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Cell-based strategies for periodontal regeneration

Xiangzhen Yan
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Cell-based strategies for periodontal regeneration

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te Zibo, P.R. China
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Cell-based strategies for periodontal regeneration

Doctoral Thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus, prof. dr. S.C.J.J. Kortmann,
according to the decision of the Council of Deans
to be defended in public on Monday, September 29, 2014
at 16.30 hours
by

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Wanxun Yang

Alexey Klymov
There is only one happiness in life -- to love and to be loved.

George Sand (1804-1876)

To my beloved family

谨以此书献给我的家人
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Chapter 1

General Introduction

Xiangzhen Yan
1. Periodontitis
The periodontium comprises the specialized tissues that both surround and support the teeth, including alveolar bone lining the tooth socket, root cementum, periodontal ligament (PDL), and part of the gingiva facing the tooth. Single or multiple components, and hence the structure of the periodontium can be damaged by periodontitis, which is a common inflammatory disease caused primarily by dental plaque microorganisms. The process of inflammation and the related degeneration of periodontal tissues ultimately may lead to periodontal defects and can potentially lead to tooth loss. Surveys have shown that approximately 48% of adults have chronic periodontitis.

2. Periodontal therapy
The goal of periodontal therapy is to prevent periodontal breakdown and to restore the destroyed tooth supporting tissues. Conventional treatment consists of scaling and root planing, which eradicate the clinical symptoms of the disease. These conventional treatments indeed prevent further disease progression, but the regeneration of already damaged periodontal tissues remains a clinical challenge. New therapeutic approaches include use of bone substitute materials and guided tissue regeneration (GTR) membranes. The purpose of using bone substitute materials is to restore the height of the alveolar bone around a previously diseased tooth. Although utilization of bone substitute materials (i.e. synthetic materials and grafts from different types of biological sources) may result in some gain in clinical attachment levels, careful histological assessment usually reveals that these materials have generally no osteoinductive capacity and become encapsulated in a dense fibrous connective tissue. Additionally, GTR has also been applied in clinical practice. The principle of GTR is to use membranes to exclude the faster proliferating epithelial cells and gingival connective tissue from entering the healing site, while promoting the slower proliferating PDL cells, cementoblasts and/or osteoblasts to repopulate the defect site. Still, the limited clinical and histological success, particularly in advanced stage periodontal defects, has led researchers to further explore alternative treatment strategies.

3. Cell-based strategies for periodontal tissue engineering
Periodontal tissue engineering, exploiting the synergistic combination of cells, scaffolds and biomolecules, has been applied as a promising alternative to conventional periodontal treatments.
Figure 1. Periodontal tissue engineering approaches for periodontal regeneration. Cells, scaffolds and biomolecules were combined and implanted to periodontal defects to regenerate the lost tissues.

3.1 Cells
It has been demonstrated that stem/progenitor cells, having the ability to self-renew and differentiate\textsuperscript{7}, are the key factors in tissue engineering\textsuperscript{8}. Since successful periodontal regeneration requires the reconstruction of new cementum, alveolar bone, and functional connective tissue fibers, stem/progenitor cells that are able to differentiate into cementoblasts, osteoblasts, and fibroblasts are needed for periodontal regeneration. Various cell types, including but not limited to periodontal ligament cells (PDLs), bone marrow mesenchymal stromal cells (BMSCs), alveolar periosteal cells (APCs), dental follicle cells (DFCs), and dental pulp cells (DPCs)\textsuperscript{9-11}, have been assessed in periodontal tissue regeneration and given rise to promising results. However, stem/progenitor cells are available in insufficient quantities to be used directly for clinical purpose. Consequently, maintaining the stemness of stem cells during \textit{in vitro} expansion has been recognized as a major challenge in stem cell-based research.

3.2 Scaffolds
Scaffolds or biomaterials offer tissue engineering a powerful tool in the form of cell and/or biomolecule delivery vehicles. Amongst the large group of biomaterials, chitosan has received major attention in tissue engineering and regenerative medicine due to its biocompatibility\textsuperscript{12}, biodegradability\textsuperscript{13, 14}, tissue regeneration capacity\textsuperscript{15}, anti-inflammatory effects\textsuperscript{16} and antimicrobial activity\textsuperscript{17}. Chitosan can be applied as a biomaterial in different forms, of which hydrogel vehicles are of great interest, as they offer several advantages including high cell seeding efficiency, easy handling and good formability to fill irregular defects\textsuperscript{18}. Chitosan hydrogels can be prepared by either chemical or physical crosslinking of chitosan chains. The
drawbacks of chemically crosslinked chitosan hydrogels include the potential cytotoxicity of applied chemical crosslinkers, such as glutaraldehyde, and the risk of altering the initial properties of chitosan through chemical modifications of the primary structure\textsuperscript{19, 20}. A possible means to avoid the aforementioned disadvantages is to prepare physically crosslinked chitosan hydrogels, which can be obtained by increasing the pH of acidic chitosan solutions without the use of chemical crosslinking agents\textsuperscript{13, 21, 22}. Recently, Chenite \textit{et al.}\textsuperscript{23} demonstrated that chitosan hydrogels can be formed homogeneously by neutralization of chitosan solutions using ammonia as produced \textit{in situ} from enzymatic hydrolysis of urea. Important aspects of this system, such as the effect of urea and urease concentrations on the kinetics of gelation and degradation were not addressed, while its potential for biomedical applications was not confirmed.

3.3 Biomolecules

Biomolecules/growth factors have an important role in regulating the proliferation, migration, and/or extracellular matrix synthesis of a variety of cell types, including those derived from the periodontium\textsuperscript{4}. The purified enamel matrix protein product, (Endogain\textsuperscript{®}; EMD), is the most commonly clinically used mix of biomolecules in periodontal practice. EMD is prepared from porcine tooth germs and although described to consist mainly of amelogenins\textsuperscript{24}, the exact content is complex and remains unknown\textsuperscript{25, 26}. Since the mechanism of EMD in promoting alveolar bone regeneration is still unclear, knowing the effect of EMD on gene expression of primary human bone cells would add important information.

4. Objectives of this thesis

The ultimate goal of periodontal therapy is the full regeneration of damaged or lost periodontal tissues to their original state and function. Therefore, the overall objective of the present thesis was to develop cell-based approaches for periodontal tissue engineering more potent for clinical periodontology. The effects of Stro-1, Wnt3a, chitosan hydrogels and EMD on cell behavior were investigated \textit{in vitro} and/or \textit{in vivo} to further understand how to transfer cell-based strategies from bench to bed-side.

More specifically, the following research questions were covered:
1. What is the efficacy of cell-based approaches in animal models for periodontal regeneration?
2. What is the current state-of-the-art in maintaining stemness of stem cells during \textit{in vitro} expansion?
3. What is the appropriate PDL cell population for clinical applications and how to improve its \textit{in vitro} expansion?
4. Can cross-linking for chitosan be enzymatically controlled and can this system be used for incorporation of PDLCS?
5. What is the in vivo biocompatibility and periodontal regenerative potential of enzymatically cross-linked chitosan hydrogels with or without incorporated PDLCS?
6. What is the mechanism of EMD in promoting alveolar bone regeneration?

5. References

Chapter 2

Cell-based approaches in the regeneration of supporting periodontal tissue: A systematic review and meta-analysis on periodontal defect models in animal experimental work

Xiang-Zhen Yan, Fang Yang, John A. Jansen, Rob B.M. de Vries, Jeroen J.J.P. van den Beucken

In preparation
Periodontitis, a multifactorial disease caused primarily by dental plaque microorganisms\textsuperscript{1}, is characterized by the destruction of the periodontium, which consists of the alveolar bone lining the tooth socket, the root cementum, the periodontal ligament (PDL), and part of the gingiva facing the tooth. The process of inflammation and related degeneration of periodontal tissues ultimately leads to periodontal defects. Without adequate treatment, periodontitis can eventually lead to tooth loss. Surveys have shown that approximately 48% of adults have chronic periodontitis\textsuperscript{1}. In addition, advanced periodontitis is more prevalent among the older age groups\textsuperscript{1,2}.

The ultimate goal of periodontal treatment is to prevent periodontal breakdown and to regenerate the destroyed periodontal tissues, including gingival connective tissue, PDL, alveolar bone and cementum\textsuperscript{3}. Conventional treatment for periodontitis consists mainly of oral hygiene instructions, scaling and root planing. This approach is usually successful in preventing further disease progression, but the regeneration of already lost tissues remains a clinical challenge\textsuperscript{6}. New therapeutic approaches include the introduction of bone grafts, alloplastic materials, guided tissue regeneration (GTR), and various growth factor-based therapies\textsuperscript{4}. Still, these strategies fail to regenerate completely and reliably all periodontal tissues damaged by severe periodontitis\textsuperscript{4,6}.

It has been postulated that stem/progenitor cells with the ability to self-renew and differentiate are the key factors in regenerative medicine\textsuperscript{7,8}. In view of this and to overcome the limitations of existing treatment, recent research has been focused on the development of cell-based approaches for periodontal regeneration. Cells can either be injected directly into the defect as a suspension or delivered by biomaterial scaffolds or cell carriers\textsuperscript{9-11}. Various cell types, including but not limited to PDL-derived cells, bone marrow mesenchymal stromal cells (BMSCs), alveolar periosteal cells (APCs), dental follicle cells (DFCs), and dental pulp cells (DPCs)\textsuperscript{12-14}, have been assessed for experimental periodontal tissue regeneration in a variety of animal models. Despite numerous publications in animal models, the efficacy of cell-based approaches for periodontal regeneration is still controversial. Some studies demonstrated that cell-based approaches had a favorable effect on periodontal tissue regeneration compared to their controls (e.g. cell-carrier alone group when cell carrier was applied)\textsuperscript{9,15-17}, while others reported that there were no significant differences between experimental groups with or without cells\textsuperscript{18-20}. 

Introduction
These conflicting results have raised doubt about the validity of cell-based approaches for the enhancement of periodontal regeneration. In order to increase the value of animal experimental work as proof-of-concept preparative evidence for clinical trials, systematic reviews have been proposed as the standard method for analyzing preclinical studies²¹. By means of a systematic review (particularly if it includes a meta-analysis), information relevant for evaluating the efficacy of treatments may be obtained that cannot be directly obtained from individual studies²²,²³. In the case of cell-based approaches for periodontal regeneration, a systematic review and meta-analyses of preclinical animal studies may provide valuable information for the implementation of cell-based approaches in clinical practice. Therefore, the purpose of this study was to systematically review and perform a meta-analysis regarding cell-based approaches for periodontal regeneration in animal studies to obtain clarity on their efficacy. In order to increase the attachment function of a tooth, the periodontal connective tissue fibers have to insert into newly formed bone. Given that alveolar bone is a main part of the tooth-supporting apparatus and new bone formation is essential to regeneration of PDL and cementum, the result of periodontal regeneration in the current meta-analysis was evaluated by new bone formation in periodontal defects.

**Materials and methods**

1. Search strategy and selection of the papers

Original articles concerning the effects of cell-based approaches in periodontal regeneration were searched using two databases, PubMed and Embase (via OvidSP). Three components were included in the search strategy: cell, periodontal regeneration, and animal (for complete search strategy see Table 1).

Search filters to detect all animal studies were utilized in both PubMed and Embase²⁴,²⁵. The search was performed on March 19, 2014. No language restriction was used and papers in other languages than English were translated by native speakers of that particular language within Radboudumc.

For the selection of studies, two investigators (XY and FY) independently screened the titles and abstracts of the publications based on the inclusion criteria. Full texts of papers that were considered eligible for inclusion were obtained for further independent evaluation.
<table>
<thead>
<tr>
<th>Component</th>
<th>PubMed</th>
<th>Embase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component 2: periodontal regeneration</strong></td>
<td>(exp periodontium) OR exp periodontal ligament/ OR exp alveolar bone/ OR exp alveolar ridge/ OR exp cementum/ OR exp alveolar bone loss/ OR (periodontium OR periodontiums OR periodontal OR PDL OR alveolar bone OR alveolar ridge OR cementum OR paradentum OR paradentums OR periodontiums OR periodontal OR PDL OR alveolar bone OR alveolar ridge OR cementum OR paradentum OR paradentums OR tooth socket OR tooth sockets OR (tooth OR dental) AND (supporting structures OR supporting structure OR ligament OR ligaments)) OR (alveolar AND atrophy) OR (alveolar AND atrophies) OR (alveolar AND resorption) OR (alveolar AND resorptions).ti,ab.) AND (exp regeneration/ OR exp healing/ OR exp rehabilitation/ OR (regeneration OR regenerations OR regenerative OR regenerated OR regenerating OR regenerate OR reconstructive OR reconstruction OR reconstructed OR reconstructions OR reconstructing OR recomposing OR recolonization OR recolonise OR recolonising OR recolonised OR recolonising OR recolonized OR recolonize OR recolonizing OR recolonized OR recolonize OR recolonizing OR OHEALING OR Haled OR heal OR Rehabilitation OR rehabilitated OR rehabilitate OR (newly OR new) AND (formed OR formation)) OR restoration OR restored OR restoring OR restored. ti,ab.)</td>
<td>Search filter for animal studies</td>
</tr>
<tr>
<td><strong>Component 3: Search filter for animal studies</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1 search strategy**
Articles were included if they studied the effects of cells on periodontal regeneration and their primary outcome measures focused on were the quantification of new bone, cementum, or PDL formation. The inclusion criteria were as follows: (1) The study was performed in animals in vivo; (2) Cell-based approaches should be used; (3) The study was an original paper (e.g. not a letter or review etc.); (4) The study assessed periodontal tissue regeneration; (5) A periodontal defect model (e.g. not subcutaneous implantation etc.) was used; (6) Not a duplicate article; only one publication was included in case a paper was found more than one time in one of the databases; (7) Data should be acquired comparatively to an appropriate control group (e.g. data from cell-carrier alone group when cell carrier was applied); (8) Histomorphometrical data should be presented for new bone, cementum or periodontal ligament formation; (9) Cells should be locally applied (e.g. not systemically applied cells). Criteria (1) to (5) were used only in initial screening phase based on titles and abstracts and criteria (1) to (9) were used in full-text selection phase. The inclusion criteria were specified in advance and documented in a protocol. Any disagreements between the two reviewers regarding inclusion of a certain publication were resolved by discussion.

tadvBMP-2, adenovirus-mediated BMP-2 gene-infected MSC+PF127 group; advbgal, adenovirus-mediated bgal gene-infected MSC+PF127 group; MSC, mesenchymal stem cell group; PF127, Pluronic F127 control group; PDLC, periodontal ligament cell; ABC, alveolar bone cell; GMC, gingival margin-derived cell; PDLSC, periodontal ligament stem cell; HA-TCP, hydroxyapatite-tricalcium phosphate; DBCB, deproteinized bovine cancellous bone; SRP, scaling and root planning; HAL, histological attachment level; JE, junctional epithelium length; CTA, connective tissue adhesion; SPDs, allogeneic stem cells isolated from miniature pig deciduous teeth; e-PTFE, e-polytetrafluoroethylene; DPSC, dental pulp stem cell; nHAC, nano-Hap-collagen; ALP, alkaline phosphatase; GF, gingival fibroblast; CDC, cementum-derived cell; ABBM, anorganic bovine bone mineral; DPSC, dental pulp stem cell; PAFSCs, periapical follicular stem cell; PRP, platelet-rich plasma; ACB, autogenous cortical bone; ECT, epithelium/connnective tissue extension; ASC, adipose tissue derived stem cell; APC, alveolar periosteal cells; AP-C-PLA, astragalus polysaccharides-chitosan/ polyactic acid; PLGA, Poly(DL-lactic-co-glycolic acid; OPG, osteoprotegerin.
Table 2: Study characteristics of the included studies.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Language</th>
<th>Species/strain</th>
<th>Age/weight</th>
<th>Sex</th>
<th>Defect type/size</th>
<th>Experimental groups</th>
<th>number of defects/group</th>
<th>Cell types</th>
<th>Cell passage number</th>
<th>Cell number/defect</th>
<th>Scaffold types</th>
<th>Duration</th>
<th>Quantification of newly formed tissues</th>
<th>Dropout/group + reason</th>
</tr>
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<tbody>
<tr>
<td>Akizuki 2005</td>
<td>English</td>
<td>Dog/beagle</td>
<td>3-year/8.8</td>
<td>F</td>
<td>Dehiscence/5 x 5</td>
<td>Cell + carrier/carrier</td>
<td>5</td>
<td>Canine PDLC</td>
<td>P4-6</td>
<td>1 x 10⁵</td>
<td>Hyaluronic acid sheets</td>
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<td>Length of NC, NB, connective tissue attachment</td>
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<td>Chen 2008</td>
<td>English</td>
<td>New Zealand white rabbit</td>
<td>3 kg</td>
<td>M</td>
<td>Alveolar bony defect/15 x 7 x 5</td>
<td>advBMP-2/advgal/MSC/ PF127</td>
<td>6</td>
<td>Rabbit BMSC</td>
<td>N</td>
<td>1 x 10⁶</td>
<td>Pluronic gel</td>
<td>6 weeks</td>
<td>Percentage of NB, NC, PDL</td>
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<tr>
<td>Dan 2014</td>
<td>English</td>
<td>Athymic rat/CBH-ru</td>
<td>12-week</td>
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<td>Blank/scaffold/GMC/ABC/PDL</td>
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<td>Minipig PDSC/PDL</td>
<td>N</td>
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<td>CaP-PCL scaffold</td>
<td>1 and 4 weeks</td>
<td>Percentage of NB</td>
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<td>Ding 2010</td>
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<td>N</td>
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<td>12 weeks</td>
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<td>Rat/SD</td>
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<td>N</td>
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<td>Scaffold/scaffold</td>
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<td>Rat BMSC</td>
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<td>12 and 24 days</td>
<td>Percentage of NB</td>
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<td>M</td>
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<td>9 - 12 months/40-45 kg</td>
<td>220-250 g</td>
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<td>7 weeks</td>
<td>10 kg</td>
<td>9.5 - 10.5 kg</td>
<td>12 - 20 months/10-14 kg</td>
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<td>Periodontitis defect/5 x 7 x 5 mm³</td>
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<td>Frenestration defect/2 x 3 mm²</td>
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<td>Class III furcation defect/5 x 5 x 4 mm³</td>
<td>3-wall infrabony defect/5 x 5 x 4 mm³</td>
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<tr>
<td>Experimental groups</td>
<td>Cell + DBCB + Cell + collagen/DBCB/collagen/SRP/blank</td>
<td>HA-TCP/HA-TCP + PDLCs/HA-TCP + SPDs</td>
<td>PDLCs + Gelfoam/Gelfoam/hair</td>
<td>Cell + clotting factors/clotting factors/blank</td>
<td>PDLCs + amnion/Amnion</td>
<td>PDLC + e-PTFE + b-TCP + cells/e-PTFE + b-TCP/e-PTFE</td>
<td>(0.2, 5, 10, 20) × 10⁶ cells</td>
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<td>Minipig PDLC/SPD</td>
<td>Rat PDLC</td>
<td>Human foreskin iPSC-MSC-like cells</td>
<td>Human PDLC</td>
<td>Canine PDLC</td>
<td>Canine periosteal cells</td>
<td>Canine BMSC</td>
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</tr>
<tr>
<td>Cell number/defect</td>
<td>2 × 10⁷</td>
<td>1 × 10⁶</td>
<td>1 × 10⁶</td>
<td>1.25 × 10⁶</td>
<td>N</td>
<td>9 × 10⁶</td>
<td>5 × 10⁶</td>
<td>(2, 5, 10, 20) × 10³</td>
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<tr>
<td>Scaffold types</td>
<td>DBCB and collagen</td>
<td>HA-TCP</td>
<td>Gelfoam</td>
<td>Fibrinogen + Amniotic membrane</td>
<td>PGA sheets</td>
<td>b-TCP</td>
<td>Atelocollagen</td>
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<tr>
<td>Duration</td>
<td>12 weeks</td>
<td>12 weeks</td>
<td>1, 2, 3, 4 weeks</td>
<td>2 weeks</td>
<td>4 weeks</td>
<td>6 weeks</td>
<td>6 weeks</td>
<td>1 month</td>
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<tr>
<td>Quantification of newly formed tissues</td>
<td>Attachment level; junctional epithelium length; connective tissue adhesion</td>
<td>Percentage of NB</td>
<td>Percentage of NB area, NB length, NC length</td>
<td>Percentage of NB</td>
<td>Percentage of NB, NC</td>
<td>Percentage of NC, PDL; NB, soft tissue, Amnion; Percentage of NC, PDL</td>
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<td>Age/weight</td>
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<td>Cell types</td>
<td>Cell passage number</td>
<td>Cell number/defect</td>
<td>Scaffold types</td>
<td>Duration</td>
<td>Quantification of newly formed tissues</td>
<td>Drop-outs/group + reason</td>
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</tr>
<tr>
<td>Khorsand 2013</td>
<td>English</td>
<td>Dog/mongrel</td>
<td>M</td>
<td>years/14‐22 kg</td>
<td>periodontal defect/3 x 5 x 8 mm³</td>
<td>Cell + carrier/cell + carrier</td>
<td>10</td>
<td>Canine DPSC</td>
<td>P3</td>
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<td>Bio‐Oss</td>
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<tr>
<td>Lang 1998</td>
<td>English</td>
<td>Minipig</td>
<td>F</td>
<td>4‐8 years</td>
<td>furcation and walled interdental</td>
<td>Cell carrier/cell + carrier</td>
<td>3 and 8</td>
<td>Minipig ABC</td>
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<td>Bone gelatin</td>
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<td>Li 2009</td>
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<td>F</td>
<td>12‐18 kg</td>
<td>Fenestration defect/5 x 5 mm³</td>
<td>Cell + carrier/cell + carrier</td>
<td>2009/9/8</td>
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<td>Li 2010</td>
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<td>N</td>
<td>10 kg</td>
<td>Class II furcation defect/N</td>
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<td>2008/8/6</td>
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<td>P3</td>
<td>3 x 10⁵</td>
<td>Collagen</td>
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<tr>
<td>Liu 2008</td>
<td>Chinese</td>
<td>Minipig</td>
<td>N</td>
<td>months/30‐40 kg</td>
<td>Periodontitis defect/3 x 7 x 5 mm³</td>
<td>Cell + carrier/cell + carrier</td>
<td>2007/7/6</td>
<td>Minipig PDLSC</td>
<td>P2‐4</td>
<td>2 x 10⁷</td>
<td>HA‐TCP</td>
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<td>Lu 2004</td>
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<td>Dog/mongrel</td>
<td>M</td>
<td>1‐2 years</td>
<td>Class II furcation defect/3.5 x 4 mm³</td>
<td>Cell + carrier/cell + carrier</td>
<td>3</td>
<td>Canine PDLSC</td>
<td>P3</td>
<td>9 x 10⁴</td>
<td>nHAC</td>
<td>4 weeks</td>
<td>5, 10, 14, 28 days</td>
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<td>English</td>
<td>Merino ewes</td>
<td>N</td>
<td>years/63.5‐72 kg</td>
<td>Dehiscence defect/10 mm deep</td>
<td>Cell + carrier/cell + carrier</td>
<td>1 and 2/no treatment</td>
<td>Human GF</td>
<td>P4‐6</td>
<td>9.5 ± 2.6 x 10⁴</td>
<td>Gelfoam</td>
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<td>weeks/230‐270 g</td>
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<tr>
<td>Age/weight</td>
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<td>1–2 years/14–22 kg</td>
<td>10 months/10 kg</td>
<td>15 kg</td>
<td>1.46 ± 0.18 years/10-20 kg</td>
<td>1.46 ± 0.18 years/10-20 kg</td>
<td>350 g</td>
<td>9-10 months/10 kg</td>
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<td>3-wall intrabony defect/3 x 4 mm²</td>
<td>3-wall intrabony defect/4 x 4 mm²</td>
<td>Periodontitis defect/3 x 3 mm²</td>
<td>Class II furcation defect/5 x 2 mm²</td>
<td>Class III furcation defect/5 x 2 mm²</td>
<td>Class II furcation defect/5 x 2 mm²</td>
<td>Class III furcation defect/5 mm height</td>
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<tr>
<td>Experimental groups</td>
<td>CDC + carrier/PDLC + carrier/carrier</td>
<td>Cell + carrier/carrier</td>
<td>PDLS/DPSC/AFSC/blank/no defect</td>
<td>Blank/PRP/ACB/PRP</td>
<td>Blank/GTR/Carrier + GTR + cell</td>
<td>Cell + carrier/carrier</td>
<td>Cell + carrier/carrier + blank</td>
<td>Cell + carrier/carrier + blank</td>
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<td>number of defects/group</td>
<td>8</td>
<td>9</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>2006/12/6</td>
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<td>Cell types</td>
<td>Canine CDC/PDLC</td>
<td>Canine BMSC</td>
<td>Canine PDLS/PDSC/P AFSC</td>
<td>Canine BMSC</td>
<td>Canine PDLC</td>
<td>Canine PDLC</td>
<td>Human BMSC</td>
<td>Canine ASC</td>
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<td>P4-5</td>
<td>P3</td>
<td>P2-3</td>
<td>N</td>
<td>N</td>
<td>P2-3</td>
<td>P4-5</td>
<td>P2</td>
<td></td>
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<td></td>
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<tr>
<td>number/defect</td>
<td>&gt; 0.75 x 10⁶</td>
<td>2 x 10⁷</td>
<td>6 x 10⁵</td>
<td>1 x 10⁷</td>
<td>3 x 10⁵</td>
<td>3 x 10⁵</td>
<td>1.5 x 10⁶</td>
<td>1.5 x 10⁷</td>
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<td>Scaffold types</td>
<td>Collagen sponge</td>
<td>ABBM</td>
<td>No</td>
<td>PRP</td>
<td>Collagen sponge</td>
<td>Collagen sponge</td>
<td>Peptide hydrogel + collagen sponge</td>
<td>PRP</td>
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<td></td>
<td></td>
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<tr>
<td>Duration</td>
<td>3 months</td>
<td>8 weeks</td>
<td>8 weeks</td>
<td>8 weeks</td>
<td>3 months</td>
<td>3 months</td>
<td>1 and 4 weeks</td>
<td>1 and 2 months</td>
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<tr>
<td>Quantification of newly formed tissues</td>
<td>Length of NB, dimension of NC</td>
<td>Percentage and length of NB, NC, PDL</td>
<td>Percentage and height of NB</td>
<td>Percentage of NB and NC</td>
<td>Length of NC, PDL, ECT, tissue free; area of NB, soft tissue, non-filled tissue</td>
<td>Length of NC, PDL, ECT, tissue free; area of NB, soft tissue, non-filled tissue</td>
<td>NB volume density, osteoclast number</td>
<td>Percentage of NB and NC</td>
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<td>Drop-outs/group reason</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
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</table>
2. Study characteristics

The following study characteristics were extracted: animal species, strain, sex, age and/or body weight of animals, defect type and size, description of experimental groups, number of defects per group, cell types, cell passage number, cell number per defect, cell carrier/scaffold types, duration of treatment, outcome measures, number of defects excluded from statistical analysis, reason for excluding samples.

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Reference</th>
<th>Language</th>
<th>Species/strain</th>
<th>Language</th>
<th>Species/strain</th>
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<tbody>
<tr>
<td>Rat/SD</td>
<td>Yang 2010</td>
<td>English</td>
<td>Nude rat</td>
<td>Yu 2013</td>
<td>English</td>
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<tr>
<td>Dog/beagle</td>
<td>Zhan 2008</td>
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<td>Dog/beagle</td>
<td>Zhao 2004</td>
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<td>Zhou 2012</td>
<td>English</td>
<td>Dog/beagle</td>
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<th>Experimental groups</th>
<th>Number of defects/group</th>
<th>Cell types</th>
<th>Cell passage number</th>
<th>Cell number/defect</th>
<th>Scaffold types</th>
<th>Duration</th>
<th>Quantification of newly formed tissues</th>
<th>Drop-outs/group + reason</th>
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<tbody>
<tr>
<td>M</td>
<td>10 kg</td>
<td>1-wall intrabony defect/5 x 5 mm³</td>
<td>PDLC + carrier/BMSC</td>
<td>4</td>
<td>Canine BMSC/PDL, BMSC</td>
<td>P3</td>
<td>5 (9-15) x 10³</td>
<td>Polyglycolic acid</td>
<td>8 weeks</td>
<td>Percentage of NB; length of NC, junctional epithelium; periodontal score</td>
<td>No</td>
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<tr>
<td>M</td>
<td>1 year/13 ± 2 kg</td>
<td>Horizontal defect/5 x 2 mm³</td>
<td>+ + +</td>
<td>10</td>
<td>Canine BMSC/PDL, BMSC</td>
<td>P3-5</td>
<td>2 x 10³</td>
<td>AP-C-PLA/PLA</td>
<td>4 and 8 weeks</td>
<td>Percentage of NB</td>
<td>No</td>
</tr>
<tr>
<td>M</td>
<td>8 weeks</td>
<td>Fenestration defect/1 x 3 mm³</td>
<td>+ + + + + + + + +</td>
<td>10</td>
<td>Canine BMSC/PDL, BMSC</td>
<td>Day 25</td>
<td>N</td>
<td>Microcarrier gelatin beads</td>
<td>3 weeks</td>
<td>Percentage of NB and NC; new ligament density</td>
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<tr>
<td>N</td>
<td>7 weeks</td>
<td>3-wall defect/2 x 1.7 mm³</td>
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<td>10</td>
<td>Canine BMSC/PDL, BMSC</td>
<td>P4</td>
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<td>Gelatin sponge</td>
<td>6 weeks</td>
<td>Percentage of NB, NC, PDL</td>
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<tr>
<td>N</td>
<td>18 months</td>
<td>Class II furcation defect/5 x 3 mm³</td>
<td>+ + + + + + + + +</td>
<td>10</td>
<td>Canine BMSC/PDL, BMSC</td>
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<td>5 x 10⁷</td>
<td>Collagen membrane</td>
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<td>Percentage of NB, NC</td>
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<td>N</td>
<td>250 - 300 g</td>
<td>Fenestration defect/3 x 2 mm³</td>
<td>+ + + + + + + + +</td>
<td>10</td>
<td>Canine BMSC/PDL, BMSC</td>
<td>P2/N</td>
<td>1 x 10⁷</td>
<td>PLGA</td>
<td>3 and 6 weeks</td>
<td>Percentage of NB, NC, connective tissue</td>
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<td>M</td>
<td>10 - 14 kg</td>
<td>Window defect/4 x 4 x 3 mm³</td>
<td>+ + + + + + + + +</td>
<td>10</td>
<td>Canine BMSC/PDL, BMSC</td>
<td>N</td>
<td>1 x 10⁸ cells/ml</td>
<td>PLGA</td>
<td>6 weeks</td>
<td>Height of NB, NC, connective tissue</td>
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<tr>
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<td>Yang 2010</td>
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<td>English</td>
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(Table 2). Bibliographic details such as author, year of publication and language were also registered.

