RASHI KHANNA-JAIN

Isolation, Characterization and Osteogenic Differentiation of Dental Stem Cells in Vitro for Bone Tissue Engineering

■

ACADEMIC DISSERTATION
To be presented, with the permission of
the board of Institute of Biomedical Technology of the University of Tampere,
for public discussion in the Jarmo Visakorpi Auditorium,
of the Arvo Building, Lääkärinkatu 1, Tampere,
on December 11th, 2012, at 12 o'clock.

UNIVERSITY OF TAMPERE

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
University of Tampere, Institute of Biomedical Technology
Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB)
Finland

Supervised by
Docent Susanna Miettinen
University of Tampere
Finland
Professor Riitta Suuronen
University of Tampere
Finland

Reviewed by
Professor Thimios Mitsiadis
University of Zurich
Switzerland
Professor Willy Serlo
University of Oulu
Finland

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 40 190 9800
taju@uta.fi
www.uta.fi/taju
http://granum.uta.fi

Cover design by
Mikko Reinikka

Acta Universitatis Tamperensis 1785
ISBN 978-951-44-8981-5 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1260
ISBN 978-951-44-8982-2 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2012

To my dear parents, beloved Anchit and precious Ayaan

Table of Contents

Tiivistelmä 6
Abstract 8
List of abbreviations 10
List of original publications 13
1. Introduction 14
2. Review of the literature 16
2.1 Stem cells 16
2.2 Human embryonic stem cells 18
2.3 Induced pluripotent stem cells 18
2.4 Multipotent stem cells 19
2.4.1 Bone marrow derived stromal stem cells 19
2.4.2 Adipose stem cells 19
2.4.3 Origin of the dental stem cells 20
2.4.3.1 Dental pulp stem cells 21
2.4.3.2 Dental Follicle stem cells 22
2.4.3.3 Dental periodontal ligament derived stem cells 22
2.5 Growth factors and hormones 24
2.5.1 Bone morphogenetic proteins 24
2.5.2 Dexamethasone 25
2.5.3 Vitamin D_3 metabolites 25
2.6 Culture media 26
2.7 Biomaterial 27
3. Aims of the study 29
4. Materials and methods 31
4.1 Isolation and culture of dental stem cells 31
4.2 Flow cytometry (III, IV) 33
4.3 Preparation of the biomaterial (IV) 33
4.4 CyQUANT® Cell Proliferation Assay (IV) 34
4.5 WST-1 (I, II, III) 35
4.6 Quantitative alkaline phosphatase activity (I, II) 35
4.7 Alkaline phosphatase staining (II, IV) 36
4.8 *In vitro* multilineage differentiation analysis (II, III) 36
4.8.1 Alizarin red staining (I, II, III) 37

4.8.2 Alcian blue staining (III) 37
4.8.3 Oil O Red staining (II, III) 37
4.9 Quantitative real time- polymerase chain reaction (I, II, III, IV) 38
4.10 1, 25 hydroxy vitamin D Enzyme immunoassay (II) 39
4.11 Immunostaining (III, IV) 40
4.12 Statistical analysis (I, II, III, IV) 42
5. Results 44
5.1 Cell proliferation and morphology 44
5.1.1 Cells treated with BMP-2 and -6 (I) 44
5.1.2 Cells treated with vitamin D_3 (II) 45
5.1.3 Cells cultured in FBS, SF/XF and HS (III, IV) 46
5.1.4 DPSCs seeded on biomaterials (IV) 47
5.2 Cell surface markers expression (III, IV) 47
5.3 Immunostaining 50
5.4 Multipotent differentiation potential of dental pulp stem cells (II, III) 51
5.4.1 Osteogenic differentiation 51
5.4.2 Adipogenic differentiation (III) 51
5.4.3 Chondrogenic differentiation 52
5.5 Early osteogenic differentiation potential of dental stem cells (I, II, IV) 53
5.6 Gene expression profile (I, II, III, IV) 54
6. Discussion 56
6.1 Optimized cell culture and isolation conditions 57
6.2 Cell surface markers expression 58
6.3 Multipotentiality of dental pulp stem cells 58
6.4 Effect of BMP-2 and BMP-6 on osteogenic differentiation 60
6.5 Effect of vitamin D_3 on osteogenic differentiation 60
6.6 The future of dental stem cell for therapies 61
7. Conclusion 63
Acknowledgements 66
References 67
Original Communications 79

Tiivistelmä

Aikuisen kantasolut ovat erilaistumattomia soluja, joilla on rajoittunut jakaantumiskyky. Viisaudenhampaasta eli kolmannesta poskihampaasta peräisin olevat hampaan kantasolut ovat uusi solutyyppi käytettäväksi regeneratiivisessa lääketieteessä. Hampaan kantasoluja saadaan pulpasta eli hammasytimestä, hampaan follikkelista sekä periodontaaliligamenttikudoksesta. Hampaan pulpan kantasolut ovat hyvin saatavilla oleva solutyyppi useisiin kudosteknologian sovelluksiin. Nämä solut ovat monikykyisiä kantasoluja, jotka voivat erilaistua mm. rusto-, rasva-, luu- ja lihassoluiksi sekä hermokudoksen solutyypeiksi. Viimeaikoina luukudosteknologia on kehittynyt yhdeksi lupaavimmista kudosteknologian muodoista. Solujen käyttöä kudosteknologiassa haittaa kuitenkin eläinperäisten aineiden, kuten naudan seerumin tarve solujen viljelyssä. Naudan seerumin kohdalla ongelmina ovat mm. vaihtelu eri valmistuserien välillä, eläinperäisten tartuntatautien välittyminen sekä immunologiset reaktiot. Näiden syiden takia tässä väitöskirjassa tarkasteltiin korvaavia vaihtoehtoja naudan seerumille hampaan kantasolujen ylläpidossa.

Tässä väitöskirjassa tutkittiin hampaan pulpan, follikkelin ja periodontaaliligamentin kantasolujen erilaistamista luusoluiksi. Töissä käytettiin mm. kasvutekijöitä kuten luun morfogeneettinen proteiini 2 ja 6 (BMP-2 ja -6), glukokortikoidi dexamethasonia (DEX) sekä D_3-vitamiinin metaboliitteja 25-hydroksivitamiini D_3 ($25OHD_3$) ja 1α,25-dihydroksivitamiini D_3 ($1\alpha,25(OH)_2D_3$). Tulokset osoittivat että BMP-2 ja -6 lisäävät periodontaaliligamentin kantasolujen luuerilaistumista vain jos ne yhdistetään muihin luusolujen muodostusta tukeviin tekijöihin, kuten dexamethasoniin, askorbiinihappoon ja β-glyserofosfaattiin.

Lisäksi havaittiin että D_3-vitamiinin metaboliitit ovat tehokkaampia kuin perinteisesti käytetty dexamethasoni pulpan ja follikkelin kantasolujen erilaistumisessa luusoluiksi.

Naudan tai ihmisen seerumia sisältäviä sekä seerumittomia ja eläinperäisistä ainesosista vapaita (SF/XF) soluviljelyelatusaineita vertailtiin pulpan kantasolujen viljelyssä. Ihmisen seerumin tai SF/XF-olosuhteiden käyttö mahdollistaisi solujen turvallisemman käytön soluhoidoissa. Lopulta tutkimme kliiniseen käyttöön soveltuvan solu-biomateriaali-yhdistelmän käyttöä luukudosteknologiassa. Tulosten mukaan pulpan kantasolut kiinnittyvät, säilyvät elinkykyisinä, jakaantuvat ja voivat erilaistua luusoluiksi käytettäessä kolmiulotteista β-TCP/P(LLA-CL) (β-tricalcium phosphate/Poly L-Lactic acid- caprolactone) biomateriaalitukirakennetta.

Tutkimuksen johtopäätöksenä on että hampaan pulpan kantasolut ovat monikykyisiä, helposti saatavilla olevia soluja tutkimustarkoituksiin. D_3-vitamiinin metaboliitit ovat tehokkaita pulpan ja follikkelin kantasolujen luuerilaistuksessa. Ihmisen seerumia sisältävissä elatusaineissa pulpan kantasolut jakaantuivat nopeasti ja erilaistuivat rasva-, rusto- ja luusoluiksi ja siten ihmisen seerumi voisi toimia naudan seerumin korvaavana ainesosana hampaan pulpan kantasolujen kasvatuksessa. Lisätutkimuksia kuitenkin tarvitaan SF/XF-olosuhteista, sillä kantasolujen jakaantuminen näissä olosuhteissa oli hidasta ja erilaistumiskyky oli heikko verrattuna muihin olosuhteisiin. Tämän väitöskirjan tutkimustuloksia voidaan hyödyntää tulevissa kudosteknologiaa ja hampaan kantasoluja hyödyntävissä hoitomuodoissa.

Abstract

Adult stem cells are undifferentiated cells found in tissues or organs that are renewable with limited replicative capacity. Dental stem cells (DSCs) derived from impacted third molar teeth are considered a new source of stem cells that could be used for regenerative medicine. The DSCs can be obtained from the dental pulp tissue, dental follicle tissue and periodontal ligament tissue. Dental pulp stem cells (DPSCs) are an accessible stem cell source with therapeutic applicability in repair and regeneration of damaged tissues. DPSCs exhibit a multipotent character as they can differentiate into chondrocytes, adipocytes, osteoblasts, myocytes, and neuronal cells as reported in the literature. Recently, bone tissue engineering has emerged as most promising approach to develop biological bone substitutes to restore, maintain and regenerate bone tissue function. However, the methods adopted for culturing and harvesting cells is a major downside due to usage of fetal bovine serum (FBS). The scientific problems encountered due to the presence of FBS are batch to batch variability, risk of possible contamination with virus, prions, bacteria, and immunogenic response to the FBS cultured cells may lead to anaphylactic or arthus like immune reaction in patients. Therefore, in this thesis alternative to animal serum for DPSCs culture was studied for its potential clinical availability.

Additionally, in this thesis, manipulation of culture condition for directing the differentiation of various DSCs such as DPSCs, Dental follicle stem cells (DFSCs) and periodontal ligament derived cells (PDLCs) to form bone forming cells was studied. The effect of growth factors and metabolites described in this work are bone morphogenetic proteins (BMP-2 and -6), vitamin D_3 metabolites 25-hydroxyvitamin D_3 ($25OHD_3$) and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) and dexamethasone (DEX). There are potential merits of the combined use of BMP-2 or BMP-6 and osteogenic supplements (OS; DEX, ascorbic acid (AA), and β-

glycerophosphate) for osteogenic differentiation of human periodontal ligament cells (PDLCs). The vitamin D_3 metabolites $25OHD_3$ and $1\alpha,25(OH)_2D_3$ as a substitute to DEX in the osteogenic supplements were found to be more effective in differentiating DPSCs and DFSCs into osteogenic lineage.

Furthermore, FBS, human serum (HS) and serumfree/xenofree (SF/XF) culture media for expansion of DPSCs was tested. By expanding DPSCs in SF/XF or in HS media, the problems including possible infections and severe immune reactions can be eliminated to use cultured stem cells safely in clinical therapies. Lastly, the appropriate combination of biomaterial, DPSCs and specific differentiation factors for functional bone tissue engineering was tested *in vitro*. Based on our findings, DPSCs cultured in HS medium attached, remained viable, proliferated and differentiated osteogenically when seeded on β-Tricalcium phosphate/Poly L-Lactic acid- caprolactone (β-TCP/P (LLA-CL)) 3D biomaterial scaffold.

In conclusion, DPSCs were found to be multipotent, easily procurable and anatomically accessible to use for our research work. DPSCs and DFSCs can be efficiently differentiated into osteogenic lineage under the influence of vitamin D_3. DPSCs isolated in HS proliferate more homogenously; differentiate into adipogenic, osteogenic and chondrogenic lineages. Thus, HS could serves as a safer alternative to FBS for DPSCs culture. However, further research is required for expansion of cells in SF/XF-M, as observed in our study the cells proliferated at a very slow rate and with minimal multilineage differentiation. These findings are essential for the future applicability of DSCs for bioengineering tissues for regenerative therapies.

List of abbreviations

AA	Ascorbic acid
AlloHS	Allogenic human serum
ALP	Alkaline phosphatase
AM	Adipogenic medium
AutoHS	Autologous human serum
BM	Basal medium
BM-MSCs	Bone marrow derived mesenchymal cells
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
CD	Cluster of differentiation
CM	Chondrogenic medium
cDNA	Complimentary deoxyribonucleic acid
DEX	Dexamethasone
DFSCs	Dental follicle stem cells
DMEM/F-12	Dulbecco's modified Eagle's medium: nutrient mixture F-12
DPBS	Dulbecco's phosphate buffered saline
DPSCs	Dental pulp stem cells
DSCs	Dental stem cells
EMD	Enamel matrix derivative
ESC	Embryonic stem cells
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FBS-M	Fetal bovine serum-medium
FCS	Fetal calf serum
FITC	Fluorescein iso thiocyanate isomer 1
HLA-DR	Human leucocyte antigen class II

HS	Human serum
HS-M	Human serum-medium
iPSCs	Induced pluripotent stem cells
MSCs	Mesenchymal stem cells
OCN	Osteocalcin
OM-DEX	Osteogenic medium containing dexamethasone
OM-VD	Osteogenic medium containing vitamin D
OPN	Osteopontin
OS	Osteogenic supplements
PDGF	Platelet derived growth factor
PDLCs	Periodontal ligament cells
PPARγ	Peroxisome proliferator-activated receptor γ
qALP	Quantitative alkaline phosphatase assay
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Riboucleic acid
RPLP0	Large ribosomal protein P0
RUNX2	Runt-related transcription factor 2
SCAP	Apical papilla
SF	Serum Free
SF/XF	Serum Free/Xeno Free
SF/XF-M	Serum Free/Xeno Free-medium
SHED	Stem cells derived from exfoliated deciduous teeth
β-gp	Beta glycerophosphate
β-TCP	Beta Tricalcium phosphate
$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
$25OHD_3$	25-hydroxyvitamin D_3

List of original publications

The present thesis is based on the following original publications:

I. **Khanna-Jain, R.**, Agata, H., Vuorinen, A., Sándor, G., Suuronen R., Miettinen, S. Osteogenic differentiation of human periodontal ligament cells induced by Dexamethasone, ascorbic acid, and β-glycerophosphate with or without bone morphogenetic proteins 2 or 6. Growth Factors. 2010 Jun 23.

II. **Khanna-Jain, R**., Vuorinen, A., Sándor, G., Suuronen, R., Miettinen, S. Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells. J Steroid Biochem Mol Biol. 2010 Aug 16.

III. **Khanna-Jain, R.,** Vanhatupa, S., Vuorinen, A., Sándor, G., Suuronen, R., Mannerstrom, B., Miettinen, S. Growth and differentiation of human dental pulp stem cells maintained in fetal bovine serum, human serum and serum-free/xeno-free culture media. J stem cell therapy and research. 2012 Aug 18.

IV. **Khanna-Jain, R.,** Mannerstrom, B., Vuorinen, A., Sándor, G., Suuronen, R., Miettinen, S. Osteogenic differentiation of human dental pulp stem cells on β-TCP/P (LLA-CL) three dimensional scaffolds. J Tissue engineering. 2012 Oct 9.

1. Introduction

Tissue engineering from cultured adult stem cells is a novel approach to restore lost tissue. Progress in cell culture techniques can give rise to a new approach for reconstruction of bone defects (Kimelman et al., 2006). Regenerative treatments for bone reconstruction require integration of inductive morphogenetic signals (such as Bone morphogenetic proteins; BMPs or Vitamin D_3 metabolites), responding stem cells and biomaterial scaffold (Seo et al., 2004). It is known that mesenchymal stem cells (MSCs) derived from impacted third molar teeth such as, dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs) and dental follicle stem cells (DFSCs) have the ability to undergo osteogenic, chondrogenic and adipogenic differentiation, as reported in previous studies (Iohara et al., 2004; Shiba et al., 2001; Yao et al., 2008). Lately, DPSCs have attracted much interest for bone tissue engineering, Laino, Papaccoio and colleagues have shown that human DPSCs were capable of differentiating into bone, which was well woven and vascularized (d'Aquino et al., 2007; d'Aquino, De Rosa, Lanza, et al., 2009; Papaccio et al., 2006). However, they differentiated the DPSCs in basal culture medium supplemented with fetal bovine serum (FBS) which is an animal derivative, leading to risk of transmission of infection to the cells through the culture media (Brunner et al.). FBS is a cocktail of various factors required for cell attachment, growth and metabolism. It will always be an ill-defined mixture of components; hence, results in lot-to-lot variability leading to varying data. Therefore, from cell biology point of view the development of xenofree cell culture system is essential. Recent advances in stem cells based therapies necessitate the demand of serum free or xenofree alternatives of human origin for autologous cell expansion and clinical grade tissue engineering.

Another aspect is the exposure of progenitor cells to inductive morphogens or hormones in appropriate biomaterial scaffold which will enable their future application in bone tissue engineering. BMPs play a critical role in tooth morphogenesis and were originally identified as proteins that induce bone formation

at extra- skeletal sites; BMP-2 induces alveolar bone formation and BMP-6 regulates osteogenic differentiation of cells (Chen et al., 1997; Chen et al., 2004). However, the effects of BMPs have been demonstrated to be inconsistent, it is therefore essential to establish a potent exogenous growth factor to induce osteogenesis. Apart from BMPs, the glucocorticoid, dexamethasone is another commonly used osteogenic inducer in combination with ascorbic acid and β-glycerophosphate (Hayami et al., 2007). Considering the possibility of glucocorticoid induced osteoporosis *in vivo* (Pierotti et al., 2008), the need for an alternative osteogenic inducer remains. It is also known that Vitamin D_3 controls the formation of mineralized tissue through regulation of the expression of various proteins involved in bone formation. Its role as a potential osteogenic inducer for MSCs has not been explored extensively (Shiba et al., 2001). Taken together, in this work we have studied the xenofree isolation, proliferation, and osteogenic differentiation and cell adhesion of DPSCs within a medical grade biomaterial. Hopefully, the results of our study would guide us to the future applicability of dental stem cells.

2. Review of the literature

2.1 Stem cells

The human body has a remarkable capacity to regenerate. Epithelial and hematologic tissues continually renew themselves even in late stages of individual's life. During histogenesis, the embryonic stem cells are responsible for tissue development as they are capable of differentiating into any cell type. Stem cells are also found in adult tissues. These cells have the ability to self renew and repair tissues. Based on these considerations, cell therapies seem to be a promising treatment option for regenerative medicine. Application of stem cells for blood disease, bone fractures, and cartilage degeneration are already in clinical use. The usage of stem cell systems as a tool for tissue engineering has great potential. Stem cells are cells that have the potential to develop into many different or specialized cell types. Stem cells are referred to as "undifferentiated" cells because they have not yet committed to a developmental path that will form a specific tissue or organ. The process of changing into a specific cell type is known as differentiation. Stem cells can be classified on the basis of their origin as totipotent, pluripotent and multipotent stem cells (Figure 1). Totipotent stem cells are obtained from 1-3 day old embryos and each cell has the ability to develop into an individual (Mitalipov and Wolf, 2009). Pluripotent stem cells are harvested from the inner cell mass of the blastocyst seven to ten days after fertilization. These cells can form any cell type or from induced adult stem cells by transfer of genes to generate pluripotent stem cells (Takahashi et al., 2007; Thomson et al., 1998). Lastly, multipotent stem cells are derived from fetal tissues and adult tissues that have limited differentiation capacity. Multipotent fetal stem cells or adult stem cells are derived from the three embryonic germ layers (ectoderm, mesoderm and endoderm) that become more and more

committed to a particular cell type for regenerating organs and tissues (Kelly et al., 2004). This review of literature focuses on multipotent adult stem cells of mesenchymal origin derived from human impacted third molar teeth for bone tissue engineering applications.

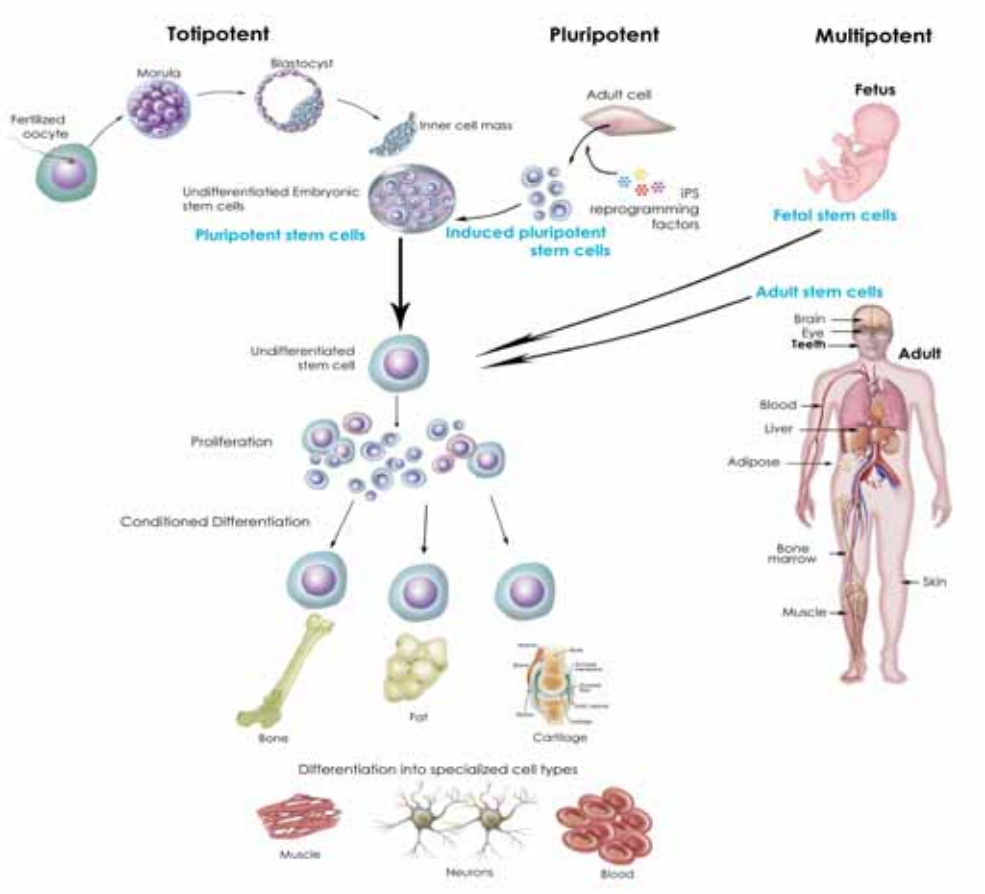

Figure 1. Different sources of stem cells divided into groups according to their differentiation capacity totipotent-, pluripotent-, induced pluripotent- and multipotent stem cells. Figure modified from pictures made by Bettina Mannerström. The original images were made by Catherine Twomey from the National Academies Understanding stem cells: An Overview of the Science and Issues, http://www.nationalacademies.org/stemcells.

