal matrix material. Even if cartilage engineering represents a promising solution, an adequate approach for the long-term regeneration of cartilage lesions has not yet been identified.

5.3 Clinical Studies

For both bone and cartilage tissue-engineering approach, to integrate opportunely differentiated MSCs with the correct scaffold would represent a promising strategy for hard tissues regeneration, generating new, cell-driven, functional tissues, rather than cell free allogenic or heterologous tissue grafts.

Since many years, clinic applied research has demonstrated a successful therapy in patients with bone defects that have received grafts with autologous and opportunely *in vitro* differentiated MSCs integrated with hydroxyapatite scaffolds. Hard tissue engineering exploiting MSCs differentiated in osteoblasts or chondrocytes and seeded on biocompatible three-dimensional scaffolds, allows tissue-like structures formation with vascular ingrowth.

At present different kinds of scaffolds are already used to regenerate degenerative or traumatic bone defects: both synthetic bone mineral matrix or bio-absorbable ones can be enriched with growth factors (BMPs) or platelet enriched plasma for more effective results [78]. The integration of differentiated MSCs with the mentioned scaffold could make regenerative therapies also more effective compared to autologous bone therapies. These treatments would have the great advantage to avoid autologous bone graft preserving the skeleton and the harvesting site surgical consequences [79]. Yet, there are some issues that have to be further investigated: MSCs from bone marrow are not easy to be isolated and expanded; other sources, as dental tissues and peripheral blood, appeared to be more convenient. Moreover pre-clinical studies on mice showed that MSCs used for bone regeneration could lead to ectopic bone formation [80].

Cartilage restoration instead requires a different approach and can be used in patients with small cartilage lesions. It can be accomplished using a variety of methods as tissue grafts (autografts or allografts) or techniques adequate to stimulate the natural repair process [2].

In order to eliminate the necessity of a donor site and the concerns associated with allogenic and autogenic implants, many attempts have been made to heal or regenerate the existing cartilage, rather than to replace it. The techniques suggested are focused on improving the intrinsic tissue regenerative properties or on chondrocytes transplantation with the objective of generating more tissue. Unfortunately, no one of these techniques has led to a complete success, especially in older patients.

The most common treatment of cartilage regeneration consists in penetrating the subchondral bone providing a scaffold for MSCs migration and their eventual dif-

ferentiation into chondrocytes and osteocytes [81]. This method leads to a large variability in the results.

Less invasive techniques are considered laser or electrical stimulation, or pharmacological agents used to stimulate chondrocytes activity [2].

Cells transplantation, using chondrocytes or undifferentiated cells, can be used to restore the mass of cartilage tissue lost. It is necessary a small tissue biopsy, the number of cells obtained is expanded in culture and then the resulting cells are placed where the defect is present. Chondrocytes transplantation is object of studies since 1968 [82, 83].

Most promising results were obtained by using a surgical method that utilizes a periosteal flap sutured on the defect as a barrier for cultivating injected chondrocytes [84]. This procedure is called ACI and results in a filling of the defect with hyaline cartilage or mixed-type neocartilage which is integrated with the host tissue [85]. After ACI first application by Brittberg in 1987, the method has evolved [86, 87], since an issue associated with the use of a periosteal membrane was often represented by hypertrophy [88].

In order to contain differentiated cells after transplantation in a defined area and let them to distribute uniformly, it is possible to use scaffolds made of porous materials instead of tissue flaps. This technique is less invasive, reduces the morbidity related to the use of a donor site and provides an anchoring substrate which is fundamental for cell adhesion processes [89].

The researchers have tried to reconstruct cartilage tissues *in vitro* through tissue engineering, a technique that combines the use of cells and biomaterials providing scaffolds on which the cells can grow in three dimensions and under physiological conditions [90].

Chondrocytes cultivated on two-dimensional cultures differentiate, tend to assume a more flattened appearance and produce Collagen I in place of Collagen II. If cultured on three-dimensional systems, the cells maintain their phenotype and their functionality [91]. The 3D culture produces microenvironments very similar to those of the native cartilage, favoring the formation of cell-cell and cell-matrix interactions, this is an advantage which distinguishes the 3D from the 2D culture. An ideal scaffold should act as a support for the cells during new cartilage formation, then replaced by the neo-synthesized matrix and, above all, should be biocompatible.

Scaffolds can be also used with no cells to promote cell migration to the purpose of regeneration enhancement.

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Chapter 6
Mesenchymal Stem Cells in Dental Applications: State of the Art and Future Insights

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6.1 Introduction

Studies on stem cells have demonstrated their capacity for organ and tissue repair with their self-renewal and differentiation features. They produce different cell types, thus providing new strategies for regenerating missing tissues and treating more diseases. Adult mesenchymal stem/stromal cells (MSCs) have been identified in several oral and maxillofacial tissues and, adequately reprogrammed, induced pluripotent stem (iPS) cells, providing an ideal source for new bio-technologies and tissue engineering, for novel tools for reconstructive surgery with regard to clinical availability and applications in dentistry.

The demand for treatment strategies of musculoskeletal tissue degenerative diseases is continuously growing, especially considering the increasing number of older people all over the world. In a recent data analysis, the number of over 65 year-olds in the USA is expected to double and the number of over 85 year-olds is projected to quadruple in the next 20 years [1]. In Germany about 1.5 million people are under medical treatment for degenerative joint diseases and every year 110,000 American people present non-healing bone defects.

For skeletal reconstruction, orthopedic surgeons have applied autologous or allogenic tissues and artificial alloplastic implants. Therefore, the surgical success was limited due to the donor site morbidity of autologous grafts, the immunogenicity of allogenic grafts and loosening of the alloplastic implants [2]. In 1968 E. Haeckel, in the "Natural History of Creation" used the term "stem" (stammzelle) for the first time to indicate ancestral unicellular organisms from which,

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it is presumed, all multicellular organisms evolve [3]. Many stem cells have been hypothesized in a wide spectrum of normal and pathological conditions. In 1961, E. McCulloch and J. E. Till, identified stem cells (SCs), in a mice model, isolated following bone marrow transplantation after massive radiotherapy [4]. A few years later, the characteristics and properties of stem cells were defined as the ability to self-renew without senescence, symmetrical self-renewal divisions when two daughter cells become stem cells, capable of limited proliferation and differentiation in highly specialized cells [5]. In addition, J. E. Till suggested that, most stem cell divisions are asymmetrical in postnatal life, yielding one stem cell and a more differentiated cell, or progenitor cell, which has limited self-renewal ability [6].

The potency of a stem cell is defined by its ability to divide and produce one to many different cell types and tissues. Also, stem cells are able to proliferate extensively before differentiation in clonogenic cells. Plasticity is based on the differentiation capability and stem cells can be divided into embryonic and adult stem cells. Embryonic stem cells, are found physiologically in a fertilized ovocyte and the cells deriving from it by successive duplications during the first days of embryonic life. In each successive duplication, stem cells undergo a gradual reduction of differentiation ability and they are divided into totipotent and pluripotent stem cells. In the embryo phase, the Zygote, formed from the merger of the male and female gamete, is a toti-potent cell, able to produce a complete organism. In humans, this capacity remains only for three subsequent cell divisions. In morula and blastula later stages of stem cell differentiation, the capacity to create various cell subtypes is preserved.

In blastula stage the Inner Cell Mass (ICM), formed from pluripotent embryoblast cells, can differentiate into any of the three germ layers: endoderm (interior stomach, gastrointestinal part, lung), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system). The ICMs are able to create a complete organism, then the other mass of cells, formed from the trophoblasts, lead to the formation of extra-embryonic tissues. In contrast, adult stem cells may be multipotent or unipotent. The multipotent stem cells, show a differentiative skill and produce different mature cells of a specific tissue or different tissues, while the unipotent stem cell, can generate only a specific type of mature cell. During different tissue development, a group of multipotent somatic stem cells, with specific tissue function, are able to proliferate and generate mature cells to increase the mass of tissue during pre- or post-natal growth, or replace the cellular death for aging, apoptosis or damage. In addition to the multipotent cells, there are also other stem cells from the low potential, defined unipotent. These groups of cells have the skill to terminally differentiate only into a single mature cell line (for example, osteoblasts can only differentiate into osteocytes) defined nullipotente [7]. In regenerative medicine, the stem cells used are classified into embryonic (ESCs) and adult (ASCs) cells with the same characteristics. In dentistry, numerous studies have been conducted in recent years, to verify the possibility of regenerating dental tissues using both adult and embryonic stem cells. The aim of our study was to evaluate the

different MSC sources and their specific characteristics, and to identify the current strengths and weaknesses of rehabilitation treatments in dentistry through "tissue engineering", for the regeneration of different human craniofacial tissues.

6.2 Mesenchymal Stem Cells (MSCs)

Tissue engineering in dentistry, is considered to be a new frontier in the regeneration of degenerative and missing oral tissues and organs. The adult mesenchymal stem cells (MSCs) have been identified in several oral and maxillofacial tissues, which suggests that the oral tissues are a rich source of stem cells, and oral and mucosal stem cells have a crucial role in tissue regeneration and health. The primary sources of stem cells are considered adult (ASC) and embryonic (ES). In addition to these stem cells, which are naturally present in the human body, induced pluripotent stem (iPS) cells have been recently generated artificially with a genetic manipulation of somatic cells [8, 9]. ES cells and iPS cells are collectively referred to as pluripotent stem cells because they can develop into all types of cells from all three germinal layers. Although most adult stem cells are multipotent, they can only differentiate into a limited number of cell types. The ASC are called somatic or postnatal stem cells and are present in a small number in many adult tissues and organs. The adult stem cells, with asymmetric division, produce cells that are identical to themselves and they are implicated in self-renewal and differentiation processes that maintain healthy tissues or repair destroyed tissues. In many oral and maxillofacial regions of various mesenchymal tissues, many specific areas of "stem cell niche" were found, that can be utilized for adult stem cell sources, collectively referred to as mesenchymal stem cells or multi-potent mesenchymal stromal cells (MSCs).

Therefore, many recent studies in the dental field, have focused attention on Pluripotent stem cells (PSC) for the unlimited self-renewal and pluripotency. The pluripotency of stem cells is the capability to generate all lineages of the mature organism in response to signals from the embryonic or cell culture environment [10]. This capacity was investigated in basic dental studies on tissue engineering to explain dental tissue development, to test innovative drugs and regenerative therapies, and to find a realistic model for biological processes of dental tissue differentiation and growth.

Mesenchymal stem cells (MSCs) were isolated, for the first time in the 70s by A. Friedstein, from the stromal component of the bone marrow, where they represent approximately 0.01% of all nucleated cells. In addition, the MSCs were isolated from different adult tissues for regenerative use from umbilical cord blood, adipose tissue, placenta and peripheral blood and from other different areas, such as dental tissue (DPSC, DFSC, APSC, PSLSC) [11]. Also the ASCs showed a proliferative capacity reduction when cultured *in vitro*, while, induced pluripotent stem cells (iPSCs), derived from reprogramming specialized adult cells, differentiate into any tissue cell line. The mesenchymal stem cells from bone marrow (BMMSCs), are involved in the

release of growth factors and cytokines, mediators and chemotactic hematopoietic functions (CSE), favoring differentiation, angiogenesis, the slowdown of apoptotic and inflammatory processes. In the MSCs it is possible to observe a unique skill of spontaneous differentiation, both *in vivo* and *in vitro*, in adipose tissue, bone tissue and cartilage tissue of mesodermal origin [12]. Some studies have also shown a plasticity of these cells, assuming their transformation towards the muscle and neuronal lineage. Mesenchymal stem cells show a high adhesive and expansive capacity *in vitro*, with high replicative potential, immunosuppressive and/or immune-modulatory functions and are able to express specific membrane markers. MSC migrate spontaneously to source tissues and also selectively towards damaged tissues (multi-organ capability/tropism) and in the damage seat, promote the regeneration of compromised tissue after paracrine secretion of anti-inflammatory and growth factors.

In 2006 the International Society for Cellular Therapy defined three characteristics for the mesenchymal stem cell definition: adhesion and growth on a plate of uncoated culture, positive expression of the markers CD90, CD73, CD105 and CD44, the negativity expression for hematopoietic markers CD34, CD45, CD11b and CD19, and osteogenic, adipogenic and chondrogenic *in vitro* differentiation capacity. Recently, other unique cell surface markers for human MSCs, such as CD271 [8] and MSC antigen-1 [13], have been reported. In addition to the use of surface marker analysis, the selection of MSCs using stable mRNA markers specifically expressed in the MSCs has been proposed [14, 15]. Also microarray technology was recently used to identify and discriminate mesenchymal stem cells with gene expression analysis in dental tissue during tissue development and growth [16]. According to the ISCT criteria, MSCs must be adherent to tissue-culture-treated plastic when maintained in standard culture conditions. Additionally, MSCs must express CD105, CD73 and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules.

Stem cells exist only in a quiescent state and at each cell cycle, by means of an asymmetric division, can give rise to a cell identical to itself, which ensures the propagation of the cellular compartment (self-renewal), tissue repair and homeostasis [5]. More growth factors and different cytokine polypeptides, play an important regulating action. Among these platelet growth factor (PDGF), transforming growth factor beta (TGF-beta), insulin-like growth factor 1 and 2 (IGF), fibroblast growth factor (FGF) and finally bone morphogenetic proteins (BMP) seem to play a crucial role during the cell and tissue proliferation and differentiation processes and show a specific action for a particular type of target cell with different specific phenotypes. Among the growth factors, PDGF, VEGF, TGFβ and IGF are the most studied and used alone or in combination with others for their intense mitogenic activity in mesenchymal cells in stimulating cell proliferation [17]. This approach also offers many therapeutic clinical benefits, reduces the risk of rejection by the patient and the ethical implications.

6.3 Regenerative Medicine

Regenerative medicine is an innovative discipline for repairing adult tissues and/or organ diseases or what has been compromised by aging, in order to create a conductive environment for biological regeneration. This aim can be achieved through the identification of the cellular source capable of best regenerating damaged tissue. In clinical practice, the stem cells are those that are closest to this model. For their special features the MSC are now considered as the most promising candidate for use in regenerative/reparative medicine, in cell therapy and tissue engineering alone or in combination with biomaterial scaffolds and growth factors. The high proliferative potential, the trophism, the anti-inflammatory ability, the possibility to dispose of off-the-shelf cells and in particular the possibility to differentiate and trans-differentiate to specialized cells, if implanted in the right context and micro-environment, mean that the MSC can be an instrument for regeneration and repair tissues damaged by trauma, degenerative or pathologies. The potentials of regenerative medicine are so immense as to make it absolutely necessary and extremely important to continue to invest in this, so fruitful, branch and to be able to identify solutions for curing that which undermines the quality of life [18].

6.3.1 Programming and Cellular Re-Programming

In nature stem cells are found only in a quiescent state and rarely undergo cell divisions. However, at each asymmetrical division of the cell cycle, a stem cell can give rise to a cell identical to itself, which ensures the propagation of the cellular compartment (self-renewal), which in turn sustains morphogenesis, the repair of fabrics and the maintenance of homeostasis [5]. The growth factors are mostly polypeptides which play an important regulating action for a particular type of target cell [17]. MSCs are cells that can be isolated from various adult tissues, ideal for regenerative therapy. However, the isolation of AMSCs is obtained through invasive procedures. The AMSCs are rare and when cultured *in vitro* show a progressively proliferative ability. Induced pluripotent stem cells (iPSCs) are cells that can differentiate into any tissue cellular line, resulting from reprogramming specialized adult cells (such as fibroblasts).

Post-implantation embryo and then the fetus still have many stem cells, although their isolation is very difficult. Among the most studied are definitely primordial germ cells (PGC, Primordial Germ Cell) that represent the differentiation stage which precedes the formation of gonads. The PGC make their appearance, in the embryo of rat and in human, in the first and third week of development respectively. If isolated from the embryo, these cells can proliferate and produce pluripotent cells called EG (Embryonic Germ cell), capable, like ES cells, to differentiate into nearly all cell types in the adult. The difficulty in obtaining them, however, hinders their use in the treatment of diseases. But it is possible to find adult stem cells in several different tissue districts: in the spinal cord, the seminiferous epithelium of the male

gonad, in the retina, in the epithelia and in the brain. If the stem cells of each of these districts are isolated and cultivated appropriately, it is possible to increase the number and differentiate them into specific cell types of the tissue district from which they derive, or even trans-differentiate them into blood or nervous tissue. However, the adult stem cells, are very difficult to isolate because they are numerically very rare; also they cannot be grown long as, after a few cell divisions, they lose their pluripotency characteristics. Embryonic stem cells, however, can be maintained in culture for many cell division cycles, even for more than 10 years, without losing their pluripotency. An alternative route to obtain stem cells is their isolation from the umbilical cord. It should however be remembered that numerical and physiological limitations are also present in this case, although to a lesser extent [19].

6.3.2 Cellular Re-Programming

Since 1997 another very promising source of stem cells has been developed. The hypothesis is based on the possibility to modify the genetic program of adult differentiated cells, which can be reprogrammed. In 2006, Shinya Yamanaka [20] demonstrated *in vitro*, a new way to 're-program' specialized adult cells into pluripotent stem cells capable of producing every cell of the body. These cells are called induced pluripotent stem cells, or iPS cells. Until now, in nature, only embryonic stem cells showed pluripotent characteristics. The Yamanaka discovery means that, at least *in vitro*, every cell in the body could be able to divide itself and could be turned into a pluripotent cell stage.

IPS cells are capable of self-renewal, which means they can divide and produce an unlimited number of themselves and show very similar characteristics to stem cells not derived from embryos in the early stages of development. In addition, when cultured under specific laboratory conditions, they can produce almost any type of specialized cell. The iPS cells and the embryonic stem cells can help us to understand the development and growth of specialized cells, and in the future could also be an unlimited source of cells to be used for tissue regeneration and tissue engineering in diseases today incurable. However, recent research suggests that certain genes in iPS cells behave differently than they do in embryonic stem cells. Probably due to an incomplete reprogramming of the cells and/or acquired genetic modifications, actually limiting their clinical use. More studies are needed to better understand how reprogramming takes place within the cell.

6.3.2.1 Bone Marrow Stem Cells (BM-MSCs)

The mesenchymal cells derived from bone marrow (BM-MSCs) are found in the stroma of the bone marrow in relatively small quantities. It has been suggested that they constitute approximately 0.001–0.01% of nucleated bone marrow cells [21]. During embryological development, the maxilla and mandible bones originate from cranial neural crest cells [22], whereas the iliac crest bone is formed by mesoderm.

These embryological origin differences may result in functional differences between orofacial and iliac crest human BMSCs and their behavior during bone tissue regeneration [23, 24]. The BM-MSCs are easily isolated using a bone marrow aspirate and can generate several colonies of stem cells, which expand after 50 duplications in 10 weeks, and they can differentiate into osteoblasts, adipocytes, chondrocytes, myocytes, astrocytes, oligodendrocytes and neurons.