3. Data extraction
The outcome measure used for the meta-analysis was the quantification of newly formed bone. For all studies, outcome data for experimental and control groups were extracted if mean, standard deviation (SD) or standard error (SE), and number of defects per group (n) were reported, or could be recalculated. If SE was reported, this SE was converted to SD for meta-analysis. If data were only presented graphically, data were re-measured based on the distances of figures using a universal on-screen digitizer software (Universal Desktop Ruler v3.6.3481, AVPSOft.com) when possible.

4. Data synthesis and statistical analysis
Data were analyzed using Review Manager Version 5.2 (Copenhagen, The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). Meta-analysis was performed for the outcome measure new bone formation, by computing the standardized mean difference (SMD). If a study contained two or more cell types, or outcomes were measured at several time points, these groups were analyzed as if they were separate experiments. However, if the same control group served more than one experimental group, the number of defects in the control group was divided by the number of experimental groups served. Forest plots were used to display individual and weighted overall effect sizes. Data were presented as SMD and 95% confidence intervals (CIs). To account for anticipated heterogeneity, a random effects model was used. I² was used as the measure of heterogeneity.

To explore possible causes for heterogeneity as well as to assess the influence of variables on cell-based strategy efficacy, subgroup analyses were performed. Only subgroups that contained more than three studies were included in the subgroup analyses, including animal species (dog, rat and minipig), sex (male and female), and cell types (PDL-derived cells and BMSC).

Publication bias was assessed by visually evaluating the possible asymmetry in funnel plots. Finally, to assess the robustness of the findings, a sensitivity analysis was performed for pooling outcome data using different units of measurement (percentage, length or volume of newly formed bone).

Results
1. Description of the included studies
The search strategy described in Table 1 retrieved 3742 papers (2011 papers in PubMed and 1731 papers in Embase). After initial screening based on titles and
abstracts, 116 papers were included for full text screening. After studying these full-text articles, 77 reports were excluded for reasons mentioned in Figure 1 and 39 studies were included (Figure 1). Three of the included 39 studies were translated, as these were published in Chinese.

Figure 1: Flow chart of study selection. The number of studies in each phase is indicated between brackets.

The characteristics of these studies are shown in Table 2, which varied substantially among the included studies. Six different animal species were used in the included studies, including 21 studies performed with dogs, 10 with rats, 5 with minipigs, and 1 with rabbits, mice, or merino ewes, respectively. Fourteen studies used only male animals; 8 studies used only female animals; 1 study used both male and female animals; and 16 papers did not mention the sex of animals. A variety of defects were induced, including surgical dehiscence (2 studies), fenestration (11 studies), intrabony (7 studies), furcation (13 studies) and defects induced by chronic inflammation (6 studies). Twelve different cell types were
evaluated in periodontal regeneration, including PDL-derived cells (19 studies), BMSCs (13 studies), gingival margin-derived cells (GMCs, 2 studies), alveolar bone cells (2 studies), stem cells isolated from deciduous teeth (1 study), iPS cells (2 studies), periosteal cells (2 studies), DPCs (2 studies), cementum-derived cells (2 studies), peripapical follicular stem cells (PAFSCs, 1 study), adipose-tissue derived stem cells (ASCs, 1 study), and follicular cells (1 study). Also the passage number and number of applied cells varied greatly between the studies, ranging from passage 1 to passage 7, and from $9 \times 10^4$ to $2 \times 10^8$ cells per defect, respectively.

Moreover, multiple types of cell carriers or biomaterial scaffolds were used in the included studies, including natural (e.g. collagen, platelet-rich plasma, etc.) and synthetic biomaterials. The included synthetic biomaterials were ceramics (e.g. hydroxyapatite-tricalcium phosphate), polymers (e.g. polyglycolic acid), and composites (e.g. apatite-coated silk).

2. Meta-analysis of outcome measure bone formation

Thirty-eight studies of the included 39 studies evaluated the effect of cell-based strategies on new bone formation in periodontal regeneration, 37 of which could be included in the meta-analysis, as in one study the number of defects per group was not reported and could not be retrieved by contacting the authors$^{38}$. The analysis contained 60 experiments or experimental groups, including data from 647 defects. In 30 experiments, the SMD and 95% CIs indicated that implantation of cells significantly increased new bone formation in the defect area (Figure 2). None of the studies reported a statistically significant negative effect of cell implantation on new bone formation. In the remaining 30 experiments, no statistically significant results were reported. Overall analysis showed that cell-based strategies enhance new bone formation in periodontal regeneration, as displayed by the global estimate SMD and its 95% CIs ($1.59 [1.20, 1.98]$). However, overall study heterogeneity was moderate to high ($I^2 = 66$).
Figure 2: Forest plot of the included studies. The forest plot displays relative weight of the individual experiments, the standardized mean difference (SMD), and 95% confidence intervals (CIs). The diamond indicates the global estimate and its 95% confidence interval.

No statistically significant differences between the subgroups were found for any of the examined variables (species, sex, cell type; Table 3). The heterogeneity
within the subgroups was in the same range as the heterogeneity of the overall analysis. Therefore, the variables examined in the subgroups cannot explain the overall heterogeneity.

Table 3: subgroup analysis of the included studies.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Studies</th>
<th>Participants</th>
<th>Effect Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>31</td>
<td>355</td>
<td>1.60 [1.06, 2.13]</td>
</tr>
<tr>
<td>Rat</td>
<td>16</td>
<td>148</td>
<td>1.09 [0.49, 1.70]</td>
</tr>
<tr>
<td>Minipig</td>
<td>10</td>
<td>108</td>
<td>2.14 [1.16, 3.12]</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>151</td>
<td>2.48 [1.53, 3.44]</td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>229</td>
<td>1.44 [0.72, 2.15]</td>
</tr>
<tr>
<td>PDL-derived cells</td>
<td>23</td>
<td>242</td>
<td>2.00 [1.28, 2.71]</td>
</tr>
<tr>
<td>BMSC</td>
<td>18</td>
<td>206</td>
<td>2.01 [1.27, 2.76]</td>
</tr>
</tbody>
</table>

The effect estimate was displayed by the standardized mean difference (SMD) and 95% confidence intervals (CIs).

3. Publication bias and sensitivity analysis
The presence of publication bias was assessed for the outcome measure of new bone formation by visual analysis of funnel plots. Figure 3 indicates that small, negative studies appeared to be underrepresented. To assess the robustness of our findings, sensitivity analysis was carried out by separately assessing the different units of measurement for expressing new bone formation (the percentage, length or volume of newly formed bone). The effect sizes did not significantly differ between these groups (data not shown).

Discussion
The aim of this study was to systematically evaluate the current evidence for the efficacy of cell-based approaches for the regeneration of periodontal supportive tissue in animal studies. The results of this systematic review and meta-analysis revealed that cell-based approaches have a favorable effect on periodontal tissue formation, as displayed by the positive effect of cell-based approaches on new bone formation. Moreover, the meta-analysis did not provide evidence for a difference between PDL-derived cells and BMSC in the enhancement of new bone formation.
Figure 3: Funnel plot of the included studies. Solid blue lines are to guide the eye. The largest studies are plotted near the average (the dashed line), and small, negative studies appeared to be underrepresented (within the red box).

The currently used approach of systematic review allowed the inclusion of a large number of studies, which enabled us to perform the meta-analysis and explore the effect of several subgroup variables. However, there are some potential limitations. First, preferably, all experiments should be performed in a similar manner when their results are being combined in a meta-analysis. However, the publications display experimental variability for the utilized animal species and sex, the periodontal defect type and size, the used cell types and passage number, the amount of cells per defect, the biomaterials applied as cell carrier, and the healing time after cell transplantation. Not surprisingly, substantial heterogeneity of the evaluated experiments was found, which might be explained in part by experimental variability. To account for the heterogeneity, a random effects model was used. Moreover, subgroup analyses (animal species, sex, and cell types) were performed in an attempt to explain this heterogeneity, but these subgroup analyses did not notably reduce the heterogeneity. Second, the results of the meta-analysis may be subject to publication bias, as visual analysis of funnel plots revealed that small negative studies appeared to be underrepresented. The true effect of cell-based approaches may therefore be smaller than the effect found in our meta-analysis. Importantly, funnel plot asymmetry can either result from non-
publication of negative results, or be caused by other factors, such as true study heterogeneity, or differences in study quality. However, the study quality assessment (assessment of risk of bias) has not been performed yet and therefore it is not sure to what extent the obtained results are overestimations of the true effect. Despite these limitations, the combined analysis of the included studies still generated extra and valuable information that could not be derived from individual studies.

One of the most important issues for clinical application of cell-based approaches is the type of cell used. As shown in this systematic review, PDL-derived cells and BMSCs are the mostly used cell types for preclinical trials and hence subgroup analyses of these two cell types were performed. Previous studies compared the regenerative potential between PDL-derived cells and BMSCs, and the results suggested that PDL-derived cells are the more suitable cell population for periodontal regeneration, as significantly more well-oriented periodontal PDL fibers, newly formed bone and cementum were observed upon transplantation of PDL-derived cells. However, the subgroup meta-analysis revealed that PDL-derived cells and BMSC had a similar effect on the enhancement of bone formation. Consequently, for the implementation of cell-based approaches in clinical practice, either PDL-derived cells or BMSC can be applied based on the specific condition of each individual patient (e.g. accessibility of cells and health condition of donor tissues).

The meta-analysis provided evidence for the enhancement of new bone formation in periodontal defects by implantation of either PDL-derived cells or BMSC. It has to be emphasized that treatment of periodontal defects requires a large number of cells (9 × 10⁴ to 2 × 10⁸ cells are needed for one defect), which sometimes would be difficult to obtain from a single human subject. Although the in vitro expansion of stem/progenitor cells is necessary, those cells typically reduce their ability to self-renew and proliferate during passaging. Therefore, other sources of stem/progenitor cell types need to be sought. Among the evaluated 12 different cell types, GMCs, iPS cells, and ASCs are potential alternative sources. GMCs, which can be readily obtained during oral surgery, have mesenchymal stem cell properties and can differentiate into osteogenic phenotype under certain inductive conditions. In addition, iPS cells, obtained after transfection of certain stem cell-related genes into adult somatic cells, have the ability to differentiate into a variety of cell types. Moreover, ASCs can be recovered easily in large numbers by liposuction under local anesthesia and show large similarity with BMSCs regarding gene expression and osteogenic capacity. Regarding these three cell types, however, only few studies were included in this systematic review (2, 2, and 1 for
GMCs, iPS cells, and ASCs, respectively). Both iPS cells\textsuperscript{17, 31} and ASCs\textsuperscript{52} have given promising results in periodontal regeneration compared to cell-carrier only groups, while GMCs\textsuperscript{18, 19} did not promote any significant periodontal regeneration. Nevertheless, to further ensure the efficacy of the aforementioned cell types in periodontal regeneration, additional studies are required in future experimental work.

Cell passage number is another important factor for clinical application of cell-based approaches. For instance, primary PDL-derived cells used at early passages have the advantage of maintaining the rich phenotypic and functional heterogeneity of fibroblasts characteristic of the original tissue\textsuperscript{63}. Though, characteristic changes of primary cells have been observed during passage. Alkaline phosphatase (ALP) activity of PDL-derived cells gradually decreased and the expression of tendo/ligamentogenesis-related genes was down-regulated as the passage number increased\textsuperscript{64}. However, in the current review, only less than half of the included studies (18 out of 39) provided information on exact cell passage number, which made it difficult to do the subgroup analysis based on cell passage numbers. Therefore, it is highly recommended that the passage number of implanted cells is clearly reported in future studies.

Animal studies from this systematic review indicate that cell-based approaches are effective for new bone formation in periodontal defects, providing some support for the implementation of cell therapy in clinical practice as a routine treatment in the future. Nevertheless, the longest evaluation time within the included studies was 12 weeks, for which long-term in vivo studies are required to ensure the pre-clinical safety. Moreover, animal models cannot mimic some fundamental features: the spontaneous emergence of periodontal defects caused by periodontitis (periodontal defects are mostly surgically generated), genetic background, and risk factors (e.g. aggressive bacterial flora, tobacco, systemic diseases of host, etc.)\textsuperscript{6}. Consequently, these features can only be evaluated in future clinical trials.

Conclusion

The current systematic review and meta-analysis indicates that, based on animal experimental work involving periodontal defect models, cell-based therapies have a favorable effect on new bone formation in periodontal defects compared to cell-carrier only therapies. Moreover, the meta-analysis showed equal performance for therapies involving PDL-derived cells or BMSC regarding the enhancement of new bone formation. These results provide important information for the implementation of cell-based approaches in clinical practice as a routine treatment in the future.
References

Chapter 3

Biomaterial strategies for stem cell maintenance during \textit{in vitro} expansion

Xiang-Zhen Yan, Jeroen J.J.P. van den Beucken, Sanne K. Both, Pi-Shan Yang, John A. Jansen, Fang Yang

Tissue Eng Part B Rev. [Epub ahead of print]
1 Introduction
Stem cells, having the ability to self-renew and give rise to multiple cell types,\(^1\) are the key factors in both developmental biology and regenerative medicine. In the last decade, an increasing interest in research on stem cells and their clinical applications has become apparent. For therapeutic applications, stem cells are firstly obtained from either early-stage embryo or adult tissues, expanded in vitro and transplanted back into patients in order to treat disease or injury (Figure 1). The most frequently studied stem cells include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), bone marrow mesenchymal stem cells (BMSCs), and adipose tissue-derived stem cells (ASCs).

![Figure 1: Schematic diagram of stem cell-based therapies. Stem cells can be obtained from either early-stage embryo or adult tissues, expanded in vitro and transplanted back to patients.](image)

Human ESCs, derived from the early-stage embryo, are one of the most widely studied cell sources for regenerative medicine (Figure 2). ESCs exhibit the ability to differentiate into variety of specialized cell types and hence represent a unique opportunity for tissue engineering and regenerative medicine. For example, controlled human ESC differentiation could result in an improved vision for patients with macular degeneration.\(^2\) However, their clinical application is mainly limited by their ethical concerns. Recently, human iPSCs, free of ethical and political issues, have been produced by transfection of certain stem cell-related
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Genes into adult somatic cells and they have gained great interest in regenerative medicine (Figure 2). These cells are similar to natural pluripotent stem cells in morphology and have the ability to differentiate into a variety of cell types. Nevertheless, one limitation of human iPSCs is that the efficiency of successfully reprogrammed cells has been incredibly low. The low efficiency rate necessitates in vitro expansion prior to clinical use of iPSCs. Other concerns involve the difficulties in homogeneous cellular differentiation to specific cell types and the in vivo properties of immortal cells such as the tumorigenic fate of teratoma-initiating iPSCs.3

Figure 2: Increasing research in developing new therapies with stem cells in tissue regeneration by using keywords "ESCs, iPSCs, BMSCs or ASCs" and "tissue regeneration" in Web of Science.

Mesenchymal stem cells (MSCs) are relatively safe and have been isolated from a variety of tissues, e.g. bone marrow,4,5 adipose tissue,6 dental pulp,7 hair follicles,8 dermis,9 heart,10 liver,11 and spleen.12 There has been an increase in adult BMSC research in tissue regeneration (Figure 2). Transplanted BMSCs can accelerate healing in human cutaneous wounds,13 repair infarcted human myocardium,4 chronic lower extremity wounds,5 and induce the formation of sufficient new bone to enable the reliable placement of dental implants.14 Nevertheless, the bone marrow harvest procedure is complex. ASCs have become one of the most popular stem cell populations in stem cell-based regeneration research (Figure 2), as
adipose tissue can be harvested in larger quantities with less invasive methods. The research to date has tended to focus on their potential for clinical applications. For instance, use of expanded ASCs is a safe and effective treatment for complex perianal fistula\textsuperscript{6} and depressed scars.\textsuperscript{15}

Beside MSCs, other somatic stem cells play essential roles in regenerative medicine. For instance, the transplantation of peripheral blood stem cell is beneficial for acute myeloid leukemia;\textsuperscript{16} transplantation of neural stem cells (NSCs) can enhance synaptic plasticity, reduce neuronal loss, and improve cognition in animal models of Alzheimer's disease;\textsuperscript{17} hematopoietic stem cell (HSC) transplantation leads to rapid improvement of clinical symptoms and quality of life in IL-10- and IL-10 receptor-deficient patients and corneal epithelial stem cell therapy using \textit{ex vivo} expanded autologous cells proves successful in the treatment of unilateral limbal stem cell deficiency.\textsuperscript{18}

Despite promising clinical applications, stem cells are usually found in low numbers, and their response to aging typically diminishes their ability to self-renew and proliferate.\textsuperscript{19} To be effective for therapeutic applications, large numbers of stem cells are needed. For example, for bone tissue engineering, 160 million cells would be required for 20 cubic centimeter of tissue engineered implant based on using 8 million cells/cm\textsuperscript{3} scaffold\textsuperscript{20,21} to gain substantial bone formation in the case of large bone defects. In the case of treating chronic ischemic heart disease by stem cell injection, lack of diffusion of the transplanted cells could also result in low cell delivery efficiency\textsuperscript{22}, thus high numbers of cells are required. Nevertheless, the fate of stem cells is doomed the same way: the pluripotency of ESCs is affected by the number of passages\textsuperscript{23} and mitochondrial dysfunction has been found to occur with prolonged culture of ESCs;\textsuperscript{24} hemangioblasts/blast cells derived from human iPSCs have been shown to exhibit limited growth and expansion capability and early senescence with decreased hematopoietic colony-forming capability;\textsuperscript{25} significant decreases in the proliferation and differentiation potential of murine and human BMSCs were observed during \textit{in vitro} expansion;\textsuperscript{26-28} the expression of stemness biomarkers in human ASCs decreased significantly during long-term manipulation, along with the decrease of differentiation ability (adipogenesis, osteogenesis, and neurogenesis).\textsuperscript{29}
Figure 3: Stem cell fate is regulated by various factors including genetic influences, cell-cell communications, growth factors and cytokines, extracellular matrix (ECM, e.g. component contents, topography/architecture), and physiochemical environment (e.g. matrix stiffness, oxygen tension, mechanical forces, electrical cues).

Taken together, the main question is how to maintain the stemness of stem cells during in vitro culture. If this problem can be solved, then a large number of high quality cells could be obtained for clinical purposes. Control of stem cell fate has been well reviewed,30-35 but unfortunately there is limited research on how to increase stem cell expansion while maintaining their potential. As shown in Figure 3, stem cell fate is regulated by varied factors including genetic influences, cell-cell communications, growth factors and cytokines, extracellular matrix (ECM; e.g. component contents, topography/architecture), and physiochemical environment (e.g. matrix stiffness, oxygen tension, mechanical forces, electrical cues). This review mainly focuses on how to maintain the stemness of stem cells by exploiting biomaterial properties.

2. Miscellaneous approaches for stem cell maintenance
Figure 3 and Table 1 show that stem cell fate is regulated by various factors and accordingly, the stemness of stem cells can be maintained by a variety of approaches including but not limited to gene transduction, growth factors and cell-cell interactions. Transduction of various genes can regulate the fate of tissue-specific adult stem cells and embryonic pluripotent cells.36-41 For example, MSCs transduced with
human telomerase (hTERT) can undergo more than 260 population doublings without losing their osteogenic potential, whereas control cells became senescence after 26 population doublings.\textsuperscript{42,43} Despite promising results, several challenges remain to be overcome. First, genetically modified cells lack the long-term expression of the transgene,\textsuperscript{44,45} which will significantly limit the clinical and research applications. Second, most current gene delivery strategies are based on viral vectors in order to achieve the stable delivery of genetic information into eukaryotic genomes, which may pose a significant risk to the patients' immune system.\textsuperscript{46} Third, with gene delivery strategies there exists the risk of interrupting intrinsic genes by random insertion of vector sequences.\textsuperscript{47,48} Moreover, the procedure of gene transduction is relatively complex. Besides gene transduction, the external signals that control stem cell fate collectively make up the stem cell microenvironment, or niche. The niche saves stem cells from depletion, while protecting the host from over-exuberant stem-cell proliferation.\textsuperscript{49} Therefore, \textit{in vitro} recapitulation of the \textit{in vivo} stem cell niche, which constitutes secreted factors, cell-cell interactions and ECM,\textsuperscript{50} provides a promising approach in reprogramming stem cells for therapeutic purposes.
# Table 1

## Miscellaneous approaches for stem cell maintenance

<table>
<thead>
<tr>
<th>Stimuli parameters</th>
<th>Cell types</th>
<th>Observations</th>
<th>References</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic modifications</td>
<td>Human and murine ESCs</td>
<td>The differentiation potential of ESCs can be maintained by the overexpression of Oct4, Sox2, Nanog, Ronin and Zfx</td>
<td>149-153</td>
<td>- Lack of the long-term expression of the transgene</td>
</tr>
<tr>
<td></td>
<td>Human MSCs</td>
<td>Knockdown of p21 enhances proliferation, and osteogenic capacity in human MSCs; MSCs transduced with hTERT can undergo more than 260 population doublings without losing their osteogenic potential</td>
<td>42, 43, 154</td>
<td>- Risk to the patients’ immune system</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Risk of interrupting intrinsic genes complex</td>
</tr>
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<td>Cell-cell communications</td>
<td>Human ESCs</td>
<td>Various types of feeder cells can support the undifferentiated growth of human ESCs</td>
<td>65, 155</td>
<td>- Risk of infectious agent contamination</td>
</tr>
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<td></td>
<td>Murine HSCs</td>
<td>Endothelial cells can retain their self-renewal and repopulation ability</td>
<td>63, 64</td>
<td>- Senescence of feeder cells</td>
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<td>Growth factors and cytokines</td>
<td>Human ESCs</td>
<td>Wnt3a and bFGF can retain human ESCs in undifferentiated state</td>
<td>55, 57, 58, 156</td>
<td>- Cumbersome, expensive and time-consuming process</td>
</tr>
<tr>
<td></td>
<td>Human MSCs</td>
<td>Wnt3a increase proliferation and inhibit osteogenic differentiation and IL-6 preserves the undifferentiated state of human MSCs</td>
<td>52, 60</td>
<td>- Risk of interrupting the genetic events of normal cells</td>
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<td>Murine HSCs</td>
<td>Wnt3a can sustain the self-renewing fate of HSCs with reduced differentiation</td>
<td>51</td>
<td>- Risk of malignancy</td>
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<tr>
<td>Biomaterials mimicking ECM components</td>
<td></td>
<td></td>
<td></td>
<td>- High production costs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>See Table 2 and 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topography/architecture</td>
<td>Murine ESCs</td>
<td>Nanofibers morphologically mimicking the ECM support the proliferation and self-renewal of murine ESCs</td>
<td>131-133</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------</td>
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<td>---------</td>
<td></td>
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<tr>
<td>Human MSCs</td>
<td></td>
<td>Nanostructured surface with square lattice symmetry can retain stem-cell phenotype and maintain the growth of human MSCs</td>
<td>134</td>
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<td>Matrix stiffness</td>
<td>Murine ESCs and muscle stem cells, human MSCs</td>
<td>Substrates which match the stiffness of their own microenvironments promote their self-renewal ability and maintain their stemness</td>
<td>140-142</td>
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<td>Oxygen tension</td>
<td>Varied types of cells</td>
<td>Hypoxia maintains undifferentiated states of embryonic, mesenchymal, hematopoietic, and neural stem cell phenotypes</td>
<td>Reviewed in 129</td>
<td></td>
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<tr>
<td>Mechanical forces</td>
<td>Human ESCs</td>
<td>Biaxial cyclic strain promotes self-renewal and retains pluripotency of human ESC</td>
<td>143</td>
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<td>Electrical cues</td>
<td>Murine NSCs</td>
<td>Continuous and defined levels of electric current promotes NSC proliferation (2 fold)</td>
<td>146</td>
<td></td>
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<tr>
<td>Human MSCs</td>
<td></td>
<td>Pulsed electromagnetic field exposure could enhance cell proliferation; Polyelectrolyte hydrogel matrices can support long-term survival and enhance proliferation (1.5 fold) of human MSCs.</td>
<td>147, 148</td>
<td></td>
</tr>
</tbody>
</table>

ESCs: embryonic stem cells; MSCs: mesenchymal stem cells; HSCs: hematopoietic stem cells; NSCs: neural stem cells; ECM: extracellular matrix; hTERT: human telomerase; IL-6: interleukin-6.
Growth factors and cytokines hold promise for the maintenance of stem cells due to their ease of application. Wnt proteins, bFGF, transforming growth factor (TGF)-beta’s and their family members, and interleukin-6 (IL-6) have shown their effects on stem cell maintenance. Yet, to maximize their clinical potential, the dosage should be optimized before their clinical administration, because the effects of growth factors on stem cells are dose dependent. For example, low doses of BMP-4 significantly increase the survival of human ASCs and retain their stemness as well as multipotency, whereas high doses increase apoptosis and reduce cell proliferation. In another respect, growth factors and cytokines may influence the genetic events of normal cells and eventually lead to malignancy. Moreover, high production costs of growth factors still exist and safe alternatives to expensive growth factors are desirable for clinical use.

The interactions between stem cells, as well as interactions between stem cells and neighboring differentiated cells, could regulate stem cell fate. However, there are still several distinct drawbacks of using cell-cell interaction strategies. First, there is a potential of infectious agent contamination when using animal-derived cells for clinical use. Second, feeder cells used for ESCs also undergo senescence and lose stem cell supportive properties after several passages. Third, this process is cumbersome, expensive and time-consuming. Last but not least, the specific mechanisms by which feeder cells support the undifferentiated growth of stem cells has not been defined. This supporting effect is likely not caused by direct cell-cell contact but by certain growth factors secreted by feeder cells. For instance, human ESCs could not be maintained by using human feeder cells lacking the ability to synthesize basic fibroblast growth factor (bFGF), as indicated by the significant decreased expressions of Oct-4 and Nanog in human ESCs cultured in bFGF-knockout group compared with those in the control.

Due to the limitations of gene transduction, growth factor, and cell-cell interaction approaches, increasing emphasis has been focused on stem cell ECM for stem cell maintenance. Decellularized ECM from porcine synovium-derived stem cells (SDSCs), a 3D matrix consisting of nanostructured fibers with collagen I as one of the major structural proteins, facilitated SDSCs regaining stem cell phenotypes. An increase in cell number from 2.7 to 14.6-fold and an enhanced chondrogenic capacity were observed compared with SDSCs plated on plastic flasks. Similar results were observed in human BMSCs and tendon stem cells (TSCs) when the cells were expanded using decellularized ECM from human BMSCs and tendon tissues respectively. The ECM which exists as a mix of several different proteins...
provides instructive cues for cell fate decisions, primarily via the integrin family of cell surface adhesion receptors. Those instructive cues can be categorized as biochemical signals (e.g. ECM components) and biophysical cues (e.g. topography and stiffness). Thus biomaterials designed by controlling parameters including components, material architecture, surface topography, mechanical and electrical properties may act as an artificial ECM to direct stem cell fate.

**Figure 4: Biomaterial strategies in vitro expansion of stem cells.** Two dimensional (2D) and three dimensional (3D) cell culture systems developed by the use of biomaterials, including both natural and synthetic origins, for the expansion of stem cells. The modifications of biomaterials include topography/architecture, mechanical properties and biochemical factors.

### 3 Biomaterials for stem cell maintenance
The use of biomaterials, from both natural and synthetic origins, to develop two dimensional (2D) and three dimensional (3D) cell culture systems for the expansion of pluripotent stem cells (Table 2) as well as adult stem cells (Table 3) is summarized in this section. The modifications of biomaterials in terms of topography/architecture, mechanical properties and biochemical factors are also described (Figure 4).

#### 3.1 Overview of biomaterials

#### 3.1.1 Natural biomaterials
In vitro cell culture is usually carried out on flat substrates, which hold the advantages as a simplified approach to identifying the effect of individual niche components on stem cell fate. The common technique of growing cells on tissue culture polystyrene is gradually being replaced by culturing cells on substrates with a more appropriate composition. Stem cells can sense and respond to ECM molecules through the interaction between integrins on the cell surface and cognate ligand-binding motifs on ECM molecules. The ECM is mainly composed of collagens, laminins, and glycoproteins serving as substrates for a variety of adhesion molecules such as integrins. The integrins are a major family of ECM receptors that transmit information from the matrix to cells, thereby playing a key role in the regulation of cell behavior, including cell survival, adhesion, proliferation, and differentiation. Designing biomaterials mimicking ECM is a relatively economic and safe approach to regulate the fate of stem cells. Considering that most cells require adhesion to an ECM for survival and growth through ECM-integrin interaction, natural biomaterials consisting ECM components have been tested for stem cell expansion.

**Matrigel**

Matrigel is a gelatinous protein mixture and commercially available product comprised of several ECM components, such as laminin, type IV collagen, and heparan sulfate proteoglycan. Matrigel served as the first feeder-free culture system in which undifferentiated human ESCs could be maintained for 130 population doublings. Human ESCs showed successful expression of pluripotency genes and the surface markers of pluripotency proteins during passaging. It should be noted that the stemness of human ESCs can be maintained on Matrigel in mouse embryonic fibroblast conditioned medium, yet ESCs on Matrigel without conditioned medium completely differentiated after two passages. Several other chemically defined culture media formulations have been developed to support the undifferentiated proliferation medium of human ESCs on Matrigel substrate in feeder-free conditions. Still, variety of growth factors and cytokines are required in these defined culture medium.