2.2 Human embryonic stem cells

The first derivation of a pluripotent mouse embryonic stem cells (ESCs) line from mouse embryo was reported in 1981 (Evans and Kaufman, 1981). ESCs can be derived from the inner cell mass of the blastocyst. These cells have unlimited self-renewal capacity and can give rise to any cell lineages. Lineage specific differentiation of ESCs can be directed under specific culture conditions and by manipulating the microenvironment (Robertson, 1990; Valdimarsdottir and Mummery, 2005). Recently, considerable attention has been devoted to directing ESC differentiation into osteogenic lineage. The results of these researches have highlighted the potential use of ESCs in the field of bone tissue engineering (Warotayanont et al., 2009; Zhang et al., 2009)

2.3 Induced pluripotent stem cells

Generation of induced pluripotent stem cells (iPSCs) holds a great promise for regenerative medicine and other aspects of clinical applications (Amabile and Meissner, 2009). Human dermal fibroblasts are the most extensively studied and feasible cell source for iPSCs generation (Takahashi et al., 2007). Other cell types which have been reprogrammed include neonatal foreskin fibroblast, mesenchymal stem cells (MSCs), amniotic fluid–derived cells, dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), and stem cells from apical papilla (SCAP) (Atari et al., 2011; Atari et al., 2012; Galende et al., 2010; Tamaoki et al., 2010). The Four factors namely c-Myc, Klf4, Oct4 and Sox2 or Lin28, Nanog, Oct4 and Sox2 are carried by viral vectors for generating iPSCs (Takahashi et al., 2007). These cells have been shown to maintain a normal karyotype and the potential to develop into all three germ layers. However, from the perspective of clinical applications, viral vectors for introducing these factors should be removed. Several approaches have been suggested to remove the vectors from the cells after they have been reprogrammed into iPSCs (Amabile and Meissner, 2009; Nakhaei-Rad et al., 2012). Due to the safety concerns related to viral vectors, iPSCs are still far from clinical use, as the current methods do not meet the GMP standards.

2.4 Multipotent stem cells

2.4.1 Bone marrow derived stromal stem cells

Bone marrow-derived mesenchymal stem cells (BM-MSCs) were first identified by Friedenstein and demonstrated that bone marrow plated in fetal calf serum (FCS) medium developed fibroblast like colonies (Friedenstein et al., 1966). The international society of cellular therapy defines MSCs or BM-MSCs on the basis of three main characteristics 1) their adhesion to plastic 2) their expression of a specific set of membrane molecules (CD73, CD90, CD105) together with a lack of expression of the hematopoietic markers CD14, CD34, and CD45 and human leukocyte antigen-DR (HLA-DR) and 3) their ability to differentiate into three different lineages such as osteogenic, chondrogenic and adipogenic (Dominici et al., 2006). Further research on BM-MSCs have shown that they are self-renewable, multipotent progenitor cells with the capacity to differentiate into lineage specific cells that form bone, cartilage, fat, tendon and muscle tissue, and possess immunosuppressive properties (Dimarakis and Levicar, 2006; Miller et al., 2008; Puissant et al., 2005). Due to certain shortcomings of obtaining the BM-MSCs that includes pain, tissue site morbidity and low cell numbers on harvest. Therefore, alternate less morbid sources of MSCs have been sought (Kobayashi et al., 2005; Miller et al., 2008).

2.4.2 Adipose stem cells

Adipose tissue has become an attractive source of stem cells, as it is an accessible and abundant adult stem cell reservoir. Adipose tissue can be obtained by suction-

assisted lipectomy (liposuction). Adipose tissue derived stem cells (ADSCs) are a heterogeneous population of cells, which after isolation are called the stromal vascularization fraction (SVF) (Gimble et al., 2007). The SVF includes the stromal cells, ADSCs that have the ability to differentiate into several lineages such as osteogenic, adipogenic, chondrogneic, and myogenic (Gimble and Guilak, 2003; Gimble et al., 2011). It has been well shown that ADSCs acts in a similar way as BM-MSCs and ADSCs showing very similar expression of the surface marker. ADSCs have been shown to be positive for a variety of markers, such as CD13, CD29, CD44, CD54 and others (Gronthos et al., 2001; Jurgens et al., 2008; Lindroos et al., 2009). Despite the similarity of the surface marker expression with BM-MSCs, it has been reported that, unlike BM-MSCs, ADSCs do not express STRO-1 (Gronthos et al., 2001; Zuk et al., 2002). Recently, ADSCs have been used by many researchers for bone tissue engineering applications. Several research reports including those from our laboratory have demonstrated that ADSCs show good adhesion, proliferation activity, and homogenous bone-like tissue formation on various biocompatible biomaterial scaffolds (Frohlich et al., 2010; Lindroos et al., 2009).

2.4.3 Origin of the dental stem cells

Tooth is a complex organ made up of hard and soft tissues namely, enamel, dentin, pulp, cementum and periodontal ligament. During the sixth week of embryogenesis, after the migration of neural crest cells into head and neck mesenchyme, the ectoderm covering the stomodeum begins to proliferate, giving rise to the dental laminae (Maas and Bei, 1997; Ten Cate, 1982). The cell differentiation and morphogenesis is regulated by the reciprocal interaction between the ectoderm and mesodermal layers, which results in the formation of a placode. Following the interaction ovoid structures begin to separate and develop into tooth germs (Thesleff et al., 1995). The tooth germ is organized into three parts namely enamel organ, dental papilla and dental follicle. The dental papilla contains cells that develop into odontoblasts, which are dentin-forming cells (Ruch, 1998; Ten Cate, 1967). Mesenchymal cells within the dental papilla are responsible for the formation of

tooth pulp. The dental follicle gives rise to three important entities: cementoblasts, osteoblasts, and fibroblasts. Cementoblasts form the cementum covering the root of a tooth (Slavkin et al., 1989). Osteoblasts give rise to the alveolar bone around the roots of teeth. Fibroblasts develop the periodontal ligaments (PDL), which connect teeth to the alveolar bone through Sharpey's fibres that insert into the cementum (Isaka et al., 2001).

DSCs can be removed from an individual's primary or permanent teeth, expanded, and put back into the same individual when repair becomes necessary. This autologous transplantation removes the need for immunosuppression. Dental tissues are the specialized tissues that do not undergo continuous remodeling as shown in bone tissue. Therefore, dental tissue derived stem cells or progenitor cells may be committed and restricted in their differentiation capacity. To date dental stem cells have been obtained from the following tissues: human stem cells from exfoliated deciduous teeth (SHED), apical papilla, dental pulp, dental follicle and dental periodontal ligament tissues (Huang et al., 2009; Morsczeck et al., 2010).

2.4.3.1 Dental pulp stem cells

The dental cavity encloses a specialized tissue type, dental pulp, that is well demarcated from the surrounding tissues and therefore, relatively easy to remove (Figure 2). Gronthos et al 2000 was the first one to show that stem cells existed in human dental pulp. The dental pulp tissue contains several types of progenitor cells, which differ in regards to the rate of proliferation, renewal ability and differentiation potential (Graziano et al., 2008; Gronthos et al., 2002). *In vitro*, DPSCs can differentiate into odontoblasts, osteoblasts, endotheliocytes, smooth muscle cells, adipocytes, chondrocytes and neurons under the influence of differentiation specific supplements (Marchionni et al., 2009; Pierdomenico et al., 2005). DPSCs have similar phenotypic characteristics as BM-MSCs based on the expression of the mesenchymal stem cell markers CD44, CD29 CD73, CD90, CD105 and the hematopoietic markers CD34, CD45 (Huang et al., 2009; Pierdomenico et al., 2005). Several studies have demonstrated that DPSCs retain their stem cell

properties following cryopreservation (Graziano et al., 2008; Woods et al., 2009; Zhang et al., 2006). Moreover, one report suggests that cryopreserving impacted third molar teeth for upto one month, maintains the stemness of the dental pulp cells (Perry et al., 2008b). Therefore, these finding of stem cells in dental pulp tissue leads to a potential stem cell source for banking. In addition, DPSCs have attracted much interest for bone tissue engineering. Laino, Papaccoio and colleagues have shown that human DPSCs were capable of differentiating into bone, which was well woven and vascularized (Laino et al., 2005).

2.4.3.2 Dental Follicle stem cells

The dental follicle is a loose connective tissue sac derived from ectomesenchymal tissues. It surrounds the developing tooth and plays an important role in coordination of tooth eruption. The dental follicle occupies the radiolucent space around the crowns of unerupted teeth (Figure 2). It is firmly attached to the surface of the crown by the reduced epithelium of the enamel organ. The thickness of the dental follicle can reach up to 5.6 mm and still maintain normal structure and organization (Morsczeck, Gotz, et al., 2005). Handa et al were the first to report stem cells in bovine dental follicle tissue. This tissue contains progenitor cells that form the periodontium such as cementum, PDL, and alveolar bone (Handa et al., 2002). DFSCs stimulated with Enamel matrix derivatives (EMD) or BMP-2 and BMP-7 expressed cementoblast markers like cementum attachment protein and cementum protein-23 (Kemoun et al., 2007). DFSCs show a typical fibroblast-like morphology and express Nestin, Notch-1, collagen type-I, bone sialoprotein (BSP), osteocalcin (OCN) and fibroblast growth factor receptor (FGFR) (Morsczeck, 2006). These cells form low numbers of adherent colonies when released from the tissue (Morsczeck, Moehl, et al., 2005).

2.4.3.3 Dental periodontal ligament derived stem cells

The periodontal ligament (PDL) connects the alveolar bone to the root cementum through its ligaments and sustains the tooth in its alveolus. The periodontal ligament tissues can be obtained from the roots of the extracted teeth (Figure 2). The tissue

contains progenitor cells that have the potential to form periodontal structures such as cementum, bone and ligament (Isaka et al., 2001). The presence of progenitor cells in the PDL tissue helps to maintain the homeostasis and regeneration of the periodontal tissue (Seo et al., 2004). It has been demonstrated that PDL progenitor cells are committed to several developmental lineages such as osteoblastic, fibroblastic, and cementoblastic (Murakami et al., 2003; Nohutcu et al., 1997). Therefore, they are a desirable source of cells for regeneration of a functional periodontal apparatus. Periodontal ligament derived stem cells (PDLSCs) have the potential to form collagen fibres and generate cementum/PDL-like structures *in vivo* (Pitaru et al., 2002). However, the clinical application of autologous PDL-derived cells is restricted due to several limitations, that is, insufficient cells availability, difficulties in harvesting, limited success and predictability of procedures developed to date.

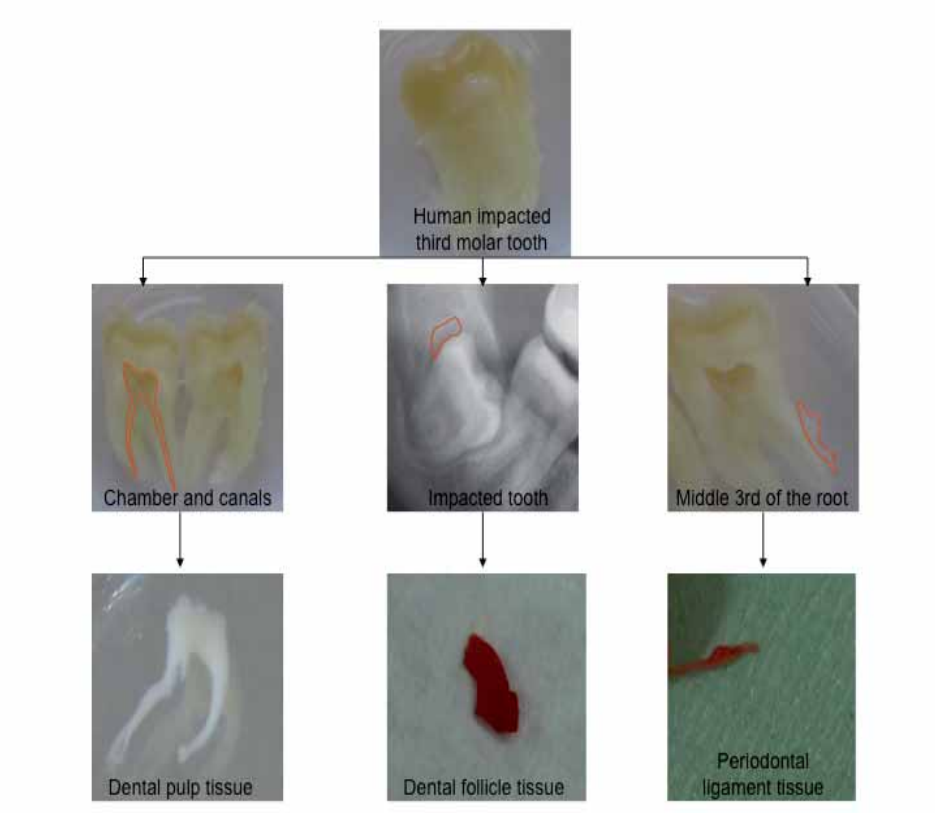

Figure 2: Anatomical location to obtain dental tissues from extracted impacted third molar tooth.

2.5 Growth factors and hormones

2.5.1 Bone morphogenetic proteins

Growth factors are biologically active polypeptide hormones that affect the immune function as well as the differentiation of cells from the epithelium, bone, and connective tissue. A major focus of periodontal research has evaluated the impact of growth factors on periodontal tissue engineering (Ripamonti, 2007). The most commonly used growth factors for periodontal tissue regeneration are Platelet derived growth factor (PDGF), Fibroblast growth factor (FGF), Transforming growth factor-beta (TGF-β) and Bone morphogenetic proteins (BMPs). BMPs have been used in preclinical and clinical trials for the treatment of large periodontal and intrabony defects around the dental implants (Blumenthal et al., 2002; Lynch et al., 1989; Nevins et al., 2005; Ripamonti and Petit, 2009) and for the regeneration of large segments of resected mandibles (Moghadam et al., 2001; Clokie and Sándor, 2008). Bone morphogenetic proteins (BMPs), originally identified as proteins that induce bone formation at extra-skeletal sites, are multifunctional growth factors that regulate the growth, differentiation, and apoptosis of various cell types, including osteoblasts, chondroblasts, neural cells, and epithelial cells (Urist 1965; Hogan 1996; Groeneveld and Burger 2000; Xiao et al. 2007). Currently, there are 14 subsets of BMPs which belong to the transforming growth factor-β superfamily. Four of them (BMP-2, -4, -6, and -7) are known as inducers of osteogenic differentiation (Lavery et al. 2008). BMP-6 has been reported to be one of the potent inducers of osteogenic differentiation in mesenchymal stem cells (MSCs; Friedman et al. 2006; Nohutcu et al. 1997; Hayami et al. 2007). However, BMP-2 is one of the most extensively studied BMPs and the combined use of BMP-2 and osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) have been shown to be able to accelerate the osteogenic differentiation of human MSCs (Jager et al. 2008). Moreover, Mizuno et al. reported that the response of BMP-2 on human MSCs varied between donors, resulting in inconsistent effect of BMP-2 on *in vitro* osteogenic differentiation of human MSCs (Mizuno et al. 2009). Similar inconsistent results have been reported between patients in clinical studies of bone regeneration by BMP-2 (Govender et al. 2002; Groeneveld and Burger 2000).

2.5.2 Dexamethasone

Dexamethasone (DEX) a glucocorticosteroid, in combination with OS is the most commonly used osteogenic inducer for human mesenchymal stem cells (MSCs) such as adipose tissue derived (Lee et al., 2009) and bone marrow derived stem cells in vitro (Beloti and Rosa, 2005). Studies have shown that human dental stem cells differentiate into osteoblasts like cells when induced by DEX (Morsczeck, Moehl, et al., 2005; Morsczeck, 2006). However, long term glucocorticosteroid exposure has deleterious effects on bone *in vivo*, resulting in glucocorticoid- induced osteoporosis (Pierotti et al., 2008).

2.5.3 Vitamin D_3 metabolites

There are some studies where 1α,25$(OH)_2D_3$ in addition to OS has been used for osteogenic differentiation of MSCs (Feng et al., 2010; Gupta et al., 2007; Zhou et al., 2006). Moreover, the role of 1α,25$(OH)_2D_3$ in tooth formation is well known from *in vivo* and clinical studies, deficiency of 1α,25$(OH)_2D_3$ results in hypocalcification of the dentin and enamel leading to unmineralized dental structure (Barron et al., 2008). Excessive doses of 1α,25$(OH)_2D_3$ cause hypercementosis, formation of pulp stones and hypercalcification in dental tissues (Giunta, 1998). Additionally, 1α,25$(OH)_2D_3$ has been reported to promote the function of osteoclastogenesis in the periodontium (Tang and Meng, 2009). Differentiation of MSCs such as those derived from adipose tissue, bone marrow tissue or from dental tissue into osteoblast like cells is induced *in vitro* by treating the cells with DEX, ascorbic acid and β- glycerophosphate as reported in many studies (Huang et al., 2006; Morsczeck, Moehl, et al., 2005). It has been reported that 1α,25$(OH)_2D_3$ may be superior to dexamethasone as an agent that induces osteogenic differentiation in human adipose derived cells (Gupta et al., 2007; Halvorsen et al., 2001; Zhou et al., 2006). However, it is essential to further elucidate the safest and most effective hormones for clinical use.

2.6 Culture media

The supplementation of basal culture media with animal serum or serum of any different origin is essential for cell growth, metabolism, and to stimulate proliferation. As stems cells are currently cultured in media containing fetal bovine serum (FBS), there is a theoretical risk of transmission of infection to the cells through the culture media (Heiskanen et al., 2007; Spees et al., 2004). In addition, it is difficult to maintain the MSCs in culture using serum, because serum contains growth factors that drive the cells to differentiate. Serum is always an ill defined mixture of components for culturing cells (Shahdadfar et al., 2005). Development of antibodies to FBS components has been demonstrated to occur *in vivo*. Metabolic uptake of nonhuman sialic acid Neu5Gc by cultured human cells from the FBS in culture medium, against which many humans possess circulating antibodies that might lead to complement activation and cell death in vivo after transplantation (Dimarakis and Levicar, 2006). We therefore need to further explore optimal culture conditions for maintaining and expanding the stem cells. The use of autologous or allogenic human serum (HS) eliminates the problem of xenogenic antibodies into patient (Aldahmash et al., 2011; Komoda et al., 2010). Furthermore, recent advances in stem cells based therapy necessitate the invention of serum-free alternatives of human origin for autologous cell expansion, reimplantation and clinical tissue engineering (Aldahmash et al., 2011; Bieback et al., 2009). Figure 3 shows the comparison between the HS and SF/XF media alternatives to FBS. Hence, before the successful widespread clinical use of DPSCs for bone tissue regeneration, xenofree media such as HS or SF/XF needs to be tested for successful proliferation, with multilineage differentiation potential and at the same time maintains the stemness of cells.

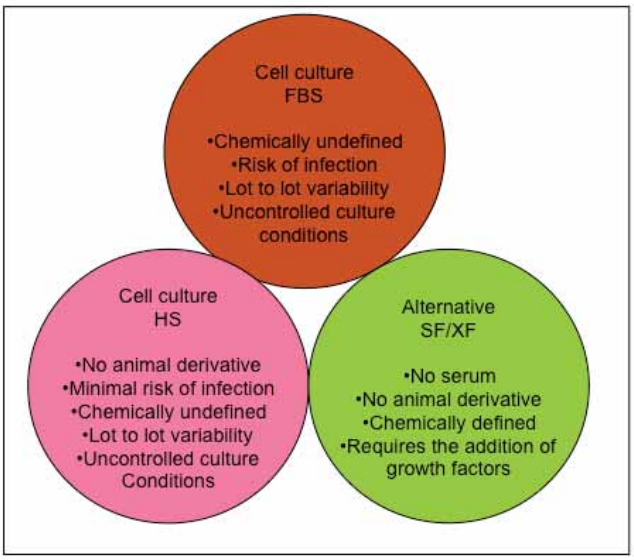

Figure 3: Comparative analysis between fetal bovine serum, human serum and serumfree/xenofree cell culture media.