BM-MSCs are a component of the HSC niche, and through the release of cytokines and growth factors contribute to the renewal, maturation and recruitment of HSC. However, the definition of MSCs is controversial because the populations of adherent cells isolated from the bone marrow are not homogeneous, and definitive markers for distinguishing MSCs have not yet been identified [25]. In 2006, the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human multipotent MSCs; notably, the ISCT termed MSCs as mesenchymal stromal cells, regardless of the tissue from which they are isolated [26]. In the second half of the nineteenth century, Cohenim hypothesized the existence of non-hematopoietic mesenchymal cells in the bone marrow, and Maximov that the blood cells derived from hematopoietic stem cells induced by bone marrow signals. While, in 1968 Tavassoli and Crosby established a high osteogenic potential in some bone marrow cells transplanted into extra-medullary sites, able to proliferate and give rise to osteoblasts and trabecular bone [27]. Only in 1970, Friedstein and coll., in an animal model, showed the osteogenic potential of BM-MSCs, associated to a minimum sub- population of bone marrow cells and the BM-MSCs skill to differentiate into bone, adipose, fibrous tissue and cartilage, totally different from the hematopoietic lineage [11]. In 1987–1988, these stem cells, were denominated “osteogenic stem cells”. Only in 1991, Caplan defined the concept of “mesenchymal stem cell” in cells isolated from aspirated bone marrow [28] and only in 1999 Pittinger demonstrated their ability to differentiate into osteogenic, adipogenic and chondrogenic cells [29].

Adult bone marrow contains rare multipotent heterogeneous progenitor cells with a high replicative and differentiative capacity to robustly form bone in vivo, which makes them an appropriate stem cell source for bone regeneration therapy [30]. A number of medium and large bone defects, due to pathological and traumatic events, present great clinical challenges commitment, for use of these cell clusters. In fact, most bone extra or intra-oral donor tissues are inadequate for obtaining quality and quantity in autologous bone grafts. Only BMSCs from the iliac crest were found to be adequate when analyzed *in vitro* for their differentiation ability in osteogenic, chondrogenic, adipogenic, myogenic or non-mesenchymal neurogenic lineages [31–33]. They have been subsequently studied *in vivo* during bone regeneration in human sinus lift procedures [34].

During the last 25 years, a great interest has been paid to the new frontier of clinical tissue engineering, to find new solutions through three-dimensional scaffolds. In an animal model, Bruder et al., have shown that the addition of a scaffold integrated with BM-MSC, supports osteogenesis much better than an “empty” scaffold. The formation of a structure of bone repair defined “callus” was also observed which was absent in the experimental trial using the scaffold in the absence of stem cells. While the normal differentiation process allows the MSCs to differentiate into fibroblasts, chondroblasts, osteoblasts and adypoblast, the stem cells derived from bone

marrow have an ability to differentiate into several very specific cell lines in a trans-differentiation, or to differentiate themselves, both to the mesodermal toward ectodermal line, as neurons and epithelial cells, or endodermal as myocytes, enterocytes and pneumocytes. In 2006 F. Scintu observed the trans-differentiation of BM-MSC as an important alternative to embryonic cells because easily to isolate and differentiate into a large group of usable cells for tissue engineering [35]. For some time, BM-MSCs have been used in different animal models *in vitro* and *in vivo* studies for the repair of critical bone defects [36]. Furthermore, BM-MSCs have been found in orofacial (maxilla and mandible) bone marrow aspirates, obtained during dental surgical procedures such as dental implant treatment, wisdom tooth extraction, extirpation of cysts and orthodontic osteotomy. Clinical observations [36, 37] and experimental animal studies [38, 39] with BM-MSCs grafted bone (membranous bone) with or without different grafting scaffolds were performed in craniofacial sites to provide better results and significantly higher resultant bone volume than bone harvested from the iliac crest or rib (endochondral bone). In other studies, BM-MSCs differentiation into osteocyte cell lines and production of polypeptides such as Osteocalcin, Osteopontin, Osteonectin, MEPE, and collagen type 1 was observed. Also, after *in vivo* transplantation with different extracellular resorbable or not scaffolds, with BM-MSCs, initial bone formation was found. Moreover, in a study conducted on baboons using allogeneic BM-MSC, no production of antibodies was observed, suggesting the complete absence of immune reaction towards these stem cells. Again, it was possible to observe the absence of immune response in patients with osteogenesis imperfecta, after transplantation of BM-MSCs.

For the first time in 2003, Shi and Gronthos [40], and Kfoury in 2015, then Scadden [41] and also Mendez-Ferrer demonstrated the osteogenic ability of bone marrow mesenchymal stem cells to facilitate bone turnover, to permit repair of the large bone lesions, participate in the *in vivo* peri-vascular cluster aggregation (Sox9+/col2+) not associated with the vascular system, and with chondrocytes to contribute to the normal turnover of cartilage and long [42]. In an animal model, the BM-MSCs have also been used for the regeneration of cartilage tissue in combination with hyaluronic acid after menisectomia. *In vivo* research on animals and humans has also studied the kidney repair process following acute events after inoculation of bone marrow reprogrammed cells of extra-renal origin. Also, in studies on NOD/SCID mice it was possible to demonstrate the restoration of renal tubular function with a reduction of morbidity and death of the animals, after human BM-MSC transplant. During osteogenesis imperfecta *in vitro* studies have shown interesting bone and local cartilage responses after BM-MSCs inoculation. Furthermore, in a murine model MSCs have shown osteogenic activity as a vehicle for gene therapy with an adenovirus carrier capable of expressing the BMP-7 peptide, suggesting a new, effective way for *in vivo* bone regeneration. In addition, in 2003 Barry in a review of the literature showed that the BM-MSCs transplantation in non-bone sites, could lead to an *in vivo* myocardium and neurological repair action [43].

In 2006 Caplain and Dennis affirmed that there is no scientific evidence on the BM-MSCs capability, when transplanted [44], to generate new bone tissues, while, numerous studies have observed an effect on all target organs where these cells were

transplanted. This effect could be tied to the paracrine response and the cells ability to feed and guide the HSC and their progeny into different biological processes. Furthermore, in 2008 Ren et al. affirmed the presence of an immune regulatory activity of BM-MSCs on the hematopoietic cells lineage, vascular structure, function and on the organ integrity and regeneration [45].

Igarashi et al. [14] showed that orofacial BMSCs have a discrete differentiation capability with distinct expression patterns for several MSC marker genes compared to tibia, femur and ilium-derived BMSCs. Moreover, Akintoye reported a higher proliferation and osteogenic differentiation capability for orofacial BMSCs compared to the iliac crest BMSCs [46]. In addition, the orofacial BMSCs upon transplantation formed more bone in a mouse model [23] and in the mandible produced larger bone nodules and more mineralized bone than BMSCs from long bones [47], while the adipogenic potential of orofacial BMSCs is less than iliac crest bone marrow MSCs (0.03–0.5 mL) [23, 48], which may decrease unfavorable fat formation during bone tissue regeneration [49, 50]. That function could be maintained during MSCs non-bone transplants, with obvious benefits for the organ function. Considering the different timing and different cellular turnover between epithelial tissue and bone, it might be assumed that the stem cells both *in vitro* and *in vivo* models, are able to regenerate bone tissue. At the same time, they would not be able to meet the same number of cellular divisions affecting epithelial stem cells or HSCs. In recent and future studies, it will be possible to expand the knowledge about the possible development of reparative tissue replacement using BM-MSCs. Bone marrow mesenchymal stem cells today remain an interesting source of great clinical interest, easily accessible, being able to differentiate into different cell populations (osteoblasts, adipocytes, chondrocytes, myocytes, astrocytes, oligodendrocytes and neurons) and regenerate bone in dentistry. Therefore, a reliable and safe cell expansion protocol should be established for the use of orofacial BMSCs in clinical trials.

6.3.2.2 Dental Tissue-Derived Stem Cells

In 2000, adult human dental stem cells were first identified in dental pulp (dental pulp stem cells; DPSCs [51], and these cells showed phenotypic characteristics similar to those of BMSCs [52]. Gronthos in 2002 first isolated stem cells directly from the dental pulp of third molars (DPSCs), showing their high osteogenic and chondrogenic differentiation capacity and, under certain conditions, even a low adipogenic potential [40]. In a mouse model the DPSCs, showed high capability of osteoblastic differentiation, forming structures similar to the tooth dentin. *In vitro*, the DPSCs proved positive for mesenchymal cell surface markers CD44, CD73, CD90, CD105, Stro1, and CD146, the ematopietic markers showed negative for CD34, CD45, CD14. Other dental MSCs, such as SHED (Stem cells extracted from the pulp of deciduous teeth) and SCAP (apical papilla stem cells), showed in animal models the ability to differentiate in an osteogenic sense and generate complex structures like dentin-pulp after a subcutaneous transplant.

Only adult stem cells derived from epithelial stem cells and MSC-like cells have been characterized in dental tissues. An adult epithelial stem cell niche located in the cervical loop of the tooth apex of teeth was first demonstrated in 1999 [53] during organ culture of the apical end of the mouse incisor. The niche contained dental epithelial stem cells, which can differentiate into enamel-producing ameloblasts. This niche may be specific to rodents because their incisors differ from all human teeth in that they erupt continuously throughout the life of the animal. Till today, no information is available for dental epithelial stem cells in humans. Mesenchymal progenitor or stem cells have long been assumed to exist in dental tissues [54, 55] periodontal tissues and dental pulp (DPSCs), regenerate or form reparative dentin by a natural process if the environmental conditions are suitable after dental treatments [56, 57]. MSC-like cells were subsequently isolated from the dental pulp of human deciduous teeth (SHED) [58]. DPSCs and SHED showed stem cell properties, such as multi-differentiation and self-renewal [49, 51, 59] and the capability to regenerate the dentin–pulp complex when transplanted into immunocompromised mice. Furthermore, an MSCs source was found in periodontal ligament stem cells (PDLSCs) from extracted tooth root surfaces, and *in vitro* used to regenerate cementum, periodontal ligament and alveolar bone in experimental animal models [60, 61] while PDLSCs from the alveolar bone surface displayed superior alveolar bone regeneration compared to the same stem cells from the root surface [62].

6.3.2.3 Mesenchymal Stem Cells from Exfoliated Deciduous Teeth (SHEDs)

SHEDs are highly proliferative mesenchymal stem cells isolated from deciduous dental pulp, able to differentiate into osteoblasts, neural cells, adipocytes, and odontoblasts and they are able to induce dentin and bone formation in animal model studies. They result positive for several mesenchymal markers including CD105, CD146, Stro-1 and CD29, and negative for CD31 and CD34. Miura in 2003, observed a greater proliferative potential in SHEDs, BMMSC and DPSCs [63]. In 2005, Shi S. and Bartold observed in an immunodeficient mice model, how DPSCs and SHEDs implanted with scaffolds of HA/TCP were able to induce a differentiation towards dentin and pulp-like structures, with an odontoblasts line around mineralized matrix [64]. Also in 2010 Sakai VT, Zhang Z, et al. in an immunocompromised animal model (mouse) found the human-like dental pulp tissue and dentin tubular formation, in SHED transplants with non-resorbable scaffolds [65].

6.4 Apical Papilla Stem Cells (SCAPs)

Mesenchymal stem cells derived from apical papilla, *in vitro* showed a specific osteoblastic, adipogenic and chondroblastic differentiation like BM-MSCs. The SCAPs are strongly positive *in vitro* for phenotipic surface markers such as CD73,

CD44, CD105, CD146 and CD166 [66]. In 2010 Mastrangelo et al. with *in vitro* studies demonstrated the high SCAP differentiation into osteoblastic like cells with bone formation. In the same study, it was possible to evaluate the high growth response of the SCAPs with VEGF during bone formation compared with only VEGF cell cultures [67]. In 2008 research conducted by Sonoyama in an animal model, evaluated that the SCAP stem cells may be suitable for the regeneration and preferentially for root repair and formation. In 2010, Huang GT, et al. have also shown the formation of new tissue like pulp with a good vascularization and a continuous layer of dentin, after transplantation of SCAP cells on synthetic poly-D,L-lactide/glycolic scaffolds, inserted in dental fragments of an immunocompromised mice model [68]. Also Laino in a study in 2011, on SCAP cultures obtained from impacted third molars, demonstrated their differentiation capability into odontoblastic like cells, an active mineralization, a specific migration potential, and *in vitro* structures similar to dentin formation [69].

Ikeda et al. [70] identified distinctive stem cells in the dental mesenchyme of the third molar tooth germ at the late bell stage (tooth germ progenitor cells (TGPCs) with a high proliferation activity and the capability to differentiate *in vitro* into lineages of the three germ layers, including osteoblasts, neural cells and hepatocytes. Stem cells from the apical papilla (SCAP) [71, 72] demonstrated better proliferation *in vitro* and better regeneration of the dentin matrix when transplanted into immunocompromised mice. These findings suggest that "developing" dental tissues may provide a better source for immature stem cells than "developed" dental tissues [73].

6.4.1 Dental Follicle Stem Cells (DFSCs)

The dental follicle (DF) is an ecto-mesenchymal derived tissue, rich in stem cell progenitors, surrounding the teeth during the growth phase until eruption into the oral cavity. Furthermore, since the DF is derived from the neural crest, the DFSCs are isolated from impacted teeth after surgical procedure [74]. The dental follicle stem cells (DFSCs) show an *in vitro* capacity to regenerate bone and periodontal tissues for treatment of craniofacial defects [75, 76]. Dental follicle stem cells (DFSCs) were first isolated in 2005 by Morsczeck et al. [77]. In 2015 during Vollkommer studies it the CD105, CD44, and CD29 surface markers positive reaction and negative reaction for CD34 and CD117 was demonstrated [78]. In 2010 Mastrangelo et al. [79] demonstrated the DFSCs strong osteogenic capacity, with *in vitro* formation of osteoblast-like cells colonies, suggesting that the DF source is suitable for repairing intra or extra-oral bone defects. In addition bovine and human DFSCs mixed with hydroxyapatite ceramic scaffolds, after subcutaneous transplantation, showed *in vivo* mineralized bone structure formation, particularly interesting for regenerative medicine [80]. Furthermore, recent studies reported the success of DFSC transplantation used in the treatment of large defects in the calvaria in immunocompromised mice.

6.4.2 Periodontal Ligament Stem Cells (PDLSCs)

McCulloch in 1985 reported the presence of stem cells within the periodontal ligament known as PDLSCs [81]. However, only in 2004 with research carried out by Seo [82] and in 2006 by Sonoyama [83] mesenchymal stem cells within the periodontal ligament were isolated from extracted teeth. The PDLSCs isolated from the periodontal ligament, showed a positive reaction in STRO-1, CD44, CD90, CD105 and CD146 markers. PDLSCs were defined as multipotent stem cells that can differentiate into osteoblasts, chondrocytes, adipocytes, neurons and hepatocytes. Also in 2007 Yokoi demonstrated the regeneration of periodontal ligament after transplanting PDLSCs into an immunocompromised mouse model [84]. Akizuki et al. applied sheets of cells of the periodontal ligament on the mesial root defects in a dog model, showing an increased periodontal tissue healing with the formation of bone, cement and periodontal ligament [85]. Ding et al. using an *in vivo* animal model (mini-pig) reported that allogeneic PDLSC is able to significantly stimulate the regeneration of the periodontal tissue and heal. Furthermore, transplanted PDLSC are able to reduce the inflammatory response during periodontitis, by suppressing the activation of B and T cell. In 2006 Rincon showed the presence of periodontal remains, Malassez cells (ERMs), within the periodontal structures in oblique histological sections. These cells are activated in response to an injury or to inflammatory processes [86]. According to the studies conducted in 2005 by Cerri and Katchburian the ERMs, after explantation and *in vitro* culturing, are capable of giving rise to squamous metaplastic cells, cystic lesions and odontogenous tumors and ameloblastic lesions [87]. Both PDLSCs and ERMs may be considered an interesting source in the regeneration of dental-periodontal complexes and periodontal ligaments after periodontitis.

6.5 Dental Pulp Stem Cells (DPSCs)

Dental pulp stem cells, were first isolated by Gronthos S, in 2000 from the dental pulp of human third molars and the they are characterized by a high proliferative rate [51]. Several studies showed the cells ability to perform an osteogenic, dentinogenic, adipogenic, chondrogenic, myogenic and neurogenic differentiation. The DPSCs provide different osteoinductive factors (bone morphogenic proteins) and their ability to generate osteoblasts has been demonstrated both *in vitro* than *in vivo*. In 2005, in a mouse model, after DPSCs immunocompromised transplant onto a scaffold of HA/TCP, Shi S. et al. observed the formation of similar dental pulp and dentin [64]. Several studies *in vitro* of the different stages of differentiation, showed the DPSCs high expression of chondrogenic markers [88, 89] and several proteins involved in melanogenesis [90]. Furthermore, the DPSCs in VEGF presence, were able to generate structures similar to capillaries [91]. In addition, *in vitro* studies after osteogenic re-programming have highlighted the DPSCs capacity to produce colonies of osteoblasts and, in the presence of VEGF, were able to produce bone matrix. This increased behavior was VEGF dose dependent [92].

Another *in vivo* study showed the capability of the hDPSCs to generate both osteoblasts and endothelial cells. Moreover in an immunocompromised mouse model the formation of bone structure with a vascular structure was reported. Zheng et al. observed in a pig model, bone tissue regeneration and repair of critical mandibular defects after DPSC transplant [93]. In other case reports, the DPSCs were used with spongy collagenous scaffolds to repair small or large defects in mandibular sites. In an immunocompromised mouse model Batuli have shown that subcutaneously transplanted DPSCs on a dentin surface formed dental-like structures after 8 weeks. In addition, within the dental-like structures, blood vessels and connective tissue, were observed indicating an initial dentin-pulp complex formation. Moreover, the Batuli research showed how transplanted DP stem cells survived only in adequate vascularized environments, on the contrary DPSCs in the absence of vascular support, undergo necrotic death or apoptosis. In 2010 Huang GT, reported dental pulp complex regeneration in root canals after SHED and DPSC transplantation [94]. The regeneration of functional dental pulp complex inside the tooth after MSC transplantation, therefore remains a major challenge for clinicians.

6.5.1 Oral Mucosa-Derived Stem Cells (OESCs)

Oral mucosa-derived stem cells are composed of stratified squamous epithelium and underlying lamina propria connective tissue, well-vascularized tissue, and the submucosa, which may normally contain, minor salivary glands, adipose tissue, neurovascular bundles and lymphatic tissues [95]. Many researchers identified in the oral mucosa, only two different types of human adult stem cells: oral epithelial progenitor/stem cells and human gingiva-derived MSCs.

The oral epithelial progenitor/stem cells (OESCs) showed a characteristic development differentiation into epithelial cells. OESCs are able to regenerate a highly stratified and well-organized oral mucosal graft *ex vivo* [96, 97], which suggests that they may be useful for intra-oral grafting [98]. The OESCs are oral keratinocytes smaller than 40 mm [99] with high clonogenicity.

Only in 2009, Zhang et al. [100] first characterized in the lamina propria of the gingiva, a subpopulation of human gingiva-derived MSCs (GMSCs). During different surgical oral treatments it is possible to obtain samples to identify high volumes of human gingiva-derived MSCs. The GMSCs in animal models have shown self-renewal properties, clonogenicity and a multipotent differentiation capability, similar to BM-MSCs. Recently, Marynka-Kalmani et al. [101] reported that a multipotent neural crest stem cell-like population, termed oral mucosa stem cells (OMSCs), can be reproducibly generated from the lamina propria of the adult human gingiva and can differentiate *in vitro* into lineages of the three germ layers. Therefore, the gingival cells inherent stemness may be an interesting research platform on the high reprogramming efficiency of gingiva-derived fibroblastic cell populations during iPS cell generation [102] and dental tissue regeneration.