For adult stem cells, Matrigel significantly improved cell expansion of MSCs by 1.6-fold through surface modification of tissue grade polystyrene. Nevertheless, Matrigel is derived from mouse tumor cells and may cause pathogen transmission to humans. Growth of stem cells under xeno-free conditions will benefit the later clinical applications.
<table>
<thead>
<tr>
<th>Biomaterials</th>
<th>Cell types</th>
<th>2D/3D</th>
<th>Feeder layer</th>
<th>Conditioned medium</th>
<th>undifferentiated cells can be maintained</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrigel, vitronectin</td>
<td>Human ESCs and human iPSCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>25 passages</td>
<td>77</td>
</tr>
<tr>
<td>Matrigel, a combination of collagen IV, fibronectin, laminin and vitronectin</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>20 passages</td>
<td>57</td>
</tr>
<tr>
<td>Matrigel</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>9 and 22 passages</td>
<td>78</td>
</tr>
<tr>
<td>Matrigel, Collagen IV</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>Yes</td>
<td>130 population doublings</td>
<td>76</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>Yes</td>
<td>7 passages</td>
<td>80</td>
</tr>
<tr>
<td>Laminin-111, 332 and -511</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>Yes</td>
<td>10 passages</td>
<td>82</td>
</tr>
<tr>
<td>Laminin-511</td>
<td>Murine ESCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>31 passages</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Human ESCs and iPSCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>20 passages</td>
<td>85</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>7 passages</td>
<td>87</td>
</tr>
<tr>
<td>Natural</td>
<td>Human iPSCs</td>
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<td>No</td>
<td>10 passages</td>
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</tr>
<tr>
<td>Calcium alginate hydrogels</td>
<td>Human ESCs</td>
<td>3D</td>
<td>No</td>
<td>No</td>
<td>260 days without passaging in the undifferentiated stage</td>
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<tr>
<td>PAS</td>
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<td>No</td>
<td>10 passages</td>
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</tr>
<tr>
<td>Heparin-binding peptides</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>17 passages</td>
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</tr>
<tr>
<td>Synthetic</td>
<td>APMAAm</td>
<td>2D</td>
<td>No</td>
<td>No</td>
<td>20 passages</td>
<td>97</td>
</tr>
<tr>
<td>PMEDSAH</td>
<td>Human ESCs</td>
<td>2D</td>
<td>No</td>
<td>No</td>
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<tr>
<td>sIPN</td>
<td>Human ESCs</td>
<td>3D</td>
<td>No</td>
<td>Yes</td>
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<td>HA</td>
<td>Human ESCs</td>
<td>3D</td>
<td>No</td>
<td>Yes</td>
<td>20 days</td>
<td>107</td>
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</table>

ESCs: embryonic stem cells; iPSCs: induced pluripotent stem cells; PAS: peptide-
acrylate surfaces; APMAAm: aminopropylmethacrylamide; PMEDSAH: poly{2-(methacryloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide}; sIPN: semi-interpenetrating polymer network; HA: hyaluronic acid;

**Table 3 Biomaterials in adult stem cell expansion**

<table>
<thead>
<tr>
<th>Biomaterials</th>
<th>Cell types</th>
<th>2D/3D</th>
<th>Observations</th>
<th>References</th>
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<td>2D</td>
<td>Improved cell expansion (1.6-fold) and enhanced neuronal differentiation</td>
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<td>Collagen types I and IV together with laminin and fibronectin</td>
<td>Human haematopoietic progenitors</td>
<td>2D</td>
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<tr>
<td>Collagen types I and IV together with laminin and fibronectin</td>
<td>Human BMSCs</td>
<td>2D</td>
<td>Enhanced proliferation capacity (2 to 4-fold) and increased chondrogenic and osteogenic potentials</td>
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</tr>
<tr>
<td>Decellularized ECM from human BMSCs</td>
<td>Human BMSCs</td>
<td>2D</td>
<td>Amplified HSCs compartment in BMSCs during culture</td>
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<td>ECM from tendon tissue</td>
<td>Human TSCs</td>
<td>2D</td>
<td>Reduced TSC population doubling time (15%) and preserve the stemness of TSCs</td>
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<tr>
<td>ECM from tendon tissue</td>
<td>Human TSCs</td>
<td>2D</td>
<td>Reduce TSC population doubling time (15%) and preserve the stemness of TSCs</td>
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<tr>
<td>Decellularized ECM from human BMSCs</td>
<td>Human BMSCs</td>
<td>2D</td>
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<td>Porcine SDSCs</td>
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<td>Human platelets lysate gel</td>
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<td>Connecting segment-1</td>
<td>Human HSCs</td>
<td>2D</td>
<td>Higher CD34⁺ cell expansion (6-fold)</td>
<td>95</td>
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<td>Synthetic</td>
<td>Human HSCs</td>
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MSCs: mesenchymal stem cells; HSCs: hematopoietic stem cells; ECM: extracellular matrix; BMSCs: bone marrow mesenchymal stem cells; TSCs: tendon stem cells; SDSCs: synovium-derived stem cells

**Collagen**
Collagen, the main component of connective tissues, is a classic natural material for tissue engineering and regenerative medicine. Collagen I, which is well defined, a component of several FDA-approved products and widely available, has recently been tested as a potential hESC growth biomatrix. Collagen I is reported to support the undifferentiated growth of human ESCs for 7 passages.\textsuperscript{80} Human ESCs grown on Collagen I processed a comparable population doubling rate as human ESCs grown on Matrigel. These cells expressed pluripotency proteins and retained a normal karyotype.\textsuperscript{80} Furthermore, collagen IV has been shown to support the undifferentiated growth of human ESCs, although the cultures on collagen IV did not contain as many undifferentiated colonies as the cultures on Matrigel.\textsuperscript{76} It has to be noted that these culture systems still need the presence of conditioned medium from feeder layers, which is full of growth factors.\textsuperscript{76,80}

For adult stem cells, tissue culture surfaces coated with collagen types I and IV together with laminin and fibronectin increased CD34\(^+\) (1.3-fold) and CD41\(^+\) (1.2-fold) cell expansions, which provide a better environment for the ex vivo expansion of haematopoietic progenitors.\textsuperscript{81}

**Laminin**

Laminin, a major component of the ECM, is found in the basal lamina. Recombinant human laminins have been investigated for stem cell culture due to the advantage that they are abundantly available and well-characterized human-origin proteins.\textsuperscript{82} More than 15 laminin isoforms have been identified\textsuperscript{83} and human ESCs, commonly expressed abundant integrin α\textsubscript{6}β\textsubscript{1}, bind predominantly to laminin-111, -332 and -511.\textsuperscript{82} Consequently, human ESCs cultured on laminin-111, -332 and -511 substrates can retain an undifferentiated state for 10 passages without feeder layers.\textsuperscript{82} However, similar as for collagen substrates, these culture systems need the presence of conditioned medium, for which they are feeder-free but not xeno-free.\textsuperscript{82} Without conditioned medium or feeder cells, Domogatskaya et al. reported that recombinant human laminin-511 but not -332, -111 alone was sufficient to maintain the self-renewal of mouse ESCs for up to 31 passages.\textsuperscript{84} Later, the same research group reported that this xeno-free system could also support the undifferentiated growth of human ESCs and iPSCs for at least 20 passages, indicating great potential for therapeutic purposes.\textsuperscript{85} In addition, laminin also benefits ex vivo culture of murine hematopoietic stem cells (HSCs) as it amplified HSCs compartment in BMSCs during culture.\textsuperscript{86}
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Vitronectin

Vitronectin, an abundant secreted glycoprotein found in serum and the ECM, was shown to promote human ESC attachment through interaction with αvβ5 integrin. Braam et al. reported that recombinant vitronectin was a functional alternative to Matrigel, supporting human ESC sustained self-renewal and pluripotency in defined, nonconditioned medium for at least 1 month (7 passages). Later other groups also reported that vitronectin could support the undifferentiated expansion of human ESCs with a variety of defined medium. The expanded human ESCs maintained differentiation capacity and expressed pluripotency markers as strongly as the human ESCs cultured on feeder layers. More recently, human iPSCs were successfully maintained their stemness for 10 passages when cultured on vitronectin coated dishes in xeno-free medium. However, the response of MSCs and ESCs is different in fate determination when cultured on vitronectin substrate. For example, contact with vitronectin promotes the osteogenic differentiation of human MSCs. Thus, the results obtained from one cell type cannot simply be transferred directly to other types of cells.

Although the results show great potential, natural biomaterials in general present several limitations when used as cell culture systems. First, they are usually not well-defined and difficult to control at the molecular level, and therefore may contribute to the variability in results. Second, the difficulty in purification and sterilization makes the manipulation more elaborate and complicated. Third, natural biomaterials may invoke immunogenic responses following implantation due to the structure similarity to biological substances. Thus, well-defined synthetic materials with controlled properties may be safer for clinical applications.

3.1.2 Synthetic biomaterials

Widespread clinical applications of stem cells require chemically defined materials that are in low-cost, with tunable physical properties and long-term stability. For that reason, defined synthetic biomaterials have been developed to replace the complicated natural materials due to the advantages of ease of scale-up and decreased risks of disease transmission from unknown pathogens. Recently synthetic peptides and polymers have been developed for stem cell expansion.

Synthetic peptides

ECM molecules are usually quite large and present diverse domains for cell adhesion. Biologically active peptides are organic compounds, which serve as a
synthetic alternative to ECM proteins. Synthetic peptides hold the advantages of stability, ease of synthesis and conjugation to materials. Melkoumian et al. reported that human ESCs could be successfully maintained on synthetic peptide-acrylate surfaces (PAS) in chemically defined xeno-free medium for over ten passages with similar phenotypic marker expression as cells cultured on Matrigel.93 Here amine-containing peptides derived from active domains of ECM including bone sialoprotein, vitronectin, fibronectin and laminin were conjugated to PAS to provide a synthetic alternative to complex ECM. At the same time, Klim et al. screened over 500 unique surfaces based on 18 bioactive peptides by using an array to present bioactive peptides either alone, in different combinations or at varying surface densities.94 The results showed that the surfaces presenting heparin-binding peptides that recognizes cell surface glycans supported the long-term growth of human ESCs and maintained their pluripotency markers for more than three months with a defined medium.94 For adult stem cells, surface-immobilization of adhesion peptides on substrate benefits ex vivo expansion of HSCs from umbilical cord blood.95,96 HSCs cultured on synthetic substrates surface-immobilized with peptides containing the connecting segment-1 binding motif (EILDVPST) showed 2 to 5-fold greater expansion of HSCs and more pluripotent colony-forming units than those on fibronectin-grafted and polyamine-grafted dishes, suggesting that the specific interaction between HSCs and connecting segment-1 helps to maintain the pluripotency of HSCs during the ex vivo expansion.96

**Synthetic polymers**

Preadsorption of proteins or peptides to substrates increases cost and limits scalability. Recently the long-term self-renewal of human ESCs was reported to be successfully maintained on hydrogel interfaces of aminopropylmethacrylamide (APMAAm) for over 20 passages in chemically-defined media without the prior attachment of any biological coatings such as peptides, proteins or Matrigel™.97 As the interface was not functionalized with proteins or peptides to promote cell adhesion, it was identified that bovine serum albumin (BSA) adsorbing from the culture media played a key role in human ESC attachment to APMAAm interfaces.97 Villa-Diaz et al. developed another standardized and fully defined synthetic polymer coating, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), which could sustain the undifferentiated growth of human ESCs in commercially available defined medium for 10 passages.98 In this case, six polymer coatings by surface-initiated graft polymerization were synthesized and tested and only PMEDSAH supported
attachment and proliferation of human ESCs. However, the mechanism of this phenomenon is not clear and the contribution of various physicochemical properties (such as wettability, stiffness, surface topography and zeta potential) of PMEDSAH still need to be determined. Those findings demonstrate that chemically-defined, synthetic biomaterials can be applied to propagate ESCs. However, no report is available concerning their effects on the self-renewal of adult stem cells based on our literature survey. Moreover, the mechanisms by which the substrates function remain largely unclear.

3.2 Biomaterials for 3D cell culture
The aforementioned 2D culture systems present useful methods for stem cell expansion, but there are certain limitations. Cells grown on 2D substrates exhibit an unnatural dorsal/ventral polarized state which is in contrast to the situation in vivo, where cells are completely embedded in a 3D microenvironment. Accordingly, 3D cell culture models, which elicit a more physiological state, provide a promising alternative for monolayer cell culture. Cells grown in a 3D environment display more similarities to in vivo cells in morphology and molecular regulation.\textsuperscript{99} Compared to the 2D culture, 3D culture provides another dimension for external mechanical inputs and cell adhesion, which affects integrin ligation, cell contraction, and associated intracellular signaling.\textsuperscript{100, 101} Thus, developing 3D culture systems have been evaluated for stem cell maintenance. In 3D cell culture models, cells are embedded in biomaterials, cultured as cellular spheroids, or grown on 3D scaffold materials.\textsuperscript{102} Biocompatible 3D scaffolds have been well reviewed by Moroni et al. for tissue engineering applications.\textsuperscript{103} However, no such scaffold has been reported to be utilized as 3D system for stem cell expansion. This is most likely because the pattern of cell seeding, attachment, and growth on these 3D scaffolds is similar to that seen on 2D surfaces. Thus this review mainly focuses on cell encapsulation and cellular spheroid methods for the expansion of stem cells.

3.2.1 Cell encapsulation
Embedding cells in semi-permeable materials is the most common approach for 3D cell culture. A completely synthesized ECM hydrogel composed of a semi-interpenetrating polymer network (sIPN) was prepared from poly(N-isopropylacrylamide-co-acrylic acid) [p(NIPAAm-co-AAc)].\textsuperscript{104} To promote cell adhesion, the polymer network was interpenetrated by polyacrylic acid-graft-ACGNGEPRGDTRYRAY-NH2 [p(AAc)-g-RGD] linear polymer chains, which binds to several integrin receptors, including $\alpha_1$, $\alpha_v$, $\beta_1$ and $\alpha_5\beta_3$.\textsuperscript{105} By varying the polymer
components and the density of the network crosslinker, sIPNs with a range of cell-adhesion ligand densities and matrix stiffness could be created. Although specific cell-matrix interactions were promoted by the peptide sequences presented in the sIPN, conditioned medium from fibroblast feeder layers was still needed to maintain self-renewal of human ESCs. In another study, hyaluronic acid (HA) hydrogel has been tested for controlling human ESC self-renewal. The rationale to choose HA was that the HA content is highly present in undifferentiated cells and decreases at the onset of differentiation. It was found that human ESCs cultured in HA could maintained their undifferentiated state, preserved their normal karyotype, and maintained their full differentiation capacity as indicated by embryoid body formation for 20 days. Afterwards, as the cells remodeled the hydrogel, colonies near the surface were released, making it difficult to accurately quantify cell proliferation as well as for the long-term maintenance of stem cells.

For the expansion of adult stem cells, Walenda et al. demonstrated a novel 3D matrix fabricated from human platelet lysate (HPL) gel for the expansion of MSCs. As HPL is human origin, it can be used as a substitute for fetal calf serum without the risk of xenogeneic immune reactions or transmission of bovine pathogens. This gel culture system facilitates a two-fold increase in proliferation rate and enhanced colony forming units outgrowth compared with tissue culture plastic for culture expansion of MSCs.

### 3.2.2 Cellular spheroids

Cellular spheroids which often mimic the in vivo situation more closely than a cell encapsulation system provide an alternative 3D culture system. Cellular spheroids are aggregate cells that grow free of foreign materials. Spheroids may contact with culture materials, but no foreign material is present within spheroids. For example, human ESCs formed spheroids by encapsulation in calcium alginate hydrogels. The encapsulated human ESCs retained an undifferentiated state and their pluripotency in a feeder layer-free and xeno-free environment for up to 260 days. ESCs increased in size and number without escaping the confines of the hydrogel for the duration of the cultures. Furthermore, these encapsulated ESCs were easily recovered from the hydrogels by using a dissolution buffer.

For adult stem cells, the methods which have been reported to induce cellular spheroids include spinner flasks and rotating wall vessel bioreactor, microwells, non-adherent surface, hanging drops, forced-aggregation technique and micropatterned surfaces. Moreover, spheroid formation of several cell types has been reported on chitosan films. The gene
expression of self-renewal markers (i.e. Oct4, Sox2, and Nanog) in MSCs and ASCs were significantly higher for the cells in spheroid in comparison to the monolayer cells,\textsuperscript{109, 120} indicating the cells in spheroid experienced a probably de-differentiating and returned to a more primitive state.\textsuperscript{109}

The 3D culture systems provide promising results for the \textit{in vitro} expansion of stem cells. However, the mechanisms of cellular interaction within these systems are not clear. It is often difficult to clarify the direct effects of the biomaterial itself from the diffusional limitations common to all 3D culture. Limitations in the diffusion of soluble factors might account for some of the differences frequently observed between 2D and 3D culture systems.\textsuperscript{124} For instance, the stem cell maintenance might be caused by the oxygen gradients (owing to the low solubility of oxygen in aqueous media) which could attribute to the higher expression of self-renewal marker genes.\textsuperscript{125, 126} All human nucleated cells can sense oxygen tension and respond to a reduced O\textsubscript{2} concentration (hypoxia).\textsuperscript{127} The conventional \textit{in vitro} cell culture is performed under 21% oxygen tension.\textsuperscript{128} On the other hand, stem cell niches are usually located in regions of low oxygen tension.\textsuperscript{125, 126} For example, the oxygen concentrations of mesenchymal, neural, and hematopoietic stem cell niches are between 1% and 8%.\textsuperscript{129} It has been well reviewed by Mohyeldin et al. that hypoxia maintains undifferentiated states of embryonic, mesenchymal, hematopoietic, and neural stem cell phenotypes.\textsuperscript{129} These studies indicate that optimization of oxygen tension for each type of stem cells is essential for their stemness maintenance during \textit{in vitro} expansion. Future work is directed to clarify the influence of biophysical and biochemical cues of biomaterials on stem cell fate determination which may help to reduce the complexity and develop advanced biomaterials for stem cell expansion.

### 3.3 Modification of biomaterials for stem cell maintenance

#### 3.3.1 Topography/architecture

The cell/material interface has been shown to exert considerable influence on stem cell function and differentiation.\textsuperscript{130} Recently, polymeric nanofibers morphologically mimicking the ECM/basement membrane were found to support the proliferation and self-renewal of mouse ESCs.\textsuperscript{131, 132} Additionally, Markert et al. screened 504 different types of topographical surface microstructures in order to identify specific topography that can support the expansion of undifferentiated murine ESCs without feeder cells or conditional medium.\textsuperscript{133} The results of this study indicated that stem cells are affected mainly by the lateral and vertical dimensions of the microstructures, while the distribution of the topographical
patterns is less important. This finding is of great importance for the design of biomaterials for stem cell maintenance. For adult stem cells, a nanostructured surface with square lattice symmetry has been identified to retain stem-cell phenotype and maintain the growth of adult MSCs. It is likely that such nanoscale features alter the interaction of integrin receptors within cell adhesions, resulting in changes in intracellular tension. Significant progress has been made in designing biomaterials to enhance stem cell differentiation. From the opposite point of view, one can take advantages from these studies and uses them to fabricate novel biomaterials to inhibit stem cell differentiation and facilitate stem cell maintenance. For example, disordered nanoscale structures can stimulate human MSCs to produce bone mineral in vitro, even in the absence of osteogenic supplements, while the ordered square nanostructures can retain their stem-cell phenotype and multipotency. Thus design of novel biomaterial platforms based on the current knowledge on biomaterials and cell differentiation may enhance the in vitro expansion of stem cells.

Taken together, these studies demonstrate that nanoscale patterning can serve as a powerful tool for the manipulation of stem cells. As most studies focused on ESCs, more research work on how to maintain the potential of tissue-specific adult stem cells by controlling the topography of their substrates should be explored in future.

3.3.2 Biomechanical factors
Stem cells can “feel” and act in response to the stiffness of the substrates. Tissue culture plates, usually used for culturing adherent cells, are much stiffer than the cell microenvironments in vivo. Substrate stiffness has been identified as a vital parameter to determine stem cell fate. For instance, rigid matrices that mimic collagenous bone are osteogenic, while soft substrates which matches the intrinsic stiffness of ESCs promote self-renewal and pluripotency of ESCs. Substrates mimicking muscle stiffness regulate self-renewal, enhance survival, prevent differentiation and promote stemness of muscle stem cells. Polyacrylamide gels that mimic the elasticity of bone marrow can maintain MSCs in a quiescent state. Accordingly, it is safe to hypothesize that stem cells cultured on substrates that imitate the stiffness of their own microenvironments may be a promising system to promote their self-renewal ability and maintain their stemness.

In addition to stiffness, there is mounting evidence that external mechanical stimulation plays critical roles in stem cell fate determination. For instance, Saha
et al. applied biaxial cyclic strain to a deformable elastic substratum upon which the human ESC colonies were cultured. Results showed that human ESC self-renewal was promoted as measured by an increase in self-renewal gene expression (Oct4 and SSEA-4); the pluripotency of human ESCs was retained as evidenced by their ability to differentiate to cell lineages in all three germ layers. Thus the application of mechanical forces may be useful towards stem cell maintenance for therapeutic applications.

3.3.3 Biochemical factors

Some biochemical factors have also been reported for the efficient in vitro expansion of stem cells. For example, short-wave UV/ozone radiation treatment on conventional tissue culture plates yields chemically defined surface with high intensities of several secondary ions (e.g., C\textsubscript{2}H\textsubscript{2}O\textsuperscript{+} and C\textsubscript{2}H\textsubscript{2}N\textsuperscript{+}) which are favorable to human ESC colony formation. This chemically defined surface generates more than three times the number of cells than feeder-containing substrates per surface area, which will finally facilitates cell therapeutic applications. Another example is that human MSCs maintained a stem cell phenotype when cultured on −CH\textsubscript{3} modified glass surfaces, but processed osteogenic differentiation when cultured on −NH\textsubscript{2} and −SH modified glass surfaces.

3.3.4 Bioelectrical factors

The behavior of in vitro cultured cells could also be influenced by bioelectrical cues through application of electrical or electromagnetic currents/fields to stimulate substrate and/or cell construct. For this purpose, Chang et al. developed a biphasic current stimulator chip for stem cell culture. This stimulator chip could generate both positive and negative currents in the same culture chamber. Further experiment indicated that this culture system could double NSC proliferation by applying continuous and defined levels of electric current. For human MSCs, pulsed electromagnetic field exposure could enhance cell proliferation during the exponential phase and it possibly resulted from the shortening of the lag phase. Recently, Lim et al. developed polyelectrolyte hydrogel based multifunctional matrices, which provide not only 3D structural support to the embedded cells but also dynamic electrical and mechanical cues to the human MSCs. These anionic hydrogels could undergo reversible, anisotropic bending dynamics in an electric field by changing the concentration of anionic groups within the hydrogel. Thanks to their close resemblance to the native cellular environment in multiple aspects, these new 3D electro-mechanical matrices were shown to support human MSC survival for at least 21 days and enhance cell proliferation by 1.5-fold.
4 Conclusions
To facilitate the translation of stem cells from bench to bedside, this manuscript reviews recent developments regarding the diverse properties of biomaterials for stem cell expansion. It has to be noted that cell fate is influenced by a variety of factors in combination. Robust, fast, safe, and cost-effective methods using defined biomaterials may reconstruct the in vivo niche of stem cells, which will finally benefit both researches and routine clinical purpose. The development of 3D culture systems by using biomaterials recapitulating the in vivo stem cell niche will probably provide better microenvironments for stem cells compared to the traditional 2D monolayer culture systems. New insights should be gained into the modifications of biomaterials such as the topography, stiffness, and surface chemistry. Though progress has been made to maintain the self-renewal ability of stem cells, we are still far from the large-scale expansion of undifferentiated stem cells for therapeutic purpose. In the future, biomaterials should be designed to present a combination of physical and chemical factors to expand stem cells. Moreover, the ultimate function of expanded cells requires in vivo evaluation to confirm their regenerative capacity, for example by transplantation of expanded cells into animal models. This needs collaboration among many disciplines including cell and molecular biology, biomaterials, pharmacology, nanotechnology and medicine.
Chapter 3

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Chapter 3
Chapter 4

Human periodontal ligament derived progenitor cells: effect of STRO-1 cell sorting and Wnt3a treatment on cell behavior

Xiang-Zhen Yan, Sanne K. Both, Pi-Shan Yang, John A. Jansen, Jeroen J.J.P. van den Beucken, Fang Yang
Introduction
Periodontitis is a multifactorial disease caused primarily by dental plaque microorganisms. Periodontitis is characterized by the destruction of the periodontium, including gingiva, periodontal ligament (PDL), cementum and alveolar bone. Without adequate treatment, periodontitis will finally lead to tooth loss, which often affects nutrition intake and self-confidence. Approximately 48% of adults have chronic periodontitis and advanced periodontitis is more prevalent among the older age groups. Current treatments are generally successful in preventing active disease, but the regeneration of the lost tissues remains a challenge. Recently, substantial progress has been made in periodontal tissue regeneration by cytotherapeutic approaches to overcome the limitations of existing procedures.

Several cell types have been used for periodontal regeneration including periodontal ligament cells (PDLCs), bone marrow stromal cells (BMSCs), alveolar periosteal cells (APCs), dental follicle cells (DFCs), and dental pulp cells (DPCs). Tsumanuma et al. transplanted PDLCs, BMSCs, and APCs in canine one-wall intrabony defects for eight weeks and results showed that significantly more newly formed cementum and well-oriented PDL fibers were formed in the PDLCs group than in the other groups. Besides, in an organ culture study performed on tooth root surfaces, new alveolar bone and PDL-like tissues were formed only by PDLCs but not by DFCs, DPCs, or BMSCs. These results indicate that PDLCs may be the most suitable cell source for periodontal tissue regeneration.

STRO-1, one of the most well-known mesenchymal stem-cell markers, has gained increasing interest in stem cell sorting over the past decade. For instance, STRO-1 has been utilized for the selection of PDL stem cells, dental pulp stem cells, and adipose-derived stem cells. STRO-1 positive PDL stem cells are usually utilized for research purpose and their potential to regenerate periodontal tissues in vivo has been reported. Since PDLCs contain subpopulations of stem cells, the heterogeneous unsorted PDLCs have also been shown to promote periodontal tissue formation. The sorted stem cells in high purity might provide a better cell source for therapeutic purposes compared with the heterogeneous unsorted cells. But STRO-1 positive cells are usually found in low numbers and therefore in vitro expansion is needed. However, the expression of STRO-1 was gradually lost during culture expansion, as suggested in previous studies. Yet, the comparison between unsorted parental cells and the expanded STRO-1 sorted cells (equal expansion as the parental cells) has never been reported. Moreover, from a practical point of view, the cell selection and expansion procedure is time-consuming. Thus, it is of importance to compare
unsorted parental cells and the expanded STRO-1 sorted cells from PDLCs in order to benefit their future clinical applications. Large numbers of cells are needed for effective therapeutic applications. For instance, 160 million cells would be required for 20 cubic centimeter of tissue engineered bone implant based on using 8 million cells/cm³ scaffold to gain substantial bone formation. PDLCs are easily accessible but the cell number is very limited from primary cell culture, and hence requires in vitro expansion before clinical applications. Yet characteristic changes of PDLCs have been observed during passaging. Alkaline phosphatase (ALP) activity of PDLCs gradually decreased as the passage number increased. Thus finding a method that can benefit the efficient in vitro expansion of PDLCs is required.

The cellular signaling pathways that control the proliferation of PDLCs are unclear. A promising candidate is the Wnt signaling, which is involved in tooth development. Wnt signaling affects tooth size and induces continuous tooth generation in mouse. As reported in a previous study, in a continuously erupting tooth, cells with the highest level of Wnt responsiveness also show the highest proliferation. Wnt3a, a representative canonical Wnt member, has been recently isolated as an active Wnt molecule. Wnt3a can enhance clonal outgrowth of neural stem cells and promote long-term expansion of mammary stem cells. Furthermore, Wnt signaling has been suggested to play an essential role in osteogenesis in vitro and in vivo. For example, Wnt3a suppressed osteogenic differentiation of MSCs. However, whether Wnt3a can directly control the proliferation and differentiation of adult human PDLCs is not known.

The aim of this study was to evaluate the effect of STRO-1 cell sorting and Wnt3a treatment on cell behavior of human PDLCs. To this end, STRO-1 positive PDLCs were sorted and then the sorted cells were expanded and compared with their unsorted parental cells in terms of proliferation, colony forming unit (CFU) and mineralization. Thereafter, human PDLCs were treated with or without Wnt3a and the cell proliferation, self-renewal, and osteogenic differentiation were evaluated. It was hypothesized that Wnt3a can benefit the efficient in vitro expansion of human PDLCs by enhancing cell proliferation and self-renewal, while inhibiting osteogenic differentiation.

**Materials and methods**

*Cell isolation*

All experiments were done by following national guidelines for working with human materials (Dutch federation of biomedical scientific societies. Human tissue and medical research: code of conduct for responsible use. Available at:
http://www.federa.org/). After patients had signed informed consent, adult PDLCs from 3 patients (20, 24 and 33 years old respectively) were obtained from healthy impacted third molars, which were routinely extracted for the prevention of third molar–related morbidity. The PDL was scraped from the middle third of the roots, dissected, and placed in a 25 cm² culture flask in proliferation medium, which contained α-MEM (Gibco) supplemented with 10% FCS (Gibco) and 100 units/ml pen/strep (Gibco). Bone was harvested from the posterior maxilla of 1 patient (41 years old) during dental implant surgery. Bone debris was retrieved from the drill surface and chopped into small pieces and put into a 50 ml tube. The tube with minced bone debris and proliferation medium was shaken vigorously and medium with human bone marrow stromal cells (hBMSCs) was collected and plated in a 25 cm² culture flask. Upon 70-80% confluency, PDLCs and hBMSCs were sub-cultured for 3 passages and afterwards cryo-preserved in α-MEM containing 20% FCS and 10% DMSO (Sigma).