2.7 Biomaterial

The gold standard to stimulate bone growth and to replace lost bone tissue is autologous bone grafting, which is the most trusted treatment modality. However, harvesting procedures involve high donor site morbidity and limited amounts of bone are available for autografting (Laurie et al., 1984). Allografts are readily available from bone banks that eliminate the pain and potential infection associated with the second surgical site necessary for autograft procedures. Large bone grafts, such as a femur for the leg or humerus for the arm, would not be possible without allografts, since such grafts cannot be obtained from the patient's own body (Calori et al., 2011). It is known that some bone grafts are osteoinductive, meaning they "signal" the patient's body to begin making new bone, promoting faster healing and better surgical outcomes (Kolk et al., 2012). But with allografts the complication

rate is high with the risks of graft-versus-host disease, transmission of infectious disease and graft failure (Forrest et al., 1992). Therefore, alternative methods including those using stem/progenitor cells have been investigated and human trials have been successfully carried out (Mesimaki et al., 2009; Sittinger et al., 2004). Human cells require interactions with the microenvironment to survive, proliferate and function, which is largely composed of the extracellular matrix proteins. In tissue engineering, these three -dimensional (3D) structures are initially provided to the stem cells by the use of biocompatible scaffolds (Lutolf et al., 2009). With the purpose of distribution of cells and osteoconductivity within a biomaterial, porous β-tricalcium phosphate (β-TCP) with good bone bonding properties is preferred (Wang et al., 2006). But, due to poor mechanical strength, β-TCP is used for bone regeneration at non-loading sites or to fill bone voids (Barrere et al., 2006). From a tissue engineering point of view, a biomaterial should have sufficient strength initially to withstand the stresses of mastication until the newly regenerated bone takes over (Sittinger et al., 2004). Moreover, the structural integrity is also crucial for the long-term success of implants in the bone (Misch, 2011). In order to achieve desirable mechanical strength for bone tissue engineering, synthetic polymer based biomaterials such as poly-L-lactic acid (PLLA) or poly caprolactone (PCL) are combined with osteoconductive bioceramics (Guarino et al., 2008; Nof and Shea, 2002). Therefore, it is important that scaffolds should be designed to provide structural integrity for the cells used in tissue engineering until newly formed tissue becomes autosustainable.

3. Aims of the study

The aims of this project were to isolate, to culture and study the osteogenic differentiation potential of dental stem cells derived from extracted human impacted third molar teeth induced by growth factors or hormones. Depending on the anatomic location, ease of availability and differentiation potential, DPSCs were chosen to be appropriate cell source for consecutive studies. In order to make DPSCs clinically accessible, human serum and serum free/ xenofree alternatives were tested for cultivation of the cells instead of animal derived serum. Finally, a clinical grade osteoconductive biomaterial was used to assess the ability of DPSCs to proliferate and differentiate into osteoprogenitors cells within the material.

The aims of the study included the following:

1) To study the effect of BMP-2 and BMP-6 on osteogenic differentiation of human periodontal ligament cells, with and without the addition of dexamethasone, ascorbic acid, and beta- glycerophosphate (**I**).
2) To study the influence of vitamin D_3 metabolites on osteogenic differentiation of human dental pulp and human dental follicle cells (**II**).
3) To study the characterization, growth and differentiation of human dental pulp cells in fetal bovine serum, human serum and serum free/xenofree media (**III**).
4) To study the osteogenic differentiation capacity of human dental pulp stem cells cultured in human serum medium on β-TCP/P (LLA-CL) biomaterial (**IV**).

4. Materials and methods

4.1 Isolation and culture of dental stem cells

Human impacted third molars were obtained with informed consent from Finnish Student Health Services, Tampere, Finland. The Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R06009), approved the collection of stem cells from teeth samples. Human dental pulp explants **(II, III, IV)**, human dental follicle explants **(II)** and human periodontal ligament explants **(I)** were obtained from partially or completely impacted third molar teeth of the patients as shown in (Figure 2), patients aged 21–26 years (23 ± 2.5 years). The dental tissue explants were brought from the health centre to the laboratory in Dulbecco's Phosphate buffered saline (DPBS; BioWhittaker Lonza, Verviers, Belgium) containing 2% antibiotics/antimycotics (a/a; 100 U/ml penicillin, 0.1mg/mL streptomycin and 0.25 µg/mL amphotericin B; Life Technologies, Paisley, Scotland, UK). The dental tissue fragments were minced by using sterilized scalpels and were then digested in collagenase type I 3mg/ml (Invitrogen) and dispase 4mg/ml (Invitrogen) for 1 hour at 37ºC. Once digestion was completed the obtained cell pellet was suspended in 3 ml of Fetal bovine serum medium (FBS-M) **(I, II)** or suspended in 600 µl of DPBS **(III, IV)**. The suspension was passed through a 100µm cell strainer (Falcon, BD Labware, Franklin lakes, NJ, USA). The isolated dental periodontal cells (PDLCs) , dental follicle stem cells (DFSCs) and dental pulp stem cells (DPSCs) were cultured in Dulbecco's modified Eagle medium (DMEM)/F-12 1:1 (Gibco Life Technologies, Paisley, UK) supplemented with 1% l-analyl-l-glutamine (Gluta-MAX I; Life Technologies), 1% a/a and 10% Fetal bovine serum (FBS; Invitrogen, Paisley, UK) (FBS-M) **(I, II, III)**. In Study **III, IV,** DPSCs were cultured in (2) (DMEM)/F-12 1:1 supplemented with Gluta-MAX I , 1% a/a and 10% allogenic Human Serum (HS; PAA Laboratories GmbH, Pasching, Austria) (HS-M) Further, DPSCs cultured and expanded in HS-M were used for testing StemPro®

Mesenchymal stem cells (MSC) Serum free/Xeno Free medium (SF/XF-M) **(III)**, culture wells were coated with CELL start (Life Technologies) to assist in cell attachment (Figure. 4). PDLCs, DFSCs and DPSCs expanded in FBS-M were harvested using 1% trypsin (Lonza/BioWhittaker, Verviers, Belgium) **(I, II, III)**. The DPSCs isolated and cultured in HS-M and SF/XF-M were harvested using TrypLE Select or Express (Life Technologies) for XF detachment of cells **(III, IV)**. Cell culture plates and T-75 culture flasks (Thermo Fischer, Nunc, and city) were monitored daily for cell growth, with medium changes taking place thrice a week.

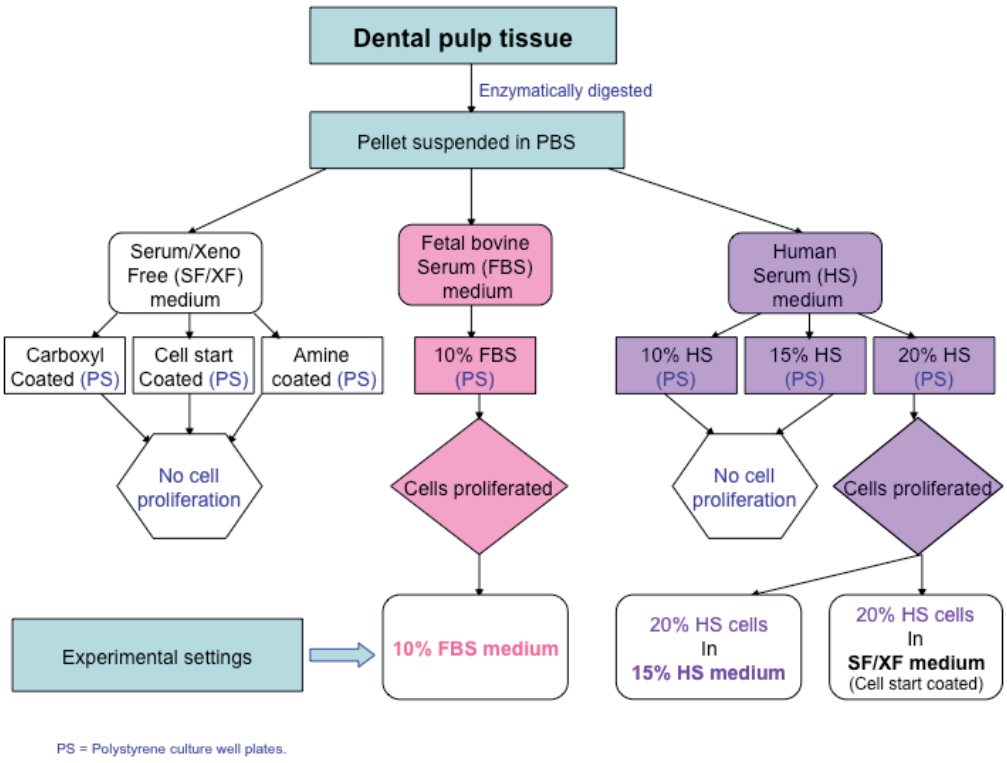

Figure 4: Flowchart showing the effect of cell culture media on proliferation (**III**).

4.2 Flow cytometry (III, IV)

DPSCs cultured in FBS-M, HS-M (**III, IV**) and SF/XF-M (**III**) and were analyzed for cell surface antigen expression by flow cytometry (fluorescence-activated cell sorting; FACS; FACSAria®; BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies (MAb) against CD29-Allophycocyanin (APC), CD44-Phycoerythrin (PE), CD90–APC, CD45–APC, CD146-PE (BD Biosciences), CD105–PE (R&D Systems Inc., Minneapolis, MN, USA), CD31– fluorescein isothiocyanate (FITC) (Immunotools GmbH, Friesoythe, Germany), and major histocompatibility class II antigen (HLA-DR)–PE (Immunotools) were used. FACS Analysis was performed on 100,000 cells/sample and the positive expression was defined as the level of fluorescence greater than 99% of the corresponding unstained cell sample.

4.3 Preparation of the biomaterial (IV)

Beta-tricalcium phosphate; β-TCP/Poly; P (L-Lactic acid; LLA/ caprolactone; CL) (ChronOS™) was kindly provided by (Synthes®, Oberdorf, Switzerland); the material is accepted for clinical use as a bone graft substitute. The biomaterial was supplied in sterile strip form with a size of 2.5 cm x 5 cm, two 3 mm thick strips and three 6 mm thick strips were provided. For the experiments, the strips were cut into 1cm x 0.8 cm pieces with scalpels in sterile conditions under the laminar flow hood as shown in Figure 5. Further, the cell-seeded scaffolds were treated with osteogenic medium (OM) containing vitamin D_3 (OM-VD) or DEX (OM-DEX) and control were cultured in human serum medium (HS-M).

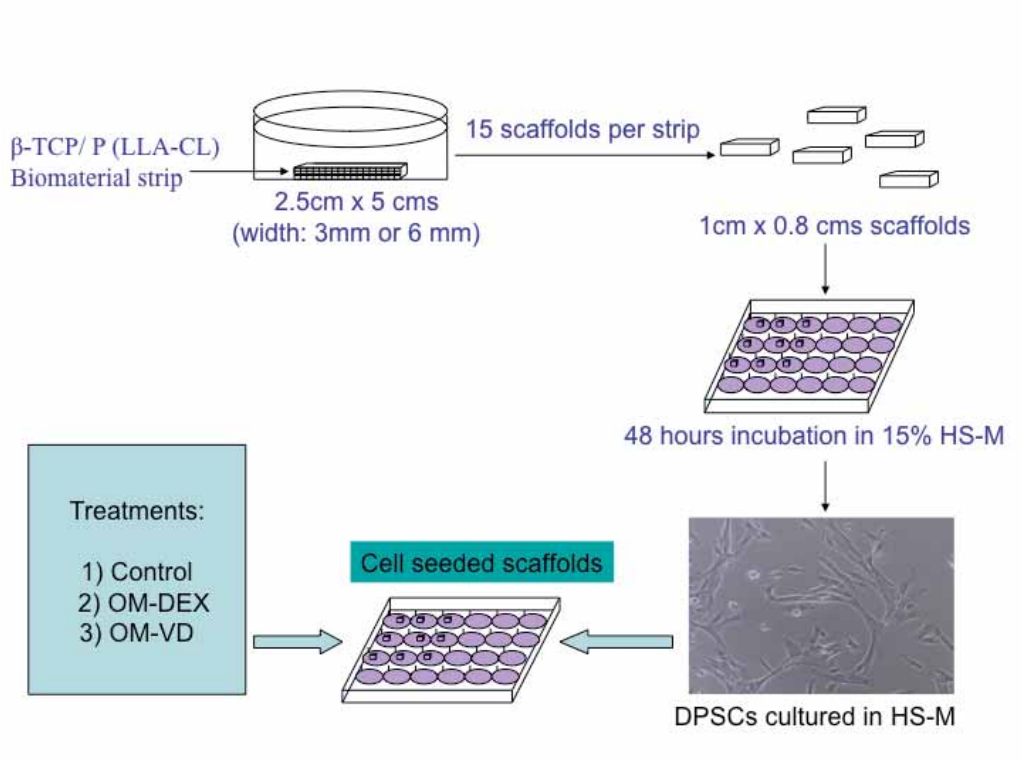

Figure 5: Representative schematic diagram showing the preparation of the biomaterial, cell seeding and treatment conditions (IV).

4.4 CyQUANT® Cell Proliferation Assay (IV)

In Study **IV**, CyQUANT® Cell Proliferation Assay Kit (CyQUANT; Molecular Probes, Invitrogen) was used according to the manufacturer's protocols to assess the cell numbers at 1, 7 and 14 days. Briefly, 500 µl of 0.1% Triton X-100 (Sigma) was pipetted through the cell seeded scaffolds and the lysed cells suspension were freezed until analysis. The CyQUANT® cell proliferation assay is based on the green fluorescence dye, CyQUANT® GR dye, which intensifies when it binds to the nucleic acid of DNA. The fluorescence, which is directly proportional to the number of cells in the sample, was measured at 480/530 nm using a microplate reader (Victor 1420 Multilabel Counter; Wallac, Turku, Finland).

4.5 WST-1 (I, II, III)

PreMix WST-1 Cell Proliferation Assay System was used to assess the cell proliferation activity. This colorimetric assay enables the measurement of the cell viability and proliferation. The assay is based on cleaving the tetrazolium salts by succinate tetrazolium reductase, which exists in the mitochondrial respiratory chain and is active only in viable cells. The enzyme activity leads to the production of formazan dye; the quantity of formazan dye is directly proportional to number of metabolically active cells. The cells were seeded on a 24-well plate at a density of 10000 cells/well **(I, II)** and 5000 cells/well **(IV)**, and the cell proliferation was assessed at 1, 7 and 14 days **(I, II)** and 1, 4, 7, 11 and 14 days **(IV)**. Cell proliferation (WST-1 absorbance) was analyzed according to the manufacturer's protocol. Briefly, WST-1 reagent was added to each well containing fresh medium (50 µl of WST-1/ 500 µl of medium in each well of 24-well plate), incubated for 60 min at 37ºC, the absorbance was measured at 450 nm using a microplate reader (Victor 1420, Finland).

4.6 Quantitative alkaline phosphatase activity (I, II)

Alkaline phosphatase (ALP) activity was analyzed with a commercially available p-nitrophenyl phosphate tablet set (Sigma, St. Louis, Missouri, USA). Briefly, each well was washed twice with PBS and p-nitrophenyl phosphate solution was added (400 µl/ well for 24-well plates). After 10 min of incubation at 37°C, conversion of p-nitrophenyl phosphate into p-nitrophenol by cellular ALP was stopped with the equivalent amount of 3 N NaOH and the absorbance of p-nitrophenol was measured at 450 nm using a microplate reader. Alkaline phosphatase-specific activity is expressed as p-nitrophenol absorbance (OD; 405 nm)/ WST-1 absorbance (OD; 450 nm), which is designed to assess the ALP activity/no. of viable cells.

4.7 Alkaline phosphatase staining (II, IV)

In Study **II and IV**, *in vitro* osteogenic differentiation capacity of the DPSCs, DFSCs at day 14 was determined by using a leukocyte ALP kit according to Sigma procedure 86 (cat. number 86R-1KT). Briefly, cells were fixed with 4% paraformaldehyde solution (PFA) for 2 mins. ALP staining solution was added following the fixation. After 15 mins of incubation in the dark, the ALP staining solution was removed and the wells were washed to remove excess stain. Thereafter, digital images were taken of the ALP stained and unstained cells.

4.8 *In vitro* multilineage differentiation analysis (II, III)

DPSCs (n = 4) were examined for their ability to differentiate toward the adipogenic, osteogenic and chondrogenic lineages. Briefly, for osteogenic and adipogenic differentiation analysis cells were seeded at a density of 5000 cells/well on a 24 well plate. After 24 hours, the differentiation media such as osteogenic medium (OM) and adipogenic medium (AM) comprising of FBS, HS or SF/XF culture conditions was added (Table 1). The chondrogenic differentiation of DPSCs was assessed by micromass cell culture method. Briefly, 100000 cells were seeded on a 24 well plate in a 10 µl volume of FBS-M, HS-M and SF/XF-M, that were left to adhere for 3 hours in an incubator prior to the addition of chondrogenic differentiation medium (CM) as described in (Table 1). For all the analysis the control cultures were maintained in FBS-M, HS-M or SF/XF-M. The SF/XF-M culture wells were pre-coated with CELLstart for osteogenic and adipogenic differentiation while for chondrogenic micro mass aggregate formation; the culture wells were not coated. All cultures were maintained for 21 days for the differentiation analysis.

4.8.1 Alizarin red staining (I, II, III)

In vitro mineralization was analyzed by alizarin red staining. For Alizarin red S staining, cells were fixed with ice-cold 70 % ethanol for 60 min at -20 ºC. Then, cells were washed twice with distilled water and stained with 40 mM Alizarin red S solution (Sigma) for 10 min at room temperature. The pH value of the solution was adjusted to 4.2 with 25% ammonium hydroxide prior to staining. After staining, excess dye was washed with distilled water and digital images of stained mineral deposits were taken.

4.8.2 Alcian blue staining (III)

After 21 days of culture, the chondrogenically induced micro masses were fixed in 4% paraformaldehyde (PFA) for 60 mins. The micro masses were then embedded in paraffin, and sectioned at a thickness of 5μm for histological evaluation. The undifferentiated and differentiated micro masses sections were stained with 0.5% Alcian blue stain and counterstained with Nuclear Fast Red solution (Biocare Medical, Concord, MA, USA). The stained micromass sections were viewed under the microscope to evaluate the proteoglycan content.

4.8.3 Oil O Red staining (II, III)

To detect adipogenic differentiation, the cells were stained with 0.3% Oil Red O-solution to detect the accumulation of extracellular lipid droplets. Briefly, cells were fixed with 4% PFA for one hour. Further the cells were rinsed with distilled water and incubated in 60% isopropanol for 5 mins. Thereafter, the cells were stained with Oil O red solution for 15 mins at room temperature. Following the staining the wells were washed thoroughly to remove the excess stain and microscopic images were taken.

Table I: Lineage specific differentiation induced by media supplements

Medium	Basal Media	Serum	Supplementation
Control (FBS-M, HS-M, SF/XF-M)	DMEM/F-12, SF/XF-M	10% FBS or 15% HS or no serum	None
Osteogenic Medium (OM; FBS-OM, HS-OM, SF/XF-OM)	DMEM/F-12, SF/XF-M	10% FBS or 15% HS or no serum	50µM L- ascorbic acid (Sigma), 10 mM beta glycerophosphate (Sigma), 100nM 1,25 hydroxy Vitamin D_3 (VD; Sigma), 1% a/a, 1% GlutaMAX
Adipogenic Medium (AM; FBS-AM, HS-AM, SF/XF-AM)	DMEM/F-12, SF/XF-M	10% FBS or 15% HS or no serum	33µM biotin (Sigma) 1µM dexamethasone (Sigma), 100 nM insulin (life technologies), 17µM pantothenate (Fluka), 1% GlutaMAX, and 1% a/a. Upon seeding of cells, 250µM of isobutylmethylxanthine (IBMX; Sigma) was added for 72 hours
Chondrogenic Medium (CM)	DMEM/F-12, SF/XF-M	No serum	Insulin Transferrin-Selenium+1 (Sigma), 50µM L- ascorbic acid, 55µM sodium pyruvate (Life Technologies), 23µM L-proline (Sigma), 1% GlutaMAX and 1% a/a. TGF-β1 (Sigma)

4.9 Quantitative real time- polymerase chain reaction (I, II, III, IV)

The total RNA was extracted by using Eurozol (Euroclone S.p.A, Pero, Italy). First-strand cDNA syntheses were performed by a High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Quantitative RT-PCR was conducted using RPLP0 (human acidic ribosomal phosphoprotein) as the house keeping gene and lineage specific primers such as for *osteogenic differentiation*: osteocalcin (OCN), osteopontin (OPN) and RUNX2, *chondrogenic differentiation*; SRY (sex determining region Y)-box 9 (SOX9) and Type X collagen alpha-1 (COL10A1) and *adipogenic differentiation*; fatty acid binding protein4 (aP2) and human peroxisome proliferator-activated receptor gamma (hPPARG) and *stemness markers;* octamer-binding transcription factor (Oct3/4) 3/4, SRY (sex determining region Y)-box (Sox2) and Nanog and *vitamin D regulating enzymes;* VDR (vitamin D receptor), CYP24 (24 hydroxylase) and 25OHD3-1α-hydroxylase (1α-hydroxylase, CYP27B1) (Table II). To exclude signals from contaminating DNA, the forward and reverse sequence of each primer were designed on different exons. The Power

SYBR Green PCR Master Mix (Applied Biosystems) was used for quantitative PCR reactions according to the manufacturer's instructions. The reactions were performed with AbiPrism 7300 Sequence Detection System (Applied Biosystems) at 95 ºC 10 min, and then 45 cycles at 95 ºC /15 s and 60 ºC /60 s. The Ct values were normalized to that of the housekeeping gene RPLP0, as described elsewhere (Pfaffl, 2001).