6.5.2 Periosteum-Derived Stem/Progenitor Cells (PMSCs)

The osteogenic capacity (osteogenesis) of the periosteum of long bones was first reported in 1932 [103]. Histologically, the periosteum is composed of two distinct layers: the outer area contained fibroblast cells and elastic fibers, and the inner area contains micro-vessels and sympathetic nerves with a high quantity of MSCs [104, 105], osteogenic progenitor cells [106, 107], osteoblasts and fibroblasts. Periosteum-derived stem/progenitor cells are able to differentiate into osteoblast, adipocyte and chondrocyte cell types. PMSCs express the typical MSC surface markers [104, 105].

Agata et al. [108] reported that human periosteal cells proliferated faster than marrow stromal cells, and subcutaneous transplants of periosteal cells treated with a combination of recombinant growth factors form more new bone than BMSCs in mice. Recent animal model studies showed the periosteum-derived stem/progenitor cells and the periosteum membrane mineralized extracellular matrix formation under the appropriate *in vitro* conditions and De Bari [109] demonstrated that single-cell-derived clonal populations of adult human periosteal cells possess mesenchymal multipotency, as they differentiate to osteoblast, chondrocyte, adipocyte and skeletal myocyte lineages *in vitro* and *in vivo*, and, when used for *in vivo* subcutaneous implantation in mice, formed ectopic bone [110]. These results suggest that PMSCs could be useful for bone and dental tissue regeneration and they have been recommended for alveolar bone augmentation with or without implant placement or in combination with bone graft surgery [111]. Additionally, cultured periosteum-derived cells have been used for alveolar ridge or maxillary sinus floor augmentation in clinical research that successfully demonstrated enhanced bone remodeling and lamellar bone formation with subsequent reliable implant insertion [112] and reduced postoperative waiting time after implant placement [113]. Therefore, the periosteum is a source of stem/progenitor cells for bone regeneration, particularly for large defects.

6.5.3 Salivary Gland-Derived Stem Cells (SGSCs)

After high or low radiotherapy all patients afflicted with head and neck neoplastic cancer suffered from an irreversible salivary gland structure and function damages with xerostomia. The existence of salivary gland stem cells has been suggested by *in vivo* studies [114, 115], but only recently Kishi [116] isolated SGSCs from rat submandibular glands and found that the cells are highly proliferative and express acinar, ductal and myoepithelial cell lineage markers. While, Lombaert [117] isolated a specific population of cells expressing stem cell markers from dissociated mouse submandibular glands, which differentiate into salivary gland duct cells capable *in vitro* of producing mucin and amylase. The salivary gland progenitor/stem cells were also isolated from swine [117] and human [116–118] salivary glands. Finally, Neumann [119] showed the long-term cryopreservation of integrin-a6b1 expressing cells as a sub-population of rat salivary gland progenitor cells. These reports suggest that the salivary gland is a promising stem cell source for future therapies especially in irradiated head and neck cancer patients.

6.5.4 Amniotic Fluid Derived Stem Cells (AF-MSCs)

Amniotic fluid is composed of embryonic and extra-embryonic cells deriving from the ectoderm, the mesoderm and endoderm able to differentiate into all tissues. Recent studies have shown the presence of undifferentiated fetal mesenchymal stem cells with a high capability to perform in bone and dental tissue. In 2003 Prusa et al. demonstrated, in the amniotic fluid, the presence of a stem cell sub-population, positive for pluripotency Oct-4 marker and mesenchymal markers CD29, CD44, CD73, CD90, CD105 [120]. Also in 2003 [121] and Tsai in 2004 [122] showed osteogenic and adipogenic differentiation of the AF-MSCs. Finally, De Coppi in 2007 [123] reported a stem cells population (AF-MSCs) able to generate and differentiate into: adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic clones, comparable to embryonic lines (mesoderm, endoderm, ectoderm) and easily accessible after amniocentesis. In regenerative medicine, the AF-MSCs showed no disadvantages such as ethical controversies, and have a high proliferative capacity, low immunological reactivity, no risk of teratomas formation and a lower risk of reaction to grafting than adult bone marrow mesenchymal stem cells.

In 2009 Peister observed *in vivo* the mineralized matrix formation after subcutaneous AF-MSCs implantation [124]. In the mouse model no significant differences could be observed between BMMSCs and AF-MSCs with polymeric scaffolds during femur bone healing. In addition, the bone growth appeared considerably higher compared to the not loaded scaffolds. Finally, the AF-MSCs are more easily isolated and show a better proliferative capacity when compared to MSCs derived from umbilical cord blood, and also show greater self-renewal and line-specific differentiation capacity than BM-MSCs. The AF-MSCs show immuno semi-allogenic quality for each parent, thus potentially useful for other family members. Numerous studies *in vitro* have shown the formation of new bone after osteogenic differentiation of AF-MSCs deposited on different natural or synthetic scaffolds such as collagen and titanium smooth, sandblasted and SLA (Sandblasted and Acid Etching), materials commonly used in dental implantology [19, 125].

6.5.5 Adipose Tissue-Derived Stem Cells (ASCs)

In 1976 adipogenic precursors of mesenchymal stem cells, derived from human adipose tissue, were isolated [126] but only in 2001, Zuk et al. identified and characterized adipose mesenchymal stem cells from human lipoaspirate adipose tissue [127]. The adipose tissue-derived stem cells (ASCs) were detected in hips, buttocks chin, upper arms and abdomen after surgical lipectomy or lipoaspirate with low donor-site morbidity [128]. More studies showed the characterization of two different types of adipocyte stem cells from subcutaneous or visceral donor sites. The stem cells derived from subcutaneous adipose tissue represent an interesting source, easily obtained and useful for many therapeutic applications [129]. The ASCs

exhibit high osteogenic capacity and several phenotypic expressions similar, but not identical, to bone marrow or dental pulp stem cells [130].

The composition of adipose tissue shows a heterogeneous cell cluster called Stromal Vascular Fraction (SVF) [129–131]. In 2001 Spiegelman and Flier classified the adipocytes in White Adipose Tissue (WAT), for the synthesis and storage of triglycerides and lipoproteins and in Brown Adipose Tissue (BAT) for metabolic action and heat generation [132]. Recent *in vitro* studies showed, under specific conditions, bone, cartilage, endothelium or adipose tissue differentiation from hASCs with or without the use of different scaffolds. In addition, the hASCs were able to release growth factors and cytokines that stimulate host tissue stem cells during healing processes. Although BM-MSC and ASC show common biological characteristics, being over 90% identical [129–133]. Indeed in culture for a long period, the human ASCs compared to BM-MSCs, appeared morphologically and genetically more stable [134] and also showed a higher proliferative and differentiation capacity [134, 135]. In recent studies it was reported that the human ASCs support hematopoiesis both *in vitro* than *in vivo* more effectively compared with BM-MSCs [136]. After fresh cell isolation the hASCs express CD34 factor. This marker decreases gradually in the later expansion phases [12, 106, 107, 137, 138], although it never disappears completely [138]. On the contrary, in BM-MSC and adult MSCs from other sources, the CD34 behavior, was stably expressed [12, 139]. In addition, ASCs expressed CD49d (integrin A4), but not CD106 [vascular cell adhesion molecule-1 (VCAM-1)], while BM-MSCs showed a strong expression [140, 141]. The ASCs displayed high levels of CD54 [ICAM-1 (ICAM-1)], while in BM-MSC only a minimal expression was observed [142, 143]. Finally, the BM-MSCs expressed the CD49f marker (integrin A6) and the Podocalyxin-like protein 1 (PODXL), while in ASCs the expression was weak [144]. However, according to some studies, the differences of immunophenotypic markers in BM-MSC and ASC, could be related to growth factors and biomaterials (scaffolds) frequently used during the trials. In other *in vitro* studies, the ASCs showed a high chondrogenic and osteogenic differentiation capability compared with BM-MSCs [135, 140, 141, 144–146]. In all *in vitro* ASCs studies, it was possible to observe a decreased osteogenic ability related to the increasing age of the donor, and this phenomenon appears more evident in female subjects. High orofacial autologous bone regeneration has been demonstrated [21, 136] and many researchers have hypothesized the use of ASCs in implant dentistry and vertical alveolar bone augmentation.

Pieri et al. [147] showed, in rabbits, a new bone formation and implant osseointegration following vertical bone calvarial augmentation with autologous ASCs and inorganic bovine bone scaffolds (Bio-Oss). In a rat experimental model, periodontal tissue regeneration has also been successfully demonstrated using ASCs [148]. Furthermore, in rat model study, the cementoblast features of ASCs were observed when cultured in dental follicle cell conditioned medium containing dentin non-collagenous proteins [149]. In studies on dogs, Ishizaka [150] showed that transplantation of ASCs, induced pulp regeneration in the root canal after pulpectomy. And Hung., in adult rabbit extraction sockets, demonstrated the ASCs capacity to

grow self-assembled new teeth containing dentin, periodontal ligament and alveolar bone with a high success rate [151]. Further studies on the isolation, characterization and application of ASCs to enhance their efficacy for bone and periodontal regeneration will provide a definitive protocol for the use of fat tissues in future clinical applications. The adipose tissue is considered an abundant source of MSCs and has been extensively studied as a stem cell source in the field of regeneration strategies in medicine, and for their typical behavior they could be considered as an alternative source for bone regeneration in dentistry.

6.6 Synovium Stem Cells (SYN-MSCs)

The synovium is a thin layer of connective tissue that lines joint surfaces and tendon sheaths, allowing articular movement, the lubrication of the articular surfaces, the nutrition of the articular cartilage, and the regulation of immune response within the joint itself. Synovial tissue shows an embryonic mesenchymal origin. Stem cells isolated from the synovium (SYN-MSC) showed chondrogenic surface markers, including SOX9, ACAN and COL1. Following the chondrogenic differentiation, the expression of these markers was significantly higher in SYN-MSCs than in BM-MSCs, while the osteogenic differentiation was lower. Numerous *in vitro* studies reported stem cells from the synovium capable of forming more colonies and a higher chondrocytic differentiation capability compared to BM-MSCs. In addition, the BM-MSCs preferentially differentiate into bone, while the SYN-MSCs differentiate preferably into adipocytes. Koga et al. with an *in vivo* rabbit model has shown massive formation of cartilage in the articular cartilage defects after MSC synovial local transplant. And in the deepest area of the defects, synovial-derived MSCs differentiated into bone, while in the superficial areas the same SYN-MSCs differentiated into chondrocytes. Such research should be confirmed by further *in vivo* studies and may suggest how the MSC-derived synovial could represent an alternative source in bone, tendon and muscle regeneration.

6.6.1 Dental Tissue Regeneration

The human body is an amazing machine containing numerous organs whose physiology is still far from being fully understood. Each organ is formed by different tissues and is able to perform one or more, often interrelated, functions. The oral cavity is a complex structure with complex functions, and the tooth, is an actual organ, formed of various heterogeneous materials, such as enamel, dentin, pulp and cement. Through the periodontal ligament they are connected to the alveolar bone and tissues which together allow the carrying out of numerous functional activities. Physiological and reparative processes are a fundamental part of life and vary greatly between individuals and between animal models and human models. While

reparative regeneration occurs naturally during normal cell turnover, it also occurs as a result of cellular or tissue damage. Unlike other organs and tissues, still little is known about the normal physiological turnover of these individual tissues and to date it is not possible to partially or totally replaced them with the same tissues when lost as a result of traumatic events, surgical or pathological. Therefore research activity is oriented in this direction. In view of these considerations, tissue engineering, based on recent advances in genetic engineering and the study of stem cells, is being given more important clinical relevance to provide important insights for improving the regeneration of the individual dental tissues. However, current dental regeneration allows only partial imitation of the natural process of development of the teeth through *in vitro* or *in vivo* studies using stem cells. Research is still needed to understand the many and complex mechanisms of cellular and tissue interaction in order to eventually reproduce single or complex tissues able to perform simple or complex functions. Moreover, since the development of teeth, as of all the organs, is characterized by, not yet finely described, endodermal, ectodermal and mesenchymal sequential interactions and the perfect mutual interaction between oral epithelium and neural crest (NC), the effort of many researchers has still not brought conclusive results. An example is the research to discover the optimal source of stem cells that have the potential to differentiate into different tissues by Thesleff and Sharpe, 1997 [152]. In this sense, the recent acquisition regarding the induced pluripotent stem (iPS), which can be genetically reprogrammed like embryonic stem cells (ESC), has significantly contributed to the understanding of processes and is having a major impact in this field. The interaction between stem cells during embryonic development stages, starts the development of various dental tissues and regulates morphogenesis of the tooth, and, although well documented by extensive research, even today not all the details of the epithelial-mesenchyme interaction are clear. In fact, according to some scientists, it would seem that during morphogenesis, mesenchymal cells respond to signals received from the dental epithelial tissue, while according to others, the interaction is mutual. Mesenchymal cells then would be able to differentiate into cementoblasts, periodontal ligament, odontoblasts, and other cells of the pulp tissue including neurons, endothelial cells and fibroblasts. At the end of tooth development, epithelial cells no longer exist, while the mesenchymal cells remain within the pulpal tissue and in the periodontal ligament. Several research groups have shown that it is possible to produce biological teeth, with appearance similar to natural teeth, on the basis of tissue-cell or cell-cell recombination using embryonic tooth germ cells [153–155]. In addition, using recombinant tissue/cell techniques, the stem cells as non-dental neural stem cells and cells derived from bone marrow (CES), have been shown to respond to inductive signals from the embryonic dental [156]. Furthermore, depending on the stage of development of the tooth germ, the dental epithelium or mesenchyme show a different inductive potential to differentiate the not-dental stem cells, into odontogenic cells. In 2007 Nakao et al. [153] et al. rebuilt, on a mouse model, a tooth using epithelial and mesenchymal cells derived from rat embryo. The tooth was able to emerge from the mouth of an immunocompromised mouse and develop into functional dental tissue. Although their approach represents a step forward in tissue engineering, this

clinical application is limited however, since it requires embryonic epithelial and mesenchymal stem cells. This branch of tissue engineering is still in its early stages and is unable to solve even just simple issues related to individual development imperfections. The most recent data from the literature suggest that adult stem cells can support, under certain conditions, some tooth development steps, which could provide a new approach to the reconstruction of dental tissues, as well as other organs, through the use of adult mesenchymal and embryonic stem cells. Many questions still remain to be clarified. However, the definition of MSC is presently controversial and new parameters, probably genetic, functional or immunological, are needed for classifying homogeneous clusters of mesenchymal stem cells. Another consideration is related to the age of the patient which is a crucial factor for the efficacy of tissue regeneration. The same attention must be paid to the processes of isolation, purification, expansion and storage of the stem cells. Therefore the stem cells obtainable during each single withdrawal must be examined to select the best source for quality and quantity of cells. Another critical point is the investigation of the differentiation and growth of the MSC fresh cluster compared with cryopreserved stem cells, and if these characteristics remain unchanged over time. With regard to source accessibility, another crucial theme is the dentistry skills needed to obtain the MSC during intra and extra-oral surgical procedures.

6.6.2 Challenges and Future Applications in Dentistry

MSCs can be identified and isolated based on their adherence to tissue-culture-treated plastic [175]. MSCs are among the most promising adult stem cells for clinical applications; they were originally found in the bone marrow, but similar subsets of MSCs have also been isolated from many other adult tissues, including skin, adipose tissue and various dental tissues [176, 177]. The cellular reprogramming shows great potential for replacing tissue in the medical field therapies, being able to use iPS cells derived from skin to produce specific healthy specialized cells to replace pathological cells or genetic diseases, without rejected by the immune system. Early research on the production and use of iPS implied permanent genetic changes which could cause the formation of teratomas, currently some researchers have developed methods of production of iPS cells without genetic modification. These new techniques are a step towards the production of iPS cells to understand the functioning of dellulare reprogramming, to learn to control the reprogrammed cells, and to secure cells for clinical applications in patients.

The stem cell accessibility represent a great challenge for dentistry of tissue regeneration because more cells sources to be used in clinical application have needed to specialistic team works and hospital structures to be realize, for example the bone marrow aspiration from the iliac crest and liposuction from extra-oral tissue is not an easy operation for dentists for dental license and the dental specialization. In contrast, orofacial bone marrow, periosteum, salivary glands and dental tissues are accessible stem cell sources for dentists; however, the isolation of stem

cells from these locations may still not be convenient because it requires surgical procedures for tooth or pulp extraction with vitality loss or an organ loss.

And, when even if impacted wisdom teeth could be a cell source, not all adults require the extraction of the third molar teeth. Moreover, only small quantities of stem cells, during surgical procedure actually can be difficult to isolate, purify and expand homogeneously. Growing evidence has demonstrated that the adult MSCs ES/iPS can be used for the autologous tailor-made cell-based in vitro oral tissue regeneration. Numerous in vitro researchers have demonstrated MSCs reliability in bone regeneration and initial encouraging results was observed in vivo dogs and murine animal model. Moreover, the actual evidence-based literature performed only BM-MSCs while too few are the studies related to other intra/extra-oral MSCs sources. The immuno-compromised animal model do not permitted to know the normal host response to the MSCs and the immuno-modulatory properties should be investigated in vivo to transplanted cells with or without biomaterials. Furthermore, stem cells characterized studies should be needed to identify the success or the failure factors for stem cell-based bone and tissue development and regeneration. However today it was needed to find an in vivo human stable experimental model to provide clinical predictable results. Moreover, the gingiva, seems to be a promising source of adult stem cells how future clinical source for dentists because rich of MSCs, easily obtainable during chair procedure with minimal discomfort from patients, and easily expanded [8, 19, 100–191] and iPS cells [102] in dentistry. Further studies are necessary to determine the regenerative abilities of gingiva-derived stem cells in oral tissues regeneration. Finally more researches are needed to define the ideal stem cells characteristics for oral tissue regeneration, to identify the specific success factors and the proper procedure to obtain and use the stem cell sources, to investigate the host immuno-responses, to reduce or neutralize the MScs transplant complications related to therapeutic effects such as angiogenesis, anti-inflammation and antiapoptosis [192], and to determinate the evidence-based practices to educate dentists and patients regarding the use of stem cells in autologous tissue repair and regenerative therapies in dentistry.