Cell characterization

After defrosting, the morphology of human PDLCs was observed by an inverse phase contrast microscope (Leica DMIL, Germany) at ×10 magnification. The self-renewal and osteogenic ability of PDLCs from the 4th passage were characterized by colony-forming unit (CFU) efficiency, ALP activity and mineralization ability (Von-Kossa staining). CFU assay was performed on day 10 in proliferation medium. ALP activity of PDLCs was measured on day 8 in osteogenic medium (proliferation medium containing 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone, all from Sigma). Human fibroblasts from foreskin (a kind gift from Department of Orthodontics and Oral Biology, Radboud University Medical Centre) and hBMSCs were used as negative and positive control for ALP activity assay. Von-Kossa staining was performed after 30 days of culture in osteogenic medium. All the assays are described in detail below.

STRO-1 fluorescence-activated cell sorting and testing

The cryo-preserved human PDLCs from 2 donors were defrosted and performed for cell sorting experiments. Single PDLC suspensions containing 10 × 10⁶ cells from the 4th passage were obtained through a 70μm cell strainer (Falcon BD, Franklin lakes, NJ, USA). Next, the cell suspensions were centrifuged at 400 g for 10 min, resuspended in 1 mL PBS/1%BSA, and preincubated for 20 min on ice. Subsequently, cells were incubated with 200μL mouse anti-human monoclonal STRO-1 IgM primary antibody (2.5 μg/10⁶ cells, R&D Systems, Minneapolis, MN) in PBS/1%BSA for 15 min on ice, and washed 3 times with PBS followed by centrifugation at 250 g for 5 minutes. The cell pellet was resuspended in 200μL PBS/1%BSA with 10μL phycoerythrin (PE)- conjugated goat anti-mouse IgM
antibody (R&D Systems, Minneapolis, MN) for 30 min on ice, washed for 3 times, resolved in 400μL of PBS, and kept on ice until sorting. For blank control, PBS was substituted for the primary and second antibodies. To confirm the specificity of primary antibody binding, non-specific mouse IgM isotype control (lambda monoclonal, abcam) which ideally matches the primary antibody’s host species was substituted for the primary antibody. All incubations were performed in the dark at 4 °C. Cells were sorted using a FACStar Plus flow cytometer (Beckton Dickinson & Co., Mountain View, CA). Positivity was defined as a level of fluorescence greater than 99% of the blank (without 1st and 2nd antibody) and negative (without 1st antibody) control. To prove the efficacy of the applied FACS sorting, sorted cells and controls (STRO-1+/STRO-1−/un-sorted cells) were evaluated by fluorescence microscopy and flow cytometry. STRO-1+ cells were collected and expanded to 10 x 10^5 cells in proliferation medium. The expanded cells were compared with their unsorted parental cells in terms of proliferation (DNA content on day 2, 4 and 6 in proliferation medium), CFU ability (day 10 in proliferation medium), and mineralization capacity (calcium content on day 30 in osteogenic medium). All the assays are described in detail below.

**Direct effect of Wnt3a on PDLCs**

Based on the STRO-1 cell sorting results, unsorted PDLCs at 4th passage were used in this study. PDLCs from 3 donors were treated with or without 50 ng/ml Wnt3a (R&D Systems). The dosage was chosen according to a previous study in which 50 ng/ml Wnt3a promoted long-term expansion of mammary stem cells. Then, DNA content, gene expression (self-renewal gene markers Oct4, Nanog and Sox2, and osteogenic gene markers ALP, Runx-2 and OC), ALP activity and calcium content were evaluated. All the assays are described in detail below.

**Functionality of PDLCs pretreated with Wnt3a**

For a long-term study, human PDLCs from passage 4 were subcultured with or without 50 ng/ml Wnt3a up to passage 9, and at each passage the cell doubling time was calculated. After pretreatment with or without Wnt3a for 5 passages, the functionality of Wnt3a-pretreated and control cells were compared in terms of proliferation (DNA content), CFU, and osteogenic differentiation (ALP activity and calcium content). All the assays are described in detail below.

**DNA assay**

After 2, 4 and 6 days of incubation in proliferation medium, samples were prepared by washing the cells layers twice with PBS and adding 1 ml of MilliQ to each well, after which repetitive freezing (-80 °C) and thawing (37 °C) cycles were performed. DNA analysis was performed via a PicoGreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands) following manufacturer’s
instructions. Briefly, 100 μL of DNA standard or sample was incubated with 100 μL of working solution for 10 min at RT in the dark. After incubation, DNA content was measured using a fluorescence microplate reader (Bio-Tek Instruments, Abcoude, The Netherlands) with excitation filter 485 nm and emission filter 530 nm.

**ALP activity**
Cells in osteogenic medium were harvested at day 7 and 10 the same way as cells for the DNA content. Then, 100 μL of substrate solution (p-nitrophenyl phosphate) was added to 20 μl of buffer (0.5 M 2-amino-2-methyl-1-propanol) and 80 μl of sample or standard in a 96-well plate. The standards were made by serial dilutions of 4-nitrophenol at final concentrations of 0–25 nM. The plate was incubated at 37°C for 1 hour. The reaction was terminated by adding 100 μL of 0.3 M NaOH. The absorbance of each well was measured in an ELISA microplate reader (Bio-Tek Instruments, Abcoude, The Netherlands) at 405 nm. ALP activity was normalized to the amount of DNA.

**Calcium content**
After 30 days of culture in osteogenic medium, cells were washed twice with PBS and then 1 ml of 0.5 N acetic acid was added to the each well. The tissue culture plate was incubated overnight on a shaking table. The calcium content was measured by the ortho-cresolphthalein complexone (OCPC) method (Sigma). For the biochemical assay, 10 μL samples or standards were incubated with 300 μL of working solution (Genzyme Diagnostics, Cambridge, MA, USA) in a 96-well plate. Standards (0-100 μg/ml) were generated using a CaCl₂ stock solution. The plate was incubated at RT for 10 min and then the absorbance of each well was measured in the ELISA microplate reader (Bio-Tek Instruments, Abcoude, The Netherlands) at 570 nm.

**Von Kossa staining**
After 30 days of osteogenic induction, cells were fixed in 10% formalin for 20 min, rinsed with MilliQ and then stained with 5% silver nitrate (AgNO₃, Merck) for 30 min. After washing with distilled water, the staining was developed with 5% sodium carbonate (Na₂CO₃, Merck), fixed with 5% sodium thiosulphate (Na₂S₂O₃, Merk) and examined by using a stereomicroscope (Leica MZ12, Germany).

**CFU assay**
Single cell suspensions (1000 cells/ml; 100 μl) were seeded into one well of a 6-well plate in proliferation medium. After 10 days, the samples were fixated with 10% formalin and stained with 0.1% toluidine blue (Sigma, Chemical Co., St. Louis, MO, USA). Colony-forming efficiency (an aggregate of ≥ 50 cells was scored as a colony) was determined by the number of colonies relative to the total number of seeded cells in each plate using a microscope (Leica DMIL, Germany).
Chapter 4

Real-time PCR
Cells in proliferation medium at day 5 (Oct4, Nanog and Sox2 gene expression) and in osteogenic medium at day 7 and 14 (ALP, Runx-2 and OC gene expression) were washed twice with PBS. Total RNA was extracted using TRizol® reagent (Invitrogen, Breda, the Netherlands) following manufacturer’s instructions. RNA concentrations and purity were determined by NanoDrop (ND-2000, Thermo Scientific). Then, the reverse transcriptase (RT) reaction of 1 μg RNA for each sample was performed using the Superscript™ III First-strand Synthesis System (Invitrogen, Breda, the Netherlands) for RT-PCR. The cDNA was amplified and gene expression was quantified with real-time PCR (BIORAD, CFX96™ real-time system). The primers used were Oct4, Nanog, Sox2, ALP, Runx-2 and OC (sequences in Table 1). The expression levels were analyzed versus the housekeeping gene GAPDH. The specificity of the primers was tested before the real-time PCR reaction. IQ SYBR Green Supermix PCR kit (BioRad, Hemel Hempstead, United Kingdom) was used for real-time measurement. The melting temperature (Tm) employed for each primer pair was 60 ºC. The gene expression was calculated using the 2^{-ΔΔCt} method and the control was used as the calibrator group.

Table 1: Human specific primer sequences used for real time qPCR

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<th>GenBank accession numbers</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
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<tr>
<td>Oct4</td>
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<td>TTGCCCTTCTGGCGCCGGTTA</td>
<td>GTCAGGCCCGTGTCGTCACTTGG</td>
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<tr>
<td>Nanog</td>
<td>NM_024865</td>
<td>TGTCCCCAAAGCTTGCCTTGCTT</td>
<td>TTCTTACCAGTCTCGTGAGGC</td>
</tr>
<tr>
<td>Sox2</td>
<td>NM_003106</td>
<td>AAAAAACAGCGCCGGACGGGT</td>
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</tr>
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<td>NM_000478</td>
<td>GGGACTGGTACTCGGATAACGA</td>
<td>CTGATATGCGAGTGTCCTTGCA</td>
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<td>Runx-2</td>
<td>NM_001015051</td>
<td>GACCAAAATGGCCTTGAAGA</td>
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<td>TAGACCCGGCCGAGAAGC</td>
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<td>GAPDH</td>
<td>NM_002046</td>
<td>CGATGCTGCGGCCTGAGTAC</td>
<td>CGTTCAGCTCAAGGATGACC</td>
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Statistical analysis
Each assay was performed in triplicate for each donor and statistical analysis was performed using GraphPad InStat (GraphPad Software, San Diego California USA). Results were statistically evaluated for each donor using an unpaired t-test (significance level, p<0.05).
Results
As all the donors showed the similar trend, only the result from one donor was presented below unless specifically mentioned.

Characterization of PDLCs
Human PDLCs were characterized on basis of their morphology, self-renewal and osteogenic ability. Microscopy observation revealed a spindle-shape cell morphology. PDLCs displayed the ability to form colonies when cultured in proliferation medium after 10 days. ALP activity was confirmed in the PDLC culture after 8 days in osteogenic medium. The ALP activity in PDLCs was in between BMSCs (positive control) and fibroblasts (negative control) cultured at the same conditions (Figure 1A). PDLCs induced mineralization as determined by Von Kossa staining after 30 days of osteogenic induction (Figure 1B).

Figure 1. Characterization of PDLCs. (A) ALP activity of the PDLCs was lower compared to BMSCs (positive control) on day 8, while fibroblasts were negative. (B) Mineral deposition was detected by a Von Kossa staining (black color) after 30 days of osteogenic induction. Error bars represent standard deviation.
Comparison between expanded STRO-1-sorted cells and unsorted parental cells
The efficiency of FACS sorting was proved by fluorescent microscopy observation and flow cytometry. The STRO-1\(^+\) cells were expanded for 4 passages to achieve 10 \(\times\) 10\(^6\) cells, the same number as their unsorted parental cells. During this expansion, the percentage of STRO-1\(^+\) cells decreased significantly from 95.3% to 2.3% (Figure 2A). Additionally, after 4 passages of \textit{in vitro} expansion, no significant difference was observed between the expanded STRO-1-sorted cells and unsorted parental cells in terms of DNA content, CFU number and calcium content (Figures 2B-D).

\[\text{Figure 2. Representative results of STRO-1 cell sorting. (A) The percentage of sorted STRO-1}^+\text{ cells decreased to 2.3% after 4 passages of \textit{in vitro} expansion. (B) No difference in proliferation was observed between the expanded STRO-1-sorted cells and the unsorted parental cells. (C) Expanded STRO-1-sorted cells displayed no difference compared to the unsorted parental cells in colony forming number. (D) No difference in calcium content was observed between expanded STRO-1-sorted cells and parental PDLCs. Error bars represent standard deviation.}\]

The direct effect of Wnt3a on PDLCs
The effect of Wnt3a on human PDLC proliferation was assessed by measuring cell DNA content and calculating cell doubling time during passaging. Wnt3a treated cells exhibited a significant increase (\(p < 0.05\); around 50% increase for day 4 and day 6 in DNA content compared to the non-treated control group; Figure 3A).
addition, Wnt3a treated cells displayed significantly shorter population doubling time when compared to untreated control cells, which were 25.46 ± 3.76 vs. 30.73 ± 0.9 hours (p < 0.05).

The self-renewal ability was evaluated by the expression of the self-renewal genes octamer-binding transcription factor 4 (Oct4), Nanog and sex determining region Y-box 2 (Sox2). The mRNA expression of Oct4 was significantly higher (p < 0.05) for the cells treated with Wnt3a than for the control cells (Figure 3B). Wnt3a treated cells also exhibited higher Nanog and Sox2 expression, but a significant difference was only observed in one donor.

Figure 3. Representative results showed Wnt3a promoted proliferation and self-renewal ability of PDLCs. (A) Wnt3a treated cells displayed a significant increase in DNA content compared to untreated cells cultured in proliferation medium. (B) Wnt3a treated cells exhibited a higher expression of Oct4, Nanog, and Sox2 after 5 days of culture. * p < 0.05; error bars represent standard deviation.

Osteogenic differentiation was assessed by gene expression of ALP, runt-related transcription factor 2 (Runx-2), and osteocalcin (OC), ALP activity, calcium content in the extracellular matrix and Von Kossa staining. PCR revealed significantly lower (p < 0.05) ALP gene expression for Wnt3a treated cells at day 7 compared to the control group (Figure 4A). For the Runx-2 and OC gene expression no significant difference was found at day 7 and day 14 between the Wnt3a treated and control groups (Figures 4A-B). ALP activity at day 7 and 10 demonstrated significantly lower (p < 0.05) levels for Wnt3a treated cells vs. untreated cells (Figure 4C). The long-term mineralization was evaluated by calcium content deposited by the cells and a Von Kossa staining after 30 days of culture. Both Wnt3a treated and untreated cells induced mineralization as determined by Von Kossa staining and no significant difference in calcium content was observed between the two groups (Figure 4D).
**Figure 4.** Representative results showed Wnt3a delayed osteogenic differentiation of PDLCs. (A) ALP mRNA was down-regulated by Wnt3a after 7 days of osteogenic induction. (B) The gene expression was equal for Runx-2 and OC between two groups. (C) ALP activity was down-regulated by Wnt3a on day 7 and 10. (D) Wnt3a has no effect on the calcium content after 30 days of osteogenic induction. * p < 0.05; error bars represent standard deviation.

**Functionality of Wnt3a pretreated PDLCs**

After passage 9, Wnt3a was no longer added to the culture medium and the proliferation, CFU and osteogenic differentiation capacity was investigated to evaluate whether the functionality of Wnt3a-pretreated cells was affected by the shorter cell doubling time. The results showed that both Wnt3a pretreated cells and control cells showed abilities to proliferate, form colonies, and osteogenically differentiate. In addition, no difference was observed between Wnt3a-pretreated cells and control cells in terms of DNA content (Figure 5A), CFU number (Figure 5B), ALP activity (Figure 5C) or calcium content (Figure 5D).
Figure 5. Representative results showed Wnt3a pretreatment did not affect functionality of PDLCs after long-term expansion. (A) No difference in DNA content was observed between Wnt3a-pretreated cells and control cells after 5 passages. (B) Wnt3a-pretreated cells displayed no difference compared to the control cells in CFU number. (C) No difference in ALP activity was observed between Wnt3a-pretreated cells and control cells. (D) No difference in calcium content was detected between Wnt3a-pretreated cells and control cells after 5 passages. Error bars represent standard deviation.

Discussion

The aim of the current study was to evaluate the effect of STRO-1 cell sorting and Wnt3a treatment on cell behavior of human PDLCs. The characteristics of unsorted parental cells and expanded STRO-1-sorted cells were compared to find the appropriate PDL cell population for clinical applications. The effect of Wnt3a on proliferation, self-renewal, and osteogenesis of PDLCs was evaluated to test the potential of Wnt3a for in vitro expansion of PDLCs. No differences were measured between the expanded STRO-1-sorted cells and unsorted parental cells in terms of proliferation, CFU, and mineralization capacity. Wnt3a promoted the proliferation and self-renewal ability of human PDLCs, as displayed by an increased DNA content, shorter population doubling time, and higher expression of Oct4. Wnt3a also stimulated the efficient in vitro expansion of PDLCs for at least 5 passages without affecting cell functionalities in terms of proliferation, CFU and osteogenic capacity.
STRO-1 positive cells are usually found in low numbers and therefore in vitro expansion is needed. Our results showed that the percentage of STRO-1 positive cells decreased significantly during culture expansion. Additionally, after 4 passages of in vitro expansion, there were no differences in proliferation, CFU ability and mineralization capacity between sorted and initially unsorted cells. A similar phenomenon has been observed with human BMSCs (unpublished data): the STRO-1 sorted BMSCs returned to the same state as their unsorted parental cells only in 7 days of in vitro expansion. Based on the results and also the time-consuming procedure of the cell selection and expansion, unsorted primary cells may provide a better cell source for future clinical applications. Therefore, unsorted PDLCs were used in the current study to test the effect of Wnt3a.

The outcome of DNA content indicates that Wnt3a enhances the proliferation of PDLCs significantly. Additionally during the long-term study, the cells were treated with or without Wnt3a for 5 passages with the same cell seeding density. Wnt3a treated cells showed more cell doublings than the control group at each passage, indicating the enhancement of Wnt3a on the proliferation of PDLCs. Oct4, Nanog, and Sox2 are key regulators essential for self-renewal of pluripotent cells. These self-renewal factors are also expressed in PDLCs and thus they were chosen in this study. Based upon the results, the self-renewal ability of PDLCs was promoted by Wnt3a as displayed by the higher expression of self-renewal gene expressions. This is consistent with one previous study in which self-renewal and gene expression were stimulated in embryonic stem cells by adding Wnt3a. These results do suggest that Wnt3a may benefit the in vitro expansion of primary cells for further cell-based clinical applications.

Wnt signaling plays an essential role in osteogenesis. Our results suggest that Wnt3a mediates some aspects of osteogenic differentiation of human PDLCs. ALP mRNA-levels and ALP-activity were reduced significantly by Wnt3a during osteogenic differentiation of human PDLCs. These observations were consistent with some previous studies. Boland et al. reported that Wnt3a suppressed osteogenic differentiation of hBMSC, with reduced ALP mRNA and ALP activity. Similarly, in another study de Boer et al. observed that Wnt3a strongly inhibited ALP expression of hBMSC. It has to be noted that for the mineralization, no inhibition effect of Wnt3a was observed after 30 days of culture, indicating Wnt3a may have a delayed effect on osteogenic differentiation of PDLCs. Although the effect of Wnt signaling on the STRO-1† cells in the efficiency of osteogenic differentiation was not evaluated here, we speculate that canonical Wnt signaling may hinder osteogenic differentiation of STRO-1† cells based on a previous study, in which the data indicate that enhanced activation of canonical Wnt signaling
inhibits osteogenic differentiation of undifferentiated MSCs, while increasing the mineralization of differentiated osteoblasts. The enhancement of mineralization of differentiated osteoblasts by canonical Wnt signaling may also explain that Wnt3a could enhance implant osseointegration \(^{25}\) and promote a better healing of a calvarial defect in adult mice \(^{24}\). The enhancement of proliferation or self-renewal by Wnt3a might be another reason for the acceleration of osteogenesis \textit{in vivo}. Recently Yamada et al. reported that XAV939, an inhibitor of canonical Wnt signaling \(^{30}\), inhibited ALP activity of hPDL-derived MSCs, indicating the promoting effect of canonical Wnt signaling pathway during the osteogenic differentiation of hMSCs \(^{31}\). However, this mechanism is not consistent with our findings, along with those of previous studies \(^{23,29}\). Our results suggest that Wnt3a, a representative canonical Wnt member, inhibited ALP activity of human PDLCs. It has to be noted that Wnt3a acts as morphogens which is a protein that can bring out differential responses in the same cell type depending on its concentration \(^{29,32}\). Recently, opposite spectrum of activity of canonical Wnt signaling in the osteogenic context of different cell types by different doses of Wnt3a has been reported \textit{in vitro} and \textit{in vivo} \(^{24}\). For instance, Wnt3a inhibits osteogenesis in juvenile calvarial osteoblasts while strongly induces osteogenesis in mature calvarial osteoblasts in a dose-dependent manner \(^{24}\). For \textit{in vivo} studies, Wnt3a treatment of calvarial defects, created in juvenile mice, enhances tissue regeneration only at low doses, whereas high doses of Wnt3a impairs calvarial healing and bone regeneration. Conversely, high doses of Wnt3a promote tissue regeneration in adult mice \(^{24}\). These findings indicate that canonical Wnt signaling can either promote or inhibit osteogenic differentiation. Therefore, the comprehensive understanding of the roles of both appropriate dose concentrations of Wnt3a treatment and endogenous activity of canonical Wnt signaling will be needed in further studies. More PDLCs with higher self-renewal ability can be obtained by using Wnt3a. To further confirm the functionality of Wnt3a expanded PDLCs, we subcultured PDLCs from passage 4 with or without Wnt3a up to passage 9, and compared the functionalities of Wnt3a-pretreated and control cells. Wnt3a promoted the rapid \textit{in vitro} expansion of PDLCs for at least 5 passages, as displayed by the shorter population doubling time, without interfering with their functionalities compared to untreated control cells. Wnt3a pretreatment resulted in much more PDLCs with similar proliferation, CFU, and osteogenic capacity as the untreated control cells, which is of importance for the clinical use of human PDLCs because a large number of cells is required for cytotherapeutic purposes. In a previous study, Zeng and Nusse reported that Wnt3a could promote long-term expansion of mammary stem cells and maintain their self-renewal ability \(^{22}\). These data indicate that Wnt3a may
be used as a useful culture supplement for the in vitro expansion of PDLCs to benefit their clinical application.

Conclusions
The proliferation, CFU and mineralization capacity of unsorted parental PDLCs and expanded STRO-1-sorted cells were compared to find the appropriate PDL cell population for future clinical applications. The results provide evidence that STRO-1-sorted human PDLCs after expansion are not superior compared to their unsorted parental cells, suggesting the time-consuming cell selection and expansion procedure can be avoided in cell-based periodontal regeneration. In contrast, Wnt3a does have an effect on human PDLCs by promoting cell proliferation and self-renewal, as displayed by an increased DNA content, a shorter cell population doubling time, and higher expression of the self-renewal gene Oct4. In addition, Wnt3a promoted the efficient in vitro expansion of human PDLCs for at least 5 passages without affecting the self-renewal and osteogenic differentiation capacity, indicating its potential for rapid and efficient in vitro cell expansion for future clinical applications.
References


Chapter 5

Enzymatic control of chitosan gelation for delivery of periodontal ligament cells

Xiang-Zhen Yan*, Arnold W.G. Nijhuis*, Jeroen J.P. van den Beucken, Sanne K. Both, John A. Jansen, Sander C.G. Leeuwenburgh and Fang Yang

* contributed equally to this work
1. Introduction

Periodontitis, initialized by the accumulation of bacterial plaque, is a common inflammatory disease. Periodontitis is characterized by irreversible loss of periodontium, which includes gingiva, cementum, periodontal ligament (PDL), and alveolar bone. Without adequate treatment, periodontitis will finally lead to tooth loss. Periodontitis is currently treated by scaling and root planning to decrease the progression of the active disease, but the regeneration of already damaged tissues remains a clinical challenge. Recently, however, cytotherapeutic approaches have been explored to overcome the regenerative limitations of existing treatment methodologies \(^1\)-\(^3\). The principle of cytotherapy is the delivery of viable cells to tissues in order to treat diseases. To this end, various cell types have been used for transplantation purposes in animal studies. Periodontal ligament cells (PDLCs), mesenchymal stromal cells (MSCs), and alveolar periosteal cells (APCs) have been transplanted using poly(glycolic) acid-based cell-sheet carriers in canine one-wall intrabony defects in order to compare the differences between cell sources for periodontal regeneration \(^1\). After eight weeks, significantly more periodontal regeneration, including newly formed cementum and well-oriented PDL fibers, was observed upon transplantation of PDLCs. In addition, nerve filaments were only formed by PDLCs. In an organ culture study performed on tooth root surfaces, new PDL-like tissues and alveolar bone were formed only by PDLCs but not by dental follicle cells (DFCs), dental pulp cells (DPCs), or MSCs \(^2\). These results indicate that proper delivery of PDLCs may be a promising treatment to regenerate periodontal tissues.

From a clinical point of view, hydrogels are particularly promising cell delivery vehicles, as they offer several advantages including easy handling, high cell seeding efficiency and good formability to irregular defects in a minimally invasive manner \(^3\). Chitosan, a cationic amino polysaccharide copolymer derived from naturally abundant chitin, has received increased attention due to its well-documented biodegradability \(^4\)-\(^5\), biocompatibility \(^6\), wound healing \(^7\), and tissue regeneration ability \(^8\). In addition, its mucoadhesive properties \(^9\), antimicrobial activity \(^10\), and anti-inflammatory effects \(^11\) render chitosan one of the most suitable candidates for treatment of periodontitis.

Chitosan hydrogels can be prepared using either irreversible, chemical crosslinking or reversible, physical crosslinking of chitosan chains. A straightforward method to obtain chemically crosslinked chitosan hydrogels is the use of dialdehyde crosslinkers, such as glutaraldehyde \(^12\). However, these crosslinkers are potentially cytotoxic when not fully consumed upon crosslinking, while chemical modifications of the primary structure of chitosan may alter its initial properties \(^13\), \(^14\). Physically
crosslinked chitosan hydrogels have been obtained without the use of additional chemical crosslinking agents by increasing the pH of acid chitosan solutions (Figure 1)\textsuperscript{4,15,16}. At low pH, chitosan is positively charged (pK\textsubscript{a}≈6.5) and soluble due to the protonation of the free amino groups as present on chitosan chains\textsuperscript{16}. When exposed to neutral or alkaline pH, the positive charge and corresponding electrostatic repulsion between the charged macromolecules is reduced, which gives rise to gelation due to hydrogen bonding and hydrophobic interactions between the chitosan chains as well as formation of chitosan crystallites\textsuperscript{17}.

\begin{figure}[h]
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\includegraphics[width=\linewidth]{figure1.png}
\caption{Schematic representation of the mechanism of chitosan gelation by increasing pH.}
\end{figure}

The most commonly used method to form physically crosslinked chitosan hydrogels involves the addition of glycerophosphate (GP) salt, which can neutralize the ammonium groups of chitosan. This method, however, necessitates high concentrations of GP, which may lead to serious cytotoxicity\textsuperscript{18,19}, whereas gelation of chitosan solutions containing cytocompatible levels of GP does not occur at physiological temperature\textsuperscript{20}. Recently, Chenite \textit{et al.}\textsuperscript{16} demonstrated that chitosan hydrogels can be formed homogeneously by neutralisation of chitosan solutions using ammonia as produced in situ from enzymatic hydrolysis of urea. Important aspects of this system, such as the relationship between pH and kinetics of gelation and degradation were not addressed, while its potential for biomedical applications was not confirmed. Therefore, we aimed to study the possibility to control chitosan gelation using enzymatic hydrolysis of urea for the delivery of PDLCs. In more detail, we aimed to examine the effect of urea and urease concentrations on
the pH and osmolarity of chitosan hydrogels as well as their gelation time, rheological properties, morphology, water content, swelling and degradation rate. Additionally, we aimed to evaluate the suitability of the optimized hydrogel for delivery of PDLCs by assessing the viability of encapsulated periodontal ligament cells and determining the capacity of released cells to form colony-forming units (CFUs), proliferate and osteogenically differentiate.

2. Experimental Section

2.1. Materials

Urease (type III from Jack beans, U1500, Sigma-Aldrich), urea (Invitrogen) and chitosan (average molecular weight 500 kDa, degree of deacetylation 85%, Heppe Medical Chitosan GmbH) were used as received. Chitosan was dissolved at a concentration of 2.5 w/v% in 0.125 M HCl (Scharlau), while phenol red (Life Technologies) was added at a concentration of 11 mg/l (similar to cell culture medium) in order to monitor pH changes and sterilized by autoclavage. Urease was dissolved in sterile phosphate buffered saline, (PBS, pH=7.4) at a concentration of 12.5-100 U/ml. Urea was dissolved at a concentration of 10 M in MilliQ water and sterilized by filtration over a 0.2 μm filter. To prepare 1.5 w/v% chitosan hydrogels, 1 ml chitosan solution was mixed with 0.67 ml urease (12.5-100 U/ml) solution at room temperature. Subsequently, 5-10 μl of the urea solution was added and mixed which resulted into urea hydrolysis, pH increase and gelation of chitosan solutions. The concentration of 1.5 w/v% was chosen according to previous studies in which chitosan hydrogels with this concentration were evaluated for tissue engineering applications 12, 21.

2.2. Optimization of the gelation kinetics

Gelation time was assessed by the inverted vial method. Briefly, 1 ml of freshly prepared hydrogel solution was placed in a 2 ml eppendorf vial. Gel transition time was determined by inverting the vials every 30 seconds and the time at which the formulation did not flow was recorded as the gelation time. This gelation time was recorded for 25 different conditions, i.e., urease concentrations of 3.75, 7.5, 15, 30 and 60 U/ml and urea concentrations of 30, 37.5, 45, 52.5 and 60 mM (n=3). After this optimization study, 6 different hydrogel compositions were selected for further investigations on the gelation, degradation and cytocompatibility of chitosan hydrogels. These hydrogels contained 45 or 37.5 mM urea and 60, 30 or 15 U/ml urease, and were supplemented with 20.8 μl (for 37.5 mM urea) or 16.7 μl (for 45 mM urea) of 10X PBS (Life Technologies) to set the osmolarity to the
physiological range. The final composition of these hydrogels and their abbreviations are given in Table 1.

**Table 1:** Compositions and abbreviations of various hydrogels.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Urea (mM)</th>
<th>Urease (U/ml)</th>
<th>Chitosan (w/v %)</th>
<th>Initial osmolarity (osm/l)*</th>
<th>Final osmolarity (osm/l)*</th>
<th>Initial pH slope (units/min)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease60-Urea45</td>
<td>45</td>
<td>60</td>
<td>1.5</td>
<td>295</td>
<td>340</td>
<td>0.18</td>
</tr>
<tr>
<td>Urease30-Urea45</td>
<td>45</td>
<td>30</td>
<td>1.5</td>
<td>295</td>
<td>340</td>
<td>0.12</td>
</tr>
<tr>
<td>Urease15-Urea45</td>
<td>45</td>
<td>15</td>
<td>1.5</td>
<td>295</td>
<td>340</td>
<td>0.10</td>
</tr>
<tr>
<td>Urease60-Urea37</td>
<td>37.5</td>
<td>60</td>
<td>1.5</td>
<td>296</td>
<td>333</td>
<td>0.17</td>
</tr>
<tr>
<td>Urease30-Urea37</td>
<td>37.5</td>
<td>30</td>
<td>1.5</td>
<td>296</td>
<td>333</td>
<td>0.14</td>
</tr>
<tr>
<td>Urease15-Urea37</td>
<td>37.5</td>
<td>15</td>
<td>1.5</td>
<td>296</td>
<td>333</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*Since urea decomposes into 2NH₃ + CO₂ the osmolarity of the hydrogels increases upon gelation (CO₂ was released during gelation and not accounted for).