Table II. Primers sequence for quantitative RT-PCR (I, II, III, IV)

Gene Name	5'-sequence-3'	Product size	Accession number	Study
RPLP0	Forward AATCTCCAGGGGCACCATT Reverse CGTTGGCTCCCACTTTGT	70	NM_001002	I, II, III, IV
OCN	Forward AGCAAAGGTGCAGCCTTTGT Reverse GCGCCTGGGTCTCTTCACT	63	NM_000711	I, II, III, IV
OPN	Forward GCCGACCAAGGAAAACTCACT Reverse GGCACAGGTGATGCCTAGGA	71	J04765	I, II, III, IV
SOX9	Forward AAAGGCAACTCGTACCCAAATTT Reverse TGATTGGCCACAAGTGGGTAA	75	NM_000346	III
COL10A1	Forward CACGCAGAATCCATCTGAGAATAT Reverse GTTCAGCGTAAAACACTCCATGAA	92	NM_000493	I, III
PPARG	Forward CAGTGTGAATTACAGCAAACC Reverse ACAGTGTATCAGTGAAGGAAT	100	NM_015869	III
AP2	Forward GGTGGTGGAATGCGTCATG Reverse CAACGTCCCTTGGCTTATGC	71	NM_001442	III
OCT3/4	Forward GACAGGGGGAGGGGAGGAGCTAGG Reverse CTTCCCTCCAACCAGTTGCCCAAAC	118	NM_002701	III
SOX2	Forward GGGAAATGGGAGGGGTGCAAAAGAGG Reverse TTGCGTGAGTGTGGATTGGTG	125	NM_003106	III
NANOG	Forward AAAGAATCTTCACCTATGCC Reverse GAAGGAAGAGGAGAGACAGT	111	NM_024865	III
ALP	Forward CCCCGTGGCAACTCTATCT Reverse GATGGCAGTGAAGGGCTTCTT	73	NM_000478.4	IV
CYP24	Forward GCCCAGCCGGGAACTC Reverse AAATACCACCATCTGAGGCGTATT	61	NM_00782	II
CYP27B1	Forward TTGGCAAGCGCAGCTGTAT Reverse TGTGTTAGGATCTGGGCCAAA	75	NM_000785	II
VDR	Forward CCTTCACCATGGACGACATG Reverse CGGCTTTGGTCACGTCACT	77	NM_00376	II
RUNX2	Forward CCCGTGGCCTTCAAGGT Reverse CGTTACCCGCCATGACAGTA	76	NM_004348	I

4.10 1, 25 hydroxy vitamin D Enzyme immunoassay (II)

The 1, 25-Dihydroxy Vitamin D_3 EIA kit (Immunodiagnostic Systems Ltd, Boldon, UK) was used to measure the conversion of $25OHD_3$ into $1\alpha,25(OH)_2D_3$ by 1α-hydroxylase in hDPCs and DFSCs in Study **II**. The cells were cultured in BM +

$25OHD_3$ (500 nM) and BM + $25OHD_3$ (500 nM) + inhibitor (ketoconazole; 10µM) for 24 hours. The control samples were maintained in BM without the addition of vitamin D_3 metabolites and inhibitor. 10,000 cells/well were seeded on a 6 well plate and after overnight incubation at 37ºC in a 5% CO2 humidified atmosphere different metabolites were added. After 24 hours the culture media for all the samples were taken for immunoextraction following quantitation by enzyme immunoassay as per manufacturer's protocol. Samples were obtained from three different patient samples for both the tissues, which were tested independently. Briefly, 100µl of the delipidated samples and controls were added to the appropriately labeled immunocapsules in duplicates per sample. Primary antibody was added to the immunoextracts and incubated overnight at +4ºC. Next day, secondary antibody was added which was followed by the addition of enzyme conjugate and tetramethylbenzidine (TMB) substrate. An acidic stop solution was added to terminate the reaction, which resulted in the color change from blue to yellow. The intensity of the yellow color is inversely proportional to the concentration of $1\alpha,25(OH)_2D_3$. The absorbance was measured within 30 min of the addition of the stop solution using a plate reader (Victor 1420) at a wavelength of 450nm. The results were calculated by measuring the absorbance of the calibrators provided with the kit and creating a calibration curve by plotting the percent bind on the y axis and the amount of $1\alpha,25(OH)_2D_3$ on the x-axis. The percent bind values for the samples were calculated and then interpolated amount of $1\alpha,25(OH)_2D_3$ using the calibration curve.

4.11 Immunostaining (III, IV)

For immunocytochemistry in Study **III**, 2500 cells/ well were plated on 48 well plates. After 3 days of culturing, cells were fixed with 4% paraformaldehyde (Fluka, Italy) containing 0,2% of TritonX-100. After fixation, cells were stained with stemness markers. Briefly, unspecific staining was blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 45 min at room temperature. The cells were washed 2-3 times with PBS. Thereafter, the primary antibodies; goat anti-octamer-binding transcription factor (Oct) 3/4, mouse anti-SRY (sex determining

region Y) box-2 (Sox2) and goat anti–Nanog (all: R&D Systems) as well as mouse anti-stage specific embryonic antigen (SSEA)-4 (Santa Cruz) in 1% BSA-PBS solution were incubated overnight with cells at +4°C. Next day, the cells were washed three times with PBS and were incubated in secondary antibodies, Alexa Fluor 488 and 568 conjugated to anti-goat and anti-mouse (Molecular Probes, Invitrogen) in 1% BSA-PBS for 1 h at room temperature. Finally, cells were washed three times with PBS, twice with water and mounted with Vectashield containing 4', 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, England) to identify nuclei. Cell samples were analyzed by using an Olympus IX51 phase-contrast microscope equipped with fluorescence unit and an Olympus DP30BW camera (Olympus).

In Study **IV**, after 14 days of inducing osteogenic differentiation in the cell seeded scaffolds using OM-DEX and OM-VD, they were immunostained with primary antibody anti-human osteocalcin (AbD serotec, immunodiagnostics Oy, Finland). The cell-seeded scaffolds cultured in HS-M were used as controls. Briefly, the cell seeded scaffolds were fixed with 4% PFA for 10 mins and then blocked against non-specific antigen binding with 10% normal donkey serum (NDS), 0.1% Triton X-100, and 1% Bovine serum albumin (BSA) in DPBS. After 45 mins of blocking, the cell seeded scaffolds were washed with 1% NDS, 0.1% Triton X-100, and 1% BSA in DPBS (washing solution). The primary antibody anti-human osteocalcin was diluted to 1:50 in the washing solution. The cells were incubated overnight at +4 °C with the primary antibody. The next day, the cells were washed with 1% BSA in DPBS and incubated for 1 hr at RT. Thereafter, cells were incubated for 1 hr in Alexa Fluor-488 (1:1000, Invitrogen) conjugated anti-mouse secondary antibody, diluted in 1% BSA in DPBS. Then, cells were sequentially washed with PBS and phosphate buffer, and mounted with Vectashield (4′, 6-diamidino-2-phenylindole; DAPI, Vector Laboratories, and Peterborough, UK). For negative controls primary antibody was omitted. Stained DPSCs within the scaffolds were imaged using a microscope equipped with a fluorescence unit and camera.

4.12 Statistical analysis (I, II, III, IV)

The statistical analyses of the results were performed with GraphPad Prism 5.01. The data is presented as mean ± standard deviation (SD) for all quantitative assays and experiments were carried out in triplicate for cells derived from three donor samples. All statistical analyses were performed at the significance level $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons.

5. Results

5.1 Cell proliferation and morphology

Dental stem cells derived from dental pulp (**II, III**), dental follicle (**II**) and dental periodontal ligament (**I**) were assessed for proliferation and morphology under various treatment conditions.

5.1.1 Cells treated with BMP-2 and -6 (I)

We investigated the effect of BMP-2+AA, BMP-6+AA, OS, OS+BMP-2, or OS+BMP-6 on the osteogenic differentiation of PDLCs. The cells exposed to BMP-2+AA or BMP-6+AA appeared as more fibroblastic and spindle-shaped. In contrast, cells cultured with OS, OS+BMP-2, or OS+BMP-6 were relatively polygonal in shape, and they started to mineralize *in vitro* as observed by phase contrast microscopy by day 14 (Figure 6). Cells cultured with BMP-2+AA or BMP-6+AA showed relatively greater cell number than control (BM) on day 7, though ($P < 0.001$) decrease in cell number was observed on day 21. In contrast, cells cultured with OS, OS+BMP-2, and OS+BMP-6 showed ($P < 0.001$) lower cell numbers on day 7.

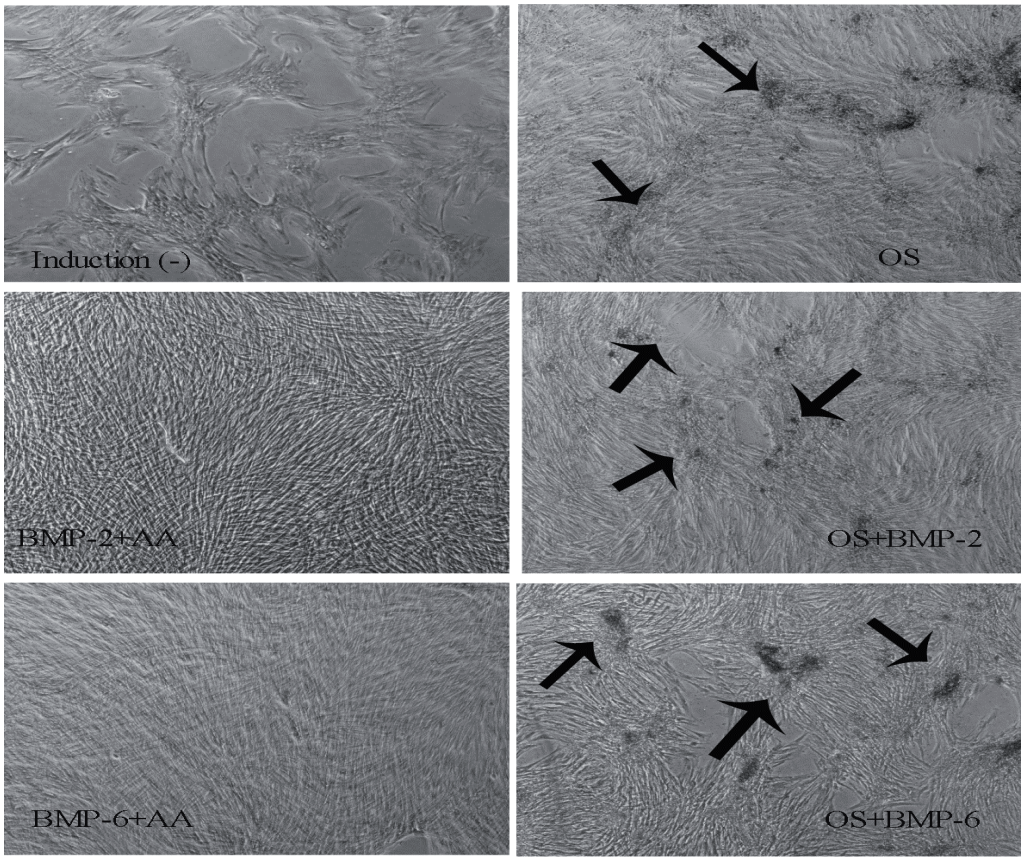

Figure 6. Representative phase contrast photographs of the cells cultured with BMP-2, BMP-6, and osteogenic supplements (day 14) Phase contrast microscopy of human PDLCs were exposed to (1) induction (-) without additives as control; (2) osteogenic supplements (OS: dexamethasone, ascorbic acid, and β-glycerophosphate); (3) BMP-2 (10ng/ ml) + ascorbic acid (AA); (4) OS + BMP-2 (10ng/ ml); (5) BMP-6 (0.1ng/ ml) + AA; or (6) OS + BMP-6 (0.1ng/ ml), and cultured for 14 days. Morphologically, cells cultured with BMP-2 + AA or BMP-6 + AA appeared as more fibroblastic and spindle cells in shape. Cells cultured with OS, OS + BMP-2, or OS +BMP-6 were relatively polygonal in shape, and they started to mineralize *in vitro* (Black arrow). Original magnification (x 40)

5.1.2 Cells treated with vitamin D_3 (II)

Time course effects of different concentrations of $1\alpha,25(OH)_2D_3$ (10 nM, 100 nM) and $25OHD_3$ (100 nM, 500 nM) at days 1, 7 and 14 on proliferation of hDPCs and

DFSCs were analyzed. The addition of vitamin D_3 metabolites, to hDPCs and DFSCs decreased cell proliferation when compared to the cells treated without the metabolites. While cell proliferation was significantly inhibited by $1\alpha,25(OH)_2D_3$ (100 nM) in DFSCs by day 14.

5.1.3 Cells cultured in FBS, SF/XF and HS (III, IV)

From day 1 to day 9, DPSCs cultured in HS-M proliferated in colonies and spherical clusters. Morphologically, cells mostly appeared spindle shaped and comprised of a homogenous cell population when viewed under the phase contrast microscope. After first passage, the cells did not proliferate in clusters; rather, cells were more spread out and proliferated uniformly. Using phase contrast microscopy, the morphology of human DPSCs expanded in FBS-M, HS-M or SF/XF-M was compared. Cells cultured in FBS appeared broader and flattened in shape, whereas cells cultured in HS-M were more fibroblastic and appeared more homogenous. Moreover, cells expanded in SF/XF-M exhibited a more flattened fibroblastic like morphology. The effects of FBS-M (10%), HS-M (15%) and SF/XF-M on DPSCs growth were analyzed following days 1, 4, 7 and 14. The cells cultured in SF/XF-M proliferated slowly in comparison to the cells cultured in FBS and HS medium that was observed from day 4. Statistical analysis revealed that cells cultured in FBS-M and HS-M proliferated significantly faster than cells cultured in SF/XF-M at day 7 to day 14 ($p < 0.001$). Moreover, no significant differences were observed between cells cultured in FBS-M and HS-M. Further, HS concentration gradient effect from 5% to 20% was evaluated on DPSCs isolated in 20%. Our results suggested that DPSCs cultured in 5% HS ($p < 0.001$) significantly lowered the proliferation in comparison to 10%, 15% and 20% HS-M. Furthermore, we decided to study the effect of HS as an additive in the concentration of 1% or 5% in SF/XF-M. Our study revealed that SF-XF/M+1%HS ($p < 0.001$) significantly increased cell proliferation in comparison to SF/XF-M alone by day 14.

5.1.4 DPSCs seeded on biomaterials (IV)

The cell-seeded scaffolds cultured in HS-M, OM-DEX and OM-VD were assessed for increase in cell numbers from day 1, day 7 to day 14. There was significant increase in cell numbers at day 7 ($p < 0.01$) and day 14 ($p < 0.001$) time points, when cells cultured in OM-VD were compared to the control HS-M at day 7 and 14. Though, there was no significant increase in cell numbers when cells were cultured in OM-DEX.

5.2 Cell surface markers expression (III, IV)

DPSCs expanded in FBS-M, HS-M and SF/XF-M were analyzed using flow cytometry against mesenchymal markers such as CD29, CD44, CD90 and CD105; hematopoietic and angiogeneic markers such as CD31, CD45, CD146 and for HLA-DR. The results showed that DPSCs cultured in different media showed positive expression for the mesenchymal marker (>50%) (Fig. 7 a, b), low expression for the CD146 marker and lacked the expression for CD31, CD45 hematopoietic markers. In addition, DPSCs cultured in FBS-M, HS-M and SF/XF-M lacked the expression of HLA-DR (Figure 7 c, d). Also, there were no statistical significant differences observed between the expression profile of cells cultured in FBS-M, HS-M and SF/XF-M as shown in (Fig. 7 b, d).

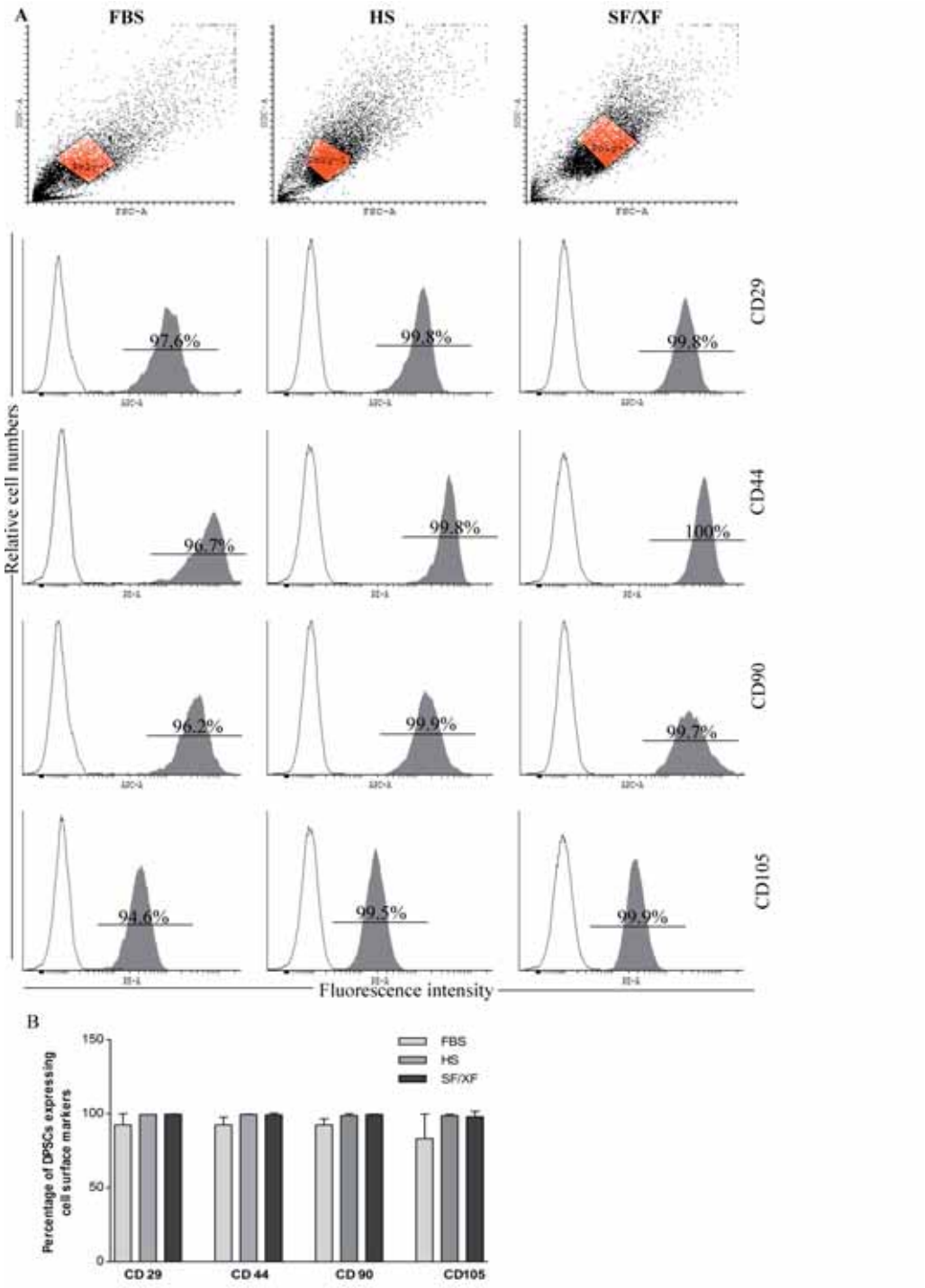

FIGURE 7 A, B: Flow cytometeric analysis of mesenchymal surface marker expression of undifferentiated DPSCs cultured in FBS, HS and SF/XF media (**III, IV**).

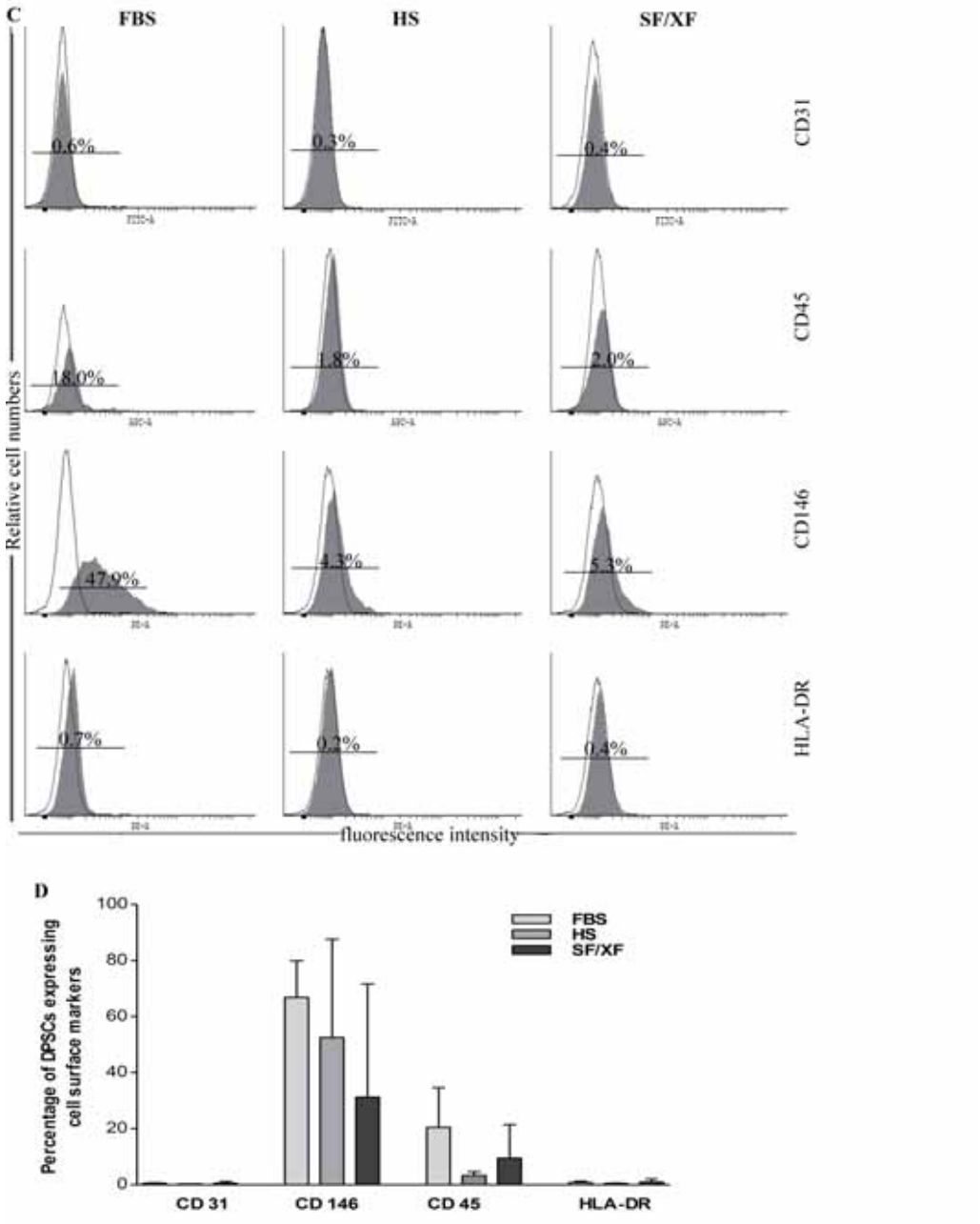

Figure 7 C, D: Flow cytometeric analysis of hematopoietic surface marker expression of undifferentiated DPSCs cultured in FBS, HS and SF/XF media (**III, IV**).