6.7 Conclusion

The stem cells self-renewal and differentiation capability is an interesting aspect of the dental tissue regeneration in combination with the biomaterial scaffolds and growth factors, to be able to get safe and predictable clinical strategies not only to repair a tissue lost to injury, cancer or oral pathology, but to made a new organ: the "bioengineering tooth". In particular, during last years, more attention was directed to the defects of hard tissue and especially to the bone defects. Infact, after tooth loss, usually the result is an horizontal and vertical bone loss [157], which limits the effectiveness of dental implant treatments and the related prosthodontic rehabilitations [158]. Therefore, the stem-cell-based regenerative technology was studied and is actually considered, a new frontier in dentistry to prevent or to treat the bone

deficits [159]. The most current research on mesenchymal stem cells are aimed at clarifying the bone regeneration and preservation of the periodontal sockets, moving from therapies based on osteoconductive scaffolds and growth factors to new therapies based on osteoinductive and osteogenic stem cells with or without bioactive materials. The stem cell technology for regenerative therapies is already available, and the MSCs already have been introduced in the clinical case studies for alveolar bone augmentation, however relatively little is known about their in vivo biology. In addition, it is crucial to verify the stem cells regenerative properties, to the possible negative effects of the transplanted cells on the host immune system, the long time work request, their high costs and unpredictable results [19, 51, 55–160]. However, the clinical evidences have showed that bone augmentation of the severely atrophic alveolar ridge, particularly vertical bone augmentation during sinus-lift, GTR or GBR procedures, cannot be easily accomplished through only autologous cancellous bone, biomaterials and therapies based on growth factors for the tipical not osteoinductive characteristics of the materials, for the osteoclasts action and immune response against the biomaterials that induce an unavoidable bone resorption. The difficulty in harvesting, the limited intraoral providing, associated with donor site morbidity observed for biomaterials and autologous grafts, have encouraged the development of alternative therapies based on stem cells transplantation into small or large defect sites could be able to respond to signaling molecules generated in the damaged microenvironment to regenerate the bone tissue. Therefore, the mesenchymal stem cells need again other researches to clarify the procedures and the specific approaches to represent a promising future strategy to achieve the regeneration of large alveolar bone defects, particularly to provide stable bone formation and to favorite the dental implant osseointegration treatments. Pre-clinical studies have showed in animal model interesting advances identifying feasible strategies to regenerate bone, periodontal ligament, salivary glands, mandible condyle, tongue and teeth [193–197]. These research advances could be used in future human clinical trials for tooth regeneration and replacement lost teeth, more difficult to achieve due to the structural complexity of the studies and for the variables presents in humans [161]. Swine model pre-clinical researches [161] demonstrated that a root/periodontal complex constructed using periodontal ligament (PDLSCs), apical papilla (SCAP), dental pulp (DPSCs) stem cells and different reasorbable/not reasorbable scaffolds, were capable to treat damaged crown or root section to provide normal tooth function with a new artificial approach [116]. The final target of most recent studies is to develop fully functioning bioengineered teeth that can replace lost teeth [162] using many different types of stem cells from mice [163], rats [164] and pigs [165]. In animal model, the head and neck mesenchymal stem cell transplantation is utilized to regenerate structures and function of salivary glands after surgery, radiotherapy and oncology [166]. In other pre-clinical model it was possible to observe the temporomandibular joint disc or condyle (condylar osteochondral defect) repair after arthritis and trauma. In a rat model tongue tissue loss from surgical resection represent a critical challenge for the complex muscle fibers, mucosa with taste buds, nervous structure and speech, swallowing and airway functions [167, 168]. The host local immune responses against stem cells and

grafting materials are highly relevant in tissue regenerative medicine, and the complete understanding of biological processes will be crucial to design new and more effective clinical strategies for oral tissue and organ regeneration. Actually, stem cells seem to present a promising strategy to achieve only the bone regeneration in small defects, particularly to provide stable and accelerated bone formation as well as enhanced osseointegration in dental implant treatments. For the small number of the human clinical studies, the small number of patients included and the traditional scaffolds used in the researchers, it is also necessary more time to establish definitive appropriate protocols for stem/osteoprogenitor cell preparation and new generation carrier scaffolds. In vivo bone regeneration clinical approaches to stem-cell-based the bone augmentation is structured in a tissue engineering approach and in a chair-side cellular grafting approach. In both approaches, bone marrow-derived MSCs (BMSCs) from the iliac crest are the most commonly used because the scientist have been shown to possess superior osteogenic ability [169, 170]. In addition, periosteum [112, 113], adipose tissue [136, 171] and dental tissue—derived stem/osteoprogenitor MSCs [172] have been applied to bone regeneration in periodontal bone loss [17], alveolar cleft osteoplasty [95], and maxillary sinus floor elevation [96–99]. The chair side cellular approach used patient-derived freshly processed bone marrow mesenchymal stem/stromal cells (MSCs), hematopoietic stem cells (HSCs), and angiogenic cells, mixed with scaffold, growth factors, and platelet-rich plasma (PRP). In the tissue engineering approach for bone augmentation, the MSCs after autologous bone marrow aspiration from the ilium or mandible, are isolated, in vitro expanded and cultured with osteogenic factors and solid or gel scaffolds to generate an osteogenic construct (tissue-engineered bone) or cell sheets as a grafting material [173]. However for many research efforts in recent years has made the clinical outcomes of ectopic bone formation by transplanted stem cells in humans [97] is not always predictive probably related to the use in the pre-clinical studies of immune-compromised animal models specially in mice. These results suggest that the T and B cells immune system, could play a key role in the success of MSC-mediated bone and other tissue regeneration. Therefore, through more attention for immunitary theme should be evaluated in future studies. It is also necessary a large and deep knowledge of new growth factors and new biomaterials resorbable or not resorbable to understand their function, turnover, relationship, immuno-response, their specific role with the different oral tissues and real long-term benefits to patients, to create a solid stem-cell-based strategies for oral tissue engineering [112, 113, 136, 169–173]. The MSCs new clinical application in the tooth regeneration scientific researches will be the identification of an appropriate autologous stem cell source in humans. In this regard, iPS cells may be an appropriate cell source because they can be differentiated to dental epithelial and mesenchymal cells [146, 174] and can be prepared from the patients' own somatic cells. Another clinical challenge of stem cells regeneration it will be the vascular and nervous pulp complex regeneration area, where even today, there are no scientific evidences. Therefore, it will be necessary a new biomaterials and growth factors knowledge to create stem-cell-based strategies for oral tissue engineering. More basic, translational researches and clinical randomized controlled trials for

longer durations are necessary, should be performed to advance the field using scientific result that can ultimately offer long-term benefits to patients. The growing demand of oral health in all over the world, gives a further thrust to research and encourages to invest new resources. Moreover, the introduction of digital 3D technologies and genomics may play a synergistic role to understand the physiological mechanisms of different oral tissues and the tooth organogenesis, structuring effective and predictable strategies to use in the "future bioengineering dentistry".

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Chapter 7
Oral-Derived Mesenchymal Stem Cells Used to Treat the Periodontal Diseases: State of the Art and New Insight

Zamira Kalemaj and Felice Roberto Grassi

7.1 Introduction

Periodontal disease is a current public health problem compromising heavily patients' quality of life. Scientific evidence on treatment of periodontal disease is associated with a high degree of variability in reported outcomes and potential efficacy. Thus, regeneration of periodontal tissues through safe and efficient treatment protocols remains an important challenge. Moreover, conventional therapies result habitually in repair rather than regeneration of deteriorated tissues. Recent development in tissue engineering and regenerative medicine has paved the path for new treatment approaches. Mesenchymal stem cells are an outstanding candidate for tissue regeneration. They are the key element of combined tissue engineering therapies for periodontal regeneration. Related scientific evidence has highlighted exciting potential therapeutic utilization accompanied by numerous limitations and future challenges. The present chapter has been assembled considering these aspects and with the hope of providing investigators with solid bases for the state of art and potential future directions of periodontal regenerative therapies.

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7.2 Periodontium and Periodontal Disease

7.2.1 Periodontium

The periodontium is a complex organ composed by different specialized tissues that supports and maintains teeth in the jaw bones. It originates from the dental follicle which is a fibrocellular layer enveloping the dental papilla and enamel organ at the bell stage of dental development [1, 2]. Formation of periodontium includes multiple differentiation and cells migration processes that take place prior and during tooth eruption [1–3].

The mature structure of the periodontium consists of four different tissues: gingiva, periodontal ligament, cementum and alveolar bone. Understanding the structure of the periodontium is of outmost importance and has relevant implications in implementing different methods of periodontal healing and regeneration. Detailed description of periodontal architecture is provided elsewhere [4, 5]. Herein we present a short description of periodontium morphology which will assist readers in better understanding the following sections.

7.2.1.1 Gingiva

Gingiva is composed by a central nucleus of connective tissue covered by a multilayer epithelial stratum. It bounds tightly to the bone and holds against the tooth through the gingival fibres which convey to the gingiva sufficient resistance to withstand the forces of mastication without distorting or breaking. The part of gingiva facing the tooth, denominated also dentogingival junction is covered by epithelium that can be divided into three functional compartments: gingival, sulcular and junctional epithelium. The underlying connective tissues can be categorized into superficial and deep compartments.

The junctional epithelium forms a collar around the cervical portion of the tooth, thus, has an essential role in sealing off periodontal tissues from the oral environment and preserving integrity of underlying periodontium structure. The cell layer in contact with tooth surface attaches by means of a structural complex called the *epithelial attachment*. The marginal part of the junctional collar is detached from the tooth surface and constitutes the bottom of the gingival sulcus.

The connective gingival tissue adjacent to the junctional epithelium is highly vascularized and rich in inflammatory cells that migrate across the junctional epithelium and gingival sulcus. The structure and physiology of the connective tissue that supports junctional epithelium is different from that of the connective tissues supporting sulcular epithelium or gingival. This difference holds important implications for understanding the progression of periodontal disease and different attempts of periodontal regeneration [4–6].

7.2.1.2 Periodontal Ligament

The periodontal ligament is a soft highly specialized connective tissue linking the tooth root surface covered by cementum to the surrounding alveolar bone. Due to the dense mass of collagen fibres, elastic fibres, rich vascular bundle and extracellular matrix, the periodontal ligament provides high absorption of occlusal and masticatory forces. It exhibits a high concentration of proprioceptive fibres responsible for mechano-responsive actions under mechanical loading [7].

Apart from the cells typically found in the connective tissue such as fibroblasts, macrophages, lymphocytes and mast cells, the cellular population of the priodontium comprises cells responsible for the turnover of surrounding tissues such as osteoblasts, osteoclast, cementoblasts and cementoclasts. Furthermore, epithelial cells, deriving from the root sheath of Hertwig, known as epithelial cell rests of Malassez can be found imbedded in the periodontium [7, 8].

Progenitor cells present in the periodontal ligament can be epithelial stem cells, generally located in the proximities of tooth apex, or mesenchymal stem cells mostly distributed around the perivascular space. Denomination and identification criteria of periodontal stem cells are to-date a controversial topic and will be discussed further in this chapter. Under different signals, progenitor cells in the periodontal ligaments can differentiate into osteoblasts, cementoblasts fibroblast or other connective tissue cells, contributing to maintenance and repair of all periodontal structure [4, 9].

7.2.1.3 Cementum

Cementum is a specialized hard connective tissue that covers the root of the tooth and anchors the final terminations of periodontal ligament fibres. It extends from the cervical part of the tooth, at the cemento-enamel junction, to the apex. The cervical portion is generally an acellular structure which is known as primary cementum and is the main site of anchorage for periodontal fibers. The apical part, termed secondary cementum is rich of cementoblasts and cementocytes entrapped in lacunae formed by their own extracellular secreted matrix. The biochemical composition and structure of secondary cementum is similar to bone structure. It contains 45–50% of inorganic elements, consisting of calcium phosphate under the form of hydroxyapatite crystals and 50–55% of organic substances, primary collagen fibres, proteins and polysaccharides [9, 10].

7.2.1.4 Alveolar Bone

The alveolar bone is the part of jaw bones that contains the alveoli (teeth sockets) where teeth are attached. It is connected to the basal bone of the jaw and consists of outer (buccal, lingual, palatal plates), central (spongiosa) and inner (bundle bone) components.

In the bundle bone are imbedded the extremities of intrinsic fibres (Sharpey's fibres) that connect teeth to alveolar bone. Being in close contact with teeth, the bundle bone exhibits a high remodelling rate, responding so to constant tooth movements during eruption, mastication or other functional displacements [11].

7.2.2 Periodontal Disease

7.2.2.1 Classification, Aetiology and Pathogenesis

Periodontal diseases comprise a variety of conditions affecting the health of periodontium. According to the European Federation of Periodontology in collaboration with American Academy of Periodontology, periodontal diseases can be broadly classified into: *gingival diseases, chronic periodontitis, aggressive periodontitis, periodontitis as manifestation of periodontal disease and necrotising periodontal diseases* [12, 13].

Apart from *gingival disease* which is localised only in the gingiva and results in reversible inflammatory reaction, all other forms of periodontal disease affect both gingiva and other underlying periodontal tissues.

Chronic periodontitis is characterized by irreversible damage in supporting alveolar bone and connective tissue attachment. Clinical patterns of disease activity range from a continuous progression of destructive phenomena over a long period to an episodic burst model that occurs in a short period [14].

Studies on etiopathogenesis of periodontal disease recognize dental plaque bacteria as key factor on initiation and progression of the inflammatory process that affects periodontal tissues. Other important factors seem to be related to genetic predispositions, host-defence mechanisms and interaction of risk factors such as smoking and obesity [14–17]. Our group of research has extensively investigated immunological mechanisms and pathways that underlie periodontal disease [18–21].

Identification of patients at risk for developing chronic periodontitis prior to disease onset remains the big challenge of the modern periodontal epidemiology research [22].

Independently from the clinical model, all chronic periodontal diseases seem to have a common physiopathological mechanism leading to periodontal tissue breakdown with tooth attachment loss.

When the integrity of the junctional epithelium is broken, the pathogens can access the underlying periodontal structures. The presence of bacteria triggers inflammatory response both directly and indirectly, by exacerbating the host's immune response. The local inflammation causes disintegration of underlying connective tissue which is responsible for the maintenance and development of junctional epithelium. The first structural changes during periodontitis consist of apical migration of junctional epithelium along the root surface, creating what is clinically know as gingival pocket. The increased free space between the periodontal structures and roots surface without proper seal from oral cavity can host more bacterial plaque, resulting in a vicious circle of increasing inflammation. Inflammatory cells

such as B-lympohocytes and T-lympocytes generate a pro-inflammatory mediator response. The normal balance of bone turnover is perturbed by the presence of bone resorption-associated biochemical mediators. Excessive bone resorption results in formation of bony periodontal pocket. Similarly, root surface resorption might occur, increasing in each case potential sites of bacterial colonisation [4, 23].

The cascade of inflammatory response that follows the penetration of pathogens beyond the junctional epithelial seal results in irreversible structural destruction which compromises heavily the integrity of the periodontium and consequently tooth stability and function. Every attempt in rebuilding the periodontium structure must restore each of the four components of periodontium.

Considering the high complexity of pathogenesis and potential interaction of numerous etiologic factors in chronic periodontitis, a thorough understanding of etiopathological mechanisms combined with an accurate diagnosis and identification of risk factors is of outmost importance for every treatment strategy.

7.2.2.2 Epidemiology

Epidemiologic data on periodontal status derived from the 2009 to 2010 analysis of the National Health and Nutrition Examination Survey (NHANES) reveals that 70% of the US adults aged 65 years or older have some form of periodontitis and that 86% show attachment loss higher or equal to 4 mm and 45% higher or equal to 6 mm [24]. Other authors report that more than 50% of adult population is affected by chronic periodontitis and severe forms of periodontitis are found in 11% of adults, making severe periodontitis the sixth most prevalent disease of mankind [25].

The World Health Organization published in 2010 a report revealing that among priority public health conditions, periodontal disease meets the criteria for consideration as a public health problem that requires action [26].

Individuals' oral health-related quality of life is severely impaired by periodontal disease [27]. Additionally, there is a mounting scientific evidence that associates periodontal disease with major systemic diseases, such as diabetes, atherosclerotic diseases, obesity and metabolic syndrome [28, 29]. Although the complex mechanisms of these associations are of no direct causality and still remain unclear, the rapidly increasing onset prevalence of systemic diseases such as diabetes [30], implies further challenges for management and treatment of chronic periodontitis.

7.3 Treatment of Periodontal Disease

7.3.1 Healing of Periodontal Wound

Healing of an wound with extensive tissue loss is generally accomplished by collagenous scar tissue which only repairs the defect but does not fully restore form and function of lost structure [31, 32]. This type of healing in a periodontal defect

consists of connective repairing tissue attached to debrided root surface and does not ensure an appropriate sealing and function of periodontium [33, 34].

A different pattern of healing following periodontal reconstructive therapy results in the formation of a long junctional epithelium which might seal periodontal components from oral pathogens but is not supported by basal connective tissue and is still considered a repairing process [35].

Contemporary periodontal treatment aims full regeneration, defined as the reproduction or reconstruction of a lost or injured part so that form and function of lost structures are restored [36].

7.3.2 Conventional Periodontal Therapy

It is scientifically proved and widely agreed that management of periodontal disease must start with a protocol of inflammation control which is indispensable for reducing the bacteria load and creating an appropriate environment for regenerative treatment. Control of inflammation is performed through mechanical scaling, curettage, open flap debridement and accurate domestic oral hygiene protocol. Once obtained an optimal control of inflammation through constant monitoring of periodontal inflammatory indexes, regeneration therapies can be performed [37, 38].

Although periodontium exhibits some intrinsic regenerative capacity, natural restitution ad integrum without intervention of external regenerative therapies cannot be expected [39].

From an historical prospective the first periodontal regenerative attempts consist of use of biomaterials such as bone grafts, bioactive materials and barrier membranes for guided tissue regeneration (GTR). The basic concept underlying the conventional periodontal regenerative therapy is to provide an appropriate environment in which cells can migrate and differentiate. Scientific evidence has proven that validity of conventional periodontal regeneration techniques is highly dependent on number of residual bony walls that provide mechanical support for bone substitutes and adequate blood supply [40].

7.3.2.1 Bone Grafts

The clinical validity and long-term performance of bone grafts has been long investigated in numerous pre-clinical and clinical trials [36, 40]. They are still an important component of the majority of bone tissue engineering techniques, providing the core scaffold element for regeneration.

Depending on their origin, bone grafts have been broadly classified into autogenous, allogenic, alloplastic and xenogenic.

Determination of appropriate structural features such as macro-, micro- and nano-geometry for bone substitutes is a fundamental requirement. Moreover,

capacity of fixation to surrounding tissues and ability of inducing tissue regeneration are two important aspects of scaffold performance, especially when considering that space maintenance and wound stability are key factors in demining success of regenerative therapy [36, 39, 41].

From a biochemical prospective they must provide an environment similar of natural extracellular matrix (ECM) that stimulates cells migration, differentiation and proliferation of periodontal cells, while preventing junctional epithelium cells down-growth.

As most of bone substitutes leak osteinductive attitude, they are mostly used in combined therapies, along with bioactive materials.

7.3.2.2 Bioactive Materials

The term "bioactive materials" is used for a large population of substances that can stimulate bone regeneration through different mechanisms. They are mainly represented by growth factors which stimulate and recruit stem cells in order to achieve regeneration. Other bioactive materials are also stem cells themselves and gene therapy agents which will be discussed further in this chapter.

The most representative growth factors used in tissue engineering of periodontal defects include platelet rich plasma (PRP), enamel matrix protein derivatives (EMD) and bone morphogenetic proteins (BMPs).

PRP consists of high concentration of autologous platelets suspended in a small volume of plasma and is often used in concomitant with bone grafts or GTR. Combination therapies are generally indicated as more satisfying compared to PRP alone. Nonetheless, it is scientifically recognized that PRP confers beneficial effects on the clinical and radiographic outcomes for regeneration of infrabony defects [42]. The effects of PRP are attributed to the angiogenic, mitogenic and proliferative abilities of growth factors such as platelet-derived growth factors (PDGF), transforming growth factor (TGF) and vascular endothelial growth factor [43, 44]. PRP does not exhibit osteinductive capacity itself but influences greatly the mitogenic capacity of osteoblasts and accelerates bone formation.