**Initial slope of the pH increase as calculated over the first 5 min (from Figure 2B).

2.3. Physicochemical characterisation

2.3.1. PH versus time

The pH was recorded to investigate the speed at which the pH changed under different gel compositions. In order to record the pH the electrode was placed in a freshly prepared mixture of starting solutions immediately after addition of the urea. The pH was recorded for 45 min at room temperature using a Meterlab PHM210 calibrated with IUPAC buffers (S11M002, S11M004, S11M007 from Radiometer analytical).

2.3.2. Rheological characterization

A rheometer (AR2000ex, TA instruments) was used to determine the viscoelastic properties of the hydrogels. All measurements were performed using a flat steel plate geometry (20 mm diameter) at 25°C within the linear viscoelastic region. Storage modulus (G’) was determined by using an oscillatory time sweep test for 45 min at a constant strain of 1% and a frequency of 1 Hz.

2.3.3 Gel morphology

Scanning electron microscopy (SEM) was performed to observe the morphology of the various hydrogels. Prior to SEM the hydrogels were lyophilized and cross-sections were cut with a surgical knife. Subsequently, the cross-sections were coated with a conductive thin film of gold and observed using a JEOL 6340F SEM.

2.3.4. Swelling ratio and water content
After gelation for 1 hour, the initial hydrogels were gently patted dry with a filter paper to remove surface water and weighed (Weight initial, $W_i$), followed by incubation in PBS at 37°C for 24h to reach the equilibrium swelling state and weighed (Weight equilibrium, $W_e$). After freeze-drying for 3 days, the weight of the dry samples was recorded (Weight dry, $W_d$). Accordingly, the swelling ratio and water content of swollen hydrogels were calculated using equation (1) and (2), respectively ($n=3$):

\[
\text{Swelling Ratio} = \frac{W_e}{W_i} \tag{1}
\]

\[
\text{Water Content} = \frac{W_e - W_d}{W_e} \times 100\% \tag{2}
\]

2.3.5. Degradation rate
Degradation of the hydrogels was determined gravimetrically according to a previous study. To mimic the in vivo situation, the hydrogels were incubated in 10 ml of PBS containing 1.5 μg/ml lysozyme (Sigma-Aldrich) at 37°C under gentle agitation (60 rpm) on a shaker platform. The medium was refreshed every 3 days and the mass of the (patted-dry) hydrogels was monitored every 6 days up to 30 days. The degradation rate was expressed as the percentage of remaining weight (relative to the initial weight).

2.4. Cellular responses in the gel
All experiments were done by following national guidelines for working with human materials (Dutch federation of biomedical scientific societies. Human tissue and medical research: code of conduct for responsible use. Available at: http://www.federa.org/). The viability of encapsulated cells and the biological behaviour of cells released upon degradation of the chitosan hydrogels were tested by using human PDLs. After patient had signed informed consent, PDL cells were harvested from an impacted third molar from 1 adult patient (18 years, female). The cell proliferation medium consisted of α-Minimal Essential Medium (α-MEM, Gibco) supplemented with 10% fetal calf serum (FCS, Gibco) and 100 units/ml penicillin/streptomycin (Gibco). The cells were cultured in proliferation medium for 3 passages and then frozen in α-MEM containing 20% FCS and 10% dimethylsulfoxide (Sigma) in liquid nitrogen. After defrosting, cells from the 4th passage were used. To test the cytocompatibility of chitosan hydrogels, 200 µl chitosan solution containing urea (37.5 mM), urease (15, 30 or 60 U/ml, dissolved in culture medium instead of PBS) and $2\times10^5$ PDLs was prepared in 48-well plates
and allowed 30 min for gelation. Afterwards, proliferation medium (500 µl) was added and refreshed after 30 and 60 min to remove possible side products of the hydrogel formation (such as NH₃), thereafter was refreshed twice per week. Cell viability was determined using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) after 3, 15 and 30 days of culture in proliferation medium. Hydrogel samples were washed in sterile PBS and incubated for 30 min at 37 °C with 2 mM calcein-AM and 4 mM ethidium homodimer in PBS solution. After incubation, hydrogel samples were rinsed again in PBS, and then observed and photographed by using Zeiss Imager Z1 together with the AxioCam MRc5 camera using AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

2.5. Cellular responses after release from the gel
During the cell culture, PDLCs were gradually released in the culture medium due to hydrogel degradation and were collected at day 3, day 15 and day 30, reseeded in tissue culture flasks and expanded for 6 days. This resulted in 8 different cell populations collected from the hydrogels (the hydrogel with 60 U/ml urease was completely degraded after 30 days), whereas parental cells of passage 4 were also included as control yielding for a total of 9 different cell populations. The biological behaviour of these 9 cell populations was evaluated in terms of capacity to form colony forming units (CFUs), proliferation, and osteogenic differentiation capacity (alkaline phosphatase activity, ALP, marker for early differentiation; mineralization, calcium content, marker for late differentiation).

2.5.1. Capacity to form CFUs
Cells were seeded into 6-well plates at a density of 100 cells per well in proliferation medium for 10 days. After 10 days, the samples were formalin-fixed (10%) for 20 min and stained with 0.1% toluidine blue (Sigma, Chemical Co., St. Louis, MO, USA) for 10 min. The number of CFUs was quantified by counting the number of colonies (diameter ≥ 2mm) in each well using light microscopy (Leica DMIL, Germany).

2.5.2. DNA content
Cells were seeded in 48-well plates at a density of 10,000 cells per well in osteogenic medium (alpha MEM supplemented with 10% FCS, 50 µg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone; all from Sigma) for 7, 10 and 14 days. Then cells were washed twice with PBS, homogenized in 1 ml of MilliQ water, frozen and thawed twice before DNA analysis. A PicoGreen
DNA kit (Molecular Probes, Leiden, The Netherlands) was used according to manufacturer's protocol. Briefly, 100 μl of DNA standards or samples were incubated with 100 μl of working solution and allowed to incubate for 10 min at RT in the dark. After incubation, DNA content was read in a fluorescence microplate reader (Bio-Tek Instruments, Abcoude, The Netherlands) with excitation filter 485 nm and emission filter 530 nm.

2.5.3. ALP activity
The same samples used for DNA assay were used for ALP activity assessment. For analysis 80 μl of sample or standard and 20 μl of buffer solution (5 mM MgCl$_2$, 0.5 M 2-amino-2-methyl-1-propanol) was pipetted into a 96-well plate, and 100μl of substrate solution (5 mM paranitrophenylphosphate) was added per well. Subsequently, the plate was incubated for 1 h at 37 °C, after which the reaction was stopped by adding 100 μl of 0.3M NaOH. Dilutions of 4-nitrophenol (0–250 μM) were used for the standard curve. The plate was read in an enzyme-linked immunosorbent assay reader (Bio-Tek Instruments) at 405 nm. ALP activity results were normalized to the amount of DNA (in nmol 4-NP/h/ng DNA).

2.5.4. Calcium content
Cells were seeded in 48-well plates at a density of 10.000 cells per well in osteogenic medium for 14 and 24 days. Then cells were washed twice with PBS and incubated with 1 ml of 0.5 N acetic acid on a shaker table overnight. The calcium content was measured by the ortho-cresolphthalein complexone (OCPC) method. Briefly, 10 μl of sample or standard were incubated with 300 μl of working solution in a 96-well plate. Working solutions consisted of 5 ml of OCPC solution, 2 ml of 8-hydroxyquinoline, 5 ml of 14.8 M ethanolamine/boric acid buffer, and 88 ml of MilliQ. The standards (0-100 μg/ml) were prepared using a CaCl$_2$ stock solution. After 10 min incubation at room temperature, the absorbance of each well was measured in the ELISA microplate reader (Bio-Tek Instruments, Abcoude, The Netherlands) at a wavelength of 570 nm.

2.6. Statistical analysis
Statistical analysis was performed using GraphPad InStat (GraphPad Software, San Diego California USA). Results were statistically evaluated via a One-way ANOVA, followed by a Tukey-Kramer Multiple Comparisons Test. Results were considered statistically significant at p-values below 0.05.
3. Results

3.1. Gelation kinetics

The gelation times of hydrogels prepared with various urease (3.75, 7.5, 15, 30 and 60 U/ml) and urea (30, 37.5, 45, 52.5 and 60 mM) concentrations are shown in Figure 2A. Gelation times ranged from 3.5±0.5 min for hydrogels containing 60 U/ml urease and 60 mM urea to 163±15 min for hydrogels containing 3.75 U/ml urease and 30 mM urea. Generally, increasing urease concentrations resulted in decreasing gelation times. In contrast, gelation times hardly depended on the concentration of urea, except for a much slower gelation observed at the lowest urea concentration of 30 mM. In Figure 2B, the pH during gelation of the chitosan hydrogels is displayed, which confirmed that the pH increased from initial values of about 6-6.5 to final values of 7-8 within 8-30 min after addition of urease. The final pH values were controlled by the amount of urea (with highest pH values observed for hydrogel containing highest urea concentrations), while the initial slope (see Table 1) of the pH increase was mainly related to the concentration of urease.

Figure 2: A: Gelation time (min) as a function of urease and urea concentration depicted as the average (large bars) and standard deviation (small lines) for n=3. B: pH as a function of hydrogels containing 37.5 or 45 mM urea and 15, 30 or 60 U/ml urease.
3.2. Physicochemical characterisation

3.2.1. Rheological characterization

Representative time sweeps of the storage modulus ($G'$) of the various chitosan hydrogels as a function of urea and urease concentration are shown in **Figure 3A**. In general, the storage moduli were higher than the loss moduli (not shown) directly after addition of urease, except for hydrogels containing urease concentrations of 15 or 30 U/ml and a urea concentration of 45 mM. The average final $G'$ after 45 min of gelation is depicted in **Figure 3B**. Storage moduli decreased with increasing urease concentration, showing highest storage moduli of up to 6200 Pa for urease concentrations of 15 U/ml and significantly lower storage moduli of 4400 and 600 Pa for hydrogels containing 30 or 60 U/ml, respectively.

![Figure 3A](image)

![Figure 3B](image)

**Figure 3**: A: Representative time sweeps of the storage modulus ($G'$) of the various hydrogels. B: $G'$ after 45 min of gelation for the different hydrogels (average for $n=3$, error bars represent standard deviation). ^, O, □, #, X and + indicate there are statistically significant differences with Urease15-Urea37, Urease15-Urea45, Urease30-Urea37, Urease30-Urea45, Urease60-Urea37 and Urease60-Urea45, respectively.
3.2.2. Gel morphology
The morphology of lyophilized and cross-sectioned hydrogels is depicted in Figure 4, which revealed a porous network for all hydrogels. Pore dimensions were up to 100 µm for hydrogels with high urease concentrations of 60 U/ml (Figure 4A and 4D), whereas pore sizes in hydrogels formed at lower urease concentrations (30 and 15 U/ml) did not exceed 20 µm (Figure 4B, 4C, 4E and 4F).

Figure 4: Scanning electron micrographs of cross-sections of Urease60-Urea45 (A), Urease30-Urea45 (B), Urease15-Urea45 (C), Urease60-Urea37 (D), Urease30-Urea37 (E) and Urease15-Urea37 (F).

3.2.3. Swelling ratio and water content
The equilibrium water content and swelling ratio are depicted in Figure 5. The water content of the hydrogels (based on the dry weight) ranged from 97-98% (Figure 5A, the hydrogels contained 1.5% chitosan and were in equilibrium with PBS, which contains about 1% dry weight). The hydrogels did not swell as evidenced by a swelling ratio between 99.8 and 100.1% for the experimental hydrogels (Figure 5B). No difference was observed among all the groups (p>0.05).
3.2.4. Degradation rate

For all hydrogels, three distinct phases could be discerned in the degradation profiles (Figure 6A). A slow initial phase of about 6 days was followed by a fast degradation between 6 and 12 days, after which an intermediate degradation rate was observed between 12 and 30 days. The percentage of remaining weight after 30 days mainly depended on the urease concentration, with degradation increasing with increasing urease concentration (Figure 6B).
Figure 6: A: Degradation curves for the various hydrogels after soaking for 6-30 days in PBS containing 1.5 μg/ml lysozyme. B: Remaining weight after 30 days of soaking (average for n=3, error bars represent standard deviation). ^, O, □, #, X and + indicate there are statistical significant differences with Urease15-Urea37, Urease15-Urea45, Urease30-Urea37, Urease30-Urea45, Urease60-Urea37 and Urease60-Urea45, respectively.

3.3. Cellular responses

3.3.1. Cellular responses in the gel

After 3, 15 and 30 days of encapsulation in the chitosan hydrogels, PDLCs were still viable as evidenced by the appearance of large number of live cells (appearing in green) and few dead cells (appearing in red; Figure 7).
3.3.2. Cellular responses after release from the gel
Since the hydrogel slowly degraded, cells gradually released and the released cells and were collected at day 3, 15 and 30 for further analysis (except for the hydrogel with 60 U/ml urease which was completely degraded after 30 days, thus hampering reliable cell collection). The collected cells were expanded for 6 days and revealed an elongated morphology during expansion. The collected cells exhibited a similar capacity to form CFUs as compared to parental control cells (Figure 8A-C). At day 7, there was no difference between parental and collected cells regarding DNA content ($p > 0.05$, Figure 8D-F). After 10 and 14 days of culture, parental cells exhibited a statistically significant higher value of DNA content compared to the cells collected after 15 and 30, but not 3 days of encapsulation (Figure 8D-F).
Figure 8: A-C: Number of colony forming units (CFUs) of the cell populations collected from the hydrogels (with 15, 30 or 60 U/ml urease) after 3 days (A), 15 days (B) and 30 days (C) depicted as average for n=3, error bars represent standard deviation. D-F: DNA content for cell populations collected from the hydrogels (with 15, 30 or 60 U/ml urease) after 3 days (D), 15 days (E) and 30 days (F), depicted as average for n=3, error bars represent standard deviation, * denotes a statistically significant difference with the corresponding parental cells. Please note that the hydrogel containing 60 U/ml urease was completely degraded after 30 days (C and F).

A continuous increase in ALP activity from day 7 to day 14 was observed for parental cells as well as all collected cells (Figure 9A-C). Except cells collected from hydrogels with urease 30 U/ml after 3 days showed higher ALP activity on day 7 compared with the parental cells (p=0.0268), there are no differences among all other groups (p>0.05, Figure 9A-C). Over the first 14 days of osteogenic induction, parental cells exhibited a higher value in calcium content than cells collected after 3 days of encapsulation, but this value was equal and lower when compared to cells collected after 15 and 30 days of encapsulation respectively (Figure 9D-F). The total amount of calcium produced by the cells was higher after 24 days compared to 14 days and there was no difference among all groups (p>0.05, Figure 9D-F).
4. Discussion
The aim of the current study was to optimize the enzymatic control of chitosan gelation for the delivery of PDLCs. By varying the urea and urease concentration, it was possible to control the pH and hence the kinetics, strength and degradation of chitosan hydrogels. PDLCs remained viable upon encapsulation within chitosan-based hydrogels for up to 30 days. Cells that were released and collected upon degradation of the hydrogels after 3, 15 and 30 days retained the capacity to form colonies and osteogenically differentiate. Consequently, chitosan-based hydrogels seem suitable for providing a continuous delivery of functional cells that can osteogenically differentiate (for example in the periodontal defect site) when released (caused by hydrogel degradation) from the hydrogel. In conclusion, it was shown that the enzymatic control over the gelation of chitosan hydrogels offers options for the delivery of PDLCs via an injectable cytotherapeutic system.

4.1. Gelation kinetics
The strong dependence of gelation kinetics on the concentrations of urea and urease was expected in view of the pH-dependent gelation of chitosan. Higher concentrations of urea and urease resulted in faster pH-increases and faster gelation. Chenite et al. observed a similar trend, despite using a different mixing method and initiation of gelation at a temperature of 4°C. In this study, the gelation was studied at RT because the clinical handling procedures before injection will be performed at RT. During future clinical applications, the injection time can be indicated either by the gelation time of each hydrogel or by the color change (from yellowish to whitish) as the pH indicator phenol red was added into the formulation. Based on the optimization of the urea and urease concentrations, six hydrogel compositions were selected for extended studies. Urea concentrations of 37.5 and 45 mM were reduced as much as possible without compromising the gelation time to minimize changes in osmolarity during gelation, while urease concentration of 15, 30 or 60 U/ml were selected to allow for gelation within a clinically handling time frame of 30 min. Final pH values were controlled by the concentration of urea as main source for alkaline ammonia groups, but did not depend on the concentration of urease, which allows for independent control over
final pH and gelation kinetics by varying the concentrations of urea and urease, respectively. This gives the opportunity to set the desired gelation time and final pH of the gel for various applications.

4.2. Physicochemical characterisation

Hydrogels formed at low urease concentrations revealed higher storage moduli, which can be explained by the fact that the slower increase of the pH resulted in slower gelation. Consequently, the formation of cohesive forces resulting from hydrophobic interactions, hydrogen bonding and chitosan crystallization proceeded more gradually, which clearly improved the efficacy of the physical crosslinking. The maximum storage modulus of about 6.4 kPa observed for hydrogels containing 45 mM urea and 15 U/ml urease was comparable to values of about 8 kPa obtained by Chenite et al. 16 who, however, did not report on the relationship between urease concentration and storage moduli. Chitosan gels prepared using enzymatic decomposition of urea exhibited considerably higher storage moduli than other physically crosslinked chitosan hydrogels comprising NaHCO₃ (G’ between 800-3000 Pa) 25, dipotassium phosphate (G’=171-481 Pa) 26 or β-Glycerophosphate (G’=3-225 Pa) 15,22,27,28. Despite these promising results, it should be emphasized that the molecular weight and degree of deacetylation of chitosan was different for all of these studies, which hampers a direct comparison between these studies 29. In general, the rheological results showed that urease concentration can be employed to tailor the gel-strength of chitosan hydrogels.

The observation that hydrogels formed at higher urease concentrations were weaker than other hydrogels was confirmed by analysis of hydrogel morphology and degradation rate. Even though lyophilization is known to affect the porosity of hydrogels made of natural polymers, such as gelatin, collagen and chitosan, this expectedly does not lead to differences between the hydrogels 3,15,30. Therefore, it can be concluded from SEM that porosity was higher for hydrogels formed at higher urease concentration, thereby compromising the resulting storage moduli as observed also for chemically crosslinked chitosan hydrogels 22,31.

The higher strength of hydrogels formed at lower urease concentration also reduced their degradation rate, which resulted in a remaining weight of up to 60% after soaking in lysosyme-containing PBS for 30 days. Previously reported values for the remaining weight of chitosan hydrogels, upon soaking in 1.5 µg/ml lysozyme containing PBS, ranged from 0% (after 10 days) for hydrogels physically crosslinked using β-glycerophosphate to 70% for chitosan hydrogels chemically crosslinked using genipin 22. Chitosan hydrogels formed using enzymatic hydrolysis of urea consequently are far more stable than chitosan hydrogels formed with β-
glycerophosphate and even approach the stability of chemically crosslinked hydrogels. It has to be noted that there were certain limitations regarding the method used to determine the degradation rate. In our as well as some previous studies, the wet weight was utilized to estimate the level of degradation. During the degradation, however, the enzymatic bond breakage of chitosan molecules can lead not only to the release of small polymer pieces from the bulk hydrogel (leading to a reduction in hydrogel weight) but also to a decrease in the cross-linking density which in turn may lead to an increase in swelling of the hydrogel (potentially resulting in an increase in wet weight of the hydrogel). Thus it is recommended to use the dry weight of the hydrogel to determine the degradation rate in future studies.

The equilibrium water content of 97-98% (by weight) was expected since the hydrogel contained 1.5% chitosan and was in equilibrium with PBS, which contains about 1% dry weight. Apparently, with the current formulation of the gel there was no driving force for water uptake or release. This conclusion is confirmed by the swelling ratios, which were between 0.998 and 1.001. This can be described as virtually no swelling, compared to for example swelling ratios of 0.4-0.6 for chitosan gels cross-linked with β-glycerophosphate. The urea/urease chitosan gels are thus stable after gelation, which is a beneficial property with respect to their proposed application injectable gel. Furthermore for the swelling ratio as well as the equilibrium water content no statistical significant differences were observed between the various experimental gels. Based on these results it was concluded that swelling ratio and equilibrium water content are intrinsic properties of these urea/urease chitosan hydrogels and were not influenced by the urea and urease concentrations.

4.3. Cellular responses
PDLcs were viable after 3, 15 and 30 days of encapsulation in chitosan hydrogels, which confirms that the current hydrogel formulation supports the encapsulation of PDLcs. More specifically, cells survived the gelation process, indicating that changes in pH and osmolarity as well as ammonia production were within a physiologically acceptable range under the current experimental conditions. Cells collected upon gradual degradation of the hydrogel proliferated upon subsequent expansion culture. No differences were observed regarding the number of CFUs for the different cell populations, evidencing that the number of proliferating cells was similar in collected and parental cells when cultured in proliferation medium. Both collected and parental cells showed osteogenic differentiation capacity as reflected by the continuous increase in ALP activity and calcium content.
Interestingly, as the encapsulation time increased, lower proliferation but higher osteogenic differentiation of the collected cells was observed. After 14 days of osteogenic induction, the DNA content of cells collected after 30 days of encapsulation was significantly lower while its calcium content was higher compared to parental cells. The accelerated mineralization capacity of PDLCs after 3D encapsulation was similar to previous studies in which gene expression of self-renewal markers (i.e. Oct4, Sox2, and Nanog) in MSCs and ASCs were significantly higher for the cells in 3D spheroid in comparison to the monolayer cells, indicating the cells in 3D environment experienced a probably de-differentiating and returned to a more primitive state. This phenomenon might be explained by the 3D encapsulation provides cells a more physiological environment, for example the low oxygen tension (hypoxia) and softer substrate stiffness, which have already been proved to play important roles in stem cell maintenance. In general, PDLCs can survive and maintain their capacity after being encapsulated in the chitosan hydrogel and hence justify future studies on the regenerative capacity of these cytotherapeutic systems in an animal model.

5. Conclusions
The current study demonstrated that physically crosslinked chitosan hydrogels for biomedical purpose can be easily fabricated via enzymatic control. The gelation time, strength and degradation rate of chitosan hydrogels can be fine-tuned by controlling the pH upon gelation through variation of the concentrations of urea and urease. PDLC remained viable and maintained their capacities to form colonies and osteogenically differentiate upon encapsulation within these hydrogels for up to 30 days. In summary, the enzymatic control over the gelation of chitosan hydrogels offers options for the delivery of PDLCs via an injectable cytotherapeutic system, indicating the potential of this system in periodontal tissue engineering applications.
References

Chapter 6

Periodontal tissue regeneration using enzymatically cross-linked chitosan hydrogels with or without cell-loading

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Submitted
1. Introduction

Periodontitis, a common inflammatory disease caused mainly by dental plaque microorganisms\(^1\), is characterized by irreversible loss of periodontal tissues, which include gingiva, alveolar bone, root cementum, and periodontal ligament (PDL). Conventional treatments for periodontitis including scaling and root planing are usually successful in preventing the progression of the active disease, but the regeneration of lost tissues remains a clinical challenge.

Recently, periodontal tissue engineering, using supportive biomaterials and stem/progenitor cells, has been applied as a promising alternative to conventional periodontal treatments\(^2\text{-}^5\). Biomaterials offer tissue engineering and regenerative medicine a powerful tool in the form of cell delivery vehicles and scaffolds. A three-dimensional scaffold, serving as a space maintainer to temporarily support stem/progenitor cell attachment, proliferation and differentiation, is an important element for tissue engineering\(^6\). However, a diseased periodontal environment usually lacks robust stem/progenitor cells. Therefore, transplantation of ex vivo expanded stem/progenitor cells is hypothesized to facilitate periodontal regeneration\(^7\).

Among various biomaterials, chitosan has received major attention in tissue engineering and regenerative medicine due to its biocompatibility\(^8\), biodegradability\(^9\text{-}^{10}\), tissue regeneration capacity\(^11\), anti-inflammatory effects\(^12\) and antimicrobial activity\(^13\). Chitosan can be applied as a biomaterial in different forms, of which hydrogel vehicles are of great interest, as they offer several advantages including high cell seeding efficiency, easy handling and good formability to fill irregular defects\(^14\). Chitosan hydrogels can be prepared by either chemical or physical crosslinking of chitosan chains. The drawbacks of chemically crosslinked chitosan hydrogels include the potential cytotoxicity of applied chemical crosslinkers, such as glutaraldehyde, and the risk of altering the initial properties of chitosan through chemical modifications of the primary structure\(^15\text{-}^{16}\). A possible means to avoid the aforementioned disadvantages is to prepare physically crosslinked chitosan hydrogels, which can be obtained by increasing the pH of acidic chitosan solutions without the use of chemical crosslinking agents\(^9\text{-}^{17}\text{-}^{18}\).

Chenite et al.\(^19\) reported that pH-induced chitosan hydrogels can be generated through homogeneous neutralization of chitosan solutions by ammonia generated \textit{in situ} from enzymatic hydrolysis of urea. In our previous work, chitosan gelation kinetics of this system were addressed\(^20\). The results demonstrated that the gelation time of chitosan hydrogels can be precisely controlled by variation of the urea and urease concentrations, which provides the opportunity to set the desired gelation time for various applications.
Regarding cell population for periodontal regeneration, various cell types have been evaluated, including bone marrow stromal cells (BMSCs), periodontal ligament cells (PDLcs), alveolar periosteal cells (APCs), dental pulp cells (DPCs) and dental follicle cells (DFCs)\(^2\), \(^4\), \(^5\), \(^21\). Tsumanuma \textit{et al}. transplanted BMSCs, PDLcs, and APCs in canine one-wall intrabony defects to compare the regenerative potential between cell sources\(^4\). After eight weeks, significantly more well-oriented periodontal PDL fibers and newly formed cementum were observed upon transplantation of PDLcs. In addition, an organ culture study performed on tooth root surfaces showed that new alveolar bone and PDL-like tissues were formed only by PDLcs but not by MSCs, DPCs or DFCs\(^5\). These results suggest that PDLcs are the most suitable cell population for periodontal regeneration.

In our previous study, the suitability of enzymatically cross-linked chitosan hydrogel was evaluated for delivery of PDLcs \textit{in vitro}\(^{20}\). The results showed that this hydrogel can encapsulate PDLcs and support their survival \textit{in vitro} for up to 30 days. In addition, PDLcs released from the hydrogel upon degradation were able to form colonies and differentiate into the osteogenic lineage. However, the \textit{in vivo} biocompatibility of this chitosan hydrogel is not clear and the regenerative capacity of this system in an animal model still needs to be confirmed. Therefore, the aim of the present study was to evaluate the biocompatibility and regenerative potential of the enzymatically cross-linked chitosan hydrogel with or without incorporated PDLcs for periodontal regeneration \textit{in vivo}. Based on our previous experiments\(^2\), \(^22\), \(^23\), a validated rat maxillary periodontal defect model was chosen and PDLcs from green fluorescent protein (GFP) transgenic rat were applied to allow cell tracing after transplantation.

### 2. Materials and Methods

#### 2.1. Materials

Chitosan (average molecular weight 500 kDa, degree of deacetylation 85%, Heppe Medical Chitosan GmbH, Halle, Germany), urea (Invitrogen, Carlsbad, USA) and urease (type III from Jackbeans, U1500-20kU, Sigma, St. Louis, USA) were used as received. Chitosan was dissolved in 0.125 M HCl at a concentration of 2.5 w/v%. Urea (50 U/ml) was dissolved in MilliQ at a concentration of 10 M and sterilized by filtration over a 0.2 μm filter. Urease was dissolved in cell culture medium, which contained alpha minimal essential medium (αMEM; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Sigma), 100 U/ml penicillin and 50 U/ml streptomycin (Gibco).

#### 2.2. Isolation of PDL cells
Primary PDLCs were retrieved from one GFP transgenic SD rat (Japan SLC Inc., Shizuoka, Japan) as described previously\(^2\). Briefly, PDLCs were derived from extracted incisors of the GFP rat. The primary cell extraction procedure was performed according to the method described by Brunette et al.\(^24\). Cells were expanded in cell culture medium. Upon 70-80% confluence, cells were subcultured for 3 passages and afterwards cryo-preserved in α-MEM containing 20% FCS and 10% DMSO until use. After defrosting, cells from the 5\(^{th}\) passage were used.

### 2.3. Cell encapsulation

Cell encapsulation was performed 1 day before in vivo implantation. Chitosan hydrogels were prepared as described previously\(^20\). In brief, 1 ml chitosan solution was mixed with 0.67 ml urease solution (50 U/ml). Subsequently, 6.25 µl of the urea solution was added and mixed, which resulted in urea hydrolysis and pH increase. Before gelation, 200 µl chitosan solution containing 37.5 mM urea and 30 U/ml urease were mixed with 50 µl proliferation medium containing 3×10\(^6\) PDLCs. After gentle mixing, 20 µl of the mixture (containing 0.24×10\(^6\) PDLCs) was quickly injected in pre-sterilized Teflon molds (Ø 2.5 mm), followed by incubation at 37\(^\circ\)C for 30 minutes. Afterwards, the newly formed gels were transferred to non-adherent tissue culture 24-well plates. Proliferation medium was added and refreshed 30 and 60 min after encapsulation.