5.3 Immunostaining

The expression of stemness markers Oct3/4, Sox2, the stage-specific embryonic antigen 4 (SSEA-4) and Nanog were evaluated by immunostaining in Study I**II.** Merging the antibody stained DPSCs with the nuclear stained images saw the expression of Oct3/4 and Sox2 in 20% HS cultured cells. In addition, Nanog, Oct3/4 and SSEA-4 were expressed in DPSCs cultured in FBS 15%, HS 20% and SF/XF media (**supplemental data III**) (Figure 8).

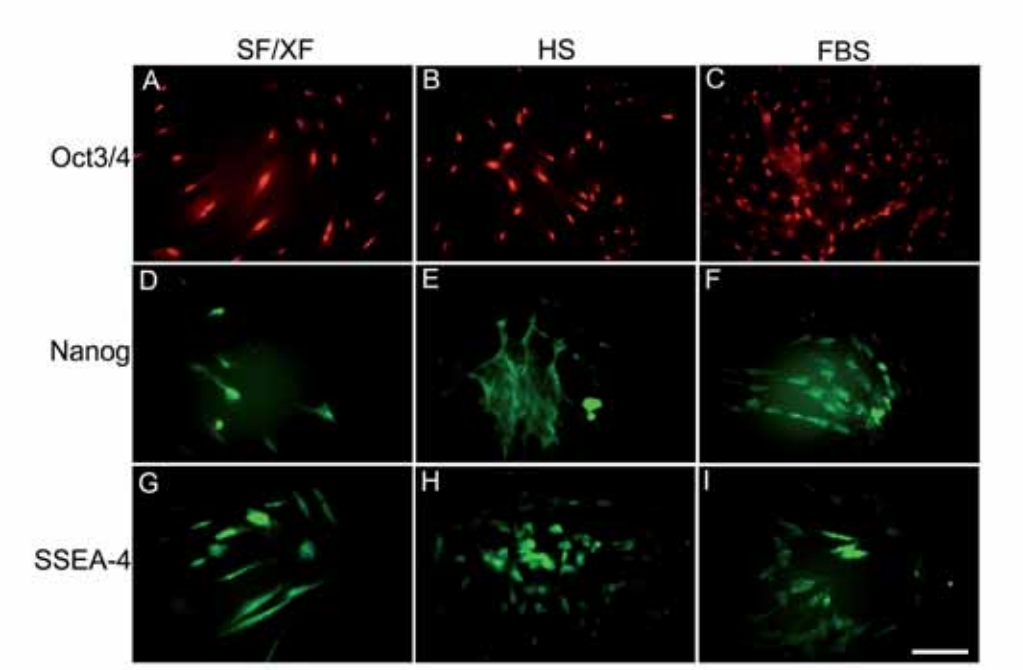

Figure 8: Oct3/4, Nanog and SSEA-4 are expressed in SF/XF, HS and FBS culture conditions. Stemness markers were analyzed by immunocytochemistry. 2500 DPSCs were cultured in SF/XF, 20% HS and 10% FBS medium on 48 well plates for 4 days, fixed, stained and analyzed as described in materials and methods. Thereafter, cells were stained with *primary antibodies*: goat anti-octamer-binding transcription factor (Oct) 3/ 4 (a, b, c; R&D Systems), goat anti–Nanog (d, e, f; R&D Systems) and mouse anti-stage specific embryonic antigen (SSEA)-4 (g, h, i; Santa Cruz), *secondary antibodies:* Alexa Fluor 488 and 568 conjugated to anti-goat and anti-mouse (Molecular Probes, Invitrogen). Scale bar =100 µm.

5.4 Multipotent differentiation potential of dental pulp stem cells (II, III)

5.4.1 Osteogenic differentiation

The DPSCs and DFSCs were induced to differentiate towards osteogenic lineage with the addition of vitamin D_3 metabolites + AA + βGP in Study **II** as shown in Figure 9 A and B. From Study **II**, the most effective concentration of vitamin D_3 1,25: 100nM was chosen, which was used in subsequent studies for inducing osteogenic differentiation. The alizarin red staining results showed that DPSCs cultured in FBS-OM and HS-OM formed mineralized matrix under the influence of vitamin D_3 containing osteogenic medium (OM-VD), however, the calcified matrix staining was more pronounced in cells cultured in HS-OM. On the other hand, cells cultured in SF/XF-OM did not mineralize in Study **III**, Figure 9 C.

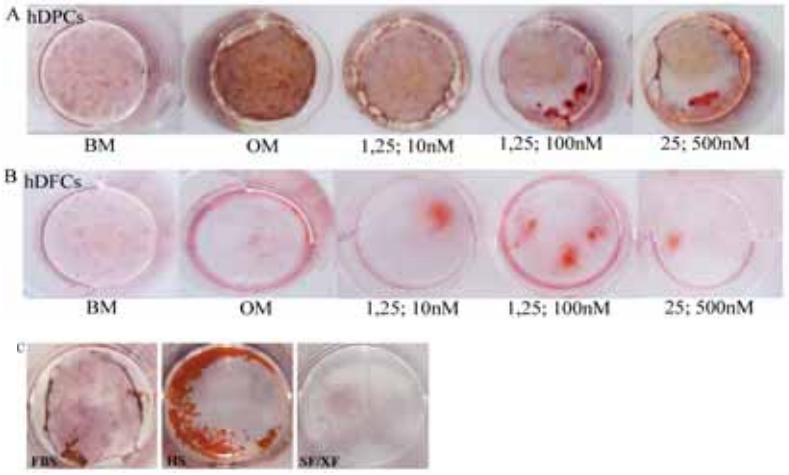

Figure 9: Osteogenic differentiation of A) DPSCs and B) DFSCs by vitamin D_3 metabolites + AA + βGP. C) DPSCs cultured in different media induced by OM-VD.

5.4.2 Adipogenic differentiation (III)

Differentiation into adipocytes was estimated after 3 weeks of culturing the cells in AM by Oil Red O staining. DPSCs cultured in HS-AM had stronger capacity to differentiate into adipocytes than cells cultured in FBS-AM and SF/XF-AM. These

results were assessed based on the higher number of accumulated lipid droplets. The potential of DPSCs to differentiate into adipocytes was also observed when the cells were cultivated in HS-M without the addition of adipogenic differentiation supplements, however, formed very few lipid droplets (Figure 10).

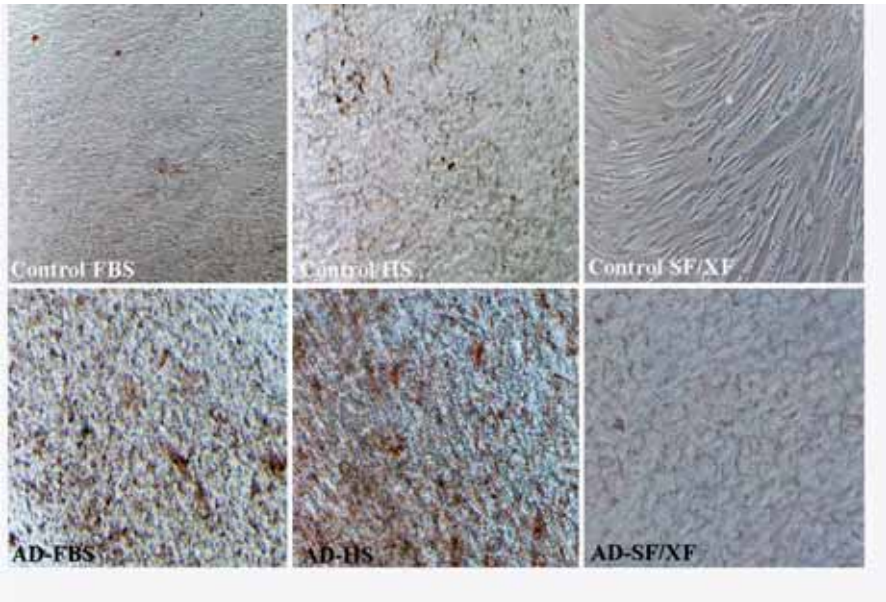

Figure 10: Adipogenic differentiation of DPSCs cultured in FBS, HS and SF/XF culture media and then induced by adipogenic medium for three weeks.

5.4.3 Chondrogenic differentiation

After 21 days, chondrogenic differentiation was estimated after staining the micromasses cultured in CM, with alcian blue stain, which stains the proteoglycan rich extracellular matrix. As shown in Figure 11, cells cultured in FBS and HS media differentiated more into chondrocytes-like cells as assessed by the stronger staining than in SF/XF-CM cultured cells.

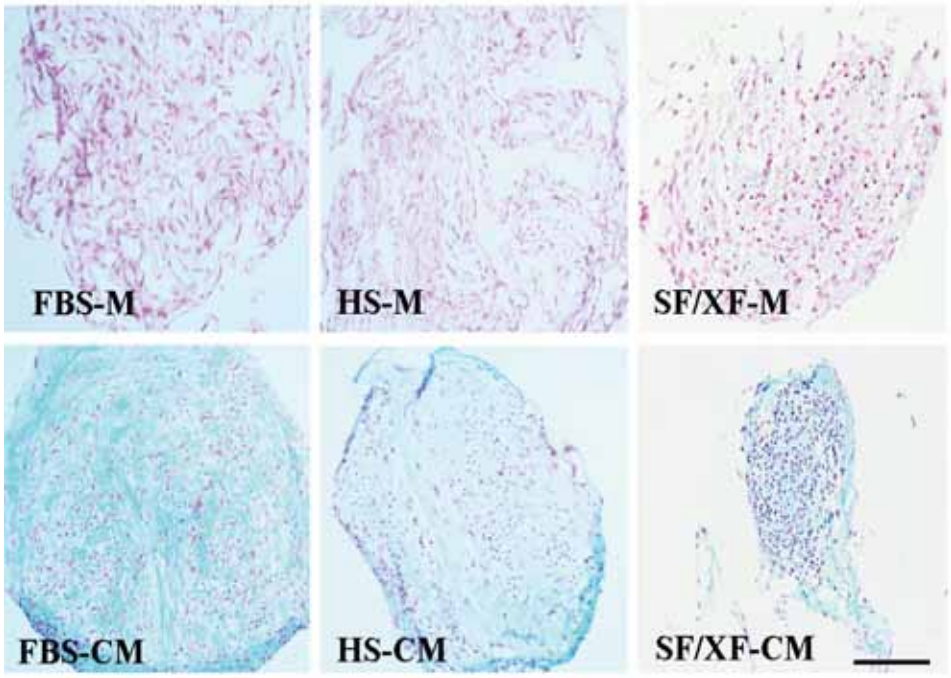

Figure 11: Chondrogenic differentiation of DPSCs cultured in FBS, HS and SF/XF media and differentiated in chondrogenic medium for 21 days.

5.5 Early osteogenic differentiation potential of dental stem cells (I, II, IV)

ALP is an early marker that indicates differentiation of cells towards osteogenic lineages (Stucki et al., 2001). In Study **I**, to investigate the effects of BMP-2 or BMP-6 on ALP activity of PDLCs, first dose-response experiments were conducted based on the results, we decided to use 10ng/ml concentration of BMP-2 and 0.1ng/ml concentration of BMP-6 for the subsequent experiments. Time course experiments of cell culture showed that when BMP-2 or BMP-6 was combined with osteogenic supplements (OS) containing DEX, ALP activity of PDLCs continued to increase from day 7 to 21. Although the greatest ALP activity was observed in cells cultured with OS+BMP-2, there were no significant differences in ALP activity among cells cultured with OS, OS+BMP-2, and OS+BMP-6 after 21 days.

Furthermore, the effects of vitamin D_3 metabolites with or without OS on osteogenic differentiation of DPSCs and DFSCs were analyzed by measuring their ALP

activities in Study **II**. Time course experiments showed significant increases in ALP activity of DPSCs on day 7 after treatment with OS + 1α,25$(OH)_2D_3$ (10 nM), OS + 1α,25$(OH)_2D_3$ (100 nM) and OS + 25OHD_3 (500 nM) in comparison to cells treated without OS and the untreated control. Following day 14, ALP activity was significantly increased in cells treated with OS + 1α,25$(OH)_2D_3$ (100 nM), OS + 25OHD_3 (500 nM) and OS + DEX (10 nM) in DPSCs. In addition, DFSCs treated with OS + 1α,25$(OH)_2D_3$ (100 nM), OS + 25OHD_3 (500nM) at day 7 significantly increased ALP activity. Following the 14 days time course, DFSCs expressed significant increase in ALP activity in cells treated with OS + 1α,25$(OH)_2D_3$ (10 nM), OS + 1α,25$(OH)_2D_3$ (100 nM); though cells treated without OS and OS + 25OHD_3 (100 nM) and OS + DEX (10nM) did not significantly increase the ALP activity of DFSCs. Human DPSCs and DFSCs treated in combination with OS and vitamin D_3 metabolites showed significant ALP activity.

In Study **IV**, the ALP activity of cells-seeded on biomaterial and cultured in HS-M, OM-DEX and OM-VD was assessed. Interestingly, ALP activity expressed by cells cultured in OM-VD was stronger than the cells cultured in OM-DEX. The control cells were cultured in HS-M and no ALP staining was detected.

5.6 Gene expression profile (I, II, III, IV)

The osteoblast genes expression pattern in DPSCs, DFSCs and PDLCs was analyzed in Study **I, II, III, and IV,** which was tested under the influence of BMP or vitamin D_3. The expressions of osteogenic, adipogenic, chondrogenic and stemness marker expression genes were tested in Study **III**. Vitamin D_3 regulating genes especially CYP24 expression and VDR were regulated by higher concentrations of 1α,25$(OH)_2D_3$ (100 nM) and 25OHD_3 (500 nM) in DPSCs and DFSCs as shown in Figure 12**.**

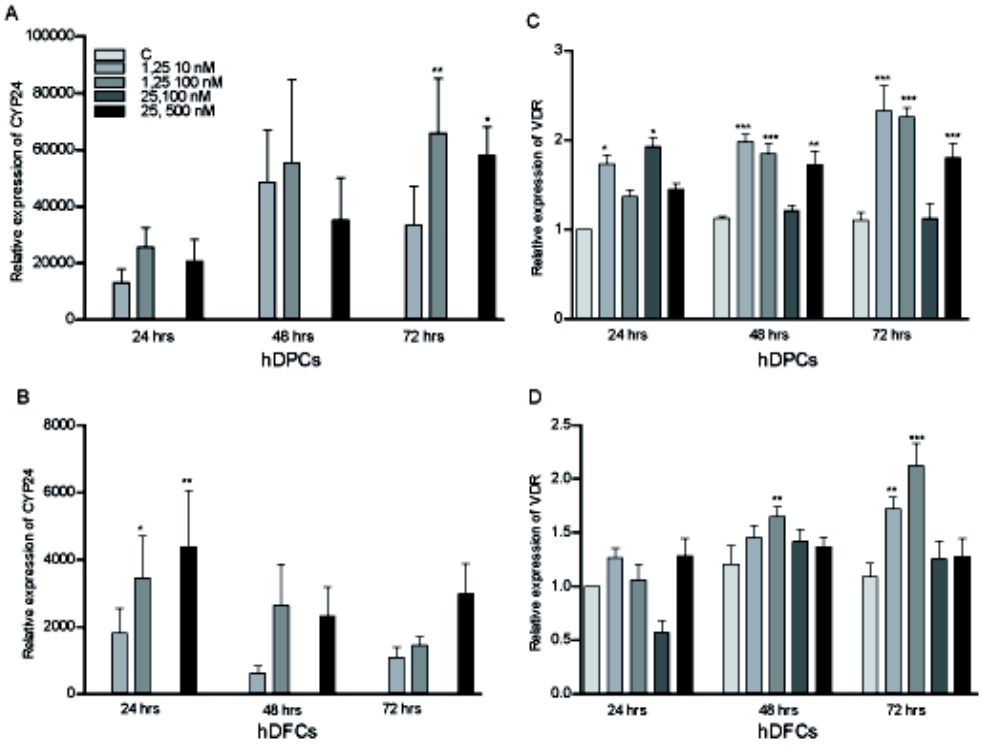

Figure 12: Relative mRNA expression of CYP24 in hDPCs and hDFCs (A and B) and VDR in hDPCs and hDFCs (C and D), cultured in Basal medium containing 1α,25$(OH)_2D_3$ (1,25; 10 nM, 100 nM) and 25OHD_3 (25; 100 nM, 500 nM) at 24, 48 and 72 hour time points. Results are reported as change in gene expression relative to untreated control at 24 hours time point and normalized to housekeeping gene hRPLP0. Columns represent the mean ± SEM of three separate experiments. The difference in relative expression is considered statistically significant when *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

6. Discussion

In this thesis we isolated stem cells from extracted impacted third molar teeth for osteogenic differentiation studies. The dental stem cells were obtained from dental pulp, dental follicle and dental periodontal ligament tissues found within and around the teeth. We have studied the effect of growth factors and hormones for *in vitro* mineralization ability of DPSCs, DFSCs and PDLCs. Following the growth factor and hormones studies; DPSCs were assessed to be most multipotent and therefore, were used to study the xenofree culture media such as serumfree/xenofree medium and human serum medium. Also, osteogenic differentiation capacity of the DPSCs cultured in human serum within a medical grade biomaterial was tested (Figure 13).

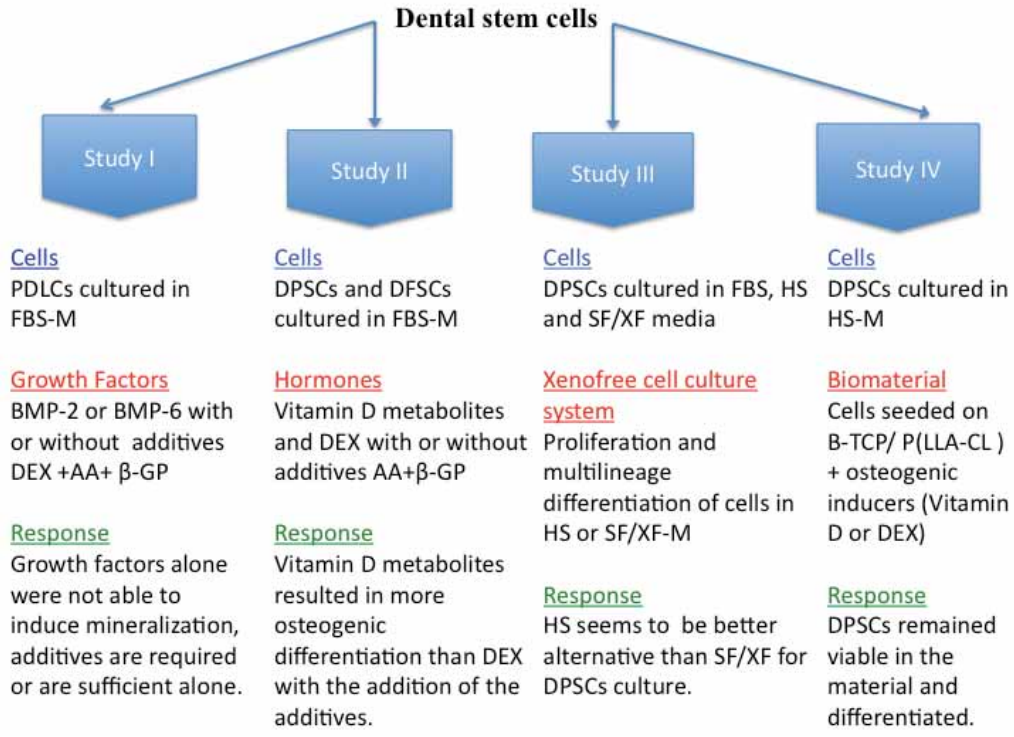

Figure 13: Representative flow chart summarizes the four studies conducted for this thesis. Periodontal ligament cells (PDLCs), Dental pulp stem cells (DPSCs), Dental follicle stem cells (DFSCs), fetal bovine serum (FBS), human serum (HS), SF/XF (serumfree/xenofree), Dexamethasone (DEX), ascorbic acid (AA), β-glycerophosphate (β-TCP).

6.1 Optimized cell culture and isolation conditions

The DSCs were enzymatically digested and isolated in FBS-M in Study **I, II, and III**. There are several promising investigations describing the role of human DPSCs for mineralized tissue regeneration, advancing their therapeutic relevance as a valuable stem cell source (Chadipiralla et al., 2010; d'Aquino, De Rosa, Laino, et al., 2009; Mori et al., 2010). Most commonly, DPSCs are cultured in FBS, which poses risk of transferring infections and induction of immune reactions upon transplantation (Dimarakis and Levicar, 2006; Spees et al., 2004). In order to facilitate the translation of DPSCs from basic biology to clinical application, the development of appropriate cell culture protocols is a relevant and critical factor. To address this clinical concern, in Study **III,** DPSCs were enzymatically isolated from dental pulp tissue of healthy individuals and suspended in PBS. The isolated dental pulp cells suspension in PBS was used to test the effect of different media on cell culture. We found that the cells directly isolated on carboxyl, CELL start and amine coated culture wells in SF/XF medium, did not proliferate. There are reports showing the effects of serum free or low serum containing media on DPSCs cultures (Hirata et al., 2010; Karbanova et al., 2010). Nevertheless, DPSCs cultured in SF/XF medium, has not been reported, so far. Additionally, cells directly isolated in 10% or 15% HS-M did not proliferate; therefore, cells were isolated in 20% HS-M. However, after first passage, 15% HS-M supported DPSCs expansion. Since, there is lack of information on the effect of HS in cultivation of DPSCs; Study **III** describes the effects of HS on DPSCs. In addition, cells isolated in 20% HS-M were able to proliferate in SF/XF-M, thus, we were able to maintain xenofree conditions for cell culture. DPSCs directly isolated in 10% FBS-M proliferated. On the other hand, AD-MSCs derived in our laboratory have been reported to proliferate better in the same SF/XF-M (Lindroos et al., 2009), this suggests variable response to SF/XF-M between different sources of MSCs. With respect to the immuno pathogenic risks posed due to addition of FBS in cell culture, our studies suggest that HS could be considered as a safer alternative for DPSCs culture. However, other SF/XF media alternative composition remains to be studied. Thus, these findings propose that HS was equally effective as FBS in supporting DPSCs proliferation, similar response has been successfully reported in other studies with

BM-MSCs and AD-MSCs (Aldahmash et al., 2011; Komoda et al., 2010; Yilmaz et al., 2008).