EMD has been extensively used in periodontal regeneration. Almost 90% of EMD is composed of amelogenins and the rest by enamelins, sheathelin and two enzymes corresponding to MMP-20 and EMSP1. Apart from regulation and maturation of hydroxyapatite crystallites in the enamel, the EMD are temporarily deposited onto the dentinal root surface and induce formation of cellular cementum [45]. EMD plays also an important role in stimulating growth and differentiation of mesenchymal cells and stem cells [46]. Clinical studies indicate that results of infrabony defects treated with EMD are similar to those of GTR therapy [36, 47]. EMD used in combination therapies seem to perform better than EMD alone [48].

BMPs form a unique group of proteins within the transforming growth factor superfamily of genes and play an essential role in bone formation induction and maintenance. BMPs have been largely investigated in sinus flor augmentation therapies and the results indicate consistent improvement in bone gain and alveolar ridge augmentation [49]. Effect of BMPs on periodontal regeneration has been evaluated in several animal models [50, 51] and clinical trials [52]. Results are promising, with relevant gain of clinical attachment and favourable bone healing [53].

7.3.2.3 Guided Tissue Regeneration

The GTR has been extensively investigated and documented over the past three decades. The technique is based on the Melcher hypothesis of wound healing and involves utilisation of a barrier membrane to isolate the healing periodontal zone from colonization of epithelium and gingival connective tissue. Barrier membranes can be both resorbable (collagen membranes, oxidized cellulose mesh, acellular dermal allografts, etc) and non-resorbable (polylactic acid derivatives, combination of polylactic, olyglycolic acid derivatives, etc), with no relevant differences in terms of clinical and histological periodontal tissue regeneration [54, 55]. Based on their origin they can also be classified into autogenous, allogenic, alloplastic and xenogenic.

Barrier membranes have shown to create an appropriate environment for periodontal tissue guided regeneration but do not directly bond to bone and most of them do not exhibit osteoinductive capacity. Hence, combined techniques that involve use of bone substitutes, collagen-based grafts or bioactive mediators have been experimented under the general denomination of guided bone regeneration (GBR) [56]. The graft materials used for GBR are mainly calcium phosphate (CaP)-based materials hydroxyapatite, biphasic calcium phosphate etc) [57, 58]. A more recent attempt for adding osteoinductive capacity to barrier membranes includes incorporation of substances such as antibiotics of growth factors that are purported to improve membrane bioactivity and facilitate early cell differentiation [59]. Treatment of an infrabony defect with bone graft and resorbable membranes as well as radiographic controls are showed in Figs. 7.1 and 7.2.

Scientific evidence on bone grafts, bioactive materials, membranes and combination therapies used for periodontal regeneration has been thoroughly and systematically reviewed in the last years [60–62]. Numerical data has been extracted and elaborated into network meta-analysis with the aim of providing a ranking of treatments and respective probability for being the best treatment. Data summary indicate that to-date, combination therapies may perform slightly better than single therapies but additional benefit seem to be small. As suggested by authors, because of the low-to-moderate general quality of the original studies, and the wide range of variation in protocol treatment, the overall quality of evidence must be considered with caution.

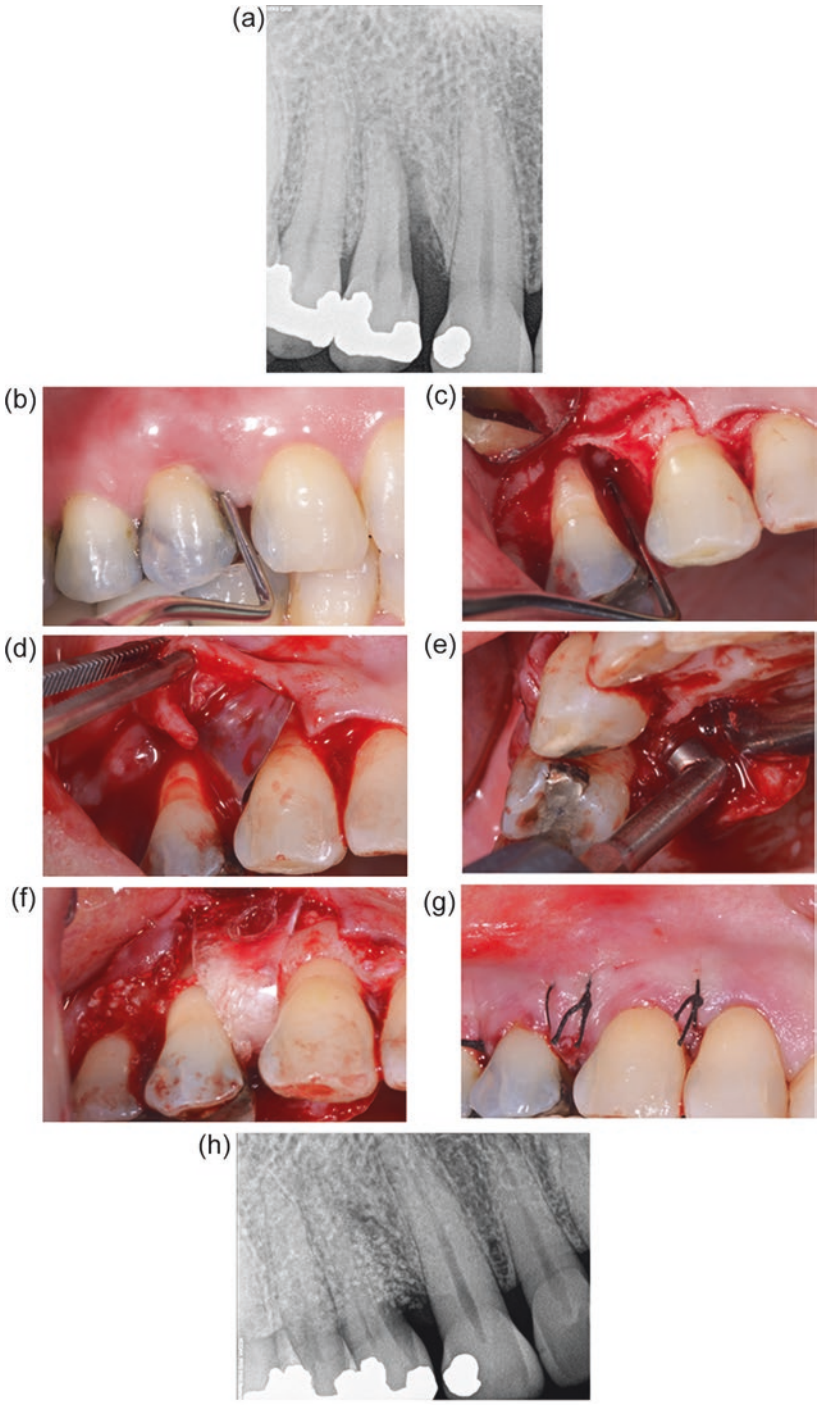

Fig. 7.1 (**a**) Initial radiographic situation of infrabony defect on maxillary premolar; (**b**) periodontal status on probing; (**c**) clinical view of infrabony defect after granular tissue removal; (**d**) resorbable membrane in situ; (**e**) resorbable membrane pin fixation; (**f**) insertion of bone graft IngeniOS B-TCP 0.025–1 mm particles and non-crystalline poly-D, L-lactic acid (PDLLA) membrane; (**g**) suturing; (**h**) post-treatment radiographic check

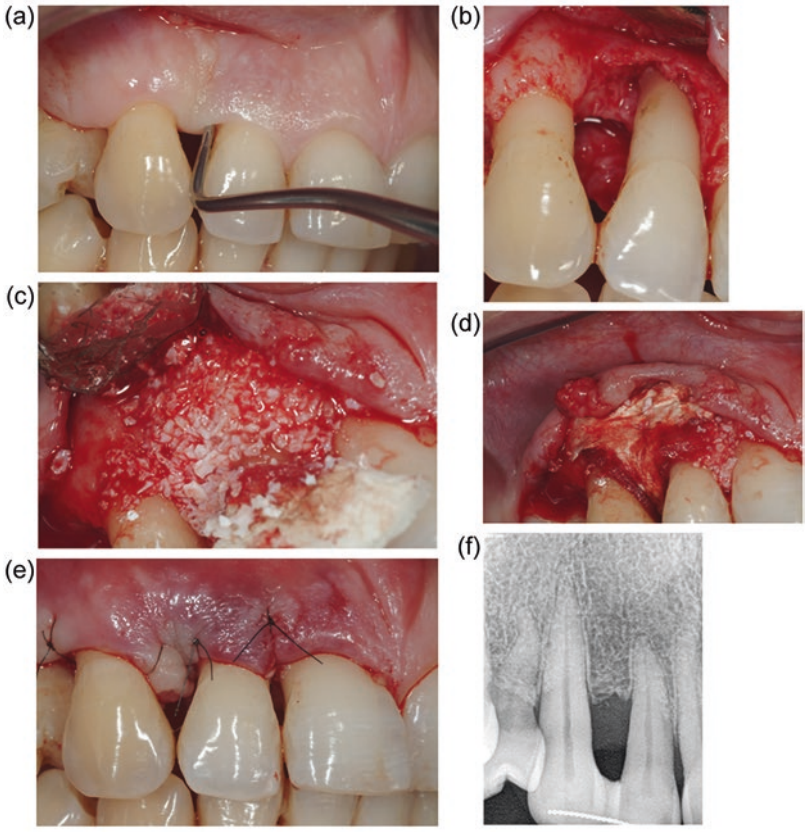

Fig. 7.2 (**a**) Clinical evaluation of infrabony defect; (**b**) Intrasurgical situation; (**c**) bone graft insertion; (**d**) resorbable membrane adaptation; (**e**) suturation; (**f**) radiographic follow-up at 8 years

7.3.3 New Frontiers in Periodontal Therapy

Over the last two decades the periodontal therapeutic strategies are based on ma more complex approach that belongs to the tissue engineering and regenerative medicine. This is a translational research area that includes a broad range of disciplines such as material sciences, biochemistry, technological manufacturing, nanotechnology and stem cells research [63]. The main focus of such multidisciplinary approach is to translate laboratory findings in tissue engineering into everyday clinical practice. Over the last years, research on nanotechnology sciences and nanotechnology-based applications has particularly emerged demonstrating promising results in therapeutic strategies of bone regeneration [64].

One important challenge of bone tissue engineering is to overcome the limitation of conventional periodontal therapies, which is their successful applicability mainly in infrabony defects, meaning vertical localized pattern of bone loss. Horizontal extended bone defects remain difficult to manage. To this end, bone tissue engineering has focused especially on construction of scaffolds which perform efficiently in reconstruction of large periodontal destruction. Conventional bone grafting materials serve the role of supporting matrix but they cannot adapt completely following a tailored desired shape. Thus, under mechanical forces they lose the initial volume and consequently one part of the final desired support [65, 66].

Tissue engineering based on three-dimensional (3D) customized printed scaffolds opens new frontiers for periodontal restoration. Scaffolds are produced based on a computer tomography scan of bone defect area [67]. Park and co-workers describe in a recent article the flow work of 3D customized scaffolds, from data imaging to oral adaptation [68].

During printing a liquid binder solution is applied onto a powder bed and can simultaneously incorporate different cell types such as proteins, DNA plasmids and living cells can be inserted. Scaffolds can be either cellular or acellular upon implantation. The cellular model incorporates osteoprogenitor cells seeding into the scaffold or encapsulated by hydrogel polymer matrix. Acellular scaffolds are free of cells but promote the recruitment of local progenitor cells. The template should demonstrate mechanical strength comparable to native bone, especially in load-bearing areas, until new tissue is formed. A scaffold should also be biocompatible and biodegradable [67].

3D printed scaffolds seem to perform successfully in large bone defects and post-extraction socket regeneration. A recent randomized controlled clinical study conducted by Goh and co-workers reported that prefabricated 3D polycaprolactone (PCL) scaffolds inserted in post-extraction sockets provide better maintenance of alveolar bone, compared to empty sockets [69].

In terms of periodontal regeneration, 3D printed scaffolds have been recently tested in animal models, showing promising results by inducing “cell homing” regeneration [70]. Multi-phasic PCL scaffolds have also been tested, allowing not only for the regeneration of periodontal fibres but also alveolar bone and cementum-like tissue [71]. Apart from PCL or other biodegradable synthetic polymers (PLA, PGA, PLGA) other bioactive materials applied into scaffolds are bioceramics [72].

When periodontal defect is located in a non-load-bearing area, better outcomes seem to be expected with the incorporation of collagen, resulting in a bioceramic/collagen mix which is the closest replicate of extracellular matrix in native bone [73].

A 3D printed scaffold has been recently used in a periodontal defect by Rasperini et al. with positive clinical findings at 6 months of follow-up [74]. Regardless, to-date scientific evidence from clinical trials is still weak and results are less promising than outcomes on animal models [67]. Consequently, there is still much work ahead to apply findings of preclinical studies into clinical everyday practice.

The most challenging development of tissue 3D printing is full organ printing or biomedical application of rapid prototyping which has the potential of surpassing traditional solid scaffold-based tissue engineering. The ultimate goal of such

approach is to fabricate 3D vascularized and fully functional human living organs, for clinical implantation [75]. Organ printing paths the way toward personalized medicine which means also customized organ regeneration. While exciting advances already exist in general medicine [76], few attempts are made for printing of oral organs [71]. Extensive research and clinical testing will be needed prior to clinical application of such revolutionary application.

The recent big steps of tissue engineering are being accompanied by promising emerging technologies that focus on delivering of bioactive substances into targeted tissues. To this end, gene therapy represents an interesting approach. It is based on a controlled delivery of bioactive proteins from genetically modified cells [39]. The secretory capacity can be conveyed to cells through viral or nonviral vectors. The produced growth factors of other bioactive proteins are delivered to the targeted tissue directly or incorporated into scaffolds or 3D printed tissues. Gene therapy has actually been largely investigated in laboratory studies [77, 78] and few in vivo studies [79]. Findings indicate good capacity of promoting periodontal ligament formation and bone repair. Further research will be needed in the field of gene therapy before translating laboratory findings into clinical intervention.

7.4 Stem Cells and Periodontal Disease

7.4.1 Introduction

Stem cells are defined as clonogenic cells which are capable of both self-renewal and multi-lineage differentiation. Through self-renewal they can give rise to other stem cells whereas through multi-lineage differentiation they differentiate into a variety of cells specialized on specific functions. They constitute an emerging line of tissue engineering and translational regenerative medicine.

Stem cells that are naturally present in the human body can be of two major types: embryonic stem cells and adult stem cells. They differ in terms of origin, location and differentiation capacity.

Embryonic stem cells are pluripotent stem cells that have the capacity of differentiating into all cell types of the body, whereas adult stem cells are multipotent stem cells that can differentiate into a limited number of cell types. Interaction among embryonic stem cells initiate developmental processes of all tissues, resulting in highly specialized organs. In a matured organism, embryonic stem cells disappear, whereas adult stem cells persist in the developed tissue to sustain the homeostasis and repair injuries.

Notably, studies on embryonic pluripotent stem cells raise ethical, practical and regulatory issues. Therefore, other sources of progenitor cells such as adult stem cells are the focus of translational medicine in assisting tissue engineering and regenerative therapies.

Adult stem cells are also called postnatal stem cells or somatic stem cells. Their origin is not clearly defined in the scientific literature. Consequently, even the denomination and classification of different types of adult stem cells is not fully standardized and remains a source of scientific debate [80].

Endogenous human adult stem cells exist naturally in different tissues of human body. Nevertheless, their low concentration implies a poor capacity of naturally regenerating degraded tissues. Stem cells-based regenerative medicine aims strengthening the therapeutic effect of stem cells through prior ex vivo expansion. Adult human stem cells are cultivated in different mediums including animal-derived mediums and human blood plasma [81–83]. The last approach is customized and potentially reduces the exposure to animal-borne pathogens.

7.4.1.1 Mesenchymal Stem Cells

Adult stem cells which are found in mesenchymal human tissues are collectively referred to as mesenchymal stem cells (MSCs) [84]. It is commonly considered that MSCs originate from mesoderm, however an emerging line of evidence supports the hypothesis that they derive from tissue-resident neural crest [80]. They can give rise to mesenchymal cells such as adipocytes, osteoblast, chondrocytes, therefore residing in many adult tissues, including adipose tissue, skin and dental tissues. Interestingly, MSCs can also differentiate into nonmesenchymal cell lineages such as endothelial cells, keratinocytes or neuronal cells.

The International Society for Cellular Therapy proposed in 2006 the minimal criteria to define MSCs. According to these criteria, independently from the tissue where MSCs are isolated, they must be adherent to tissue-culture-treated plastic in culture conditions. They must also be able to differentiate into mesenchymal cells in vitro and must specific surface markers such as CD105, CD73, CD90, CD271 and MSC antigen-1 [83].

MSCs can be delivered directly or through scaffolds into the deteriorated tissue. Because of their mesenchymal lineage plasticity and sensitivity to local hormonal stimulations, they may induce immunomodulation, neovascularization or tissue regeneration [85, 86].

Pittenger and co-workers firs isolated MSCs from bone marrow of iliac crest and demonstrated their multipotency after culture expansion and differentiation into osteogenic, adipogenic and chondrogenic cell lineages [87]. Since then, MSCs have been identified and isolated from multiple adult tissues, with special attention to bone marrow and orofacial tissues which seem to be a rich source of MSCs.

To date, the stem cells most commonly used and better documented for periodontal bone regeneration are bone marrow-derived mesenchymal stem cells (BMMSCs). Their principal source in dental patients is the iliac crest [84]. BMMSCs modulate lymphocyte activity by being well accepted by immune system in allogenic settings. This immunologic consideration has increased the interest and scientific investigation on their regenerative potential. Problems related to bone BMMSCs

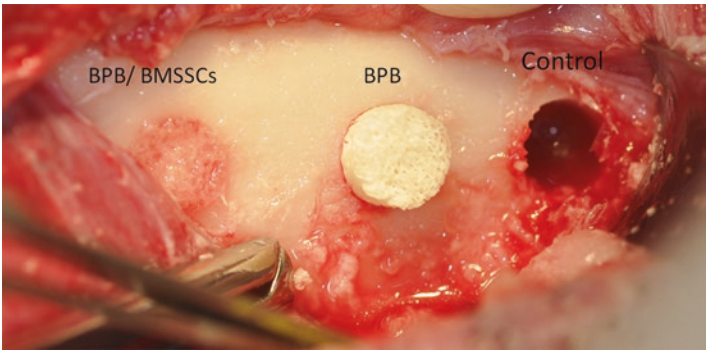

Fig. 7.3 Three critical-size circular defects. Clinical situation during surgery

harvesting consist of high invasiveness for the donor and also of potential direct correlation to patient age, which seem to make MSCs less effective in bone formation [88].