### 2.4. In vitro assays

Cell survival was determined using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) after 1 day of culture. Hydrogel samples were washed in PBS and incubated for 30 min in PBS solution with 2 mM calcein-AM and 4 mM ethidium homodimer at 37 °C. After incubation, samples were rinsed again in PBS, and then photographed using a Zeiss Imager Z1 equipped with an AxioCam MRc5 camera and operated using AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

For histological analysis, samples were fixed in 10% phosphate buffered formalin for 2 hours. Thereafter, samples were dehydrated in graded ethanol and embedded in paraffin. After deparaffinization in xylene and rehydration through graded series of ethanol, sections of 6 µm were cut with a microtome (Leica RM2165, Nussloch, Germany).

### 2.5. Animals

Twelve healthy male athymic nude rats (Hsd:RH-Foxn1nu, Harlan Laboratories, Horst, the Netherlands) were used as the recipient animals. The Animal Ethical Committee of Radboud University Nijmegen approved the study protocol (RU-DEC-2013-143). All procedures were in accordance with the national guidelines for the
care and use of laboratory animals. The recipient rats were 6-week old at the start of the experiment and had a known specific-pathogen free (SPF) status.

2.6. Surgical procedures
To minimize peri- and postoperative pain, rats received subcutaneous injection of carprofen \((5 \text{ mg kg}^{-1}; \text{Rimadyl, Pfizer Animal Health BV, Capelle aan de IJssel, the Netherlands})\) pre-operatively and on the first 2 days postoperatively. After intubation, general anesthesia was maintained with a mixture of nitrous oxide, 2.5% isoflurane, and oxygen via a constant volume ventilator. Bilateral intrabony three-wall periodontal defects were created mesially to both maxillary first molars as described previously\(^2,\,25\) (Figure 1 A-F).

**Figure 1.** Surgical procedure and schematic drawing of histomorphometrical analysis: (A) Preoperative general observation; (B) Flaps were raised to expose the root surface and alveolar bone; (C) The created defect; (D) Hydrogel placement in the defect; (E) Flaps were repositioned; (F) Flaps were closed with resorbable sutures; (G) Schematic drawing illustrating the histometric analysis of periodontal tissue regeneration.

With the aid of 2.5× magnifying loupes and strong light, a 3-mm-long full thickness incision was made along the alveolar ridge. A local adrenaline-containing dental anesthetic (Ultracain D-S\(^\circ\), Aventis Pharma BV, Gouda, the Netherlands) was applied to reduce bleeding. Flaps were raised to expose the root surface and alveolar bone. Subsequently, a three-wall intrabony defect was created along the
root surface using a piezoelectric device (OTS B-tip; Ø1.7 mm; Piezosurgery®, Mectron, Carasco, Italy). Thereafter, the remaining alveolar bone, PDL, and cementum were carefully removed from the root surface using a less abrasive OP5-tip. This tip was also used to create the final defect (W × L × D; 2 × 2 × 1.7 mm³), with monitoring the dimensions by a periodontal probe. Thereafter, defects were rinsed with sterile saline and dried with sterile gauze. Subsequently, the defects were randomly left empty (n=8; EMPTY), treated with either chitosan hydrogel only (n=8; CHIT) or chitosan hydrogel encapsulated with 0.24×10⁶ rat GFP⁺ PDLCs (n=8; CHIT+CELL). Finally, flaps were repositioned and sutured with resorbable sutures (Vicryl® 5-0, Ethicon Products, Amersfoort, the Netherlands).

2.7. Histological preparation
After 4 weeks, rats were deeply anesthetized prior to 10% formalin cardiovascular perfusion. Complete maxillae were harvested and excess tissues were removed. Subsequently, the maxillae were split into two parts through the palatal median line and fixed in buffered 10% formaldehyde for 24 hours. Afterwards, specimens were decalcified in 10% EDTA at room temperature. Decalcification end point was confirmed using X-ray. Thereafter, specimens were dehydrated in a graded series of ethanol and embedded in paraffin. Mesio-distal sections (6 μm thickness) were cut with a microtome (Leica RM2165, Nussloch, Germany). Sections were stained with haematoxilin and eosin (HE) for general tissue survey. For ligament and epithelial tissue observation, adjacent sections were stained with Azan staining and Elastica-van Gieson (EVG) staining, respectively.

2.8. Immunohistochemistry
GFP-positive cells from in vitro and in vivo samples were detected by immunohistochemical staining using an anti-GFP antibody (rabbit IgG, fraction, 1:500; Molecular Probes, Eugene, OR, USA). Specimens from previous work² were used as positive control. After sections were deparaffinized, rehydrated, and rinsed in PBS, antigens were retrieved with sodium citrate buffer at 70 °C for 10 minutes. Then, sections were pre-incubated in 10% normal donkey serum to inhibit non-specific staining for 60 min at room temperature. Next, sections were incubated with primary antibody (GFP, 1:500, rabbit anti IgG fraction; Molecular Probes) or PBS (negative control) overnight at 4°C in a humid atmosphere. Subsequently, sections were rinsed with PBS and incubated with a biotin-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Then, sections were immersed in ABC complex solution in the dark for 45 min at room temperature. The final visualization of reaction products was performed by treatment of 3,3’ diaminobenzidine (DAB) substrate (Envision kit; DakoCytomation,
Glostrup, Denmark) for 10 min at room temperature. The nuclei were slightly counter-stained with haematoxylin.

2.9. Histomorphometry
For the quantitative analysis of each specimen, up to 4 sections from the middle third of the defect area were evaluated. Sections were photographed using a Zeiss Imager Z1 equipped with an AxioCam MRc5 camera operated with AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH). Area and linear measurements were performed on images of histological sections by a blinded assessor (XY) using Image J image-analysis software (National Institute of Health, Bethesda, Maryland, USA), as previous described² (Figure 1 G). The following quantitative results were calculated based on the formulations:

\[
\text{Relative alveolar bone height} = \frac{L_{ac}}{L_{bg}}
\]

(1)

where \(L_{ac}\) is the length of the mesial molar from apex to cusp (Figure 1G ②), and \(L_{bg}\) is the length of bone gap which was assessed as the distance between the regenerated bone level and the top of the defect (Figure 1G ①).

\[
\text{Relative new bone area} = \frac{A_{nb}}{A_{td}}
\]

(2)

where \(A_{nb}\) is the area of new bone formation (Figure 1 G, area within green frames), and \(A_{td}\) is the area of the total defect (Figure 1 G, area within red frame).

\[
\text{Relative epithelial downgrowth} = \frac{L_j}{L_{ac}}
\]

(3)

where \(L_j\) is the length of the junctional epithelium from the cemento-distance from enamel junction to the apical extent (Figure 1G ③), and \(L_{ac}\) is the length of the mesial molar from apex to cusp (Figure 1G ②).

\[
\text{Relative functional ligament length} = \frac{L_{ff}}{L_{ca}}
\]

(4)

where \(L_{ff}\) is the length of functional ligament (Figure 1G ④), and \(L_{ca}\) is the distance from the cemento-enamel junction to the apical end of the defect (Figure 1G ⑤). Collagen fiber bundles were defined to be functional if the angle between the long axis of the main fibers and the root surface was larger than 60°⁴,₂⁶.

2.10. Statistical analysis
Statistical analysis was carried out using GraphPad InStat (GraphPad Software, San Diego California USA). Results were statistically evaluated by One-way ANOVA, followed by a Tukey-Kramer Multiple Comparisons Test. Results were considered statistically significant at \(p < 0.05\).

3. Results
3.1. Cell viability in the chitosan hydrogel
After 1-day culture, chitosan hydrogels encapsulated with PDLC were processed with LIVE/DEAD viability assay. The results showed that PDLCs were viable, as evidenced by the appearance of large numbers of live cells (appearing in green) and only few dead cells (appearing in red).

3.2. General observations animal experiment
One sample from EMPTY and one sample from CHIT were lost after one rat died during surgery. The remainder of the rats showed uneventful healing and gained weight during the 4-week study in a similar fashion. No clinical signs of inflammation were observed in any of the experimental groups, neither throughout the post-operative period, nor at tissue harvest.

3.3. Descriptive histology
In the HE-stained overview sections, two specimens from CHIT+CELL and one specimen from EMPTY were lost during histological processing. Consequently, data were obtained from 19 out of 22 retrieved surgically created periodontal defect specimens (Table 1).

| Table 1: Number of defects, harvested and evaluated specimens for different groups. |
|-------------------------------------------------|--------------------------|-------------------------------------------------|
| Number of defects | Number of harvested specimens | Number of evaluated specimens |
| EMPTY | 8 | 7 (1 lost during surgery) | 6 (1 lost during histological processing) |
| CHIT | 8 | 7 (1 lost during surgery) | 7 |
| CHIT+CELL | 8 | 8 | 6 (2 lost during histological processing) |

In general, the apical end of cementum/ligament removal was observed in all experimental groups (Figures 2 and 3). The healed defects were occupied by epithelium, connective tissue, and newly formed alveolar bone. In some specimens, newly formed cellular cementum (Figure 2B), PDL (Figure 2B), and ankylosis (Figure 3B) was observed. The newly formed cellular cementum was limited to the apical part of the defect. Occasionally, the presence of resorption lacunae was detected on the surface of root dentin (Figure 3B). None of the specimens showed complete periodontal regeneration. For all CHIT and CHIT+CELL specimens, chitosan hydrogel remnants were observed (Figure 3C and 3E). None of the specimens showed signs of inflammation or adverse tissue responses.
Figure 2. Histological observations of the different treatment groups (HE staining): (A) EMPTY; (C) CHIT; (E) CHIT+CELL. Higher magnifications of panels A, C, and E are shown in panels B, D, and F, respectively.

For EMPTY, all specimens exhibited new bone formation. On the denuded dentin surface, resorption lacunae were detected in 2 and ankylosis was observed in 5 out of 6 specimens. Some specimens (2 out of 6) exhibited new cellular cementum formation, which was limited to the apical part of the defect. Relatively loosely packed fibrous connective tissue was observed running parallel to the dentin surface (Figure 4A and 4B).

For CHIT, light microscopic analysis showed that the chitosan hydrogels were largely degraded (Figure 3C) without any adverse reaction in the surrounding tissue. Penetration of connective tissue into the remaining fragments was observed (Figure 3D). Newly formed bone was observed in all specimens, mainly originating from the apical part of defects. Beside new bone formation being present in the defect margin areas, new bone tissue was observed in the middle of
the defect area as islets, which were scattered amongst the remnants of scaffold material (Figure 2D). Along the dentin surface, resorption lacunae were detected in 1, ankylosis in 3, and new cellular cementum formation in 3 out of 7 specimens. In addition, the supracrestal connective tissues exhibited functional orientation of the fibers, which obliquely inserted into the dentin surface (Figure 4D).

**Figure 3.** Histological observations of the different treatment groups (EVG staining): (A) EMPTY; (C) CHIT; (E) CHIT+CELL. Higher magnifications of panels A, C, and E are shown in panels B, D, and F, respectively.

Specimens from CHIT+CELL showed comparable histological results to CHIT. Newly formed bone was mainly observed at the mesial/apical defect edges. Immature bone islets were scattered amongst the remnants of scaffold material. Along the dentin surface, resorption lacunae were detected in 2, ankylosis in 3, and new cellular cementum formation in 2 out of 6 specimens. Moreover, functional orientation of fibers was observed in the supracrestal connective tissues (Figure 4F).
Figure 4. Representative images for ligament formation from three treatment groups (Azan staining): (A) EMPTY; (C) CHIT; (E) CHIT+CELL. Higher magnifications of panels A, C, and E are shown in panels B, D, and F, respectively.

3.4. GFP immunohistochemistry
The immunohistochemical staining succeeded in demonstrating GFP signal in the positive control (Figure 5A). The negative control showed no specific immunoreactivity in the related tissues (Figure 5B). Examination of the immunohistochemically stained sections revealed that PDLCs embedded in chitosan hydrogel were GFP-positive prior to implantation (Figure 5D). At 4 weeks
post-transplantation, no GFP signal was detected in any of the in vivo specimens (Figure 5C).

**Figure 5.** Anti-GFP immunohistochemical staining: (A) GFP+ cells were visualized by brown staining in the positive control; (B) The negative control showed no specific immunoreactivity; (C) At 4 weeks posttransplantation, no GFP signal could be detected in the in vivo specimens; (D) PDLCs were GFP positive before implantation.

### 3.5. Histomorphometrical analysis of periodontal regeneration

Histomorphometrical measurements of periodontal regeneration are presented in Figure 6. The relative alveolar bone height was 6.1 ± 1.4, 5.9 ± 4.0, and 4.6 ± 1.4, for EMPTY, CHIT, and CHIT+CELL, respectively, with no statistical difference between the experimental groups (p > 0.05). Comparable values (p>0.05) of relative alveolar bone area were measured for all groups, ranging from 0.3 ± 0.1 for EMPTY, 0.2 ± 0.1 for CHIT, to 0.2 ± 0.1 for CHIT+CELL. The relative epithelial downgrowth in EMPTY, CHIT, and CHIT+CELL was 0.2 ± 0.1, 0.2 ± 0.1, and 0.2 ± 0.1, respectively, without significant differences between the experimental groups (p > 0.05). For relative functional ligament length, the Azan stained sections showed that around 9% of denuded root surface in the EMPTY (0.09 ± 0.04) was covered by functional collagen fibers. In contrast, sections from CHIT (0.35 ± 0.09) and CHIT+CELL (0.38 ± 0.19) showed a significantly (p < 0.01) higher relative functional ligament length than EMPTY. In addition, CHIT demonstrated a comparable score.
for relative functional ligament length to the CHIT+CELL, with more than 35% of the denuded root surface covered by functional collagen fibers.

**Figure 6.** Histomorphometrical measurements. There was no statistical difference between all groups in terms of alveolar bone height (A), alveolar bone area (B) and epithelial downgrowth (C). Samples from EMPTY showed a significantly negative effect with respect to the functional ligament measurements than the CHIT and CHIT+CELL groups (D). **p <0.01.

4. Discussion
The objective of the current study was to evaluate the in vivo biocompatibility and periodontal regenerative potential of enzymatically cross-linked chitosan hydrogels with or without incorporated PDLCs. To this end, GFP-positive PDLCs were encapsulated in chitosan hydrogels and transplanted into a rat periodontal defect model. The results from this study demonstrate that enzymatically cross-linked chitosan hydrogels are biodegradable. In addition, this hydrogel was well-tolerated in the periodontal defect, as there were no undesirable local responses observed in the recipient rats. Moreover, the hydrogel was favorable to induce functional orientation of fibers compared to empty controls in the current rat model. PDLCs remained viable upon encapsulation within the chitosan hydrogels
before transplantation, although no GFP signal was detected at 4 weeks post-transplantation in the defect region. To explore the efficacy of chitosan hydrogel as a potential implantation material in periodontal regeneration, an intrabony defect with limited spontaneous healing was used\textsuperscript{25}. This defect was surgically created, which is dissimilar to naturally occurring periodontitis caused by plaque. However, compared to plaque-induced periodontal breakdown, surgically prepared defects help keep the initial conditions of different experimental groups almost identical, which increases the reproducibility of the model system. Many reports have shown that chitosan-derived materials have high biocompatibility\textsuperscript{27-30}. Chitosan hydrogels used in the current study were fabricated using urea and urease. According to our previous work\textsuperscript{20}, chitosan hydrogel containing 37.5 mM urea and 30 U/ml urease was used since its gelation time (11.7 ± 0.6 min) is suitable for clinical handling of cell encapsulation. Our previous work has demonstrated that the urea can be totally converted by hydrolysis within 15 min\textsuperscript{20}. However, the \textit{in vivo} influence of the remaining urease in this hydrogel are not clear. This study proved that enzymatically cross-linked chitosan hydrogel also showed good biocompatibility, as there were no undesirable local responses observed in the recipient rats. Light microscopy analysis demonstrated that this hydrogel was largely degraded \textit{in vivo} after 4 weeks and there was no inflammatory cell infiltration around the grafted material, indicating there was no adverse effect from the limited amount of remained urease. Our results further confirmed that enzymatically cross-linked chitosan hydrogel is a promising biomaterial for periodontal tissue regeneration based on several findings. First, CHIT displayed superior development of the ligament structure compared to EMPTY. For measurements on the ligament structure, the quantity as well as the orientation of newly formed collagen bundles were taken into account as reported previously\textsuperscript{3,31}. The insertion of functionally oriented collagen bundles into hard tissues may lead to improved sensing and protection toward mechanical load from mastication\textsuperscript{32}. Second, CHIT exhibited relatively less ankylosis (3/7 samples) compared to EMPTY group (5/6 samples). Ankylosis is a pathologic fusion of the dentin or cementum of a tooth root to the alveolar bone\textsuperscript{33}. Ankylosis may occur when primary proliferation of cells from alveolar bone takes place, for which the speed of PDLCs in repopulating the root surface determines whether or not ankylosis occurs\textsuperscript{30}. Therefore, our results indicated that this chitosan hydrogel might enhance periodontal ligament regeneration by allowing/promoting repopulation of PDLCs on the root surface. Third, the chitosan hydrogel used in the present study supports new bone formation, as new bone tissue were formed as
islets, which were scattered amongst the remnants of scaffold material. A previous study demonstrated that chitosan-collagen composite but not collagen alone was able to stimulate ectopic bone formation in a subcutaneous location, indicating the possible osteoinductive property of chitosan\textsuperscript{34}. Taken together, these above findings suggest that this chitosan hydrogel may be useful for applications in periodontal regeneration.

Our previous in vitro study\textsuperscript{20} demonstrated that enzymatically cross-linked chitosan hydrogel can support PDLC survival in vitro for up to 30 days, which justifies the evaluation of this chitosan-based hydrogel as a cell delivery system for periodontal regeneration in an animal model. However, at the end of the 4-week transplantation period, no GFP signal was detected in any of the in vivo specimens, indicating that there are no exogenous healing cells in the defect area anymore. This result is different from our previous study, in which GFP-positive staining of implanted PDLCs was detected after 6-week implantation using a gelatin sponge as a cell carrier material\textsuperscript{9}. As the same PDLCs and defect model were used in this study, this discrepancy is most likely caused by the difference in cell carriers. As GFP-positive cells were only detected in 4 out of 8 specimens in our previous study\textsuperscript{9}, the cells encapsulated in chitosan hydrogels most likely migrated out of the defect after implantation in vivo. Similarly, Tour et al. reported that implanted PDLCs decreased in number with time in a defect after transplantation in a calvarial defect model, from around 240 GFP\textsuperscript{1} cells/mm\textsuperscript{2} defect area after 1 week to single or no positive cells detected after 10 weeks\textsuperscript{35}. Taken together, these findings suggest that transplanted cells tend to vanish with time after implantation.

Considering that there are no differences in periodontal regeneration between CHIT+CELL and CHIT, additional work on the localization and contribution of transplanted cells to tissue regeneration are required.

The limitations of this study include the small model size and only one time point of observation. Due to the difficulty in handling specimens with small defect size, 3 out of 22 samples were lost during histological processing. Although this model is suitable for initial studies, the biocompatibility and regenerative capacity of this chitosan hydrogel should be corroborated in a larger animal model in the future. A larger animal model would provide higher comparability with the human situation and thus would facilitate the translation from bench to bedside more optimally. In order to clarify the exact fate of transplanted PDLCs delivered by chitosan hydrogels, additional experiments with different observation periods are also necessary.
Conclusion
Enzymatically cross-linked chitosan hydrogel was demonstrated to display high biocompatibility and biodegradability. In addition, the implantation of hydrogel alone promotes functional ligament formation and reduces the incidence of ankylosis in the current rat model, indicating the application potential of this hydrogel for periodontal tissue regeneration. In contrast, no signal from transplanted PDLCs could be detected at the end of 4-week transplantation period, indicating that there are no exogenous healing cells in the defect area anymore.
Chapter 6

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Chapter 7

The effect of enamel matrix derivative (Emdogain®) on gene expression profiles of human primary alveolar bone cells

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1. Introduction

The purified enamel matrix protein product, (Emdogain®; EMD), is commonly used in periodontal practice. EMD is prepared from porcine tooth germs and although described to consist mainly of amelogenins^1^, the exact content is complex and remains unknown. The effect of EMD on periodontal ligament (PDL) cells has been examined in different in vitro studies, which found that EMD stimulates cellular proliferation, migration, alkaline phosphatase (ALP) activity, mineralized nodule formation and transforming growth factor β1 (TGF-β1) production^2^-^5^ and ^6^ - ^8^ . In addition, a few studies have focused on the bone regenerative properties of EMD.

It is evident that the effect of EMD on proliferation and differentiation varies among different cell sources, but still marked effects of EMD on different osteoblastic cell lines have been reported. Yoneda et al.^6^ used two mouse osteoblastic cell lines and found no effect for EMD with the ST2 cell line, but with the KUSA/A1 cell line enhanced cell proliferation, ALP activity and mineralized nodule formation were observed. Other studies found different responses of osteoblastic cell lines with EMD^7^-^10^ . Keila et al.^7^ performed a study with rat bone marrow cells and observed an increase in the osteogenic capacity (ALP activity and mineralized nodule formation) of the cells in the presence of EMD. Schwartz et al.^8^ found that EMD stimulated the proliferation, but not the differentiation, of immortalized preosteoblastic 2T9 cells, and inhibited the proliferation and stimulated differentiation of osteoblast-like MG63 (osteosarcoma) cells. They also found that the proliferation and differentiation of normal human osteoblast NHOst (unprocessed human bone marrow cells) cells increased. Hama et al.^10^, however, found that EMD inhibited ALP activity, mRNA expression of osteocalcin and core binding factor, and decreased bone nodule formation of rat carvarial cells. When reviewing previous literature it is evident that some researchers used calvarial cells^9,10^ to examine the osteogenic capacity, while others used bone marrow cells^7^.

Moreover, different animals were used for cell retrieval. All these discrepancies in primary conditions can have severe implications for the final results. Because of the great differences between outcomes of in vitro studies, their validity in terms of determining the effect of EMD on primary human alveolar bone cells, which are present in human periodontal and periimplant bony defects, is questionable. Moreover, since it is still not completely known which factors make up EMD^11,12^, one can hardly conclude about possible pathways of EMD on different cell types (pathway = “The cascade of interaction between different genes of human bone cells modulated by the treatment of Emdogain”). Therefore we hypothesize that knowing the effect of EMD on RNA expression of primary human cells would add important information.
The micro-array technique is commonly used for m-RNA expression profiling. With this technique it is possible to monitor expression levels for all genes simultaneously to study the effects, for example, of certain treatments. Therefore the aim of the study was to analyze the effect of EMD on expression profiles of human alveolar derived bone cells with the help of the micro-array, quantitative polymerase chain reaction (qPCR), and enzyme-linked immunosorbent assay (ELISA) in order to discover the pathways of EMD. With a complete understanding of the mechanism of EMD, a further development of a standardized product in order to achieve similar or better treatment outcome compared to EMD would be possible.

2. Materials and Methods
2.1. Cell isolation
After patients had signed informed consent, bone was harvested from 7 non-smoking patients with good general health (ASA score I) during dental implant surgery (surplus material that otherwise would have been thrown away). All patients were of female gender with an age range of 20 to 37 years. The bone debris was harvested from the posterior maxilla in 3 cases and from the posterior mandible in 4 cases. Bone debris was retrieved from the drill surface and stored in sterile phosphate-buffered saline (PBS). The bone debris was washed extensively with PBS to remove undesirable components (blood cells, fatty tissue etc). Thereafter, the bone particles were placed in a 25 cm² culture flask. The flask was put up side down, and 5 ml of culture medium (a-MEM, supplemented with 10% foetal calf serum (FCS) (Gibco BRL, Life Technologies B.V. Breda, the Netherlands), 50 μg/ml ascorbic acid, 3 μg/ml fungizone, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) was added. The flask was placed into a humidified incubator set at 37°C and 5% CO₂ for 3 hours to allow for attachment of the bone debris to the culture flask surface. Then, the flask was gently rotated so that the debris was immersed into the culture medium. The cultures were left untouched for a week, then inspected twice a week to see cell outgrowth. As soon as cell outgrowth was observed, medium was changed every 2-3 days.
2.2. Cell seeding
When cells grown from the explanted bone material had reached subconfluence, the cells surrounding the explants were detached using trypsin-EDTA (Gibco) and cells from four patients were subcultured in one 25 cm² culture flask and two 175 cm² flasks. Cells from other three patients were subcultured in one 25 cm² culture flask and one 6-well plate in triplicate. The latter were cultured up to subconfluence. Subsequently the cells in the “control” flask or well from each
patient was cultured in previously used growth medium, and the cells of the “test” flask or well of each patient was cultured in the same medium with the addition of 100 µg/ml Emdogain® (Straumann AG, Waldenburg, Switzerland), until 24 hours thereafter RNA was extracted and cell culture supernates were stored at -80°C for further ELISA assays.

2.3. Von Kossa staining
To verify that the primary human bone cells of each patient after culture and single passaging still were able to produce mineralized tissue, one 25 cm² flask was cultured under mineralization inducing conditions: using α-MEM medium supplemented with 10% FCS (Gibco), 50 µg/ml ascorbic acid, 50 µg/ml gentamycin, 10 mM sodium β-glycerophosphate and 10⁻⁸ M dexamethasone (Sigma). The cells were incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37°C. The medium was changed every 2-3 days.

The cells were allowed to grow for three weeks, thereafter Von Kossa staining was performed in order to identify the formation of calcium phosphate in the matrix as follows: cells were fixed with 2% glutaraldehyde, stained with fresh 5% silver nitrate (AgNO₃), washed with distilled water, developed with 5% sodium carbonate (Na₂CO₃) in 25% formalin, and fixed with 5% sodium thiosulphate (Na₂S₂O₃). Finally, the samples were examined with a light microscope.

2.4. RNA isolation
RNA was isolated from the cells of the two flasks or six wells of each patient (with or without EMD) with the aid of an RNA-isolation kit (RNeasy kit; Qiagen, Venlo, the Netherlands) following manufacturer’s instructions. Cells were disrupted by adding lysis buffer. After isolation, the RNA pellet was dissolved in RNase-free water and stored at -80°C until RNA quality control and subsequently micro-array and qPCR was performed. The RNA samples were analysed separately, thus 8 micro-arrays have been performed, i.e. one control and one EMD sample from each of the four patients. The RNA samples from the other patients were further assessed with qPCR to externally validate the micro-array data.

2.5. RNA quality control and Affymetrix micro-array
The RNA integrity was verified by using the 2100 Bioanalyzer (Agilent), and samples with a 28S:18S ratio <1.5 were excluded. RNA samples (30 ng in 1 µl water) were incubated for 2 minutes at 70°C in thin-wall 0.5 mL PCR tubes. Samples were cooled followed by immediate analysis with RNA 6000 Nano LabChip kits on the Agilent 2100 bioanalyzer according to the manufacturer’s instructions. In the Affymetrix standard protocol, an initial cycle of reverse transcriptase (RT) converts RNA into cDNA using random primers linked with the T7 promoter. This was followed by second-strand cDNA synthesis. The double-stranded cDNA was
then used as a template for in vitro transcription with T7 RNA Polymerase, which produces many copies of the cRNA that are reverse complementary to the original RNA molecules. In the second cycle of cDNA synthesis, random primers were used to reverse transcribe the cRNA to obtain single-stranded DNA. The DNA was then fragmented and labeled in preparation for hybridization.

After two RT cycles, the final single-strand DNA product was in the same orientation as original RNA. Based on annotation databases, the probes were pre-manufactured to be reverse complementary to RNA sequences, so that the labeled product could hybridize with them.

Two µg of total RNA was labeled according to the GeneChip Whole Transcript (WT) Sense Target Labeling Assay as provided by the manufacturer (Affymetrix, Santa Clara, CA, USA), and hybridized to Human Exon 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) overnight before scanning in an Affymetrix GCS 3000 7G scanner. Quality and reliability of the micro-array performed was assessed using the Area Under the Curve of a Receiver Operator Characteristics (AUC) scores (expresses how many of 1000 positive control probesets were indeed positive and how many of 1000 negative control probesets were indeed negative).

2.6. Reverse transcriptase PCR and real-time PCR

Five genes of particular interest were selected for data validation by qPCR. After the extraction of mRNA, the first strand reverse transcriptase (RT) PCR was performed using the Superscript™ III First-strand Synthesis System for RT-PCR (Invitrogen) according to manufacturers’ protocol.

The cDNA was then amplified and specific gene expression was quantified in a real-time PCR. For this reaction, 12.5 µL master mix, 2 µL DNA, 3 µL primer mix (1.5 µL forward primer and 1.5 µL reverse primer) and 7.5 µL RNase free water was mixed and centrifuged. Subsequently the PCR was performed in a Real-Time PCR reaction apparatus (BIORAD) with the desired temperatures. The used primers were from Prostaglandin-endoperoxide synthase-2 (also known as Cyclooxygenase 2; COX-2), Interleukin 7 (IL-7), biglycan (BGN), latent TGF β binding protein (LTBP), Prostaglandin E receptor 2 (EP2), glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (sequences can be found in Table 1). The expression levels were analyzed versus the housekeeping gene GAPDH. Specific sense and antisense primers for the genes were designed according to published cDNA sequences of GenBank. The specificity of the primers was tested separately before the real-time PCR reaction. Each sample was done in duplicate and expressed as mean ± standard deviation. The RNA-negative control consisted of only milli-Q water that was reverse transcribed and amplified in parallel with the cell samples. The expression of the tested genes was calculated via the $2^{-\Delta\Delta Ct}$ method. 