6.2 Cell surface markers expression

DPSCs expressed CD29, CD44, CD90 and CD105 mesenchymal markers which are involved in MSCs migration, cell-cell matrix interaction and cell adhesion (Goodison et al., 1999; Lindroos et al., 2009) and moderately expressed CD45 but lacked expression of CD31 hematopoietic markers and HLA-DR (Study **III, IV**). Strikingly, we did not observe high variation in the expression of mesenchymal markers in DPSCs cultured in FBS, HS and SF/XF media conditions. This is especially important, since slower proliferation and limited multilineage differentiation ability of cells cultured in SF/XF-M was observed in Study **III**. Additionally, expression of stemness markers such as Oct3/4 and Sox2 were expressed in 20% HS isolated cells. The functional importance of SOX2 and NANOG genes in altering the progenitor status has also been clearly demonstrated (Hanna et al., 2010; Ratajczak et al., 2008; Takahashi and Yamanaka, 2006; Yu et al., 2007; Zuba-Surma et al., 2009). NANOG has been reported to be a key gene for maintaining pluripotency, as shown by the capacity for multilineage differentiation and perpetual self-renewal of cells expressing this gene. Moreover, SSEA-4 expression was also analyzed, which is an embryonic glycolipid antigen commonly used as a marker for undifferentiated pluripotent human embryonic stem cells and cleavage to blastocyst stage embryos, that also identifies the adult mesenchymal stem cell population. Oct3/4, Nanog and SSEA-4 were expressed in cells cultured in FBS 20%, HS 15% and SF/XF medium.

6.3 Multipotentiality of dental pulp stem cells

In Study **II**, DPSCs cultured in FBS medium were tested for their ability to differentiate into three lineages. More extensively and comparatively, in Study **III**,

cells were cultured in FBS, HS or SF/XF differentiation media and assessed for osteogenic, adipogenic and chondrogenic differentiation potential *in vitro*. There are several studies reporting the mineralization potential of DPSCs induced by DEX osteogenic medium supplemented with FBS (Alge et al., 2010; Lindroos et al., 2008; Pierdomenico et al., 2005; Zhang et al., 2006), as also shown in our previously published data (Khanna-Jain et al., 2010). However, in our studies **II, III and IV**, DPSCs were induced to differentiate osteogenically in FBS or HS medium containing $1\alpha,25(OH)_2D_3$ instead of DEX as an osteogenic inducer. We have shown that mineralized tissue formation was induced by HS-OM supplemented with $1\alpha,25(OH)_2D_3$, which differentiated DPSCs towards mineral nodule formation within β-TCP/P (LLA-CL) biomaterial in Study **IV.** This was evaluated by ALP staining as alizarin red stains the β-TCP particles within the biomaterial scaffold used. However, in Study **I, II, III**, to ensure the osteogenic differentiation of DPSCs, DFSCs and PDLCs mineralized matrix was stained with alizarin red, a specific stain to qualitatively detect calcification *in vitro* (Lazcano et al., 1993). Moreover, SF/XF medium did not support the osteogenic differentiation of DPSCs (Study **III**), although AD-MSCs proliferated and differentiated in SF/XF osteogenic medium as reported (Lindroos et al., 2009). The reason for this could be the absence of unknown growth factors in the SF/XF medium, essential for DPSCs differentiation.

Furthermore, chondrogenic differentiation of DPSCs cultured in FBS (**Study II supplementary data, III**) was exhibited, as shown in previous studies (Alge et al., 2010; Tomic et al., 2011). We have reported for the first time in Study **III** that cells cultured in HS and SF/XF also have the ability to form chondrocyte-like cells.

In Study **II supplementary data**, cells cultured in FBS medium differentiated into adipocytes. Moreover, it is widely known that DPSCs differentiate into adipocytes in FBS adipogenic medium (Gronthos et al., 2000; Zhang et al., 2006), but to our knowledge this is the first time that adipogenic capacity of DPSCs has been shown in HS and SF/XF supplemented medium. Research in Study **III**, under the influence of HS and SF/XF adipogenic medium resulted in lipid accumulation, but adipogenic differentiation was more pronounced in cells cultured in FBS or HS as observed by oil O red staining, which revealed intracellular fat droplets (Hopkins et al., 2010).

6.4 Effect of BMP-2 and BMP-6 on osteogenic differentiation

BMPs are known to be powerful osteogenic inducers and are involved in tooth morphogenesis (Aberg et al., 1997; Xiao et al., 2007). BMPs have failed to induce osteogenic differentiation in rat PDL cells (Rajshankar et al., 1998). In contrast, an *in vivo* study reported that BMP-6 increased bone and cementum formation in a rat model (Huang et al., 2005). Our results in Study **I**, however, demonstrated that addition of BMP-6 to OS or BMP-6 alone did not enhance osteogenic differentiation of PDLCs. The reason for this discrepancy could be due to difference in the responsiveness to BMP-6 between species, which needs to be further elucidated. Considering the inconsistent response to BMPs, Study **I** highlights the potential merits of OS in osteogenic differentiation of PDLCs. In fact, there are many reports showing the osteogenic potential of PDLCs under the influence of OS alone (Hayami et al., 2007; Kuru et al., 1999; Nohutcu et al., 1997). The synergistic effect of the combined use of OS and BMP-2 in promoting osteogenic differentiation has been reported in human MSCs (In Sook et al., 2008; Jager et al., 2008). Our study revealed that cells cultured with OS+BMP-6 as well as OS+BMP-2 showed relatively greater ALP activity than cells cultured with OS alone at 21 days time point, though amongst them there were no significant differences in the *in vitro* mineralization ability (**Study I**).

6.5 Effect of vitamin D_3 on osteogenic differentiation

So far studies towards the direct effects of vitamin D_3 on osteogenic differentiation of isolated human DPSCs and DFSCs have been limited. To confirm osteogenic capacity of the DPSCs and DFSCs, ALP expression was studied in Study **II**. ALP is a membrane bound enzyme and is an early marker of osteogenic differentiation (Wennberg et al., 2000). It was reported that the addition of $1\alpha,25(OH)_2D_3$ metabolite to OS + DEX significantly increased ALP activity of DPSCs (Tonomura et al., 2007). Here, we have shown that $1\alpha,25(OH)_2D_3$ (10 nM, 100 nM)+ OS and DEX +OS increased the ALP activity in DPSCs. Additionally, for the first time we have shown that $25OHD_3$ (500 nM) also increased the ALP activity in DPSCs and

DFSCs. There are several studies reporting the osteogenic potential of DPSCs by showing increased ALP activity (Perry et al., 2008a; Tonomura et al., 2007). Previous reports regarding DFSCs, suggest that, DEX + OS stimulated ALP activity and mineralization (Jin et al., 2008; Morsczeck, Moehl, et al., 2005). However, the effect of the vitamin D_3 metabolites on ALP activity and mineralization of DFSCs was reported for the first time in Study **II**. Here we report that, DFSCs formed mineralized matrix when treated with vitamin D_3 metabolites in the presence of OS. Interestingly, $1\alpha,25(OH)_2D_3$ had more pronounced effect on mineralization and ALP activity than DEX (10 nM) in combination with OS in DFSCs (**Study II**). These results highlight that vitamin D_3 could be used as an alternative to DEX for *in vitro* mineralization considering the potential catabolic effects of DEX when used *in vivo* (Pierotti et al., 2008).

6.6 The future of dental stem cell for therapies

Various dental stem cells, such as DPSCs, SHED, PDLCs, and DFSCs have been examined *in vitro* and *in vivo* for their potential (Huang et al., 2009). Different studies *in vitro* and *in vivo* have provided evidence that DPSCs have mesenchymal cell characteristics based on their ability to differentiate into cartilage, bone, adipocytes, muscle tissues and neural cells (De Rosa et al., 2011; Laino et al., 2006; Marchionni et al., 2009). Primarily, DPSCs have been reported to have the potential to treat several pathologies requiring bone tissue growth and repair in clinics. Dental pulp could be considered one of the major sites for collection of mesenchymal stem cells, as the process is non-invasive and tissue sacrifice is very low. As reported transplantation of new-formed bone tissue obtained from DPSCs led to formation of vascularized adult bone and well integrated between the graft and the surrounding tissues *in vivo* (Graziano et al., 2008; Laino et al., 2005; Laino et al., 2006). Additionally, DPSCs have been reported to have immuno suppressive activity, similar to that found in BM-MSCs (Pierdomenico et al., 2005). While DPSCs will play a fundamental role in future for various human tissues regeneration, they will also have great potential use in the regeneration of teeth. Autologous DPSCs

collected from dental pulp tissue of the permanent teeth have been used for pulp tissue engineering. Dental pulp cells were grown on polyglycolic acid (PGA) that formed pulp-like tissue both *in vitro* and *in vivo* model. However, the implantation of stem cells seeded on scaffold into the root canal for pulp tissue regeneration may compromise the vitality (Casagrande et al., 2011). Despite the significant interest in this field, no clinical trials have been performed for dentin-pulp repair and regeneration (Huang et al., 2010; Iohara et al., 2011). Furthermore, several *in vivo* and *in vitro* investigations have been reported using human PDLCs to regenerate target cells like osteoblasts, adipocytes, cementoblasts, chondrocytes, and periodontal tissue (Kawanabe et al., 2010; Seo et al., 2004). These cells could also be used for engineering a bio-tooth, where DSCs must work together in a spatially and temporally controlled manner. Two methods have been described; one is application of cells in carrier material *in vitro* under the influence of a stimulus supporting tissue regeneration. Another method is the combination of dental epithelial and mesenchymal cells *in vivo* (Wang and Wang, 2008*)*. However, making a bio-tooth with masticatory function and supportive tissues from dental stem cells involves several hindrances including stemness maintenance of stem cells, dental morphogenesis, tooth type determination, controlled bio-tooth growth and eruption, the formation of vascularized pulp with neural tissues and lastly overcome the host-graft immunorejection in the jaws (Yu, Jin, et al., 2008; Yu, Shi, et al., 2008). Therefore, the usage of DSCs for tissue engineering has great potential. However, the outcome of all the tissue engineering approaches using autologous cell treatments greatly depends on the patient selection. The identification of appropriate indications and selection of patients are important for the evaluation of the outcome of the stem cells based treatments.

7. Conclusion

In this thesis, DSCs derived from dental pulp, dental follicle and dental periodontal ligament were differentiated into osteogenic lineage using growth factors or hormones or combination of both. DPSCs are multipotent and have tremendous differentiation potential as reported in the literature. Therefore, DPSCs were isolated, cultured and differentiated in xenofree media for their potential clinical applications in future. Finally, for functional analysis of DPSCs cultured in HS-M, were differentiated in medical grade material β-TCP/P (LLA-CL).

Based on the four studies, the following conclusions can be drawn:

- Study **I**, The addition of BMP-2 or BMP-6 growth factors to L-ascorbic acid-2-phosphate, dexamethasone and β-glycerophosphate (OS) does not enhance the osteogenic differentiation of human PDLCs, however, osteogenic supplements alone induced mineral nodule formation.

- Study **II**, Vitamin D_3 metabolites $1\alpha,25(OH)_2D_3$ (10, 100 nM) or $25OHD_3$ (500 nM) synergistically with L-ascorbic acid-2-phosphate and β-glycerophosphate resulted in osteogenic differentiation in the DPSCs and DFSCs cultures. Vitamin D_3 could be used as an alternative to DEX for *in vitro* mineralization considering the potential catabolic effects of DEX when used *in vivo*.

- Study **III**, DPSCs can be safely isolated, cultured, differentiated and maintain their stemness in medium containing human serum (HS-M), therefore, HS could serve as a safer alternative to FBS for cell therapies. However, serum free/xenofree-medium needs to be further tested for DPSCs isolation, expandability and differentiation efficiency to be available for clinical use.

- Study **IV**, DPSCs cultured in HS-M proliferated and differentiated in β-TCP/P (LLA-CL) scaffolds, and differentiated osteogenically under the influence of VD and DEX osteogenic medium.

Acknowledgements

First of all, I would like to sincerely thank my supervisor, Docent Susanna Miettinen, for her guidance and support during my PhD studies. Her foresight of frontier science, tender attention to students and patience, always inspired and encouraged me over my 4-years of doctoral studies. I, thank you, for always being there to offer a hand, whenever I had problems related with research or practical situations.

I owe my sincere gratitude to Prof. Riitta Suuronen my co-supervisor and Prof. George Sándor my mentor for their immense support and believing in my abilities throughout my PhD study.

I would like to greatly thank the external reviewers, Prof. Willy Serlo and Prof. Thimos Mitsiadis, for the critical analysis and valuable comments to improve my thesis.

I would like to thank all the co-authors included in the publications for this dissertation; it has been a pleasure learning different attributes of science from all of you: Annukka Vuorinen, Sari Vanhatupa, Hideki Agata, Bettina Mannerström.

I am truly grateful and extend my thanks to all the laboratory technicians in Adult stem cell group for their skillful assistance in culturing cells, FACS analysis and gene expression analysis: Miia Juntunen, Minna Salomäki, Anna-Maija Honkala and Sari Kallioski.

I warmly thank all my work colleagues in Adult stem cells group and friends in Regea for their support. I also value all the good times spent together with all of you during my PhD studies.

I owe my greatest thanks, to my spouse Dr. Anchit Khanna, for being my strength and all the encouragement throughout. I would also like to thank my younger sister Rakhi for being by my side in the time of need and helping me with my submissions. Finally, I am very thankful to my parents, parents-in-law and siblings Pridhi, Ashna and Armaan for their constant love, care, and support**.**

This work was financially supported by TEKES, the Finnish Funding Agency for Technology and Innovation, the competitive research funding of the Pirkanmaa Hospital District and University of Tampere.

References

Aberg T, Wozney J, Thesleff I (1997): Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. Dev Dyn 210: 383-396.

Aldahmash A, Haack-Sorensen M, Al-Nbaheen M, Harkness L, Abdallah BM, Kassem M (2011): Human Serum is as Efficient as Fetal Bovine Serum in Supporting Proliferation and Differentiation of Human Multipotent Stromal (Mesenchymal) Stem Cells In Vitro and In Vivo. Stem Cell Rev.

Alge DL, Zhou D, Adams LL, Wyss BK, Shadday MD, Woods EJ, Gabriel Chu TM, Goebel WS (2010): Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. J Tissue Eng Regen Med 4: 73-81.

Amabile G, Meissner A (2009): Induced pluripotent stem cells: current progress and potential for regenerative medicine. Trends in molecular medicine 15: 59-68.

Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferres Padro E, Giner L, Casals N (2011): Isolation of pluripotent stem cells from human third molar dental pulp. Histology and histopathology 26: 1057-1070.

Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez DA, Barajas M, Carrasco M, Jung HS, Hernandez-Alfaro F, Casals N, Prosper F, Ferres Padro E, Giner L (2012): Dental Pulp of the Third Molar: A New Source of Pluripotent-like Stem Cells. Journal of cell science.

Barrere F, van Blitterswijk CA, de Groot K (2006): Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. Int J Nanomedicine 1: 317-332.

Barron MJ, McDonnell ST, Mackie I, Dixon MJ (2008): Hereditary dentine disorders: dentinogenesis imperfecta and dentine dysplasia. Orphanet J Rare Dis 3: 31.

Beloti MM, Rosa AL (2005): Osteoblast differentiation of human bone marrow cells under continuous and discontinuous treatment with dexamethasone. Braz Dent J 16: 156-161.

Bieback K, Hecker A, Kocaomer A, Lannert H, Schallmoser K, Strunk D, Kluter H (2009): Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells 27: 2331-2341.

Blumenthal NM, Koh-Kunst G, Alves ME, Miranda D, Sorensen RG, Wozney JM, Wikesjo UM (2002): Effect of surgical implantation of recombinant human bone morphogenetic protein-2 in a bioabsorbable collagen sponge or calcium phosphate putty carrier in intrabony periodontal defects in the baboon. Journal of periodontology 73: 1494-1506.

Brunner D, Frank J, Appl H, Schoffl H, Pfaller W, Gstraunthaler G Serum-free cell culture: the serum-free media interactive online database. Altex 27: 53-62.

Calori GM, Mazza E, Colombo M, Ripamonti C (2011): The use of bone-graft substitutes in large bone defects: any specific needs? Injury 42 Suppl 2: S56-63.

Casagrande L, Cordeiro MM, Nor SA, Nor JE (2011): Dental pulp stem cells in regenerative dentistry. Odontology 99: 1-7.

Chadipiralla K, Yochim JM, Bahuleyan B, Huang CY, Garcia-Godoy F, Murray PE, Stelnicki EJ (2010): Osteogenic differentiation of stem cells derived from human periodontal ligaments and pulp of human exfoliated deciduous teeth. Cell Tissue Res 340: 323-333.

Chen D, Harris MA, Rossini G, Dunstan CR, Dallas SL, Feng JQ, Mundy GR, Harris SE (1997): Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. Calcif Tissue Int 60: 283-290.

Chen D, Zhao M, Mundy GR (2004): Bone morphogenetic proteins. Growth Factors 22: 233-241.

d'Aquino R, Graziano A, Sampaolesi M, Laino G, Pirozzi G, De Rosa A, Papaccio G (2007): Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. Cell Death Differ 14: 1162-1171.

d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V, Papaccio G (2009): Human dental pulp stem cells: from biology to clinical applications. J Exp Zool B Mol Dev Evol 312B: 408-415.

d'Aquino R, De Rosa A, Lanza V, Tirino V, Laino L, Graziano A, Desiderio V, Laino G, Papaccio G (2009): Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. Eur Cell Mater 18: 75-83.

De Rosa A, Tirino V, Paino F, Tartaglione A, Mitsiadis T, Feki A, d'Aquino R, Laino L, Colacurci N, Papaccio G (2011): Amniotic fluid-derived mesenchymal

stem cells lead to bone differentiation when cocultured with dental pulp stem cells. Tissue Eng Part A 17: 645-653.

Dimarakis I, Levicar N (2006): Cell culture medium composition and translational adult bone marrow-derived stem cell research. Stem Cells 24: 1407-1408.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006): Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317.

Evans MJ, Kaufman MH (1981): Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154-156.

Feng Y, Sun Y, Jia W, Zhang C (2010): Platelet-rich plasma and 1,25(OH)(2) vitamin D (3) synergistically stimulate osteogenic differentiation of adult human mesenchymal stem cells. Biotechnol Lett.

Forrest C, Boyd B, Manktelow R, Zuker R, Bowen V (1992): The free vascularised iliac crest tissue transfer: donor site complications associated with eighty-two cases. Br J Plast Surg 45: 89-93.

Friedenstein AJ, Piatetzky S, II, Petrakova KV (1966): Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16: 381-390.

Frohlich M, Grayson WL, Marolt D, Gimble JM, Kregar-Velikonja N, Vunjak-Novakovic G (2010): Bone grafts engineered from human adipose-derived stem cells in perfusion bioreactor culture. Tissue engineering. Part A 16: 179-189.

Galende E, Karakikes I, Edelmann L, Desnick RJ, Kerenyi T, Khoueiry G, Lafferty J, McGinn JT, Brodman M, Fuster V, Hajjar RJ, Polgar K (2010): Amniotic fluid cells are more efficiently reprogrammed to pluripotency than adult cells. Cellular reprogramming 12: 117-125.

Gimble JM, Guilak F (2003): Differentiation potential of adipose derived adult stem (ADAS) cells. Current topics in developmental biology 58: 137-160.

Gimble JM, Katz AJ, Bunnell BA (2007): Adipose-derived stem cells for regenerative medicine. Circulation research 100: 1249-1260.

Gimble JM, Grayson W, Guilak F, Lopez MJ, Vunjak-Novakovic G (2011): Adipose tissue as a stem cell source for musculoskeletal regeneration. Frontiers in bioscience 3: 69-81.

Giunta JL (1998): Dental changes in hypervitaminosis D. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 85: 410-413.

Goodison S, Urquidi V, Tarin D (1999): CD44 cell adhesion molecules. Mol Pathol 52: 189-196.

Graziano A, d'Aquino R, Laino G, Papaccio G (2008): Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Rev 4: 21-26.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000): Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 97: 13625-13630.

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM (2001): Surface protein characterization of human adipose tissue-derived stromal cells. Journal of cellular physiology 189: 54-63.

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002): Stem cell properties of human dental pulp stem cells. J Dent Res 81: 531-535.

Guarino V, Causa F, Taddei P, di Foggia M, Ciapetti G, Martini D, Fagnano C, Baldini N, Ambrosio L (2008): Polylactic acid fibre-reinforced polycaprolactone scaffolds for bone tissue engineering. Biomaterials 29: 3662-3670.

Gupta A, Leong DT, Bai HF, Singh SB, Lim TC, Hutmacher DW (2007): Osteo-maturation of adipose-derived stem cells required the combined action of vitamin D3, beta-glycerophosphate, and ascorbic acid. Biochem Biophys Res Commun 362: 17-24.

Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM (2001): Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng 7: 729-741.

Handa K, Saito M, Tsunoda A, Yamauchi M, Hattori S, Sato S, Toyoda M, Teranaka T, Narayanan AS (2002): Progenitor cells from dental follicle are able to form cementum matrix in vivo. Connect Tissue Res 43: 406-408.

Hayami T, Zhang Q, Kapila Y, Kapila S (2007): Dexamethasone's enhancement of osteoblastic markers in human periodontal ligament cells is associated with inhibition of collagenase expression. Bone 40: 93-104.

Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, Mikkola M, Olsson C, Miller-Podraza H, Blomqvist M, Olonen A, Salo H, Lehenkari P, Tuuri T, Otonkoski T, Natunen J, Saarinen J, Laine J (2007): N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells 25: 197-202.

Hirata TM, Ishkitiev N, Yaegaki K, Calenic B, Ishikawa H, Nakahara T, Mitev V, Tanaka T, Haapasalo M (2010): Expression of multiple stem cell markers in dental pulp cells cultured in serum-free media. J Endod 36: 1139-1144.

Hopkins PM, Kermeen F, Duhig E, Fletcher L, Gradwell J, Whitfield L, Godinez C, Musk M, Chambers D, Gotley D, McNeil K (2010): Oil red O stain of alveolar

macrophages is an effective screening test for gastroesophageal reflux disease in lung transplant recipients. J Heart Lung Transplant 29: 859-864.

Huang GT, Shagramanova K, Chan SW (2006): Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro. J Endod 32: 1066-1073.

Huang GT, Gronthos S, Shi S (2009): Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88: 792-806.

Huang GT, Yamaza T, Shea LD, Djouad F, Kuhn NZ, Tuan RS, Shi S (2010): Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. Tissue Eng Part A 16: 605-615.

Huang KK, Shen C, Chiang CY, Hsieh YD, Fu E (2005): Effects of bone morphogenetic protein-6 on periodontal wound healing in a fenestration defect of rats. J Periodontal Res 40: 1-10.

In Sook K, Yoon Mi S, Tae Hyung C, Yong Doo P, Kyu Back L, Insup N, Franz W, Soon Jung H (2008): In vitro response of primary human bone marrow stromal cells to recombinant human bone morphogenic protein-2 in the early and late stages of osteoblast differentiation. Development, Growth & Differentiation 50: 553-564.

Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A (2004): Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2. J Dent Res 83: 590-595.

Iohara K, Imabayashi K, Ishizaka R, Watanabe A, Nabekura J, Ito M, Matsushita K, Nakamura H, Nakashima M (2011): Complete Pulp Regeneration After Pulpectomy by Transplantation of CD105(+) Stem Cells with Stromal Cell-Derived Factor-1. Tissue Eng Part A 17: 1911-1920.

Isaka J, Ohazama A, Kobayashi M, Nagashima C, Takiguchi T, Kawasaki H, Tachikawa T, Hasegawa K (2001): Participation of periodontal ligament cells with regeneration of alveolar bone. J Periodontol 72: 314-323.

Jager M, Fischer J, Dohrn W, Li X, Ayers DC, Czibere A, Prall WC, Lensing-Hohn S, Krauspe R (2008): Dexamethasone modulates BMP-2 effects on mesenchymal stem cells in vitro. J Orthop Res 26: 1440-1448.

Jin ZL, Zhang YK, Sun HY, Lin Z, Bi YC, Duan YZ, Ding Y (2008): Osteogenic-related gene expression profiles of human dental follicle cells induced by dexamethasone. Acta Pharmacol Sin 29: 1013-1020.

Jurgens WJ, Oedayrajsingh-Varma MJ, Helder MN, Zandiehdoulabi B, Schouten TE, Kuik DJ, Ritt MJ, van Milligen FJ (2008): Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell and tissue research 332: 415-426.

Karbanova J, Soukup T, Suchanek J, Pytlik R, Corbeil D, Mokry J (2010): Characterization of Dental Pulp Stem Cells from Impacted Third Molars Cultured in Low Serum-Containing Medium. Cells Tissues Organs.

Kawanabe N, Murata S, Murakami K, Ishihara Y, Hayano S, Kurosaka H, Kamioka H, Takano-Yamamoto T, Yamashiro T (2010): Isolation of multipotent stem cells in human periodontal ligament using stage-specific embryonic antigen-4. Differentiation; research in biological diversity 79: 74-83.

Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK (2004): Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proceedings of the National Academy of Sciences of the United States of America 101: 11839-11844.

Kemoun P, Laurencin-Dalicieux S, Rue J, Farges JC, Gennero I, Conte-Auriol F, Briand-Mesange F, Gadelorge M, Arzate H, Narayanan AS, Brunel G, Salles JP (2007): Human dental follicle cells acquire cementoblast features under stimulation by BMP-2/-7 and enamel matrix derivatives (EMD) in vitro. Cell Tissue Res 329: 283-294.

Khanna-Jain R, Vuorinen A, Sandor GK, Suuronen R, Miettinen S (2010): Vitamin D(3) metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells. J Steroid Biochem Mol Biol 122: 133-141.

Kimelman N, Pelled G, Gazit Z, Gazit D (2006): Applications of gene therapy and adult stem cells in bone bioengineering. Regen Med 1: 549-561.

Kobayashi T, Watanabe H, Yanagawa T, Tsutsumi S, Kayakabe M, Shinozaki T, Higuchi H, Takagishi K (2005): Motility and growth of human bone-marrow mesenchymal stem cells during ex vivo expansion in autologous serum. J Bone Joint Surg Br 87: 1426-1433.

Kolk A, Handschel J, Drescher W, Rothamel D, Kloss F, Blessmann M, Heiland M, Wolff KD, Smeets R (2012): Current trends and future perspectives of bone substitute materials - From space holders to innovative biomaterials. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery.

Komoda H, Okura H, Lee CM, Sougawa N, Iwayama T, Hashikawa T, Saga A, Yamamoto-Kakuta A, Ichinose A, Murakami S, Sawa Y, Matsuyama A (2010): Reduction of N-glycolylneuraminic acid xenoantigen on human adipose tissue-derived stromal cells/mesenchymal stem cells leads to safer and more useful cell sources for various stem cell therapies. Tissue Eng Part A 16: 1143-1155.

Kuru L, Griffiths GS, Petrie A, Olsen I (1999): Alkaline phosphatase activity is upregulated in regenerating human periodontal cells. J Periodontal Res 34: 123-127.

Laino G, d'Aquino R, Graziano A, Lanza V, Carinci F, Naro F, Pirozzi G, Papaccio G (2005): A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res 20: 1394-1402.

Laino G, Graziano A, d'Aquino R, Pirozzi G, Lanza V, Valiante S, De Rosa A, Naro F, Vivarelli E, Papaccio G (2006): An approachable human adult stem cell source for hard-tissue engineering. J Cell Physiol 206: 693-701.

Laurie SW, Kaban LB, Mulliken JB, Murray JE (1984): Donor-site morbidity after harvesting rib and iliac bone. Plast Reconstr Surg 73: 933-938.

Lazcano O, Li CY, Pierre RV, O'Duffy JD, Beissner RS, Abell-Aleff PC (1993): Clinical utility of the alizarin red S stain on permanent preparations to detect calcium-containing compounds in synovial fluid. Am J Clin Pathol 99: 90-96.

Lee SY, Lim J, Khang G, Son Y, Choung PH, Kang SS, Chun SY, Shin HI, Kim SY, Park EK (2009): Enhanced ex vivo expansion of human adipose tissue-derived mesenchymal stromal cells by fibroblast growth factor-2 and dexamethasone. Tissue Eng Part A 15: 2491-2499.

Lindroos B, Maenpaa K, Ylikomi T, Oja H, Suuronen R, Miettinen S (2008): Characterisation of human dental stem cells and buccal mucosa fibroblasts. Biochem Biophys Res Commun 368: 329-335.

Lindroos B, Boucher S, Chase L, Kuokkanen H, Huhtala H, Haataja R, Vemuri M, Suuronen R, Miettinen S (2009): Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy 11: 958-972.

Lutolf MP, Gilbert PM, Blau HM (2009): Designing materials to direct stem-cell fate. Nature 462: 433-441.

Lynch SE, Williams RC, Polson AM, Howell TH, Reddy MS, Zappa UE, Antoniades HN (1989): A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration. Journal of clinical periodontology 16: 545-548.

Maas R, Bei M (1997): The genetic control of early tooth development. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists 8: 4-39.

Marchionni C, Bonsi L, Alviano F, Lanzoni G, Di Tullio A, Costa R, Montanari M, Tazzari PL, Ricci F, Pasquinelli G, Orrico C, Grossi A, Prati C, Bagnara GP (2009): Angiogenic potential of human dental pulp stromal (stem) cells. Int J Immunopathol Pharmacol 22: 699-706.

Mesimaki K, Lindroos B, Tornwall J, Mauno J, Lindqvist C, Kontio R, Miettinen S, Suuronen R (2009): Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. Int J Oral Maxillofac Surg 38: 201-209.

Miller JP, Perry EH, Price TH, Bolan CD, Jr., Karanes C, Boyd TM, Chitphakdithai P, King RJ (2008): Recovery and safety profiles of marrow and PBSC donors: experience of the National Marrow Donor Program. Biol Blood Marrow Transplant 14: 29-36.

Misch CM (2011): Maxillary autogenous bone grafting. Oral Maxillofac Surg Clin North Am 23: 229-238.

Mitalipov S, Wolf D (2009): Totipotency, pluripotency and nuclear reprogramming. Advances in biochemical engineering/biotechnology 114: 185-199.

Mori G, Centonze M, Brunetti G, Ballini A, Oranger A, Mori C, Lo Muzio L, Tete S, Ciccolella F, Colucci S, Grano M, Grassi FR (2010): Osteogenic properties of human dental pulp stem cells. J Biol Regul Homeost Agents 24: 167-175.

Morsczeck C, Gotz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, Sippel C, Hoffmann KH (2005): Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol 24: 155-165.

Morsczeck C, Moehl C, Gotz W, Heredia A, Schaffer TE, Eckstein N, Sippel C, Hoffmann KH (2005): In vitro differentiation of human dental follicle cells with dexamethasone and insulin. Cell Biol Int 29: 567-575.

Morsczeck C (2006): Gene expression of runx2, Osterix, c-fos, DLX-3, DLX-5, and MSX-2 in dental follicle cells during osteogenic differentiation in vitro. Calcif Tissue Int 78: 98-102.

Morsczeck C, Vollner F, Saugspier M, Brandl C, Reichert TE, Driemel O, Schmalz G (2010): Comparison of human dental follicle cells (DFCs) and stem cells from human exfoliated deciduous teeth (SHED) after neural differentiation in vitro. Clinical oral investigations 14: 433-440.

Murakami Y, Kojima T, Nagasawa T, Kobayashi H, Ishikawa I (2003): Novel isolation of alkaline phosphatase-positive subpopulation from periodontal ligament fibroblasts. J Periodontol 74: 780-786.

Nakhaei-Rad S, Bahrami AR, Mirahmadi M, Matin MM (2012): New windows to enhance direct reprogramming of somatic cells towards induced pluripotent stem cells. Biochemistry and cell biology = Biochimie et biologie cellulaire 90: 115-123.

Nevins M, Giannobile WV, McGuire MK, Kao RT, Mellonig JT, Hinrichs JE, McAllister BS, Murphy KS, McClain PK, Nevins ML, Paquette DW, Han TJ, Reddy MS, Lavin PT, Genco RJ, Lynch SE (2005): Platelet-derived growth factor stimulates bone fill and rate of attachment level gain: results of a large multicenter randomized controlled trial. Journal of periodontology 76: 2205-2215.

Nof M, Shea LD (2002): Drug-releasing scaffolds fabricated from drug-loaded microspheres. J Biomed Mater Res 59: 349-356.

Nohutcu RM, McCauley LK, Koh AJ, Somerman MJ (1997): Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization in vitro. J Periodontol 68: 320-327.

Papaccio G, Graziano A, d'Aquino R, Graziano MF, Pirozzi G, Menditti D, De Rosa A, Carinci F, Laino G (2006): Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. J Cell Physiol 208: 319-325.

Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, Hockema JJ, Woods EJ, Goebel WS (2008a): Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. Tissue Eng Part C Methods 14: 149-156.

Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, Hockema JJ, Woods EJ, Goebel WS (2008b): Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. Tissue engineering. Part C, Methods 14: 149-156.

Pfaffl MW (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP (2005): Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 80: 836-842.

Pierotti S, Gandini L, Lenzi A, Isidori AM (2008): Pre-receptorial regulation of steroid hormones in bone cells: insights on glucocorticoid-induced osteoporosis. J Steroid Biochem Mol Biol 108: 292-299.

Pitaru S, Pritzki A, Bar-Kana I, Grosskopf A, Savion N, Narayanan AS (2002): Bone morphogenetic protein 2 induces the expression of cementum attachment protein in human periodontal ligament clones. Connect Tissue Res 43: 257-264.

Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla L, Blancher A (2005): Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol 129: 118-129.

Rajshankar D, McCulloch CA, Tenenbaum HC, Lekic PC (1998): Osteogenic inhibition by rat periodontal ligament cells: modulation of bone morphogenic protein-7 activity in vivo. Cell Tissue Res 294: 475-483.

Ripamonti U (2007): Recapitulating development: a template for periodontal tissue engineering. Tissue engineering 13: 51-71.

Ripamonti U, Petit JC (2009): Bone morphogenetic proteins, cementogenesis, myoblastic stem cells and the induction of periodontal tissue regeneration. Cytokine & growth factor reviews 20: 489-499.

Robertson EJ (1990): Derivation and maintenance of embryonic stem cell cultures. Methods in molecular biology 5: 223-236.

Ruch JV (1998): Odontoblast commitment and differentiation. Biochemistry and cell biology = Biochimie et biologie cellulaire 76: 923-938.

Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S (2004): Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364: 149-155.

Shahdadfar A, Fronsdal K, Haug T, Reinholt FP, Brinchmann JE (2005): In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem Cells 23: 1357-1366.

Shiba H, Uchida Y, Kamihagi K, Sakata M, Fujita T, Nakamura S, Takemoto T, Kato Y, Kurihara H (2001): Transforming growth factor-beta1 and basic fibroblast growth factor modulate osteocalcin and osteonectin/SPARC syntheses in vitamin-D-activated pulp cells. J Dent Res 80: 1653-1659.

Sittinger M, Hutmacher DW, Risbud MV (2004): Current strategies for cell delivery in cartilage and bone regeneration. Curr Opin Biotechnol 15: 411-418.

Slavkin HC, Bringas P, Jr., Bessem C, Santos V, Nakamura M, Hsu MY, Snead ML, Zeichner-David M, Fincham AG (1989): Hertwig's epithelial root sheath differentiation and initial cementum and bone formation during long-term organ culture of mouse mandibular first molars using serumless, chemically-defined medium. Journal of periodontal research 24: 28-40.

Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, Hsu SC, Smith J, Prockop DJ (2004): Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 9: 747-756.

Stucki U, Schmid J, Hammerle CF, Lang NP (2001): Temporal and local appearance of alkaline phosphatase activity in early stages of guided bone regeneration. A descriptive histochemical Study In humans. Clin Oral Implants Res 12: 121-127.

Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007): Induction of pluripotent stem cells from fibroblast cultures. Nature protocols 2: 3081-3089.

Tamaoki N, Takahashi K, Tanaka T, Ichisaka T, Aoki H, Takeda-Kawaguchi T, Iida K, Kunisada T, Shibata T, Yamanaka S, Tezuka K (2010): Dental pulp cells for induced pluripotent stem cell banking. Journal of dental research 89: 773-778.

Tang X, Meng H (2009): Osteogenic induction and 1,25-dihydroxyvitamin D3 oppositely regulate the proliferation and expression of RANKL and the vitamin D receptor of human periodontal ligament cells. Arch Oral Biol 54: 625-633.

Ten Cate AR (1967): A histochemical study of the human odontoblast. Archives of oral biology 12: 963-970.

Ten Cate AR (1982): Development of the dentofacial complex. Dental clinics of North America 26: 445-459.

Thesleff I, Vaahtokari A, Partanen AM (1995): Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. The International journal of developmental biology 39: 35-50.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998): Embryonic stem cell lines derived from human blastocysts. Science 282: 1145-1147.

Tomic S, Djokic J, Vasilijic S, Vucevic D, Todorovic V, Supic G, Colic M (2011): Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. Stem Cells Dev 20: 695-708.

Tonomura A, Sumita Y, Ando Y, Iejima D, Kagami H, Honda MJ, Ueda M (2007): Differential inducibility of human and porcine dental pulp-derived cells into odontoblasts. Connect Tissue Res 48: 229-238.

Valdimarsdottir G, Mummery C (2005): Functions of the TGFbeta superfamily in human embryonic stem cells. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica 113: 773-789.

Wang FM, Qiu K, Hu T, Wan CX, Zhou XD, Gutmann JL (2006): Biodegradable porous calcium polyphosphate scaffolds for the three-dimensional culture of dental pulp cells. Int Endod J 39: 477-483.

Wang SL, Wang XJ (2008): [Tooth regeneration--dream to reality]. Hua xi kou qiang yi xue za zhi = Huaxi kouqiang yixue zazhi = West China journal of stomatology 26: 115-117.

Warotayanont R, Frenkel B, Snead ML, Zhou Y (2009): Leucine-rich amelogenin peptide induces osteogenesis by activation of the Wnt pathway. Biochemical and biophysical research communications 387: 558-563.

Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, Millan JL (2000): Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res 15: 1879-1888.

Woods EJ, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS (2009): Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. Cryobiology 59: 150-157.

Xiao Y-T, Xiang L-X, Shao J-Z (2007): Bone morphogenetic protein. Biochemical and Biophysical Research Communications 362: 550-553.

Yao S, Pan F, Prpic V, Wise GE (2008): Differentiation of stem cells in the dental follicle. J Dent Res 87: 767-771.

Yilmaz M, Ovali E, Akdogan E, Durmus A, Sonmez M, Dikmen T, Omay SB (2008): Autologous serum is more effective than fetal bovine serum on proliferation of bone marrow derived human mesenchymal stem cells. Saudi Med J 29: 306-309.

Yu J, Jin F, Deng Z, Li Y, Tang L, Shi J, Jin Y (2008): Epithelial-mesenchymal cell ratios can determine the crown morphogenesis of dental pulp stem cells. Stem cells and development 17: 475-482.

Yu J, Shi J, Jin Y (2008): Current approaches and challenges in making a bio-tooth. Tissue engineering. Part B, Reviews 14: 307-319.

Zhang H, Fraser ST, Papazoglu C, Hoatlin ME, Baron MH (2009): Transcriptional activation by the Mixl1 homeodomain protein in differentiating mouse embryonic stem cells. Stem cells 27: 2884-2895.

Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA (2006): Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue Eng 12: 2813-2823.

Zhou YS, Liu YS, Tan JG (2006): Is 1, 25-dihydroxyvitamin D3 an ideal substitute for dexamethasone for inducing osteogenic differentiation of human adipose tissue-derived stromal cells in vitro? Chin Med J (Engl) 119: 1278-1286.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002): Human adipose tissue is a source of multipotent stem cells. Molecular biology of the cell 13: 4279-4295.

Original Communications

Journal of Steroid Biochemistry & Molecular Biology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells

Rashi Khanna-Jain[a,*], Annukka Vuorinen[a], George K.B. Sándor[a,d,e], Riitta Suuronen[a,b,c], Susanna Miettinen[a]

[a] REGEA, Institute for Regenerative Medicine, University of Tampere and Tampere University Hospital, Biokatu-12, 33520 Tampere, Finland
[b] Department of Eye, Ear and Oral Diseases, Tampere University Hospital, Finland
[c] Department of Biomedical Engineering, Tampere University of Technology, Finland
[d] Department of Oral and Maxillofacial Surgery and Pediatric Oral and Maxillofacial Surgery, University of Toronto, Canada. The Hospital for Sick Children and Bloorview Kids Rehab, Toronto, Canada
[e] Oral and Maxillofacial Surgery, University of Oulu, Oulu, Finland

ARTICLE INFO

Article history:
Received 23 February 2010
Received in revised form 9 August 2010
Accepted 10 August 2010

Keywords:
$1\alpha,25(OH)2D_3$
$25OHD_3$
Dental pulp
Dental follicle
Osteogenic differentiation
L-Ascorbic acid-2-phosphate
β-Glycerophosphate

ABSTRACT

Vitamin D_3 metabolites regulate the bone metabolism and $1\alpha,25$-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) is known to play an important role in teeth mineralization. However, little is known about the potential of vitamin D as an osteogenic inducer in human dental pulp (hDPCs) and dental follicle cells (hDFCs) *in vitro*. Therefore, we investigated the effects of vitamin D_3 metabolites $1\alpha,25(OH)_2D_3$ and 25-hydroxyvitamin D_3 ($25OHD_3$) on proliferation and osteogenic differentiation of hDPCs and hDFCs *in vitro*. We also examined whether vitamin D_3 metabolic enzymes were regulated in hDFCs and hDPCs. Cell proliferation was decreased by both metabolites in hDPCs and hDFCs. Vitamin D_3 metabolites increased ALP activity and induced mineralization when osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) were added, though the expression of osteocalcin (OC) and osteopontin (OPN) were regulated without the addition of OS. CYP24 and CYP27B1 expressions were upregulated by vitamin D_3 metabolites and $25OHD_3$ was converted into $1\alpha,25(OH)_2D_3$ in the culture medium. These results confirm that $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (500 nM) can be used as osteogenic inducers synergistically with osteogenic supplements for differentiation of hDPCs and hDFCs. Furthermore, our findings strengthen our knowledge about the role of hDPCs and hDFCs as vitamin D_3 target cells.