Recently our group of study has experimented a protocol for stem cells differentiation toward osteoblastic phenotype on an animal model [89]. Bone marrow was harvested from tibiae of adult mini-pigs and BMMSCs were seeded on a bone porcine block (BPB). Critical-size defects on mandible of each mini-pig were implanted with the bone porcine blocks impregnated with BMMSCs (BPB/BMMSCs) and other defects were implanted with bone porcine blocks solely (BPB) or left to heal naturally (control) (Fig. 7.3). Histomorphometric analysis performed 3 months later indicated higher concentration of lamellar bone and lower concentration of marrow spaces in the BPB/BMMSCs group as compared to BPB group or control group. This data demonstrate that BPB used as scaffold to induce bone regeneration might benefit from addition of BMMSCs.

Other sources of BMMSCs are found into orofacial bones. Autologous grafts from jaw bones have been successfully used in various clinical observations indicating better performance than grafts of endochondral origin such as iliac crest. Differences in origin between different types of bone may result in functional differences between BMMSCs. Studies on animal models report higher differentiation capacity and lower adipogenic potential for orofacial BMMSCs which are positive indicators of their regenerative potential [90, 91]. However, the quantity of BMMSCs harvested from orofacial bones is inferior compared to that of iliac crest bone and still, invasiveness remains an important issue to consider during intervention. Furthermore, there is an important concern related to the role of MSCs in sarcomagenesis observed especially in animal studies. To date, scientific evidence is still weak and further studies investigating MSCs transformation and sarcomagenesis induction would provide essential insight for future directions of MSCs research [92].

7.4.1.2 Epithelial Stem Cells

Adult stem cells can also be progenitors of epithelial tissues which gives them the name of epithelial stem cells (ESCs). They are mainly localized in the basal layer of epithelial tissues such as skin and mucosa and contribute to their physiological renewal and replacement of damaged or dead superficial layers throughout of life [93]. In vitro cultivation of ESCs show high clonogenicity and proliferative capacity. Under cultivation conditions they are also able to form stratified cell sheets. Based on this, identification of ESCs is performed through their self-renewing capacity. Numerous studies have confirmed that transplanted cell sheets of oral ESCs are able to repair epithelial tissues in the body [94, 95]. However, other studies have investigated carcinogenicity of ESCs and have reported potential development of multilineage tumors, inviting investigators to thoroughly test tumorigenic properties of ESCs prior to transplantation [96, 97].

7.4.2 Oral-Derived Mesenchymal Stem Cells and Periodontal Regeneration

Oral MSCs can be found both in dental tissues and external to dental tissues. The MSCs in dental tissues include dental pulp stem cells (DPSC) and stem cells from exfoliated deciduous teeth (SHED). MSCs can also be found in immature dental structures such as dental follicle (DFSCs) and apical papilla (SCAP). MSCs external to dental tissues include periodontal ligament stem cells (PDLSC), gingiva stem cells (GMSCs) and adipose tissue stem cells (ASCs). According to their specific origin, oral-derived MSCs exhibit different behaviour when transplanted subcutaneously into immunodeficient animals or in specific culture environments [93]. Thus, their origin is an important element in determining the therapeutic choice and clinical validity.

7.4.2.1 Dental Tissue-Derived MSCs

DPSCs and SHED demonstrate the capacity of differentiating into dental tissues. Notably, these cells have the specific ability to regenerate the dentin-pulp complex but also neuronal tissues and cartilage.

The first human DPSCs were harvested from dental pulp of impacted third molars [98]. Six weeks after transplantation into immunocompromised rodents, investigators found a dentin-pulp-like complex. Since then various animal studies have been conducted, experimenting different protocols of isolation, expansion and maintenance for clinical safety and effectiveness [99]. Results indicate important potential of DPSCs in differentiating into dentin-pulp-like tissues. Moreover, important progress has been done in improving angiogenesis inside the limited root canal

space, providing essential blood supply for DPSCs [100]. Nevertheless, to date scientific background of DPSCs utilized for dentin-pulp complex regeneration comes from animal studies which do not directly translate into clinical application. Several scientific groups report ongoing clinical trials, which indicate that potential clinical use of DPSCs for dental tissue regeneration might be a close prospective [93].

Apart from the primary utility of DPSCs in dental tissues regeneration, their interesting osteoblastic phenotype expression has also been investigated. We analysed the expression of typical osteoblast markers such as alkaline phosphatase, collagen type I, osetopontin and osteocalcin in DPSCs cultured in osteogenic medium [101, 102]. Our data indicate that specific stimulation and differentiation of DPSCs result in expression of osteoblastic phenotype and therefore in potential source for periodontal bone regeneration. Other similar investigations have reported that DPSCs successfully differentiate towards osteoblasts promoting osteogenesis and bone tissue engineering [103, 104].

Interestingly SHED can also induce formation of bone-like matrix being a suitable source for periodontal bone regeneration [105]. Hypothetically this potential can be explained by their capacity in forming bone during deciduous tooth permutation.

7.4.2.2 Immature Tissue-Derived MSCs

The dental follicle is an immature structure that contains the developing tooth and gives origin to the periodontal ligament. There is abundant scientific evidence that DFSCs located in impacted or not erupted teeth have the ability to differentiate into different lineages, including osteoblasts and odontoblasts [106].

Our research team recently reported a study on osteogenic differentiation of DFSCs and adhesion extracellular matrix proteins [107]. We characterised DFSCs for expression of adhesion proteins and examined their pattern during osteogenic differentiation. The observed expression pattern reflected the mesenchymal origin of DFSCs and confirmed their osteoblast-like features. In a previous study we had already investigated the differentiation capacity of DFSCs analysing osteoblastic markers [108]. We demonstrated that DFSCs differentiated into osteoblast-like cells, expressing typical osteoblastic markers such as alkaline phosphatase and collagen I. Extensive laboratory research data seem to support the fact that DFSCs are a precious source of periodontal regeneration [109]. Their good accessibility through third molar bud extraction is an additive value to their potential clinical usage. Figure 7.4 demonstrates the radiologic imaging of third molar bud and its clinical extraction.

The other interesting source of stem cells in immature dental tissues comes from the apical part of the root of developing teeth (apical papilla). SCAP demonstrate good proliferation and regeneration of dentin matrix and dentin-pulp complex [110]. Some authors have argued that MSCs from immature tissues might provide a better source for differentiation and self-renewal than MSCs form mature oral tissues. Still, further research must be conducted especially in terms of appropriate identification of immature tissue-derived MSCs, culture medium and potential side effects on human tissues.

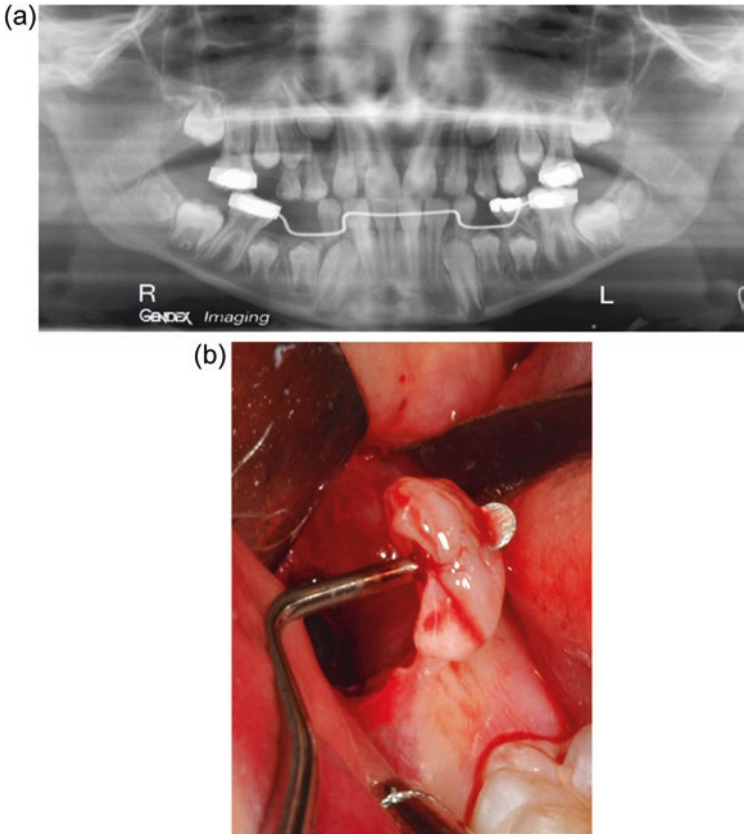

Fig. 7.4 (**a**) Radiographic estimation of third molar bud stage; (**b**) third molar bud extracted

7.4.2.3 Non Dental Tissue-Derived MSCs

Endogenous MSCs isolated in the periodontal ligament (PDLSCs) are logically the first choice for MSCs-based periodontal regeneration. Seo et al. after isolation and expansion of PDLSCs reported these cells to have similar features to BMMSCs [111]. Other studies confirmed the steaminess nature of PDLSCs by observing expression of markers such as CD 146, CD 44 and scleraxis. Numerous in vitro and in vivo animal studies have been carried out for assessing the regenerative capacity of PDLSCs. Results reveal important potential of PDLSCs in generating cementum and periodontal ligament-like structures similar to natural periodontium [112–114]. Moreover, allogeneic transplantation of PDLSCs under the form of cell sheets were well accepted by the host organism of a miniature swine resulting in a periodontal regeneration similar to that induced by autologous transplant [115]. Similarly, Tsumanuma et al. reported that allogeneic PDLSCs sheet promote periodontal tissue regeneration without side effects [116]. Menicanin et al. investigated PDLSCs

behaviour in a mice model. They reported that PDLSCs exhibit the capacity for long-term survival, self-renewal and regeneration of multiple tissue types in vivo [117]. Recently a randomized clinical trial evaluating safety and efficacy of PDLSCs in treating periodontal defects appeared in the literature [118]. Authors included a total of 30 periodontally compromised patients who received either GTR + PDLSCs + Bioss or GTR+ Bioss (control group). After 12 months of follow-up no significant differences in terms of magnitude of bone regeneration were observed between the two groups. Authors reported no safety problems that could be attributed to the presence of PDLSCs. This is an initial important clinical result that needs to be followed by other trials of larger sample size and longer follow-up. Despite the encouraging results of animal studies, there is still a poor scientific evidence of PDLSCs application in human studies and further research is needed.

GMSCs are considered a valid alternative source for periodontal regeneration [119]. Yu et al. investigated transplantation of GMSCs in class III furcation defects in a dog model. Results showed that GMSCs were able to differentiate into osteoblasts, cementoblasts and fibroblasts, regenerating entirely the damaged periodontal tissue [120]. Recently, regenerative potential of GMSCs was investigated in a porcine experimental periodontitis model in vivo [121]. GMSCs were combined with short-term releasing IL-1ra hyaluronic acid based hydrogel synthetic extracellular matrix. Results showed newly formed bone, cementum and periodontal ligament fibres. Several studies have been undertaken with the aim of comparing MSCs deriving from gingival tissue (GMSCs) to those deriving from periodontal ligament (PDLSCs) [122, 123]. Results suggest that both gingiva and periodontal ligament are similarly important sources of MSCs for periodontal regeneration. Interestingly Yang et al. reported that GMSCs exhibit fewer inflammation-related changes in terms of osteogenic potential in vitro and bone formation in vivo, compared to PDLSCs.

When compared to BMSCs, they display a stable morphology, faster proliferation and steady MSC characteristics with extended passaging. These results indicate that human gingiva is a better source of MSCs than bone marrow. Moreover, they can be found in abundance and can be easily isolated from the oral cavity, all aspects that make them an efficient source for potential clinical application.

Adipose tissue is considered an important source of stem cells and has been thoroughly investigated in regenerative medicine. ASCs can be extracted from numerous sites such as arms, abdomen and chin through interventions of low morbidity. Mesimaki et al. were the first to use autologous ASCs for producing ectopic bone in microvascular reconstruction surgery [124]. Later there has been an emerging scientific evidence of successful transplantation of ASCs for bone-like tissue regeneration [125]. ASCs have also been used successfully for periodontal regeneration in animal models [126]. More recently, combined therapies such as double-layer 3D scaffolds and ASCs have been tested and resulted in good promotion of osteogenic differentiation of ASCs and consequent bone regeneration [127]. Despite of the highly promising evidence for regeneration capacity of ASCs and positive experience in general medicine clinical application, dental clinical trials are fundamental for therapeutic application.

7.4.2.4 Induced Pluripotent (iPS) Stem Cells

Induced Pluripotent (iPS) Stem Cells are somatic cells reprogramed to an embryonic state via transduction and expression of selective transcription factors [128]. Thus, iPS have the capacity of differentiating into all derivative of the three primary germ layers, which means they can produce all tissues and organs. In dentistry, iPS have been generated from various oral MSCs and fibroblasts, showing high reprogramming efficiency. iPS cells have been tested in a mouse model, where used in conjunction with enamel matrix derivatives induced complete periodontal regeneration [129]. However, despite the great pluripotent capacity of iPS, there are several limitations that render their usage still challenging.

The first one consist of epigenetic memory of former phenotype that might result in limited differentiation potential. To overcome this scientific research on more specific markers of pluripotency has been conducted and is still going on.

Another important drawback of iPS cells usage is the capacity of residual undifferentiated iPS cells to uncontrollably proliferate into teratomas. This critical issue has been addressed through several approaches including selective ablation, suicide genes and antibody therapies [130, 131]. Another interesting alternative to this aspect is induction of iPS cells to differentiate into MSC-like cells. It seems that iPS-MSC-like cells are emerging as a promising new stem cell population including the advantages of both iPSC and MSC and eliminating the tumorigenic capacity [132, 133]. Hynes et al. tested iPS-MSC-like cells in a periodontal defect rat model. Results indicated significant increase of the amount of newly formed mineralized tissue and good regeneration [134]. This is a promising research line for appropriate integration of iPS cells into tissue regeneration. Still, scientific evidence permitting a thorough understanding of iPS cells potentials and risks for clinical usage is lacking. Future studies will need to deal especially with controversial effects and post-transplantation behaviour of iPS cells.

7.5 Practical Issues

7.5.1 Accessibility and Identification

Identification and isolation of MSCs remains a relevant challenge. Since these cells exhibit various differentiation states and are heterogeneous, distinction between true “stem cells”, progenitor cells and fibroblasts is not easily achieved. Moreover, considering that to date there is no unique surface molecule to identify MSCs from oral tissues, markers such as Oct-4, SSEA-4, CD29, CD 44 are used to identify the stem nature of these cells.

It is necessary to effectively purify and identify these cells in order to prevent unexpected or insufficient clinical results.

7.5.2 Biological and Technical Challenges

There is some scientific evidence that suggests that transplanted cells may die quickly, reduce their steaming capacity or migrate [135]. One important challenge related to stem cells transplantation is the duration of *in vivo* progenitor activity. This aspect is largely influenced by pre-culture conditions such as culture medium characteristics. Unfortunately to date there is no scientific evidence on optimum pre-culture conditions for MSCs.

Another major problem with transplanted cells is survival into the transplanted site [136]. To overcome this and support cells survival, a sufficient vascular supply is important. Moreover, a good interaction between host and donor stem cells, the so-called host-donor cross-talk is important for modulating cell-mediated immune response [137]. This cross-talk plays a key role also in the local immune response to grafted stem cells. Moreover, this aspect is the main determinant on observed differences between animal models and human models. Since animal experiments are generally performed on immunocompromised rodents their results cannot be directly transferred to human therapeutic application. Further research is indispensable in order to evaluate human host-donor cells interaction, local immune response and events following cells transplantation.

Emerging scientific evidence reveals that oral-derived MSCs exhibit satisfying immunomodulatory properties which makes them a potential immunotherapeutic tool for inflammation-related diseases [138, 139]. Systemic infusions of MSCs have been used for treatment of diseases such as colitis, chronic obstructive pulmonary diseases, sepsis, autoimmune diseases, allergic reactions and graft-vs-host disease.

In periodontal therapy, PDLSCs have shown to possess low immunogenicity and marked immunomodulation via PGE2-induced T-cell anergy. Experiments on a minipig model revealed that PDLSCs can repair allogeneic bone defect without immunological rejections [115]. This advantageous behaviour of oral-derived MSCs and PDLSCs especially, paves new ways for their successful utilization in periodontal regeneration.

7.5.3 MSCs Banking for Innovative Therapies

Oral-derived MSCs are extensively investigated as a therapeutic source for numerous systemic and autoimmunological diseases. Use of patient's own stem cells would result in a personalized medical treatment that would benefit especially from an immunological prospective. To this end, dental stem cells banking, that is the cryopreservation or magnetic freezing of oral tissues for potential future usage in regenerative therapy, has become a reality in several countries [93, 137]. Oral MSCs can be easily harvested and cryopreserved for long periods, serving a major reservoir for necessary future medical treatments. Considering the direct association between prevalence of periodontal disease and age, banking of MSCs derived from deciduous teeth or wisdom teeth would be a valid source for periodontal regenerative therapy through autologous stem cells.

7.6 Conclusions

Emerging technologies and exciting advances in materials science are paving the way to tissue engineering aiming complete regeneration of organs both structurally and functionally. Stem cells are an essential component of tissue engineering, contributing through their progenitive and immunomodulatory capacity. Over the last two decades, potentials and limitations of stem cells have been extensively investigated, mainly in laboratory and animal models. Utilization of MSCs in periodontal therapy has already resulted in promising findings that need to be further elucidated in order to translate MSCs-based therapy into clinical practice.

Future research on MSCs-based therapeutic approaches to rebuild periodontal tissues will need to consider the following issues: (1) survival of cells and efficient expression of programmed proliferative capacity; (2) appropriate pre-culture treatment and scaffold-based combined therapies; (3) control of adverse effects such as migration, missed differentiation and tumorigenesis; (4) interaction between host cells and transplanted MSCs.

The last but not the least, new technologies must make available favourable cost-effective therapies considering also patient-related aspects and socio-economic components.

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Chapter 8
Stem Cells and Low-Level Laser Therapy (LLLT): State of the Art and Future Application

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8.1 Introduction

8.1.1 Mesenchymal Stem Cells

Multipotent mesenchymal stromal cells (commonly referred as Mesenchymal stem cells; MSCs) are the most frequently used cell population in tissue engineering because of its multilineage potential, multiple sources and ability to self-renew [1].

The International Society for Cellular Therapy (ISCT) proposed the following minimum criteria to clearly define the MSCs: (1) the adherence to plastic surfaces under standard cell culture conditions; (2) the expression of cell surface markers, such as CD90, CD73, and CD105, and the lack of expression of CD14, CD34, CD45, CD79, or CD19 and HLA-DR, and (3) the capability to differentiate at least into chondrogenic, osteogenic, and adipogenic lineages [2].

Bone marrow-derived mesenchymal stem cells (BMMSCs) are being considered as a gold standard [3, 4]. However because of the difficulty to harvest a sufficient cell number as well as the pain and morbidity involved during the harvesting procedure, researchers have been exploring other sources/locations for MSCs. Many anatomical locations have been researched to yield MSC populations [1, 5].

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Besides the advantages, MSCs show also a limited proliferation ability, since they go into senescence after a long-term culture or after many population doublings in culture conditions, due to telomeres shortening [5].

The capacity of MSCs to differentiate into cell lineages and develop teratomas—a preserved tumor that contains normal three germ layer-tissue and organ parts—is a reason to consider them as multi-potent progenitor cells suitable for regenerative therapy.

Nowadays, one of the potential sources identified was the dental/oral tissues. Research on using MSCs of dental origin has increased exponentially in the last decade [6, 7].