Chapter 7
Table 1: Primer sequences for real time quantitative PCR

<table>
<thead>
<tr>
<th></th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
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<tbody>
<tr>
<td>COX-2</td>
<td>TGCATTCTTTGCCCAGCCTGA</td>
<td>AAAGGCCAGTTTACGCTGT</td>
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<td>IL-7</td>
<td>TGCCCTAATCCGTTTGGACCATGGT</td>
<td>ACACGAACTTTAGCTGCATCTCTCCAC</td>
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<tr>
<td>BGN</td>
<td>TGGTCCCTTCCATCTCTCGAAGCTG</td>
<td>GACTGCTGTCCTTGGGTTTTGC</td>
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<td>ILTBP-2</td>
<td>AAGGGGAAAGGGAGGGTTGCATAA</td>
<td>GGGGGATGGTAAAGTTCTCCGATGGT</td>
</tr>
<tr>
<td>EP2</td>
<td>CGCGTGACACTTTCCGCTT</td>
<td>AAGTAGGGGTGCCCAGATCGAGA</td>
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<td>GAPDH</td>
<td>ATGCCAGTGAGCTTCCTCCGTTGCAGC</td>
<td>TGGATCTCTGGAAGACTCATGAC</td>
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</tbody>
</table>

2.7. ELISA
The levels of IL-7 and PGE2 in the culture medium were detected by ELISA, following the manufacturer’s protocol (R&D Systems). Briefly, 200 μl sample or standard were added to each well and incubated overnight at room temperature. After a total of 6 washes, 200 μl of IL-7 conjugate solution was added and incubated for 2 hours at room temperature. Wells were washed a further six times and incubated for 45 min at room temperature with 50 μl substrate solution. Then the color development was initiated by adding 50 μl of Amplifier Solution. Forty-five minutes later, the reaction was stopped by adding 50 μl of stop solution to each well and the absorbance was read at 490 nm.

2.8. Data analysis
All genes which showed at least an average two fold difference (up or down regulation) with a p-value of ≤ 0.01 of expression between conditions were imported into Ariadne Genomics (Ariadne, Rockville, MD, USA). This computer software browses the Gene Ontology database, the Human Protein Reference Database, the Kyoto Encyclopedia of Genes and Genomes database, and the Prolexys HyNet protein interaction database in order to find known pathways or known interactions of selected genes. The differently expressed genes, as mentioned above, were finally transferred into the Database for Annotation, Visualization, and Integrated Discovery (DAVID; www.david.abcc.ncifcrf.gov). DAVID computed the percentages of differently expressed genes involved in the three general Gene Ontology (GO)-term categories (BP, CC, MF), and different
terms (only terms of level 1 specifications had been analyzed); out of these percentages p-values were generated and corrected for multiple testing (Benjamini Hochberg). Also the Gene Ontology (GO) approach was used. The GO project has developed three structured controlled vocabularies (ontologies) that describe gene products in terms of their associated biological process (BP), cellular components (CC), and molecular functions (MF) in a species independent manner with five term levels of specification. Statistical analysis for qPCR was performed using an unpaired t-test. Calculations were performed in InStat (v. 3.05 GraphPad Inc, San Diego, CA).

3. Results
3.1. Von Kossa staining
Routine microscopy showed after approximately 10 days that first mineral nodule formation was visible. At day 21 Von Kossa staining showed the whole flasks were homogenously covered with calcified nodules (Figure 1).

![Figure 1: Micrograph of Von Kossa stained cells after 21 days of culture. (Magnification: 10x)](image)

3.2. Micro-array analysis
In the Quality control of the micro-array, all our samples had AUC scores of at least 0.90 up to 0.93. Since 0.85 is regarded as the minimum reliable AUC score, evidently all samples could be utilized for further analysis. Differences of at least 2
fold changes and a p-value of ≤ 0.01 between the conditions were detected in the expression of 66 genes (see Appendix for the entire gene list). Of these genes, 41 were ≥ 2 times higher and 25 were ≥ 2 times lower expressed after EMD treatment compared to control treatment.

3.3. Pathway analysis
All genes differently expressed between the conditions in Ariade Genomics software were analyzed for possible interaction in known pathways (Figure 2). Two previously described interactions between genes were found. Names, protein function and interactions of all genes involved in these two pathways are depicted in Table 2. Unfortunately protein function and interaction of the genes shown in Figure 2 are not well understood; therefore information of function and interaction of the genes is in most cases very rare and not well explained. However, the genes encoding for COX-2, EP2, and IL-7 are known to be associated with bone resorption.

Figure 2: Pathway analysis. Two known interactions between genes were identified. Note that red highlighted genes were higher, blue highlighted genes were lower expressed after EMD treatment.
Table 2: Genes involved in the two interactions found.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>GenBank</th>
<th>Gene name</th>
<th>Protein function</th>
<th>Interaction</th>
</tr>
</thead>
</table>
| IL-7         | NM_000880 | Interleukin 7                  | Hematopoietic cytokine; involved in immune response and brain tumor growth        | • IL-7 → SLC2A1  
• positive effect on regulation and expression  
• IL-7 induced Glut1 glucose transporter expression and glucose uptake |
| SLC2A1       | NM_006516 | Solute carrier family 2        | Transporter for glucose and other hexoses                                        | • ATG1 → PTGS2  
• positive effect on expression  
• ATG1 → PTGS2  
• positive regulation effect |
| ATF1         | NM_005171 | Activating transcription factor 1 | Important transcript factor in COX-2 gene expression                             | • PAWR → PTGS2  
• positive effect on expression  
• PAWR → PTGS2  
• positive regulation effect |
| PAWR         | NM_002583 | PRKC, apoptosis, WT1, regulator | May play a role in apoptosis                                                      | • TLR 3 → PTGS2  
• positive effect of expression  
• TLR 3 → PTGS2  
• positive effect of expression |
| TLR 3        | NM_003265 | Toll-like receptor 3           | Involved in immune response                                                      | • COMP → ACTG2  
• negative effect on regulation  
• COMP reduced renal tissue levels of TGF-beta1, and actin, gamma2 |
| COMP         | NM_000095 | Cartilage oligomeric matrix protein | Noncollagenous extracellular matrix protein                                     | • COMP → ACTG2  
• negative effect on regulation  
• COMP reduced renal tissue levels of TGF-beta1, and actin, gamma2 |
| ACTG2        | NM_001615 | Actin, gamma 2, smooth muscle actin | Involved in various types of cell motility, and maintenance of the cytoskeleton | • COMP → ACTG2  
• negative effect on regulation  
• COMP reduced renal tissue levels of TGF-beta1, and actin, gamma2 |

3.4. Gene Ontology analysis

The up-regulated genes, after EMD treatment, associated with the level one terms of the three GO categories are depicted in the Appendix (individual genes were only presented if their level one term remained significant after correction for multiple testing correction). However, only the terms “proteinaceous extracellular matrix” and “extracellular matrix” of the category “Cellular Components” remained statistical significant after multiple testing correction (Table 3). Individual genes contributing to these terms are presented in Table 4. The down-regulated genes associated with the level one terms of the three GO categories, after EMD compared to control treatment, are listed in the Appendix. Again, after multiple testing correction just the term “trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity” of the Category “Molecular Function” remained statistically significant (Table 5). Genes contributing to that term are aldo-keto reductase family 1, member c1, member c2, and member c3. Markedly down-regulated individual genes, not contributing to any significant annotation term, but of particular interest are the genes encoding for COX-2, EP2, and IL-7 as mentioned above.
Table 3: Up-regulated genes associated with GO category Cellular Component. Note that the DAVID Gene software computed the percentages of differently expressed genes involved in the different terms and out of these percentages p-values were generated and corrected for multiple testing (Benjamini Hochberg correction).

<table>
<thead>
<tr>
<th>Category</th>
<th>Term</th>
<th>%</th>
<th>p-value</th>
<th>Benjamini Hochberg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular Component</td>
<td>proteinaceous extracellular matrix</td>
<td>16.0</td>
<td>1.8E-5</td>
<td>1.6E-2</td>
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<tr>
<td>Cellular Component</td>
<td>extracellular matrix</td>
<td>16.0</td>
<td>2.0E-5</td>
<td>8.7E-3</td>
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<tr>
<td>Cellular Component</td>
<td>extracellular region part</td>
<td>18.0</td>
<td>7.2E-4</td>
<td>1.9E-1</td>
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<td>Cellular Component</td>
<td>extracellular region</td>
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<td>Cellular Component</td>
<td>collagen</td>
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<td>Cellular Component</td>
<td>extracellular matrix part</td>
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<td>3.2E-2</td>
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</table>

Table 4: Genes contributing to the terms “proteinaceous extracellular matrix” and “extracellular matrix” of the category “Cellular Component”

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>GenBank</th>
<th>Gene name</th>
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<tbody>
<tr>
<td>2970942</td>
<td>NM_000493</td>
<td>collagen, type X, alpha 1</td>
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<tr>
<td>3571944</td>
<td>NM_000428</td>
<td>latent transforming growth factor beta binding protein 2</td>
</tr>
<tr>
<td>3501219</td>
<td>NM_001846</td>
<td>collagen, type IV, alpha 2</td>
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<tr>
<td>3852381</td>
<td>NM_024825</td>
<td>hypothetical protein fli23447</td>
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<tr>
<td>2865327</td>
<td>NM_001884</td>
<td>hyaluronan and proteoglycan link protein 1</td>
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<tr>
<td>3855218</td>
<td>NM_000095</td>
<td>cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>3193482</td>
<td>NM_000093</td>
<td>collagen, type V, alpha 1</td>
</tr>
<tr>
<td>3995633</td>
<td>NM_001711</td>
<td>Biglycan</td>
</tr>
<tr>
<td>3632907</td>
<td>NM_003612</td>
<td>semaphorin 7a, gpi membrane anchor</td>
</tr>
</tbody>
</table>

Table 5: Down-regulated genes associated with GO category Molecular Function.

<table>
<thead>
<tr>
<th>Category</th>
<th>Term</th>
<th>%</th>
<th>p-value</th>
<th>Benjamini Hochberg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Function</td>
<td>Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity</td>
<td>7.7</td>
<td>5.7E-6</td>
<td>1.6E-2</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>Oxireductase activity, acting on the CH-CH group of donor, NAD or NADP as acceptor</td>
<td>7.7</td>
<td>2.3E-4</td>
<td>2.8E-1</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>Oxireductase activity</td>
<td>17.9</td>
<td>1.6E-3</td>
<td>7.8E-1</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>Oxireductase activity, acting on the CH-CH group of donors</td>
<td>7.7</td>
<td>2.0E-3</td>
<td>7.6E-1</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>Bile acid binding</td>
<td>5.1</td>
<td>2.8E-3</td>
<td>7.4E-1</td>
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<tr>
<td>Molecular Function</td>
<td>3-alpha-hydroxysteroid dehydrogenase activity</td>
<td>5.1</td>
<td>2.8E-3</td>
<td>7.4E-1</td>
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<tr>
<td>Molecular Function</td>
<td>Oxireductase activity, acting on the CH-OH group of donor, NAD or NADP as acceptor</td>
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<td>Molecular Function</td>
<td>Carboxylic acid binding</td>
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<td>Molecular Function</td>
<td>Oxireductase activity, acting on the CH-OH group of donor</td>
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<td>9.9E-1</td>
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<tr>
<td>Molecular Function</td>
<td>Aldo-keto reductase activity</td>
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<tr>
<td>Molecular Function</td>
<td>Catalytic activity</td>
<td>35.9</td>
<td>4.4E-2</td>
<td>1.0E0</td>
</tr>
</tbody>
</table>
### Appendix: Differently expressed genes between test and control treatment.

Note that the positive or negative prefix indicates whether the genes are higher or lower expressed after EMD treatment.

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>GenBank</th>
<th>Gene name</th>
<th>p-value</th>
<th>Average fold-change</th>
</tr>
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<tr>
<td>2429371</td>
<td>NM_005725</td>
<td>tetraspanin 2</td>
<td>0.006358</td>
<td>5.521438</td>
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<tr>
<td>3855218</td>
<td>NM_000095</td>
<td>Cartilage oligomeric matrix protein</td>
<td>0.00228</td>
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<td>3802602</td>
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<td>Cadherin 2, type 1, N-cadherin (neuronal)</td>
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<td>4.02636</td>
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<td>3538087</td>
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<td>dapper, antagonist of beta-catenin, homolog 1</td>
<td>0.007082</td>
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<tr>
<td>3911217</td>
<td>NM_020182</td>
<td>transmembrane, prostate androgen induced RNA</td>
<td>0.000816</td>
<td>3.316139</td>
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<td>Gene Symbol</td>
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Chapter 7

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3.5. Real-time quantitative PCR analysis

The results from the q-PCR demonstrated the same trend between two different donors and the expression of four regulated genes (COX-2, IL-7, BGN, EP2) out of five correlated well with the regulation found in the micro-array data. As shown in Figure 3, EMD treatment significantly up-regulated the gene expression for BGN and the down regulation of COX-2, IL-7, and EP2 were also observed.

![Figure 3: Relative gene expression by qPCR, in separate donor from the microarray study. Influence of EMD on gene expression evaluated after 24 hours of cell-culture stimulation. Values were normalized to GAPDH. EMD treatment significantly up-regulated the gene expression for BGN and the down regulation of COX-2, IL-7, and EP2 were also observed. The results demonstrated that the expression of four regulated genes (COX-2, IL-7, BGN, EP2) out of five correlated well with the regulation found in the micro-array data. *p ≤ 0.05, n = 3.](image-url)

3.6 ELISA

Quantification of PGE$_2$ at the protein level was impossible as amounts were below the detection limits of ELISA testing. However the significant decrease of IL-7 level in the supernate of EMD-treated cells after 24 hours of cell-culture stimulation was confirmed (Figure 4).
Figure 4: Protein level of IL-7 assessed after 24 hrs by ELISA testing, in separate donors from the microarray study. IL-7 was decreased significantly in the supernate of EMD-treated cells. *p < 0.001, n = 3.

4. Discussion
Although EMD proteins are clinically employed in periodontal treatment, the exact components are unknown. Thus, one can hardly conclude through which component or components EMD exerts its clinical effects. Additionally, confusing data from in vitro studies utilizing different cell sources do not provide conclusive additional information. Therefore, the aim of the current study was to analyze the effect of EMD on expression profiles of human derived bone cells on gene and protein level, in order to discover the pathways of EMD. This type of data can be used in follow-up studies to produce (purified) products with similar or enhanced treatment efficacy compared to EMD. The data of this study represent the first step in this process.

First some technical remarks should be made regarding the study set-up. The long-term response to EMD is important to explain its clinical function in vivo. This study, however intended to investigate which genes of primary human bone cells are affected by EMD as a direct result of the treatment. Therefore 24 hours of culturing with either test or control medium was chosen. Once a genetic pathway has been activated to altered expression, it is likely that this pathway will be continued.
Until now, there are no other array data on EMD and human bone cells available in literature. Still, some parameters of the present study can be compared with literature. Various concentrations of EMD were used in different studies investigating the effect of EMD on human osteoblasts. While EMD (100 µg/ml) increased the proliferation of primary human osteoblasts, it caused a dose-dependent (25, 50, 100 µg/ml EMD) decrease in human osteosarcoma cell number (MG63). In contrast to its effect on cell number, ALP activity in this latter study was significant at 50 and 100 µg/ml. Galli et al. investigated the effect of EMD in concentrations of 20, 50 and 100 µg/ml on primary human alveolar osteoblasts, and reported that cell growth was significantly increased with all EMD concentrations. However, ALP activity, Osteocalcin production, and matrix mineralization were significantly enhanced only when EMD concentrations larger than of 50 µg/ml were used. The above reported evidence led to the decision to use an EMD concentration of 100 µg/ml in the present study.

A further technical decision was the mode of application of the EMD. Most studies like Jiang et al. and Yoneda et al. diluted EMD in culture media and found that EMD had an enhanced effect on osteoblastic cells. In contrast, van den Dolder et al. investigated the effect of EMD on the cell growth and differentiation of osteoprogenitor cells from rat bone marrow when EMD was coated onto well-plates. Such an approach perhaps is more similar to clinical application of EMD; however, no significant effect of EMD could be determined in their study, hence we did not follow this route.

Regarding our results, a clinical significant effect of EMD might only be prevalent with the up-regulated genes involved in the GO-terms “proteinaceous extracellular matrix” and “extracellular matrix” of the category “Cellular Components” since these terms remained statistical significant after correction for multiple testing. In other words, statistically significant more genes are amongst the up-regulated genes after EMD treatment involved in the GO-terms “proteinaceous extracellular matrix” and “extracellular matrix” compared to the genes of the whole human genome associated with the same GO-terms. Hence, there seems to be an effect of EMD on extracellular matrix formation of human bone cells. Regardless of the major importance of extracellular matrix in wound healing and subsequently in bone regeneration, some of these genes contributing to the GO-terms mentioned above have known effects in particular on bone formation. The gene encoding for BGN was amongst the GO-term genes encoding for “extracellular matrix”. There is growing evidence that BGN is highly expressed in extracellular bone matrix and there seems to be an important role for BGN in influencing bone cell differentiation and proliferative activity. BGN activates extracellular signal-regulated kinase (Erk) and
Smad pathways to induce osteoblast differentiation 17 and also its deficiency increases osteoclast differentiation and activity 18.

Amongst the down-regulated genes, COX-2, EP2, and IL-7 are of particular interest. Previous study reported that administration of COX-2 inhibitors resulted in significant reduction of the losses of alveolar bone 19. Pathway analysis revealed that the gene encoding for the enzyme Prostaglandin-endoperoxide synthase 2 has a central role of previously described interactions between differently expressed gene products. Prostaglandins (PGs) are mainly produced in the bone by osteoblasts and stimulate both bone formation and resorption 20-22. Among several PGs produced, PGE2 is a major product. Phospholipase A2 is responsible for the production of arachidonic acid out of membrane phospholipids. The conversion of arachidonic acid to PGE2 is catalyzed by COX-2 induced in response to IL-1 23. In vitro, PGE2 production primarily leads to bone resorption 22, 24 by stimulating adenylate cyclase in osteoblasts, inducing osteoclast formation and stimulating bone resorption 24, 25.

Another gene product interfering with the function of PGE2, seems to be affected by EMD is EP2. The effects of PGE2 appear to be mediated by G protein-coupled receptors, which activate adenylate cyclase. Four classes of receptors for PGE2 have been identified, of which two, the EP2 and EP4 receptors, act by stimulating cAMP production 21. Bone cells and bone marrow cells can express both EP2 and EP4 receptors 26, 27. The role of EP2 in the formation of osteoclast-like cells has been studied using cells from the EP2 receptor knockout (KO) mice 28. The results showed that osteoclastogenesis was impaired in EP2 KO mice and the major defect appeared to be in the capacity of osteoblastic cells to stimulate osteoclast formation and in the response of osteoclastic lineage to PGE2, suggesting that EP2 receptors play an important role in osteoclast formation.

The expression of both COX-2 and EP2 seem to be reduced after treatment of EMD, suggesting that EMD tips the balance between bone formation and bone resorption towards a more anabolic effect. On the other hand, as mentioned above, PGE2 stimulates bone formation as well, which also seems to be mediated by the EP2 receptor 29-31.

Furthermore an interesting gene found to be differently expressed is that of IL-7. IL-7 is a potent stimulator for B- and T- Leukocyte cell growth 32, but has also been reported to act as an osteoclastogenic cytokine 33. IL-7 promotes osteoclastogenesis by upregulating key T cell–derived osteoclastogenic cytokines, including receptor activator of NF-κB ligand (RANKL) 34. IL-7 also increases the number of early precursors of the B cell lineage (B220+ cells) in the bone marrow 33, a population that has been suggested to have the capacity to differentiate into osteoclasts 35. Thus IL-7 may also induce bone loss by increasing the pool of osteoclastic precursors.
Although as mentioned above there are no micro-array data published on the effects of EMD on bone cells, during our study also micro-array data became available concerning the effect of EMD on periodontal ligament cells: Barkana et al. 36 explanted human periodontal ligament cells, and cell cloning was performed after which clones were classified into fibroblastic and mineralized tissue forming according to their capacity to express alkaline phosphatase and form mineralized tissue. All cell cultures were grown for 7 days, with and without EMD added to the medium. Following RNA extraction, expression profiling was performed by hybridization with a DNA micro-array. Strikingly, both studies find involvement of IL-7 and COX-2. Barkana et al. 36 found significant up-regulation of COX-2 and IL-7 sensitivity in the periodontal ligament, while in our study down-regulation of the IL-7 itself and COX-2 occurs in primary human bone cells. Also the lower IL-7 protein level was observed in the supernate of EMD-treated cells. This finding evidently could be an allocative function; however further research also has to prove this assumption. In summary, EMD seems to tip the balance between bone formation and bone resorption towards a more anabolic effect by interaction of the PGE2 pathway and inhibition of IL-7 production. In addition the results of the present study indicate that EMD has an effect on extracellular matrix formation of human bone cells, in particular on bone matrix formation and on proliferation and differentiation.

5. Conclusion
With the micro-array technique all genes involved in EMD action on bone cells, were identified. This represents the first step determining the compounds through which EMD reaches its clinical effectiveness. IL-7 as the most important genes was validated by q-PCR and ELISA. Our results allow for further in vitro and in vivo studies with the gene products and progression to the development of alternative products for bone augmentation and periodontal diseases.
References


Chapter 7
Chapter 8

Summary, closing remarks and future perspectives
1. Summary and address to the aims

The aim of the current thesis was to develop cell-based approaches for periodontal tissue engineering more potent for clinical periodontology. In chapter 1, a general introduction on cell-based strategies for periodontal tissue engineering and a description of the aims of this thesis are presented. Thereafter, each following chapter is focused on a separate research question related to aspects of periodontal tissue regeneration, covering cell-based, material-based, and preclinical animal experimental work. This summary addresses the aims as described in the first chapter in successive order.

1. What is the efficacy of cell-based approaches in animal models for periodontal regeneration?

Recently, substantial progress has been made in periodontal regeneration by cytotherapeutic approaches. Various cell types have been assessed in periodontal tissue regeneration in animal models and promising results have been reported. Nonetheless, no systematic assessment of the relevant literature has been undertaken, yet. The aim of Chapter 2 was to systematically evaluate the available evidence for the efficacy of cell-based approaches for periodontal regeneration from animal studies involving periodontal defect models. The results of this systematic review and meta-analysis revealed that cell-based approaches have a favorable effect on new bone formation in periodontal defects. Moreover, the meta-analysis did not reveal significant differences between periodontal ligament (PDL)-derived cells and bone marrow mesenchymal stromal cells (BMSCs) in the enhancement of bone regeneration. The results of Chapter 2 provide important information for the implementation of cell-based approaches in clinical practice as a routine treatment in the future.

2. What is the current state of the art in maintaining stemness of stem cells during in vitro expansion?

Although cell-based approaches have a favorable effect on periodontal tissue regeneration, stem/progenitor cells are in general available in insufficient quantities to be used directly for clinical applications without intermediate in vitro cell expansion procedures. Even though this in vitro expansion of undifferentiated stem cells is necessary, stem cells typically diminish their ability to self-renew and proliferate during passaging. Consequently, maintaining the stemness of stem cells has been recognized as a major challenge in stem cell-based research. Chapter 3 reviewed the latest developments in maintaining the self-renewal ability of stem
cells during in vitro expansion. Stem cell fate is regulated by various factors including genetic influences, cell-cell communications, growth factors and cytokines, extracellular matrix (ECM; e.g. topography/architecture), and physiochemical environment (e.g. matrix stiffness, oxygen tension, mechanical forces, electrical cues). Chapter 3 mainly focuses on how to maintain the stemness of stem cells by exploiting biomaterial properties. The use of biomaterials, from both a natural or synthetic origin, to develop two dimensional (2D) and three dimensional (3D) cell culture systems for the expansion of pluripotent stem cells as well as adult stem cells is summarized in this chapter. The modifications of biomaterials in terms of topography/architecture, mechanical properties and biochemical factors are also described. Further, this chapter highlights what should be the focus for future studies using stem cells for regenerative applications.

3. What is the appropriate PDL cell population for clinical applications and how to improve its in vitro expansion?

STRO-1 positive PDLCs and unsorted PDLCs have demonstrated potential for periodontal regeneration, but the comparison between unsorted cells and the expanded STRO-1 sorted cells has never been reported. Additionally, Wnt3a is involved in cell proliferation, therefore may improve in vitro PDLC expansion. The aim of Chapter 4 was to evaluate the effect of STRO-1 cell sorting and Wnt3a treatment on cell behavior of human PDLCs. To this end, the proliferation, colony forming unit (CFU) and mineralization capacity of unsorted parental PDLCs and expanded STRO-1-sorted cells were compared to find the appropriate PDL cell population for future clinical applications. Thereafter, PDLCs were treated with or without Wnt3a and the cell proliferation, self-renewal, and osteogenic differentiation were evaluated. The results of Chapter 4 showed that expanded STRO-1-sorted cells and unsorted parental cells behave similarly in terms of proliferation, CFU, and mineralization capacity. Wnt3a enhanced the proliferation and self-renewal ability of PDLCs significantly as displayed by higher DNA content values, a shorter cell population doubling time, and higher expression of the self-renewal gene Oct4. Moreover, Wnt3a promoted the expansion of human PDLCs for 5 passages without affecting cell proliferation, CFU and osteogenic capacity. These results indicate that expanded STRO-1-sorted PDLCs are not superior to unsorted parental cells. On the other hand, Wnt3a promotes the efficient PDLC expansion and retains the self-renewal and osteogenic differentiation capacity.

4. Can cross-linking for chitosan be enzymatically controlled and can this system be used for incorporation of PDLCs?
The aim of Chapter 5 was to optimize enzymatic control over gelation of chitosan-based hydrogels for the delivery of PDLCs. By varying the urea and urease concentration, it was possible to control the pH and hence the gelation time, osmolarity, visco-elastic properties and degradation of chitosan hydrogels. After optimization of chitosan gelation, 3 different hydrogel compositions (37.5 mM urea and 15, 30 or 60 U/ml urease) were selected for further investigations on PDLCs behavior. PDLCs remained viable upon encapsulation within chitosan-based hydrogels for up to 30 days. Cells that were released and collected upon degradation of the hydrogels after 3, 15 and 30 days retained the capacity to form colonies and differentiate into the osteogenic lineage. Consequently, chitosan-based hydrogels seem suitable for providing a continuous delivery of functional cells that can differentiate (for example in the periodontal defect site) when released (caused by hydrogel degradation) from the hydrogel. The results of Chapter 5 indicate that the enzymatic control over the gelation of chitosan hydrogels offers options for the delivery of PDLCs.

5. What is the in vivo biocompatibility and periodontal regenerative potential of enzymatically cross-linked chitosan hydrogels with or without incorporated PDLCs?

The previous chapter showed that the enzymatic control over the gelation of chitosan hydrogels offers options for the delivery of PDLCs. Chapter 6 aimed to evaluate the in vivo biocompatibility and periodontal regenerative potential of the enzymatically cross-linked chitosan hydrogels with or without incorporated PDLCs. To this end, chitosan hydrogels, with (n=8; CHIT+CELL) or without (n=8; CHIT) fluorescently-labeled PDLCs, were prepared and transplanted into rat intrabony periodontal defects; untreated defects were used as empty controls (n=8; EMPTY). After 4 weeks, maxillae were harvested, decalcified and used for histological, histomorphometrical and immunohistochemical assessment. The results showed that PDLCs remained viable upon encapsulation within chitosan hydrogels before transplantation. Histological analysis demonstrated that the chitosan hydrogels were largely degraded after 4 weeks of implantation, without any adverse reaction in the surrounding tissue. In terms of periodontal regeneration, alveolar bone height, alveolar bone area and epithelial downgrowth were comparable for CHIT, CHIT+CELL as well as EMPTY groups. In contrast, both CHIT and CHIT+CELL showed a significant increase in functional ligament length compared to EMPTY. From a cellular perspective, the contribution of chitosan hydrogel incorporated cells to the periodontal regeneration could not be ascertained, as no signal from transplanted PDLCs could be detected at 4 weeks post-transplantation. The results of Chapter 6
demonstrated that chitosan hydrogels are highly biocompatible, biodegradable, and improve periodontal regeneration in terms of functional ligament length. Further work on the use of chitosan hydrogels as cell carriers is required.

6. What is the mechanism of Emdogain® in promoting alveolar bone regeneration?
Emdogain® (EMD) is frequently used in regenerative periodontal treatment. Understanding its effect on gene expression of bone cells would enable to establish new products and pathways promoting bone formation. The aim of Chapter 7 was to analyze the effect of Emdogain® on expression profiles of human derived bone cells. The results of Chapter 7 showed that EMD treatment significantly up-regulated gene expression for bone formation-related genes and down-regulated gene expression of bone resorption-related genes. In addition, the results indicated that EMD possibly has an effect on gene expression for extracellular matrix formation of human bone cells, in particular on bone matrix formation and on proliferation and differentiation. In conclusion, the micro-array identified gene expression changes induced by EMD, particularly those with interest for bone cells. These results can contribute to establish new products and pathways promoting bone formation.

2. Closing remarks and future perspectives
The goal of the current thesis was to develop cell-based approaches for periodontal tissue engineering more potent for clinical periodontology. First, our systematic review and meta-analysis (Chapter 2) revealed that cell-based approaches have a favorable effect on new bone formation in periodontal defects. However, the main obstacle for the clinical application of cell-based approaches is that stem cells diminish their ability to self-renew during in vitro expansion. Consequently Chapter 3 reviewed the latest developments in maintaining the self-renewal ability of stem cells during in vitro expansion. Based on this review, Chapter 4 further investigated the effects of Stro-1 and Wnt3a on cell behavior of human PDLCs. The results indicate that expanded STRO-1-sorted PDLCs showed no superiority compared to their unsorted parental cells. On the other hand, Wnt3a promotes the efficient PDLC expansion and retains the self-renewal and osteogenic differentiation capacity. Next, in order to develop a cell delivery system for periodontal regeneration, Chapter 5 and Chapter 6 evaluated enzymatic cross-linked chitosan hydrogels for the delivery of PDLCs in vitro and in vivo. Chapter 5 showed that PDLCs remained viable upon encapsulation within chitosan-based hydrogels and cells that were released from the hydrogels retained the capacity to
osteogenically differentiate. **Chapter 6** demonstrated that chitosan hydrogels displayed high biocompatibility and biodegradability *in vivo* and the implantation of hydrogel alone promotes functional ligament formation. In contrast, no signal from transplanted PDLCs could be detected at the end of 4-week transplantation period, indicating that there are no exogenous healing cells in the defect area anymore. **Chapter 7** further investigated the mechanism of EMD in promoting alveolar bone regeneration, which can contribute to establish new products and pathways promoting bone formation.