8.1.2 Dental Derived-Mesenchymal Stem Cells

Dental tissues are specialized tissues and they do not undergo continuous remodeling as has been indicated in other bony tissues; therefore, stem cells that are obtained from dental-tissue might show a restricted differentiation capacity compared to BM-MSC [8, 9].

Dental pulp stem cells (DPSC) are amongst different human dental stem and progenitor cells that have been isolated and characterized to date [10]. DPSC possess self-renewal and differentiation capacity. Human pulp cells can differentiate into odontoblastic cells in vitro, possessing polarized cell bodies and the ability to accumulate mineralized nodules [11–13]. Although dental-tissue-derived stem cells are obtained from specialized tissue and they are most potent for differentiation into odontogenic cells, DPSC also have the potential to differentiate to other cells such as adipocytes and neurons [14]. Recently, it has been revealed that DPSC have the potential to give rise to chondrocytes, osteoblasts and myocytes *in vitro* [15, 16]. To date, the regenerative application of MSC that are obtained from dental pulp involves regeneration of the whole tooth and partial bony substrate of the oral cavity in the process of maxillofacial surgical interventions [17, 18].

The osteogenic differentiation potential of the cells isolated from dental follicle (DF) has been investigated by Mori et al. [19]. This study has revealed that stemness markers are released by dental bud stem cells. Upon differentiation, these cells have been shown to express osteoblastic biomarkers such as collagen I and alkaline phosphatase (ALP) which indicates their commitment to osteoblast-like lineage [19]. Moreover, a recent report involving the role of integrins and cadherins in differentiation of dental bud stem cells has unraveled a crucial role for integrin αVβ3 during differentiation of these stem cells into osteoblasts [20]. The data elucidates the impact of extracellular matrix (ECM) proteins in directing stem cell fate towards bone formation. [20].

Recent studies have identified a new human dental derived stem cell population: human periapical cyst mesenchymal stem cells (hPCy-MSCs) have been isolated in inflammatory periapical cysts and have demonstrated to be highly clonogenic and able to differentiate towards the most common mesodermal lineages, as well as

towards the neurogenic phenotype: this last skill has been reported in a study reporting that the hPCy-MSCs exposed to neurogenic differentiation medium showed a surprising upregulation of a comprehensive set of proteins and genes that characterize the neuronal cells [21]. These new MSC population might be another optimal source of neural/glial cells for cell-based therapies to treat neurologic diseases [22].

A significant body of literature has been published in the past five years on various types of dental MSCs and its applications in fracture healing as well as regenerative bone formation interventions due to disease or loss of the tissue [7, 8, 18, 23, 24].

However there is still limited evidence regarding the capacity of dental MSCs for bone regeneration. An in depth review and understanding of preclinical *in vitro* and *in vivo* studies is a pre-requisite to assess the efficacy of dental MSCs and to translate their use into the clinics [25].

8.1.3 Low-Level Laser Therapy (LLLT)

Within this context Low-level laser therapy (LLLT) has been used in several *in vitro* experiments in order to stimulate cell proliferation.

LLLT is used considering its photobiological effects, interesting for this treatment, as the pain control, the anti-inflammatory effect (mediated by the microvascularization increase on area and by the local vasodilatation), the increase of collagen synthesis and the fibroblasts proliferation, the increase of ATP (adenosine triphosphate) production, the increase of mitochondrial activity, further the increase of strength of the scar tissue [26–34].

This capacity of accelerating the healing process is most likely related to the finding that LLLT promotes cell proliferation.

However, the underlying molecular mechanisms for this process are still not completely understood [35]. It has been suggested that the energy of the laser is absorbed by intracellular chromophores and converted into metabolic energy, which is then used by the mitochondrial respiratory chain to produce ATP and increasing DNA activity and the synthesis of RNA and proteins [36].

Nevertheless, biostimulation is not always observed because a variety of factors influence this process [37].

In this respect, *in vitro* biostimulation depends on laser-related parameters such as wavelength, dose, power and time of irradiation, [38–40] type of cell irradiated [37], and the physiological characteristics of the cells at the time of irradiation [39, 40].

As a consequence of these factors, the interaction of laser light with cells and tissues can stimulate or inhibit cell proliferation.

Because the proliferation of MSCs is usually slow and the yield of these cells after first harvest is low, a therapeutic tool that increases their proliferation without causing molecular damage while maintaining their specific characteristics is important for effective clinical application of these cells.

The visible, infrared or ultraviolet spectra of light can be used for LLLT and little is known about the effect of laser therapy on MSCs [41, 42].

Wave length used in LLLT irradiation varies between 600 and 1000 nm with an energy density of 0.04–60 J/cm^2. Different laser light sources, like helium-neon and gallium-aluminium-arsenide (GaAlAs), are being frequently used in clinical studies such as: surgical treatments of oral lesions, uncovery of implants, bacteria reduction in root canals or periodontal pockets and dentine hypersensitivity reduction. Diode lasers are known to have a high penetration depth compared to other laser types [41].

In fact, in the light of future cell therapy protocols, LLLT therapy would allow significant increase of the primary number of stem cells before differentiation, thus increasing the amount of viable differentiated cells for tissue engineering and regenerative process, especially from a simple available and accessible MSCs source such as the dental derived stem cells.

8.2 State of the Art

In vitro pre-clinical research is the basic foundation for any new therapeutic approach. Although it may not replicate a dynamic environment, *in vitro* research provides valuable information for future research steps.

Several protocols were used to irradiate the cells, with variations on wavelength, power density, radiation time, and state of light polarization.

On one hand, most studies demonstrated an increase in the proliferation rate of the irradiated cells, on the other hand the majority of the articles lacked randomization, blinding, sample size calculation and repetition of the experiments. This affects the scientific validity of experimental results.

A systematic review on the effect of LLLT on MSCs recently published has concluded that the laser therapy positively influences the *in vitro* proliferation of stem cells studied [41].

Systematic reviews provide the best evidence on the effectiveness of a procedure and permit investigation of factors that may influence the performance of a method. To the best of our knowledge, international scientific literature lack of systematic reviews that evaluated the effects of LLLT only on dental-derived mesenchymal stem cells.

Table 8.1 describes relation to the cell types studied there were four studies on the DPSCs, two using SHEDs and one PDLSCs [42–49].

Most studies used only one cell strain; whereas Pereira et al. [43] isolated six strains of DPSCs (three from healthy and three from inflamed dental pulp tissues) and Turrioni et al. [45] isolated three strains of SHEDs. Stem cell characterization in the seven studies was done with different markers and different methods for detecting those markers. The following stem cell markers were used: STRO-1; CD-146; CD29/integrin, CD44, Nanog and OCT3/4; Human MSC analysis kit (CD105, PerCP-Cy5.5, CD73-APC, CD90-FITC and negative controls), among others.

Those markers were detected in flow cytometry analysis or by using the Dynabead isolation method [43] or immunofluorescence [45]. Stem cell osteogenic and adipogenic differentiation fulfilled the stem cell characterization in two studies [44, 46].

Table 8.1 Experimental parameters and main results of dental derived MSCs, LLLT irradiated

Authors	Stem cells sources	Stem cell markers; Method	(1) Cell plating; (2) Distance between wells; (3) Darkness	Type of evaluation; Method; condition	Time points of evaluation	Main results
Eduardo et al. [43]	Human dental pulp DPSC	CD29, CD45, CD34, SH2 (CD105), SH3, and SH4; flow cytometry analysis	(1) 96-wells; (2) Not informed; (3) Yes	Proliferation; MTT assay (absorbance); nutritional deficit	20, 24, 48, and 72 h after the first irradiation	• The cells responded positively to PBM • The power of 20 mW was more effective than 40 mW • Laser higher growth than non-lased
					0, 6, 12, 18, 24, 30 and 36 h after first irradiation	
Pereira et al. [44]	Human dental pulp DPSC 6 strains: from Healthy (3) and from inflamed tissues (3)	STRO-1 enriched cells were isolated by using Dynabead isolation method	(1) 96-wells; (2) Not informed; (3) Not informed	Proliferation; MTT assay (absorbance) Normal culture condition	24, 48, 72 and 96 h after irradiation	• There was no difference in cell growth between PBM and control groups
				Differentiation; Odonto-osteogenic Alizarin red (absorbance) Mineralizing and regular (control) media	3 weeks	• There was no difference in the relative production of mineralized nodules between PBM and control groups
Soares et al. [45]	Human periodontal PDLSC	CD29/integrin; Flow cytometry Differentiation in osteogenic and adipogenic; Checked in light microscopy	(1) 96-wells and 24-wells; (2) one or three wells; (3) Not informed	Viability; MTT assay (absorbance) and Proliferation; Trypan blue assay (Cell counting in Neubauer's chamber)	0, 24, 48, and 72 h after the first irradiation	• In both analysis (Viability and proliferation) Cultures irradiated with 1 J^2 presented higher number of viable cells than all other groups in 48 and 72 h

(continued)

Table 8.1 (continued)

Authors	Stem cells sources	Stem cell markers; Method	(1) Cell plating; (2) Distance between wells; (3) Darkness	Type of evaluation; Method; condition	Time points of evaluation	Main results
Turrioni et al. [46]	Human dental pulp SHED	STRO-1, CD44, CD146, Nanog and OCT3/4; Immunofluorescence	(1) 24-well culture; (2) None; (3) Yes	Differentiation; alkaline phosphatase (ALP) activity, total protein (TP) production, and collagen synthesis gene expression (qPCR)	72 h	
Arany et al. [47]	Human dental pulp DPSC; mouse and rodent cells or pulp tissue	CD44, CD90, CD106, CD117 and Stro-1; negative CD45; Western Blot and Immunofluorescence	(1) At least, 96-wells, 35- and 60-mm dishes, (2) Not informed, (3) Used card sheet (black background)	Reactive Oxygen Species (ROS) quantification; latent TGF-β activation verified by ELISA; Luciferase activity of TGF-β reporter; differentiation assays; inhibitory/stimulatory and loss of function studies (conditional knockout); *in vivo* experiments; others	Varied according to the experiment (Immediately or 5 min after irradiation; 24 h; 3–21 days; 8–12 weeks for *in vivo*)	
Diniz et al. [48]	Human dental pulp SHED	STRO-1 and CD-146; Flow cytometry analysis	(1) 96-wells plates; (2) one well; (3) Not informed	Survival; MTT (absorbance) Culture medium conditioned by dental adhesives	24 h after irradiation	

Authors	Stem cells sources	Stem cell markers; Method	(1) Cell plating; (2) Distance between wells; (3) Darkness	Type of evaluation; Method; condition	Time points of evaluation	Main results
Zaccara et al. [49]	Human dental pulp DPSC	Human MSC analysis kit: CD105, PerCPCy5.5, CD73-APC, CD90-FITC and negative controls; Flow cytometry Differentiation in osteogenic (von Kossa) and adipogenic (Oil red O)	(1) 96-wells and 24-wells; (2) one or three wells; (3) Not informed	Viability; MTT assay (absorbance) and Proliferation; Trypan blue assay (Cell counting in Neubauer's chamber)	24, 48, 72 and 96 h	• At 72 h there were more viable cells in both lased groups than in control
				Apoptosis (flow cytometry) FITC	24 and 72 h	• No differences between lased and control groups
				Annexin V/Dead Cell apoptosis kit cell cycle (flow cytometry)	24, 48, 72 and 96 h	• No differences between lased and control groups

The cell plating for the irradiation experiments were mostly done in 96-wells plates. The cells were plated in the wells prior to irradiation, except in the study published by Pereira et al. [43] where the irradiation was done in the cell suspension prior to cell plating. In three studies there was a distance between the irradiated wells [44], in order to avoid overexposure of the cells in the contiguous wells.

Darkness during irradiation to avoid effects of light other than the laser or led used was applied only in three studies [43, 45, 46].

The effects of LLLT on the dental MSCs studied were mostly on the cell viability and proliferation. Some studies also analyzed the odonto/osteogenic differentiation of the cells. The methods used were mitochondrial activity (viability) using the MTT assay, or Trypan blue dye exclusion assay (proliferation) using the Neubauer's chamber cell counting. Zaccara et al. [48] also analyzed the effect of LLLT on apoptosis using a FITC Annexin V/Dead Cell apoptosis kit and flow cytometry and also the cell cycle. These analyses were done mostly until 96 h after the last irradiation with small variations between studies.

For differentiation, deposition of mineralized nodules (Alizarin red), alkaline phosphatase (ALP) activity, total protein production, and collagen synthesis gene expression (qPCR). The results of the differentiation assays where mineral deposition was searched were analyzed in 3 weeks; whereas when the ALP activity was examined the 72 h post irradiation was the analyzing period [45].

For the main results the studies that evaluated viability and proliferation showed that the cells responded positively to LLLT [42, 43, 45, 46].

Eduardo et al. [42] showed that the power of 20 mW was more effective than 40 mW. Pereira et al. [43] found no differences in cell growth between lased and non-lased cells. Positive effects of LLLT on cell differentiation was reported by Turrioni et al. [45], whereas Pereira et al. [43] did not find any effect.

Arany et al. [46] used a more elaborated experimental design with techniques *in vitro* complemented by a translational study. These authors identified a role for TGF-β1 in mediating laser-induced dental tissue regeneration. Moreover, they reported that LLLT generates ROS and directs stem cell differentiation.

In a recent systemic review of Borzabadi-Farahani [50], an electronic search of literature was conducted (2000–2016) on PubMed, Web of Science, and Scopus databases. Search terms included low-level light therapy, low-level laser irradiation, low-level light irradiation, LLLT, humans, adolescent, adult, cells, cultured, periodontal ligament, dental pulp, stem cells, dental pulp stem cells, mesenchymal stem cells, periodontal ligament stem cell, deciduous teeth, cell proliferation, adult stem cells, radiation, and proliferation.

The literature search in this study identified 165 studies with 6 being eligible for inclusion; all used diode lasers; five studies used InGaAIP diode lasers; four used 660 nm, and the other two applied 810 or 980 nm wavelength LLLT. The distance between the dental MSCs and the laser spot ranged between 0.5 and 2 mm. The time intervals of cell proliferation analysis ranged from 0 h to 7 days after LLLT. After 660 nm LLLT, an increase in the dental MSC's proliferation was reported [DMSCs extracted from dental pulp of deciduous teeth (two irradiations, 3 J/cm^2, 20 mW was more effective than 40 mW), adult teeth (two irradiations, 0.5 and 1.0 J/cm^2,

30 mW), and from adult periodontal ligament (two irradiations, 1.0 J/cm^2 was more effective than 0.5 J/cm^2, 30 mW)]. Similarly, an increase in the proliferation of DMSCs extracted from dental pulp of adult teeth was reported after 810 nm LLLT (seven irradiations in 7 days, 0.1 and 0.2 J/cm^2, 60 mW) or 980 nm LLLT (single irradiation, 3 J/cm^2, 100 mW). However, 660 nm LLLT in one study did not increase the proliferation of dental MSCs (single irradiation, energy densities of 0.05, 0.30, 7, and 42 J/cm^2, 28 mW).

In this scenario, LLLT-DPSCs have to be considered ideal stem cells to be used for tissue engineering since they are characterized by a high proliferation rate, multidifferentiation ability, easy accessibility, high viability, opportunity to be safely cryopreserved and expression of mesenchymal markers.

In a study from Ballini et al. [51], both irradiated and not-irradiated cultures of human DPSCs showed a good growth activities without any lack during the entire observation period and it should be noted that the application of laser irradiation did not cause cell damage.

In treated cultures respect to control group, it was possible to observe an increase of proliferation through the use of a marker of osteoblasts, the alkaline phosphatase, an early marker of osteoblasts differentiation.

In all experiments RT-PCR shown a down-regulation of RUNX-2 (gene that codifies for a transcription factor that promotes maturation and cellular differentiation), index of differentiation and stop of progression of cellular cycle in G1 phase (proliferation), with an increase in irradiated DPSCs at 12 h (Table 8.2).

In addition OSTERIX shows the same expression, being RUNX-2 transcribed upstream of OSTERIX.

The results of cytofluorimetric analysis of cellular cycle underlined that, compared to controls, a single laser stimulation induces changes in cell turnover so that a greater percentage of osteoblasts from DPSCs enters the G2 phase of cellular cycle, expression of an increasing of proliferation [51].

Several clinical trials are running to identify different aspects of MSC application in terms of safety and efficacy.

The recent 4-year reports regarding LLLT application to increase MSCs proliferative and differentiation potential were summarized in Table 8.3 [51–67].

As of date (21 October 2016), a total number of 660 clinical studies were found that involve mesenchymal stem cells for different clinical phases, but only 1 for both MSC and LLLT, moreover actually not yet recruiting (Table 8.4).

8.3 Actual and Future Applications

Lasers have been used for a long time in dentistry and low-energy laser therapy has also been used in this domain of medicine.

Except for application of LLLT mentioned above: treatment of temporomandibular disorders (TMD), wound healing and improving recovery after procedure of insertion of implant, LLLT is used in the treatment of dentin hypersensitivity [68].

Table 8.2 RUNX-2 and OSTERIX gene expression in treated and untreated cells after 3, 6 and 12 h from DPSCs under LLLT stimulation

RUNX2		A	B	ΔCT_A	ΔCT_B	$2-\Delta\Delta CT_{medi}$
CTR	GAPDH	26.03	25.39	25.31	16.55	
	RUNX2	51.34	41.94			
Treated						
3 h	GAPDH	25.63	25.57	25.61	21.04	ΔCT3H−ΔCTCTRL = 0.004
	RUNX2	51.24	46.43			
6 h	GAPDH	25.32	26.44	28.71	21.40	ΔCT6H−ΔCTCTRL = 0.034
	RUNX2	54.03	47.84			
12 h	GAPDH	25.85	26.57	28.86	23.38	ΔCT12H−ΔCTCTRL = 0.008
	RUNX2	54.71	49.95			
OSTERIX		A	B	ΔCT_A	ΔCT_B	$2-\Delta\Delta CT_{medi}$
CTR	GAPDH	21.53	21.21	26.50	28.27	
	OSX	48.03	49.48			
Treated						
3 h	GAPDH	21.01	21.36	30.37	28.99	ΔCT3H−ΔCTCTRL = 0.006
	OSX	51.38	50.35			
6 h	GAPDH	20.78	21.23	30.58	31.19	ΔCT6H−ΔCTCTRL = 0.013
	OSX	51.36	52.42			
12 h	GAPDH	22.42	21.38	30.54	31.64	ΔCT12H−ΔCTCTRL = 0.096
	OSX	52.96	53.02			

RT-PCR: The value $2-\Delta\Delta CT$ is compared with benchmarks:
≤0.5: Down-regulated gene
≥1.5: Up-regulated gene

Table 8.3 A registered clinical trial on the basis of MSCS and laser therapy as the relevant therapeutic tool (www.clinicaltrials.gov)

	Recruitment	Title, conditions and intervention	
1	Not yet recruiting	Non-randomized, Open-labeled, Interventional, Single Group, Proof of Concept Study With Multi-modality Approach in Cases of Brain Death Due to Traumatic Brain Injury Having Diffuse Axonal Injury	
		Condition:	Brain death
		Interventions:	Biological: BQ-A Peptide Extract; Biological: Mesenchymal Stem Cells; Device: Transcranial Laser Therapy; Device: Median Nerve Stimulator

LLLT is widely used in the prevention and treatment of oral mucositis caused by chemotherapy, radiotherapy and chemoradiotherapy used as a treatment for various types of cancer (including those outside the head and neck). Recently, there were created two meta-analysis consisting guidelines for dosing and preferred parameters of laser radiation. Gautam et al. [69], as a result of their meta-analysis study, made a new recommendation for the use of LLLT as a prevention of mucositis in adult patients receiving hematopoietic stem cell transplantation conditioned with high-dose chemotherapy, with or without total body irradiation. Authors suggest that

Table 8.4 The effect of LLLT on the MSCs proliferation and differentiation (literatures published in recent 4 years)

Authors	Brief description
Park et al. [53]	LLLT enhanced angiogenic effect of adipose-derived stromal cells (ASCs) spheroid in hind limb ischemia mice. LLLT is an effective biostimulator of spheroid ASCs in tissue regeneration that enhanced the survival of ASCs and stimulated the secretion of growth factors in the ischemic hind limb
Farfara et al. [54]	MSCs were stimulated by LLLT in order to affect neurological behavior and beta-amyloid burden in progressive stages of Alzheimer's disease mouse model
Yang et al. [55]	LLLT was applied as an adjunct therapy for MSCs transplantation on the functional recovery of crushed sciatic nerve in rats
Wu et al. [56]	LLLT increased the intracellular level of cAMP, which acts to downregulate NF-B transcriptional activity
Nagata et al. [57]	The combination of bone marrow aspirate/LLLT yielded significantly greater bone formation in surgically created critical-size defects in rat calvaria
Manuguerra-Gagné et al. [58]	A laser-induced model of open angle glaucoma (OAG) was used to evaluate the potential of bone marrow cell populations and the mechanisms involved in tissue repair. Laser-induced tissue remodeling as a method of targeting effector cells into damaged tissues was also evaluated
Lipovsky et al. [59]	The ability of broadband visible light illumination to promote proliferation of MSCs was evaluated
Giannelli et al. [60]	The effects of LLLT on mouse MSCs proliferation were investigated underlying cellular and molecular mechanisms, focusing the attention on the effects of laser irradiation on Notch-1 signal activation and membrane ion channel modulation
Choi et al. [61]	Adipose-derived mesenchymal stem cells- (ASCs-) seeded acellular dermal matrix was used with LLLT to repair bone defect
Alexandrov et al. [62]	Terahertz (THz) laser irradiation of MSCs can cause specific catalytic changes in cellular function that are closely related to the gene expression and differentiation state
Wu et al. [63]	The change in mRNA expression in rat MSCs after LLLT and the associated molecular mechanisms were investigated
Wu et al. [64]	LLLT induced IGF1 expression to promote both the proliferation and osteogenic differentiation of MSCs, whereas it may induce BMP2 expression primarily to enhance osteogenic differentiation
Wang et al. [65]	MicroRNA-193 proliferation effects for bone MSCs were revealed after LLLT through inhibitor of growth family, member 5
Soleimani et al. [65]	The influence of LLLT at different energy densities on MSCs differentiation into neuron and osteoblast was examined
Saygun et al. [66]	LLLT increased the proliferation of osteoblast cells and stimulated the release of bFGF, IGF-1, and IGFBP3 from these cells
Fekrazad et al. [67]	Application of cultured autologous bone marrow mesenchymal stem cells (BMSCs) with scaffold and LLLT on the repair of articular cartilage defects in rabbits

(continued)

Table 8.4 (continued)

Authors	Brief description
Ballini et al. [52]	LLLT increased the proliferation of osteoblast cells and stimulated the down-regulation of RUNX-2 , with an increase in irradiated Dental Pulp Stem Cells (DPSCs) at 12 h

those kinds of patients should receive laser radiation with a wavelength at 650 nm, power of 40 mW and energy density 2 J/cm^2. They also suggest that LLLT in prevention of oral mucositis in patients undergoing radiotherapy head and neck cancer should have a wavelength of around 632.8 nm. Unfortunately, in the case of other patients, no guidelines can be recommended, but it still may be assumed that LLLT is effective. Bensadoun and Nair [70] as a result of their meta-analysis recommend the use of laser irradiation in the wavelength range from 630 to 670 nm and from 780 up to 830 nm, suggested power output between 10 and 100 mW and energy density 2 up to 3 J/cm^2 for prophylaxis and a maximum 4 J/cm^2 for therapeutic effect. They assume that the treatment should be repeated each day or with a one-day break until results appear.

Today, researchers are conducting intensive basic and clinical research in the area of laser medicine and photobiology, with the goal of developing new diagnostic and therapeutic modalities.

Because stem cells isolated from different sources usually present a low yield and low proliferation rate, low-level laser irradiation may be a useful tool for tissue engineering using stem cells. In this respect, laser therapy permits a significant increase in the initial number of stem cells before differentiation, thus increasing the number of differentiated cells for tissue engineering and regenerative and healing processes [71].

The biological mechanisms underlying such responses significantly differ by the type of laser, target of cells, and other experimental conditions. With the appropriate use of LLLT, the proliferation rate of cultured cells, including MSCs, can be increased, which would be very useful in tissue engineering and regenerative medicine [72]. We must accumulate a systematic knowledge base by carefully analyzing the experimental data currently available, as well as data collected in the future.

It can be concluded that the laser therapy positively influences the *in vitro* proliferation of stem cells studied worldwide, being necessary to carry out further experiments on other cell types and to uniform the methodological designs.

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Chapter 9
The Human Periapical Cyst-Mesenchymal Stem Cells (hPCy-MSCs): The New Challenge of "Waste Medicine" in Regenerative Dentistry

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9.1 Background

Regenerative medicine is a modern branch of biomedicine that created the enormous therapeutic potential in the past decade.

The field of Regenerative Medicine and Stem Cell has received a great boost from the recent observation that, by gene transfer using viral vectors with a few genes, it may be possible to reprogram adult human somatic stem cells or non-human adult multipotent stem cells, up to become embryonic-like stem cells. These important scientific discoveries have generated a complex legislative and economic context. In fact, when performing a cell therapy, the stem cells will legally constitute medication, and therefore must be isolated, characterized, expanded and cryopreserved in a manner strictly codified international (good manufacturing practice "GMP").

In the literature, it is well known that the mesenchymal differentiation process involves the replication of stem cells and their entrance into pathways of different cells lines lead, then, to the production of bone tissue, cartilage, muscle, etc. For many years, the bone marrow was considered the main source of Mesenchymal

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Stem Cells (MSCs) able to respond to stimuli and to repair damaged tissues. However due to the invasiveness of the bone marrow aspiration and the gradual reduction with age in the number of cells, the researchers have been directed towards the search for new sources of MSC. In addition, the cellular matrix and the microenvironment play a major role in addressing these multipotent cells towards differentiation line or another. In particular, the level of vascularization and the consequent tissue oxygenation determine the choice between chondrogenic or osteogenic lineage. In vivo, the proximity between cells determined by the molecular weight of proteoglycan and the absence of oxygenation determines the MSC differentiation into cartilage; while the presence of vessels and the consequent oxygenation determines the shift of the differentiation of MSCs into the osteogenic lines.

The searching for alternative biological sites, easily accessible, free of morbidity for the patient, rich in stem cells, is the current challenge of regenerative medicine. Human periapical inflammatory tissues that are formed resulting from endodontic infection are usually termed periapical granuloma, and the condition is referred to as apical periodontitis. This pathological condition, if untreated, can lead to a periapical cyst.

9.2 The Human Periapical Cyst-Mesenchymal Stem Cells (hPCy-MSCs)

The research team of "Tecnologica Research Institute" and "Calabrodental" investigated about the way to determine if cells isolated from human periapical inflammatory cyst were able to express mesenchymal stem-like properties. The results of these studies were published in the publication "*Cells Isolated from Human Periapical Cysts Express Mesenchymal Stem Cell-like Properties*" [1].

In this paper, the authors shared to the scientific community the results of their studies, demonstrating that the dental periapical cysts contain in their wall several cell typologies, including mesenchymal stem cells; these findings were important to improve the field of the dental-derived stem cells [2, 3].

The dental periapical inflammatory cysts, also known as apical-radicular cysts, are those space-occupying formations that usually develop as a result of pulpal necrosis and that are often discovered by the dentist during the observation of a common orthopanoramic radiography; dental periapical cysts are among the most common dental radiographic findings, because of the ease of their development, but they are not usually dangerous from a histopathological point of view, however, they are commonly identified as a clinical finding to be eradicated as soon as possible, often together with the necrotic tooth that has made them develop.

Human periapical cystic tissue, obtained from healthy volunteers donors, were subjected to three washes in sterile phosphatase buffer saline (PBS, Invitrogen) where antibiotics were added. Cells from human periapical cystic wall were isolated and characterized, by means of flow cytofluorimetry: such cells expressed MSC-

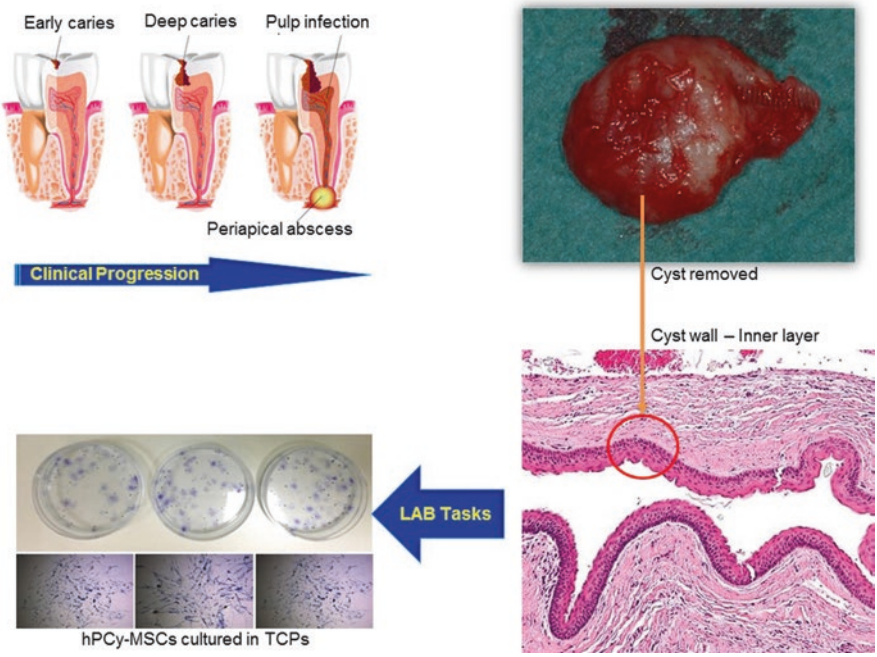

Fig. 9.1 Workflow from clinical sign to the "in vitro" activity: hPCy-MSCs are isolated from the cystic wall with mechanical and enzymatic techniques

like properties, according to the scientific agreements reported in the literature: the authors termed them "human Periapical Cyst-Mesenchymal Stem Cells" (hPCy-MSCs), in consideration of the site of harvesting (Fig. 9.1).

In this context, Marrelli et al. demonstrated that cells isolated from human periapical inflammatory cysts display MSC-like properties, as evidenced by the expression of the MSC markers $CD13^+$, $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD45^-$, STRO-1^+, $CD146^+$ as well as their self-renewal capability and osteogenic and adipogenic potential.

Several studies were carried out on such new MSCs, to investigate their behaviour with respect to the well-known Dental Pulp stem cells (DPSCs) [4]. Their potential was clear, thus, they are constantly cited in the scientific literature by several renamed research groups. As a consequence of this discovery, it's actually possible to successfully isolated and characterised hPCy-MSCs from human periapical cysts, without to recourse to the removal of other biologically healthy tissues, since human periapical cysts are commonly removed by surgeons, to prevent the development of other pathological conditions.

9.3 The Challenge of "Waste Medicine"

Regenerative medicine is a discipline aimed to repair or regenerate tissues or entire organs who have suffered from severe damages, such as an heart stroke or as a bone trauma, so to promote a replacement of the proper anatomy and function of such regenerated tissues.

The goal that regenerative medicine aims can be achieved through the identification of cells that can replace those lost or organ parts have faced degenerative disease, and among them certainly have a key role in the stem cells, however, stem cells are present in biological tissues vital, often poorly accessible and in any case in such a context that any taking of tissue would result in an injury to a component "healthy" patient who runs the taking: to understand this concept, think of the procurement of stem cells from the spinal cord through lumbar puncture, i.e. the removal of stem cells from adipose tissue that involves the surgical access to the structures of subcutaneous fat.

Regenerative medicine is a branch booming, thanks to new technologies have emerged in the past five years, to a greater awareness of some of the features of biology and physiology of mesenchymal stem cells and a basic research strongly oriented towards this subject from the potentialities still only minimally explored.

The forecast of the economic impact of this sector is extremely relevant, if we think it will certainly cover areas such as reconstructive surgery and, in some cases, functional prostheses of lost and damaged organs.

The policy regulating the use of biological tissues in research studies is quite complex and based on worldwide regulations but also on national specific laws. Biological samples collected for a biopsy are simple to obtain, however it often happens that the histological investigations need a smaller amount of the harvested tissue and such tissues become automatically a biological waste.

In 2013 Marrelli et al. disclosed to the scientific community the results of a study that showed how periapical dental cysts contain in their wall a number of cells, including mesenchymal stem cells.

Periapical inflammatory dental cysts are neoformations that usually develop as a result of necrosis of the pulp of the tooth, they are often discovered by the dentist during the observation of a common orthopanoramic dental radiography. Therefore, we can affirm absolutely that the periapical cysts do not have a biologically important function in our body, in fact, usually the cysts are stored between the organic waste, with the exception of those cases in which you require a possible histological diagnostic study, however, that, given the usual kindness of the cystic behavior is almost never carried out. The possibility of obtaining mesenchymal stem cells from a "biological waste" puts us in a position to really take advantage of a source "alternative" to zero biological cost. To date, the most common sources of adult stem cells were identified usually in the bone marrow, adipose, and recently tissue, in some intra-oral structures such as dental pulp or dental papilla; however, all these biological structures are absolutely vital, and their removal requires a "living costs", although limited and acceptable. The human periapical cyst-Mesenchymal Stem

Cells (hPCy-MSCs) are the alternative that fully embraces the modern concept of "biological waste medicine": we can achieve the regeneration of any biological tissue, such as the heart, liver or bone, by using an intriguing source of stem cells from discharged tissues that patients will not use anymore; similarly to what happens in any other recycling process.

Marrelli et al. published "Human Periapical Cyst–Mesenchymal Stem Cells Differentiate Into Neuronal Cells", where the authors were able to induce hPCy-MSCs, obtained from a biological waste as is commonly considered the benign odontogenic inflammatory cyst, to differentiate towards neuronal phenotype [5].

This research makes us able to introduce a broad concept: the experimental medicine free of biological costs, that leads to the creation of several "model-diseases" aimed to experiment, with drugs and experimental formulations that today require studies "in-vitro", on biological-models developed with the tissues engineering, starting from cells taken from discharged tissues. In addition, we should consider that the study of some pathologies of complex organs, such as brain, it needs to resort to a biopsy on the brain of sick patients to study the changes in cells, in response to drugs, moreover, the neurons are tricky to seed and a proper surface [6, 7] is needed to ensure a effective cell growth: now, it could be not anymore necessary to arrive at the anatomical site of the damage: by means of a simple harvesting of intraoral tissue, easily accessible, we can induce the differentiation of adult-SCs into several phenotypes, until they become an useful brain-like model. We can also induce some specific diseases and, therefore, we can accordingly test new therapies without any bioethical implications, with a strong reduction of iatrogenic morbidity and with the possibility to improve the costs management of health governance in all the countries involved in these experimental model. The "biological waste medicines" is a concept that ties together biology, medicine, bioethics and health economics, therefore, should be encouraged in order to stimulate a more patient-friendly point of view, and certainly more politically correct and economically sustainable.

9.4 Conclusion

Up to now, the most common sources of adult stem cells were usually identified in bone marrow, adipose tissue, and, recently, in some structures such as intraoral dental pulp. The dental inflammatory periapical cysts are usually not dangerous from a histopathological point of view, on the contrary, they represent a neoformation absolutely free of utility for the patient, in fact, usually the cysts are stored in biological waste: the possibility of obtaining mesenchymal stem cells from a "biological waste" puts us in a position to really take advantage of an "alternative" source, at practically zero biological cost. The so called "waste medicine" could have many applications towards the regenerative strategies with medical applications. This could be the future challenge to achieve the goals of excellence in healthcare with a reduction of the costs of NHS.

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Postface

Gianpaolo Papaccio

The Future Insights on Dental Stem Cells

Dental stem cells are a promising challenge for the functional reconstruction of different tissues of human body, especially for craniofacial tissues and bone. Undoubtedly, there is a need to develop regenerative therapies for patients with congenital anomalies and orofacial defects. The advantages in the use of stem cells derived from tooth home are related to their non-tumorigenic phenotype, differentiation capabilities and plasticity, high proliferation potential, cryopreservation which allows to obtain patient-specific cells for autologous transplantation, simplicity in tooth extraction protocol and cell expansion without morbidity and mortality. Despite this, there are many obstacles and issues regarding cell-based approaches. An important issue in autografts regards the limitation of stem cell number obtained from biopsies. Therefore, the cells need to be processed *ex vivo* before transplantation. These procedures, though effective and increasingly standardized, expose the graft recipient to several risks including infection, hematoma or nerve damage. Moreover, the use of cell cultures could lead to genetic and epigenetic instability of cells.

Several bone defects include surgical procedures that use bone grafts as tissue substitutes, or barrier membranes. In some cases, these procedures are associated with a wide range of grafting materials including hydroxyapatite, tricalcium phosphate and PLGA. Therefore, the paracrine/autocrine effects and the interactions with the host and immune response following cell transplantation also need to be taken into consideration. As a consequence, there is a strong need to develop new approaches for the use of stem cells in Regenerative Medicine. One idea would be to recruit and stimulate resident stem cells to differentiate or to produce factors that can physiologically promote tissue regeneration. In the case of dental stem cells, the

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thoughts go to the regeneration of the craniofacial body area. In particular, in face region, mandible and maxillary bones often undergo re-absorption, following degenerative diseases such as mandible necrosis, periodontal syndromes or tumour resections. Avoiding cell processing ex vivo that takes long time and high costs, new procedures could consist in the activation of resident dental stem cells and in their differentiation into bone tissue. The alternate approach could be to stimulate resident stem cells to produce the factors that can induce or ameliorate the differentiation. In this way, in the case of tooth engineering, the possibility of autologous cell replacement and the usage of cells naturally occurring in the site of injury may minimize the risk of side effects in patients. New accurate and detailed studies must be performed to understand and exploit the potentials of endogenous dental stem cells and their factors. In addition, the new so-called intelligent growth factor-loaded scaffolds could stimulate endogenous dental stem cells. In this way, the use of factors and biomaterials could support and ameliorate the native processes of tissue repair. Pre-clinical studies, now ongoing, will clarify whether these procedures can be used in clinical field to promote tissue regeneration without invasive instruments for human patients.

Index

M. Tatullo (ed.), *MSCs and Innovative Biomaterials in Dentistry*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-3-319-55645-1