At this point the question remains on how to progress cell-based tissue engineering strategies into clinical practice. For this to happen, a number of further investigations need to be considered. First, although our meta-analysis provided evidence for the enhancement of the regeneration of supportive periodontal tissue (i.e. bone) by implantation of PDL-derived cells and BMSC, it has to be emphasized that the treatment of periodontal defects needs a large number of cells, which sometimes would be difficult to obtain from a single subject. Although the *in vitro* expansion of stem/progenitor cells is necessary, those cells typically reduce their ability to self-renew and proliferate during passaging. Accordingly, other sources of cell types that can be easily obtained in large numbers need to be sought and further efforts should be made in maintaining the self-renewal ability of stem cells during *in vitro* expansion. According to our systematic review, iPS cells and adipose-tissue derived stem cells (ASCs) are potential alternative sources, as they can be obtained in large numbers and they have given promising results in periodontal regeneration. However, only two animal studies are available in which iPS cells and ASCs were indeed used for clinical application. Therefore, additional studies are required to further ensure the efficacy of the aforementioned cell types in periodontal regeneration. Regarding *in vitro* expansion of stem/progenitor cells, new insights should be gained into the modifications of biomaterials such as the topography, stiffness, and surface chemistry to present a combination of physical and chemical factors to expand stem cells. In addition, the development of 3D culture systems using biomaterials recapitulating the *in vivo* stem cell niche will probably provide superior microenvironments for stem cells compared to the traditional 2D monolayer culture systems.

Second, for successful cell-based approaches, it is important to develop strategies that enable the survival of implanted cells. Although our results demonstrated that enzymatically cross-linked chitosan hydrogel supports PDLC survival *in vitro* for up to 30 days, at the end of the 4-week transplantation period, no GFP signal was detected in any of the *in vivo* specimens, indicating that there are no exogenous
healing cells in the defect area anymore. Similarly, Tour et al. reported that implanted PDLCs decreased in number with time in a defect after transplantation in a calvarial defect model, from around 240 GFP\(^+\) cells/mm\(^2\) defect area after 1 week to single or no positive cells detected after 10 weeks\(^5\). Taken together, these findings suggest that transplanted cells tend to vanish with time after implantation. This phenomenon may be partially explained by the migration of the cells out of the defect, as chitosan hydrogels were largely degraded after 4-week implantation \textit{in vivo}, or the immediate cell death after implantation due to poor vascularization inside the entire cell-based construct\(^6,7\). For the former reason, in future studies, it is of importance to enhance cell adhesion and proliferation, e.g. by incorporating cell adhesive biomolecules, and/or to decrease chitosan hydrogel degradation, e.g. by adjusting urease concentration or developing more slowly degradable materials such as hydrogel-fiber composite. For the latter reason, future studies should be directed to how to enhance cell survival after implantation. One possible approach is to simulate vascularization in cell-based constructs by coculture approaches of PDLCs with angiogenic cells (e.g. endothelial cells)\(^8\). Another way to maintain cell survival after implantation is to generate bone tissue via the endochondral route—the pathway that resembles endochondral ossification by first forming a cartilage template \textit{in vitro} and subsequently allowing the cartilage to be replaced by bone \textit{in vivo} \(^9-13\). The rationale behind this approach is that chondrocytes are able to survive with limited nutrition and oxygen. Secondly, chondrocytes can secrete vascular endothelial growth factor (VEGF) in the hypertrophic stage, which is beneficial for blood vessel in-growth\(^14\).

Third, biomolecules/growth factors have an important role in regulating the proliferation, migration, and/or extracellular matrix synthesis of a variety of cell types, including those derived from the periodontium\(^15\). EMD is commonly used in periodontal practice since it has already been proven to improve periodontal regeneration by a systematic review and meta-analysis\(^16\). Nevertheless, the exact constituents of EMD are unknown, and therefore one can hardly conclude through which component or components EMD exerts its clinical effects. Our results showed that EMD seems to regulate bone formation and bone resorption towards a more anabolic effect by interaction of the prostaglandin E\(_2\) (PGE\(_2\)) pathway and inhibition of interleukin-7 (IL-7) production. This represents the first step determining the compounds through which EMD reaches its clinical effectiveness. Our results allow for further \textit{in vitro} and \textit{in vivo} studies with the gene products and progression to the development of alternative products for bone augmentation and periodontal diseases.
In summary, recent advances in the knowledge of stem cells, biomaterials and biomolecules provide exciting opportunities for clinical translation to novel therapies. Further studies are required to develop new methods to maintain the stemness of stem cells in vitro and to determine the long-term safety and efficacy of ex vivo expanded stem cells\textsuperscript{17}. In addition, suitable carriers and inductive factors that are able to help stem cells integrate into the surrounding environment for the reconstruction of functional periodontal tissues will need to be developed\textsuperscript{18}. The ultimate function of expanded cells requires in vivo evaluation to confirm their regenerative capacity, for example by transplantation of expanded cells into animal models. This needs collaboration among many disciplines including cell and molecular biology, biomaterials, pharmacology, nanotechnology and medicine.
References

Chapter 8

Samenvatting, afsluitende opmerkingen en toekomstperspectie
1. Samenvatting en evaluatie van de doelstellingen
Het doel van dit proefschrift was het ontwikkelen van cel-gebaseerde mogelijkheden voor parodontale tissue engineering met meer potentie voor klinische parodontologie. In hoofdstuk 1 wordt een algemene introductie gegeven over cel-gebaseerde strategieën voor parodontale tissue engineering alsmede een beschrijving van de doelen van dit proefschrift. Ieder volgend hoofdstuk is vervolgens gericht op een aparte onderzoeksvraag gerelateerd aan aspecten van parodontale weefselregeneratie, waaronder cel-gebaseerde, materiaal-gebaseerde en preklinisch/dierexperimenteel werk. Deze samenvatting evalueert de doelstellingen als beschreven in het eerste hoofdstuk.

1. Wat is de werkzaamheid van cel-gebaseerde parodontale regeneratie in diermodellen?
Recent is aanzienlijke vooruitgang geboekt in de parodontale regeneratie met behulp van cytotherapeutische behandelingen. Verschillende celtypes zijn onderzocht voor parodontale weefselregeneratie in diermodellen en veelbelovende resultaten zijn gepubliceerd. Desalniettemin is tot dusver geen systematisch onderzoek uitgevoerd op basis van relevante literatuur. Het doel van Hoofdstuk 2 was om het beschikbare bewijs ten aanzien van de werkzaamheid van cel-gebaseerde behandelingen in diermodellen met parodontale defecten systematisch te evalueren. De resultaten van deze systematische review en meta-analyse toonden dat cel-gebaseerde behandelingen een positief effect hebben op nieuwe botvorming in parodontale defecten. De meta-analyse zag voorts geen verschil tussen parodontaal ligament (PDL) cellen en mesenchymale stamcellen uit beenmerg (BMSCs) ten aanzien van de verhoogde botregeneratie. De resultaten van Hoofdstuk 2 verschaffen belangrijke informatie voor de implementatie van cel-gebaseerde behandelingen in de klinische praktijk als een routine behandeling voor de toekomst.

2. Wat is de huidige state-of-the-art voor behoud van de stemness van stamcellen gedurende in vitro expansie?
Hoewel cel-gebaseerde behandelingen een gunstig effect hebben op parodontale weefselregeneratie zijn stam-/voorlopercellen normaliter in onvoldoende aantallen aanwezig voor direct gebruik in klinische toepassingen zonder tussenliggende in vitro celexpansie procedures. Ongeacht de noodzaak tot in vitro expansie verliezen stamcellen hun vermogen zichzelf te vernieuwen en te groeien tijdens doorzetten in celkweek. Derhalve is het behoud van stemness van stamcellen aangemerkt als een belangrijke uitdaging in stamcel-gebaseerd
onderzoek. Hoofdstuk 3 biedt een overzicht van de laatste ontwikkelingen ten aanzien van het behoud van het vermogen zichzelf te vernieuwen voor stamcellen gedurende in vitro expansie. Het lot van stamcellen wordt gereguleerd door verschillende factoren, waaronder genetische invloeden, cel-cel communicatie, groeifactoren en cytokines, extracellulaire matrix (ECM; bijv. topografie/architectuur) en de fysisch-chemische omgeving (bijv. matrix stijfheid, zuurstofspanning, mechanische krachten en elektrische signalen). Hoofdstuk 3 richt zich voornamelijk op hoe de stemness van stamcellen te behouden via eigenschappen van biomaterialen. Het gebruik van biomaterialen (zowel natuurlijk als synthetisch) voor de ontwikkeling van 2-dimensionale (2D) en 3-dimensionale (3D) celkweek systemen voor de vermeerdering van pluripotente en adulte stamcellen wordt in dit hoofdstuk samengevat. De aanpassing van biomaterialen via topografie/architectuur, mechanische eigenschappen en biochemische factoren worden tevens beschreven. Tenslotte toont dit hoofdstuk wat de focus dient te zijn voor toekomstige studies die stamcellen gebruiken voor regeneratieve toepassingen.

3. Wat is de geschikte PDL celpopulatie voor klinische toepassingen en hoe kan de in vitro expansie ervan verbeterd worden?

STRO-1 positieve PDL cellen en niet-geselecteerde PDL cellen hebben aangetoond potentie te hebben voor parodontale regeneratie, maar de directe vergelijking tussen deze cellen is nooit gemaakt. Daarnaast is Wnt3a betrokken bij celproliferatie en zou dus de in vitro expansie van PDL cellen kunnen verbeteren. Het doel van Hoofdstuk 4 was om te evalueren welk effect STRO-1 cel selectie en Wnt3a behandeling hebben op het celgedrag van humane PDL cellen. Hiertoe werden proliferatie, colony forming unit (CFU) en mineralisatie-capaciteit van ongeselecteerde parentale PDL cellen en van vermeerderde STRO-1 geselecteerde PDL cellen vergeleken teneinde de geschikte PDL cel populatie te vinden voor toekomstige klinische toepassingen. Vervolgens werden PDL cellen behandeld met of zonder Wnt3a en vergeleken wat de proliferatie, zelfvernieuwing en osteogene differentiatie was van deze cellen. De resultaten van Hoofdstuk 4 toonden dat vermeerderde STRO-1 geselecteerde en ongeselecteerde parentale cellen eenzelfde gedrag vertonen ten aanzien van proliferatie, CFU en mineralisatie-capaciteit. Wnt3a verhoogde significant de proliferatie en zelfvernieuwing van PDL cellen, aangetoond via hogere DNA-hoeveelheid waardes, kortere verdubbelingstijden en hogere expressie van het zelfvernieuwingsgen Oct4. Daarnaast stimuleerde Wnt3a de expansie van humane PDL cellen gedurende 5 passages zonder effecten op celproliferatie, CFU en osteogene capaciteit. Deze
resultaten tonen dat vermeerderde STRO-1 geselecteerde PDL cellen niet superieur zijn aan ongeselecteerde parentale cellen en dat Wnt3a efficiënte PDL cel expansie en behoud van zelfvernieuwing en osteogene differentiatie capaciteit stimuleert.

4. Kan crosslinken van chitosan enzymatisch worden gecontroleerd en kan dit systeem gebruikt worden voor de incorporatie van PDL cellen?
Het doel van Hoofdstuk 5 was om de enzymatische controle over gelering van chitosan-gebaseerde hydrogelen voor afgifte van PDL cellen te optimaliseren. Door de concentraties van ureum en urease te variëren, bleek het mogelijk om de pH, geleringstijd, osmolariteit, visco- elastische eigenschappen en degradatie van chitosan hydrogelen te controleren. Na optimalisatie van de gelering van chitosan, werden 3 hydrogel systemen (37.5 mM ureum met 15, 30 of 60 U/ml urease) geselecteerd voor vervolgonderzoek naar het gedrag van PDL cellen. Na inkapseling in chitosan-gebaseerde hydrogelen bleken PDL cellen voor meer dan 30 dagen levensvatbaar. De cellen die afgegeven werden na degradatie van de hydrogelen op 3, 15 en 30 dagen behielden hun vermogen om kolonies te vormen en te differentiëren richting botvormende cellen. Daaruit kon geconcludeerd worden dat chitosan-gebaseerde hydrogelen geschikt lijken voor een continue afgifte van functionele cellen (via degradatie), welke kunnen differentiëren (bijv. in een parodontaal defect) richting een bepaald celtype. De resultaten van Hoofdstuk 5 tonen daarmee dat de enzymatische controle over chitosan gelering mogelijkheden biedt voor de afgifte van PDL cellen.

5. Wat is de in vivo biocompatibiliteit en de parodontaal regeneratieve potentie van enzymatisch gecrosslinkte chitosan hydrogelen met of zonder PDL cellen?
Het vorige hoofdstuk toonde dat enzymatische controle over de gelering van chitosan hydrogelen mogelijkheden biedt voor de afgifte van PDL cellen. Hoofdstuk 6 had als doel om te evalueren of dit systeem (met of zonder PDL cellen) in vivo biocompatibel is en bijdraagt aan parodontale regeneratie. Hiertoe werden chitosan hydrogelen met (CHIT+CELL; n=8) of zonder (CHIT; n=8) fluorescent-gelabelde PDL cellen gemaakt en geïmplanteerd in parodontale defecten in ratten; onbehandelde defecten fungeerden als lege controles (EMPTY; n=8). Na 4 weken werden de bovenkaken verzameld, gedecalcificeerd en gebruikt voor histologie, histomorfometrie en immunohistochemie. De resultaten toonden dat PDL cellen levensvatbaar blijven na inkapseling in chitosan hydrogelen voorafgaand aan implantaatie. Histologische analyse toonde dat de chitosan hydrogelen grotendeels gedegradeerd waren na 4 weken, zonder enige indicatie
van weefselreacties in het omlijvende weefsel. Ten aanzien van parodontale regeneratie, alveolaire bothoogte, alveolair botgebied en epitheliale ingroei waren de resultaten vergelijkbaar tussen de groepen. Echter, zowel CHIT als CHIT+CELL toonden een significant langer functioneel ligament ten opzichte van EMPTY. Vanuit een cel-perspectief kon een bijdrage van geïncorporeerde cellen hieraan niet met zekerheid vastgesteld worden, aangezien geen signaal van geïmplanteerde PDL cellen kon worden gedetecteerd op 4 weken na implantatie. De resultaten van Hoofdstuk 6 toonden dat chitosan hydrogelen zeer biocompatibel en biodegradeerbaar zijn en parodontale regeneratie bevorderen ten aanzien van de lengte van het functionele ligament. Verder onderzoek naar het gebruik van chitosan hydrogelen als cel-dragers blijft noodzakelijk.

6. Wat is het mechanisme waardoor Emdogain® alveolaire botregeneratie bevordert?

Emdogain® (EMD) wordt veelvuldig gebruikt voor regeneratieve parodontale behandelingen. Wetens wat het effect ervan is op genexpressie in botcellen zou kunnen leiden tot nieuwe producten en wegen ter bevordering van botvorming. Het doel van Hoofdstuk 7 was om te analyseren wat het effect is van Emdogain® op expressie profielen van humane botcellen. De resultaten van Hoofdstuk 7 tonen dat behandeling met EMD zorgt voor een significante verhoging van genexpressie van aan botvorming gerelateerde genen en verlaging van genexpressie van aan botresorptie gerelateerde genen. Tevens suggereerden de resultaten dat EMD mogelijk een effect heeft op genexpressie gerelateerd aan extracellulaire matrix vorming door humane botcellen, vooral op botmatrix vorming en cel proliferatie en -differentiatie. Samengevat kan gesteld worden dat de uitgevoerde micro-array EMD-afhankelijke verschillen in genexpressie heeft aangetoond, en dan met name de genen belangrijk voor botcellen. Derhalve kunnen deze resultaten gebruikt worden om nieuwe producten en manieren te ontwikkelen ter bevordering van botvorming.

2. Afsluitende opmerkingen en toekomstperspectief

Het doel van het onderhavige proefschrift was om cel-gebaseerde strategieën te ontwikkelen voor parodontale tissue engineering met meer potentie voor de klinische parodontologie. Als start toonde onze systematische review en meta-analyse (Hoofdstuk 2) dat cel-gebaseerde strategieën een positief effect hebben op nieuwe botvorming in parodontale defecten. Echter, een belangrijk obstakel voor de klinische toepassing van cel-gebaseerde strategieën is het vermijden van het vermogen tot zelfvernieuwing van stamcellen gedurende in vitro expansie. Derhalve werd in Hoofdstuk 3 een terugblik geworpen op de laatste
ontwikkelingen ten aanzien van het behoud van zelf vernieuwend vermogen van stamcellen gedurende in vitro expansie. Gebaseerd op deze terughouding ging Hoofdstuk 4 verder in op de effecten van STRO-1 en Wnt3a op gedrag van humane PDL cellen. De resultaten toonden aan dat vermeerderde, STRO-1 geselecteerde PDL cellen niet superieur zijn ten opzichte van ongeselecteerde parentale cellen. Daarentegen bleek dat Wnt3a efficiënte expansie van PDL cellen bevordert alsook het behoud van zelf vernieuwend vermogen en osteogene differentiatie capaciteit. Teneinde een cel-afgifte systeem te ontwikkelen voor parodontale regeneratie onderzochten Hoofdstuk 5 en Hoofdstuk 6 enzymatisch gecrosslinkte chitosan hydrogelen voor de afgifte van PDL cellen in vitro en in vivo. Hoofdstuk 5 toonde dat PDL cellen levensvatbaar blijven gedurende inkapseling in chitosan-gecrosslinkte hydrogelen en dat afgegeven cellen hun vermogen tot osteogene differentiatie behouden. Hoofdstuk 6 toonde dat chitosan hydrogelen zeer biocompatibel en biodegradeerbaar zijn in vivo en dat implantatie van deze hydrogel de vorming van functioneel ligament bevordert. Echter, er werd geen signaal van geimplanteerde PDL cellen waargenomen na 4 weken implantatie, hetgeen suggerereert dat er geen exogene cellen meer in het defect aanwezig zijn. Hoofdstuk 7 richtte zich op het werkingsmechanisme van EMD ten aanzien van de bevordering van alveolaire botregeneratie, welk kan bijdragen aan de ontwikkeling van nieuwe producten en manieren om botvorming te bevorderen. Momenteel blijft de vraag hoe cel-gebaseerde tissue engineering strategieën richting klinische praktijk kunnen worden gebracht. Om dit mogelijk te maken dienen een aantal extra onderzoeken te worden betracht. Hoewel onze meta-analyse heeft bewezen dat cel-gebaseerde strategieën de regeneratie van ondersteunend parodontaal weefsel (bot) verbeteren, dient te worden benadrukt dat de behandeling van parodontale defecten grote aantallen cellen behoeft, welke mogelijk moeilijk te verkrijgen zijn van een en hetzelfde individu. Zelfs wanneer in vitro expansie de benodigde aantallen zou opleveren, zullen dit veelal cellen zijn met een verminderd vermogen tot zelfvernieuwing en proliferatie vanwege doorzetten tijdens celkweek. Het zou daarom gewenst zijn om alternatieve cellen te kunnen gebruiken die wel gemakkelijk en in voldoende grote aantallen te verkrijgen zijn. Tevens zou verder gekeken dienen te worden naar mogelijkheden om zelfvernieuwing van stamcellen gedurende in vitro expansie te behouden. Uit ons systematisch review blijkt dat iPS cellen en MSCs uit vetweefsel mogelijk als alternatief kunnen dienen, aangezien deze cellen in grote aantallen verkregen kunnen worden en bemoedigende resultaten hebben laten zien in parodontale regeneratie. Echter, slechts 2 dierstudies zijn beschikbaar waarin iPS cellen en MSCs uit vetweefsel werden gebruikt voor klinische toepassingen.
Hieruit blijkt dat extra studies noodzakelijk zijn om de werkzaamheid van deze celtypes voor parodontale regeneratie te kunnen verzekeren. Ten aanzien van in vitro expansie van stam-/voorlopercellen is meer inzicht nodig met betrekking tot modificaties van biomaterialen, zoals topografie, stijfheid en oppervlaktechemie, om een juiste combinatie van fysische en chemische factoren aan te bieden voor celvermeerdering. Bovendien zal de ontwikkeling van 3D kweeksystemen die de stamcel niche benaderen waarschijnlijk een superieure micro-omgeving opleveren ten opzichte van 2D monolaag kweeksystemen.

Ten tweede, voor succesvolle cel-gebaseerde benaderingen is het belangrijk om strategieën te ontwikkelen die de overleving van geimplanteerde cellen mogelijk maken. Hoewel onze resultaten aantonen dat enzymatisch gecrosslinkte chitosan hydrogelen de overleving van PDL cellen in vitro tot 30 dagen ondersteunen, werd aan het eind van de 4 weken implantatie periode geen GFP signaal gedetecteerd in de in vivo samples, wat aangeeft dat er geen exogene cellen in het defect gebied meer aanwezig waren. Ook Tour et al. hebben beschreven dat het aantal geïmplanteerde PDL cellen daalde met de tijd in een defect na implantaat in een calvariaal defect model: van rond 240 GFP⁺ cellen/mm² defect gebied na 1 week tot enkele of geen positieve cellen na 10 weken⁵. Al met al suggereren deze bevindingen dat getransplanteerde cellen de neiging hebben om te verdwijnen met de tijd na implantaat. Dit verschijnsel kan gedeeltelijk worden verklaard door de migratie van de cellen uit het gebrek, aangezien chitosan hydrogelen grotendeels waren gedegradéerd na 4 weken implantaat in vivo, of de directe celdood na implantaat vanwege slechte vascularisatie in het gehele cel-gebaseerde construct⁶.⁷. Ten aanzien van de eerste reden is het van belang voor toekomstige studies om celadhesie en proliferatie te versterken, bijv. door het incorporeren van celadhesie biomoleculen en/of chitosan hydrogel degradatie te verlagen, bijv. door aanpassing van de urease concentratie of het ontwikkelen van langzamer afbreekbare materialen zoals hydrogel-vezel composit. Ten aanzien van de laatste reden moeten toekomstige studies worden gericht op hoe de overleving te verbeteren na implantaat. Een mogelijke benadering is om vascularisatie te genereren door co-kweek van PDL cellen met angiogene cellen (bijv. endotheelcellen)⁸. Een andere manier om cel overleving te verbeteren na implantaat is het genereren van botweefsel via de endochondrale route: deze weg van endochondrale ossificatie gebruikt eerst een sjabloon kraakbeen in vitro en vervolgens vervanging van het kraakbeen door bot in vivo⁹‐¹³. Het idee hierachter is dat chondrocyten kunnen overleven met beperkte voeding en zuurstof. Ten tweede kunnen chondrocyten vasculaire endotheliale groefactor (VEGF)

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uitscheiden tijdens de hypertrofische fase, hetgeen gunstig is voor groei van bloedvaten\textsuperscript{14}.

Ten derde hebben biomoleculen/groeifactoren een belangrijke rol bij het reguleren van de proliferatie, migratie en/of extracellulaire matrix synthese van verschillende celltypes, inclusief die afgeleid van de parodontium\textsuperscript{15}. EMD wordt vaak gebruikt in de parodontale praktijk, aangezien op basis van een systematische review en meta-analyse is bewezen dat het parodontale regeneratie verbetert\textsuperscript{16}. Toch zijn de exacte bestanddelen van EMD onbekend, en dus kan men nauwelijks aangeven welke component (of componenten) EMD deze klinische effecten geeft. Onze resultaten toonden dat EMD botvorming en botresorptie lijkt te reguleren naar een anabole werking door wisselwerking van de prostaglandine E2 (PGE2) route en remming van interleukine-7 (IL-7). Dit vertegenwoordigt de eerste stap richting het bepalen van de bestanddelen waarmee EMD klinische effectiviteit bereikt. Onze resultaten laten verdere in vitro en in vivo studies toe met de genproducten en progressie naar de ontwikkeling van alternatieve producten voor botaugmentatie en parodontale ziekten.

Samenvattend kan gesteld worden dat de recente (kennis)ontwikkelingen op het gebied van stamcellen, biomaterialen en biomoleculen interessante mogelijkheden bieden voor de klinische translatie richting nieuwe therapiën. Verder onderzoek is nodig om nieuwe methoden voor de stemness van stamcellen in vitro te handhaven en de veiligheid en werkzaamheid van ex vivo geëxpandeerde stamcellen lange termijn vast te stellen\textsuperscript{17}. Daarnaast zullen geschikte dragers en inductieve factoren die kunnen helpen stamcellen te integreren in de omgeving voor de wederopbouw van functionele parodontale weefsels moeten worden ontwikkeld\textsuperscript{18}. De uiteindelijke werking van geëxpandeerde cellen gebiedt in vivo evaluatie om hun regeneratieve capaciteit te bevestigen, bijvoorbeeld door transplantatie van geëxpandeerde cellen in diermodellen. Dit verdient samenwerking tussen vele disciplines, zoals cel- en moleculaire biologie, biomaterialen, farmacologie, nanotechnologie en geneeskunde.
Chapter 8

References

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APCs</td>
<td>Alveolar periosteal cells</td>
</tr>
<tr>
<td>APMAAm</td>
<td>Aminopropylmethacrylamide</td>
</tr>
<tr>
<td>ASCs</td>
<td>Adipose-tissue derived stem cells</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BGN</td>
<td>Biglycan</td>
</tr>
<tr>
<td>BMSCs</td>
<td>Bone marrow mesenchymal stromal cells</td>
</tr>
<tr>
<td>BP</td>
<td>Biological process</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CC</td>
<td>Cellular components</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CIs</td>
<td>Confidence intervals</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DFCs</td>
<td>Dental follicle cells</td>
</tr>
<tr>
<td>DPCs</td>
<td>Dental pulp cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMD</td>
<td>Emdogain</td>
</tr>
<tr>
<td>EP2</td>
<td>Prostaglandin E receptor 2</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>EVG</td>
<td>Elastica-van Gieson</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehydes 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMCs</td>
<td>Gingival margin-derived cells</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GP</td>
<td>Glycerophosphate</td>
</tr>
<tr>
<td>GTR</td>
<td>Guided tissue regeneration</td>
</tr>
<tr>
<td>HE</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HPL</td>
<td>Human platelet lysate</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IL-7</td>
<td>Interleukin 7</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>iPSCs</td>
<td>induced pluripotent stem cells</td>
</tr>
<tr>
<td>LTBP</td>
<td>latent TGF-β binding protein</td>
</tr>
<tr>
<td>MF</td>
<td>molecular functions</td>
</tr>
<tr>
<td>MSCs</td>
<td>mesenchymal stem cells</td>
</tr>
<tr>
<td>NSCs</td>
<td>neural stem cells</td>
</tr>
<tr>
<td>OC</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>OCPC</td>
<td>ortho-cresolphthalein complexone</td>
</tr>
<tr>
<td>PAFSCs</td>
<td>periapical follicular stem cells</td>
</tr>
<tr>
<td>PAS</td>
<td>peptide-acylate surfaces</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>PDLCs</td>
<td>periodontal ligament cells</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PGs</td>
<td>prostaglandins</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of NF-κB ligand</td>
</tr>
<tr>
<td>SDSCs</td>
<td>synovium-derived stem cells</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SMD</td>
<td>standardized mean difference</td>
</tr>
<tr>
<td>SPF</td>
<td>specific-pathogen free</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor β1</td>
</tr>
<tr>
<td>TSCs</td>
<td>tendon stem cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>αMEM</td>
<td>alpha minimal essential medium</td>
</tr>
</tbody>
</table>
Acknowledgements

List of publications

Curriculum Vita
Acknowledgements

Before I arrived in Nijmegen, the Netherlands to me was only about drinking beers on Friday evenings, cycling through the tulip fields, and screaming for goals dressed in orange. Today, four years later, the Netherlands for me is more about scheduling agenda appointments, embracing and learning about different cultures, and of course the directness - always speaking their minds😊. Without these merits, and more importantly the support and help from so many people, this book would never have been finalized smoothly. At the end of this unforgettable PhD journey, I would like to express my sincere gratitude to the following people.

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Acknowledgments, publications and CV

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List of Publications


Yan XZ, van den Beucken JJ, Cai X, Yu N, Jansen JA, Yang F. Periodontal tissue regeneration using enzymatically cross-linked chitosan hydrogels with or without cell-loading. submitted.


Curriculum Vitae

Xiangzhen Yan (闫香珍) was born on March 03, 1984 in Zibo, China. In 2002, she was enrolled in School of Stomatology, Shandong University, where she received basic education and graduated with a Bachelor degree in 2007. In the same year she was recommended to enter the graduate school in Department of Periodontology (Head Prof. dr. Pishan Yang), Shandong University, where she was awarded her first Doctor degree in Clinical Science of Stomatology in 2013.

In October of 2010, she joined a cooperative project financially supported by China Scholarship Council, and started her second PhD study in Medical Science in the Department of Biomaterials (Head Prof. dr. J.A. Jansen) at Radboudumc, the Netherlands. Her main research interests are in the area of bone and periodontal tissues regeneration utilising tissue engineering based-treatment approaches. The results of her PhD studies are described in this thesis and are presented as several publications in top 25% scientific journals. Some of the results were also presented in several international conferences among which were: International Society of Differentiation Conference 'Stem Cells, Development and Regulation' (NIRM, Amsterdam, 2012), Tissue Engineering and Regenerative Medicine International Society-Asial Pacific (TERMIS-AP, Shanghai, 2013), and the 5th international tissue engineering conference (Kos, 2014), where she received an Aegean Conference Travel Award.

As of October 2014 she is enrolled as a dentist in the Affiliated Stomatlogy Hospital of Tongji University, Shanghai, China. In her future clinical work, she aims to achieve better periodontal regeneration for patients by applying her research experience in clinics.
Gravitation cannot be held responsible for people falling in love.
Albert Einstein (1879-1955)

Science may set limits to knowledge, but should not set limits to imagination.
Bertrand Russell (1872 - 1970)

You see things; and you say, 'Why?' But I dream things that never were; and I say, "Why not?"
George Bernard Shaw (1856 - 1950)

Always be a first-rate version of yourself, instead of a second-rate version of somebody else.
Judy Garland (1922-1969)

The mind is its own place, and in itself can make a heaven of hell, a hell of heaven.
John Milton (1608-1674)

Total absence of humor renders life impossible.
Colette (1873 - 1954)

Do the thing you fear, and the death of fear is certain.
Ralph Waldo Emerson (1803-1882)

I never think of the future - it comes soon enough.
Albert Einstein (1879 - 1955)