1. Introduction

Vitamin D_3 metabolites such as $1\alpha,25$-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) and 25-hydroxycholecalciferol ($25OHD_3$) are key regulatory factors of bone metabolism [1–3]. The circulating $25OHD_3$ metabolite is hydroxylated to $1\alpha,25(OH)_2D_3$ by $25OHD_3$-1α-hydroxylase (1α-hydroxylase, CYP27B1) in the kidneys and other vitamin D target organs [4]. The biological effects of vitamin D_3 can be mediated by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors that functions as a transcription factor or the action of vitamin D_3 might be mediated non-genomically via a different receptors such as membrane-associated rapid response steroid receptor [5–7]. The actions of $1\alpha,25(OH)_2D_3$, as well as $25OHD_3$, are inactivated by 24-hydroxylase (CYP24) in the kidneys and in the other vitamin D_3 target tissue. CYP24 is a mitochondrial enzyme which catalyses the hydroxylation and thereby inactivates $1\alpha,25(OH)_2D_3$ while $25OHD_3$ is converted to $24,25(OH)_2D_3$ [8]. The expression of the CYP24 gene has been used as an indicator of transcriptional activity of vitamin D_3 metabolites [9].

Dental tissues derived cells are a source of multipotent mesenchymal stem cells that can be differentiated into osteogenic, chondrogenic, adipogenic and neurogenic cell types *in vitro*, as reported in other studies including one from our group [10,11]. These cells can be isolated from impacted human third molar teeth based on their anatomic locations and expression of stem cell markers. Human dental pulp cells (hDPCs) reside in the central cavity of the teeth and are a source of progenitor cells that can undergo differentiation towards odontoblastic, osteoblastic, neurogenic and adipocytic cell types *in vitro* [12]. It has been reported that stem cells derived from dental pulp are able to differentiate into osteoblastoids under high serum conditions and are a potential source of autologous bone produced *in vitro* [13,14]. Recent studies have identified dental follicle cells (hDFCs) which are isolated from the connective tissue surrounding the developing tooth germ before tooth eruption [15]. This tissue contains progenitor cells that give rise to the periodontium including cementum, periodontal ligament cells (PDL), and alveolar bone [16].

* Corresponding author. Tel.: +358 04 1901789; fax: +358 3 35518498.
E-mail address: rashi.khanna-jain@regea.fi (R. Khanna-Jain).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.08.001

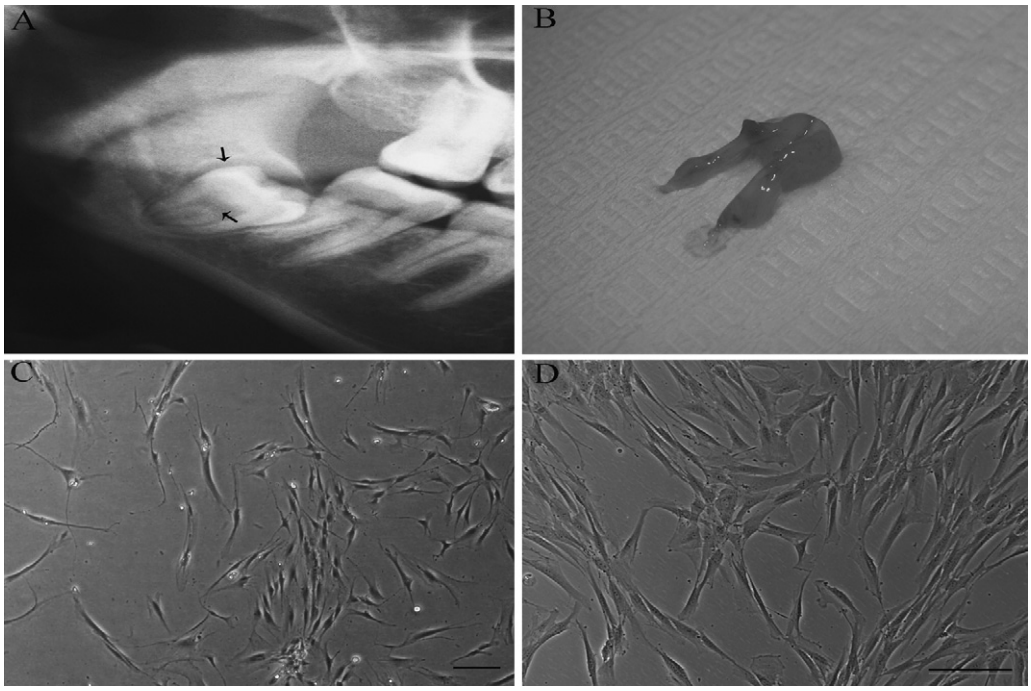

Fig. 1. Anatomical location and morphological appearances of human teeth derived cells. Radiographic image (A) showing anatomic location of dental follicle (arrow above) or dental pulp (arrow below) tissues and (B) human dental pulp tissue obtained from the pulp chamber and canals. Light microscopic appearance of (C) hDFCs (scale bar = 1 mm) and (D) hDPCs (scale bar = 100 μM).

Dexamethasone (Dex) a glucocorticosteroid, in combination with osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) is the most commonly used osteogenic inducer for human mesenchymal stem cells (MSCs) such as adipose tissue derived [17] and bone marrow derived stem cells *in vitro* [18]. Studies have shown that human dental follicle derived cells differentiate into osteoblasts like cells when induced by Dex [19,20]. Glucocorticosteroid, however have deleterious effects on bone *in vivo*, resulting in glucocorticoid-induced osteoporosis [21]. There are some studies where $1\alpha,25(OH)_2D_3$ in addition to OS has been used for osteogenic differentiation of MSCs [22–24]. However, the effects of vitamin D_3 metabolites to induce osteogenic differentiation in hDPCs and hDFCs is not yet clearly understood *in vitro*. Moreover, the role of $1\alpha,25(OH)_2D_3$ in tooth formation is well known from *in vivo* and clinical studies, deficiency of $1\alpha,25(OH)_2D_3$ results in hypocalcification of the dentin and enamel leading to unmineralized dental structure [25]. Excessive doses of $1\alpha,25(OH)_2D_3$ cause hypercementosis, formation of pulp stones and hypercalcification in dental tissues [26]. Additionally, $1\alpha,25(OH)_2D_3$ has been reported to promote the function of osteoclastogenesis in the periodontium [27]. Taken together, we hypothesized that cells derived from dental tissues when treated with vitamin D_3 metabolites might optimally induce osteogenic differentiation *in vitro*.

Therefore, this study was designed to understand the role of different concentrations of $25OHD_3$ and $1,25(OH)_2D_3$ on proliferation and osteogenic differentiation of hDPCs and hDFCs *in vitro*. In this series of investigations, we also tested whether vitamin D_3 metabolic enzymes in hDFCs and hDPCs were regulated *in vitro*.

2. Materials and methods

2.1. Cell isolation and culture

Human impacted third molars were obtained with informed consent from Finnish Student Health Services, Tampere, Finland. The collection of stem cells from tooth samples was approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R06009). Human dental pulp and dental follicle tissue explants were obtained from partially or completely impacted third molar teeth of 12 patients, patients aged 21–26 years (23 ± 2.5 years). The tooth samples were brought from the health centre to the laboratory in Phosphate buffered saline (PBS; BioWhittaker Lonza, Verviers, Belgium) containing 2% antibiotics/antimycotics (a/a; 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B; Invitrogen, Paisley, Scotland, UK). The dental tissues were isolated in the laboratory under laminar hood; the teeth were cleaned with PBS before isolating cells. The human dental pulp and follicle tissue explants were derived from third molar teeth based on their anatomic location as shown in (Fig. 1A and B). Dental Pulp tissues were obtained by exposing the pulp chamber of the teeth and dental follicle from the tissues surrounding the mineralized tooth. Following the dental pulp and follicle tissue extraction, the tissue fragments were minced using sterilized scalpels. Tissues were then digested in collagenase type I 3 mg/ml (Invitrogen) and dispase 4 mg/ml (Invitrogen) for 1 h at 37 °C. Once digestion was completed the obtained cell suspension was passed through a 70 μm cell strainer (Falcon, BD Labware, Franklin lakes, NJ, USA) and cells were seeded in 6 well culture plates (Nunc, Roskilde, Denmark) in basic cell culture medium (BM) consisting of DMEM/F-12 1:1 (Invitrogen), 10% FBS (Invitrogen), L-glutamine (GlutaMAX I; Invitrogen), and 1% antibiotics/antimycotic (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B; Invitrogen), and then incubated at 37 °C in 5% CO_2. After 14 days of culture, cells were detached using trypsin in PBS (Lonza) and then cells were expanded in 75 cm^2 culture flasks (Nunc, Roskilde, Denmark) containing BM. Cell culture plates and flasks were monitored daily for cell growth, with medium changes taking place three times per week. All assays were performed using cells between passage 3 and 4 and experiments were repeated using cells derived from 3 different donors each for hDPCs and hDFCs.

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

Table 1
Primers sequence for quantitative RT-PCR.

Name	5′-Sequence-3′	Product size	Accession number
RPLPO	Forward AATCTCCAGGGGCACCATT Reverse CGTTGGCTCCCACTTTGT	70	NM_001002
Osteocalcin	Forward AGCAAAGGTGCAGCCTTTGT Reverse GCGCCTGGGTCTCTTCACT	63	NM_000711
Osteopontin	Forward GCCGACCAAGGAAAACTCACT Reverse GGCACAGGTGATGCCTAGGA	71	J04765
CYP24	Forward GCCCAGCCGGGAACTC Reverse AAATACCACCATCTGAGGCGTATT	61	NM_000782
CYP27B1	Forward TTGGCAAGCGCAGCTGTAT Reverse TGTGTTAGGATCTGGGCCAAA	75	NM_000785
VDR	Forward CCTTCACCATGGACGACATG Reverse CGGCTTTGGTCACGTCACT	77	NM_000376

2.2. Cell proliferation assay

Cell proliferation assay was done to measure the viability and induction of cell proliferation by the action of vitamin D_3 metabolites alone, such as $1\alpha,25(OH)_2D_3$ (Sigma–Aldrich, MO, USA) and $25OHD_3$ (Sigma) on hDPCs and hDFCs. The cells were treated with different concentrations of $1\alpha,25(OH)_2D_3$ (1,25; 10 and 100 nM) and $25OHD_3$ (25; 100 and 500 nM) as follows. Human DPCs and hDFCs each were seeded at 10,000 cells/well in 24-well plates in BM with different concentrations of vitamin D_3 metabolites. The control samples were maintained in BM and blank values were also measured for non-specific binding. The culture media was changed after 3 and 4 days for each media concentration. The plates were incubated at 37 °C in a 5% CO_2 containing humidified atmosphere. Cell numbers and viability were quantified at 1, 7 and 14 days time points using the colorimetric reagent WST-1 (Takara Bio Inc., Otsu, Japan). The absorbance was measured directly with a plate reader Victor 1420 (Perkin Elmer life Sciences, Turku, Finland) using wavelength of 450 nm.

2.3. Alkaline phosphatase activity

To further investigate the combined effect of osteogenic supplements (OS; 50 μM L-ascorbic acid 2-phosphate (AA; Sigma) and 10 mM β-glycerophosphate (Sigma)) and vitamin D_3 metabolites on hDPCs and hDFCs, cells were plated in 24-well plates at a density of 10,000 cells/well, respectively, and incubated for 24 h in BM. Thereafter, cells were cultured in BM containing $1\alpha,25(OH)_2D_3$ (10 and 100 nM) or $25OHD_3$ (100 and 500 nM) with and without OS. As a control, cells were cultured in BM and for comparing the effects of different metabolites cells were cultured in BM containing dexamethasone (Dex) 10 nM + OS. Culture medium was replaced with fresh medium every 3 and 4 days. After 7 and 14 days of culture, cell proliferation and alkaline phosphatase (ALP) activity were analyzed with a commercially available *p*-nitrophenyl phosphate tablet set (Sigma, St. Louis, MO, USA) and cell proliferation kit (Premix WST-1 Cell Proliferation Assay System; Takara Bio Inc., Shiga, Japan), with modifications [28]. Cell proliferation (WST-1 absorbance) was analyzed according to the manufacturer's protocol. Briefly, WST-1 reagents was added to each well containing fresh medium (50 μl of WST-1/500 μl of medium in each well of 24-well plate), incubated for 60 min, the absorbance was measured at 450 nm using a microplate reader (Victor 1420, Finland). After WST-1 analysis, each well was washed twice with PBS and *p*-nitrophenyl phosphate solution was added (400 μl/well for 24-well plates). After 10 min of incubation at 37 °C, conversion of *p*-nitrophenyl phosphate into *p*-nitrophenol by cellular ALP was stopped with the equivalent amount of 3N NaOH and the absorbance of *p*-nitrophenol was measured at 450 nm using a microplate reader. Alkaline phosphatase-specific activity is expressed as *p*-nitrophenol absorbance (OD; 405 nm)/WST-1 absorbance (OD; 450 nm), which is designed to assess the ALP activity/no. of viable cells.

2.4. Mineralization assay (alizarin red staining)

The cell culture conditions used were similar as described for ALP activity. After 21 days of cell culture in 24-well plates, *in vitro* mineralization was analyzed by alizarin red staining. For alizarin red S staining, cells were fixed with ice-cold 70% ethanol for 60 min at −20 °C. Then, cells were washed twice with distilled water and stained with 40 mM Alizarin red S solution (Sigma) for 10 min at room temperature. The pH value of the solution was adjusted to 4.2 with 25% ammonium hydroxide prior to staining. After staining, excess dye was washed with distilled water and digital images of stained mineral deposits were taken.

2.5. Real-time quantitative PCR

Next, we analyzed the time course effect of the vitamin D_3 metabolites without OS at mRNA level. Human DPCs and hDFCs were seeded at a density of 10,000 cells/well in 6 well plate with different concentrations of $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) in BM. The control samples were maintained in the BM. The Total RNA was extracted at 24, 48, 72 h time points by Eurozol (Euroclone S.p.A, Pero, Italy). First-strand cDNA synthesis were performed by a High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Real-time quantitative PCR (qRT-PCR) was conducted using osteocalcin (OC), osteopontin (OPN), and RPLP0 (human acidic ribosomal phosphoprotein), VDR (vitamin D receptor), CYP24 (24-hydroxylase) and $25OHD_3$-1α-hydroxylase (1α-hydroxylase, CYP27B1) primer sequences as shown in (Table 1). To exclude signals from contaminating DNA, the forward and reverse sequence of each primer was designed on different exons. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for Quantitative PCR reactions according to the manufacturer's instructions. The reactions were performed with AbiPrism 7300 Sequence Detection System (Applied Biosystems) at 95 °C 10 min, and then 45 cycles at 95 °C/15 s and 60 °C/60 s. The Ct values for OC, OPN, VDR and CYP24 were normalized to that of the housekeeping gene RPLP0, as described elsewhere [29].

2.6. 1,25-Dihydroxy vitamin D_3, enzyme immunoassay (EIA)

The 1,25-dihydroxy vitamin D_3 EIA kit (Immunodiagnostic Systems Ltd, Boldon, UK) was used to measure the conversion of $25OHD_3$ into $1\alpha,25(OH)_2D_3$ by 1α-hydroxylase in hDPCs and hDFCs. The cells were cultured in BM + $25OHD_3$ (500 nM) and BM + $25OHD_3$ (500 nM) + inhibitor (ketoconazole; 10 μM) for 24 h. The control samples were maintained in BM without the addition of vitamin D_3 metabolites and inhibitor. 10,000 cells/well were

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

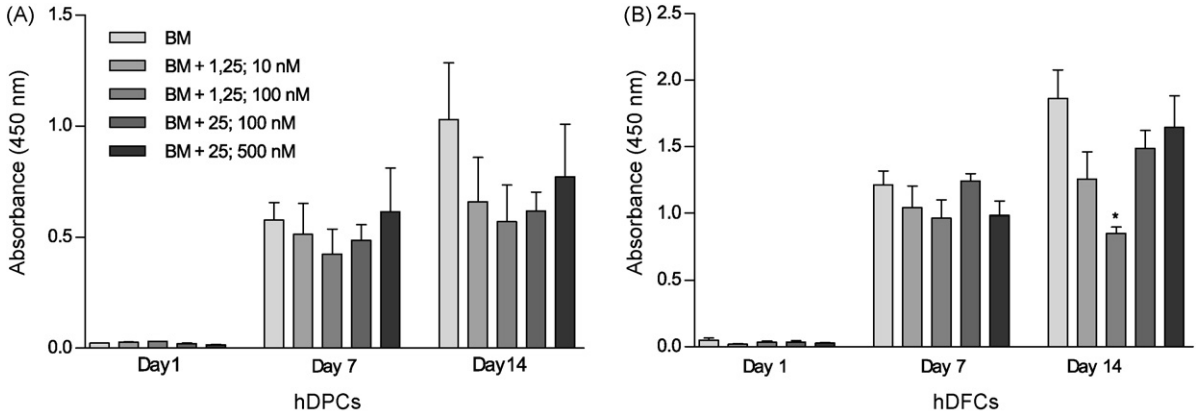

Fig. 2. Effect of $1\alpha,25(OH)_2D_3$ and $25OHD_3$ on cell proliferation. Cell numbers were analyzed in (A) hDPCs and (B) hDFCs at 1, 7 and 14 days time periods. Columns represent mean ± SEM ($n = 3$). Statistically significant difference when treated samples were compared to the control of each time point, $^*p < 0.05$.

seeded in 6 well plate and after overnight incubation at 37 °C in a 5% CO_2 humidified atmosphere different metabolites were added. After 24 h the culture media for all the samples were taken for immunoextraction following quantitation by enzyme immunoassay as per manufacturer's protocol. Samples were obtained from three different patient samples for both the tissues which were tested independently. Briefly, 100 μl of the delipidated samples and controls were added to the appropriately labeled immunocapsules in duplicates per sample. Primary antibody was added to the immunoextracts and incubated overnight at +4 °C. Next day, secondary antibody was added which was followed by the addition of enzyme conjugate and tetramethylbenzidine (TMB) substrate. An acidic stop solution was added to terminate the reaction which resulted in the color change from blue to yellow. The intensity of the yellow color is inversely proportional to the concentration of $1\alpha,25(OH)_2D_3$. The absorbance was measured within 30 min of the addition of the stop solution using a plate reader (Victor 1420) at a wavelength of 450 nm. The results were calculated by measuring the absorbance of the calibrators provided with the kit and creating a calibration curve by plotting the percent bind on the *y*-axis and the amount of $1\alpha,25(OH)_2D_3$ on the *x*-axis. The percent bind values for the samples were calculated and then interpolated amount of $1\alpha,25(OH)_2D_3$ using the calibration curve.

2.7. Statistical analysis

The statistical analyses of the results were performed with GraphPad Prism 5.01. The data is presented as mean ± standard error of the mean (SEM) for all quantitative assays and experiments were carried out in triplicate for cells derived from three donor samples. All statistical analyses were performed at the significance level $p < 0.05$. One-way analysis of variance (ANOVA) with Dunnett's post hoc test for multiple comparisons was used for the analysis.

3. Results

3.1. Cell morphology

Human DPCs and hDFCs were isolated by enzyme-digestion method. Both cell types exhibited initial triangular, stellate or spindle shape cell morphology after initial plating. The time required to form confluent cell cultures varied from 1 to 2 weeks for hDPCs and hDFCs. The hDFCs appeared spindle or stellate in shape as observed under phase contrast microscope (Fig. 1C). After first passage, the hDPCs appeared stellate in shape or some cultures formed patterns of net like structure as shown by phase contrast microscope (Fig. 1D).

3.2. Cell proliferation effect (days 1, 7 and 14)

Time course effects of different concentrations of $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) at days 1, 7 and 14 on proliferation of hDPCs and hDFCs were analyzed. The addition of vitamin D_3 metabolites, to hDPCs and hDFCs induced decrease in cell proliferation when compared to the cells treated without the metabolites, as shown in (Fig. 2A). While cell proliferation was significantly inhibited by $1\alpha,25(OH)_2D_3$ (100 nM) in hDFCs by day 14 (Fig. 2B).

3.3. Osteogenic differentiation

3.3.1. Alkaline phosphatase activity (days 7 and 14)

The effects of vitamin D_3 metabolites with or without OS on osteogenic differentiation of hDPCs and hDFCs were analyzed by measuring their ALP activities. Time course experiments showed significant increases in ALP activity of hDPCs on day 7 after treatment with OS + $1\alpha,25(OH)_2D_3$ (10 nM), OS + $1\alpha,25(OH)_2D_3$ (100 nM) and OS + $25OHD_3$ (500 nM) in comparison to cells treated without OS and the untreated control. Following day 14, ALP activity was significantly increased in cells treated with OS + $1\alpha,25(OH)_2D_3$ (100 nM), OS + $25OHD_3$ (500 nM) and OS + Dex (10 nM) in hDPCs, as shown in (Fig. 3A and B).

In addition, hDFCs treated with OS + $1\alpha,25(OH)_2D_3$ (100 nM), OS + $25OHD_3$ (500 nM) at day 7 significantly increased ALP activity. Following the 14 days time course, hDFCs expressed significant increase in ALP activity in cells treated with OS + $1\alpha,25(OH)_2D_3$ (10 nM), OS + $1\alpha,25(OH)_2D_3$ (100 nM); though cells treated without OS and OS + $25OHD_3$ (100 nM) and OS + Dex (10 nM) did not significantly increase the ALP activity of hDFCs (Fig. 3C and D). Human DPCs and hDFCs treated in combination with OS and vitamin D_3 metabolites showed significant ALP activity.

3.3.2. Mineralization

Treatment with vitamin D_3 metabolites in combination with OS promoted biomineralization of hDPCs and hDFCs as shown in (Fig. 4A and B). Human DFCs and hDPCs exposed to $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) without OS did not induce matrix mineralization (data not shown). There were differences in the intensities of alizarin red staining between cells derived from different donors.

3.3.3. The expression of bone markers at mRNA level (24, 48 and 72 h time points)

To observe the time course effect of vitamin D_3 metabolites on bone markers such as OPN and OC at mRNA level qRT-PCR was done. OC expression was up regulated by all the concentra-

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

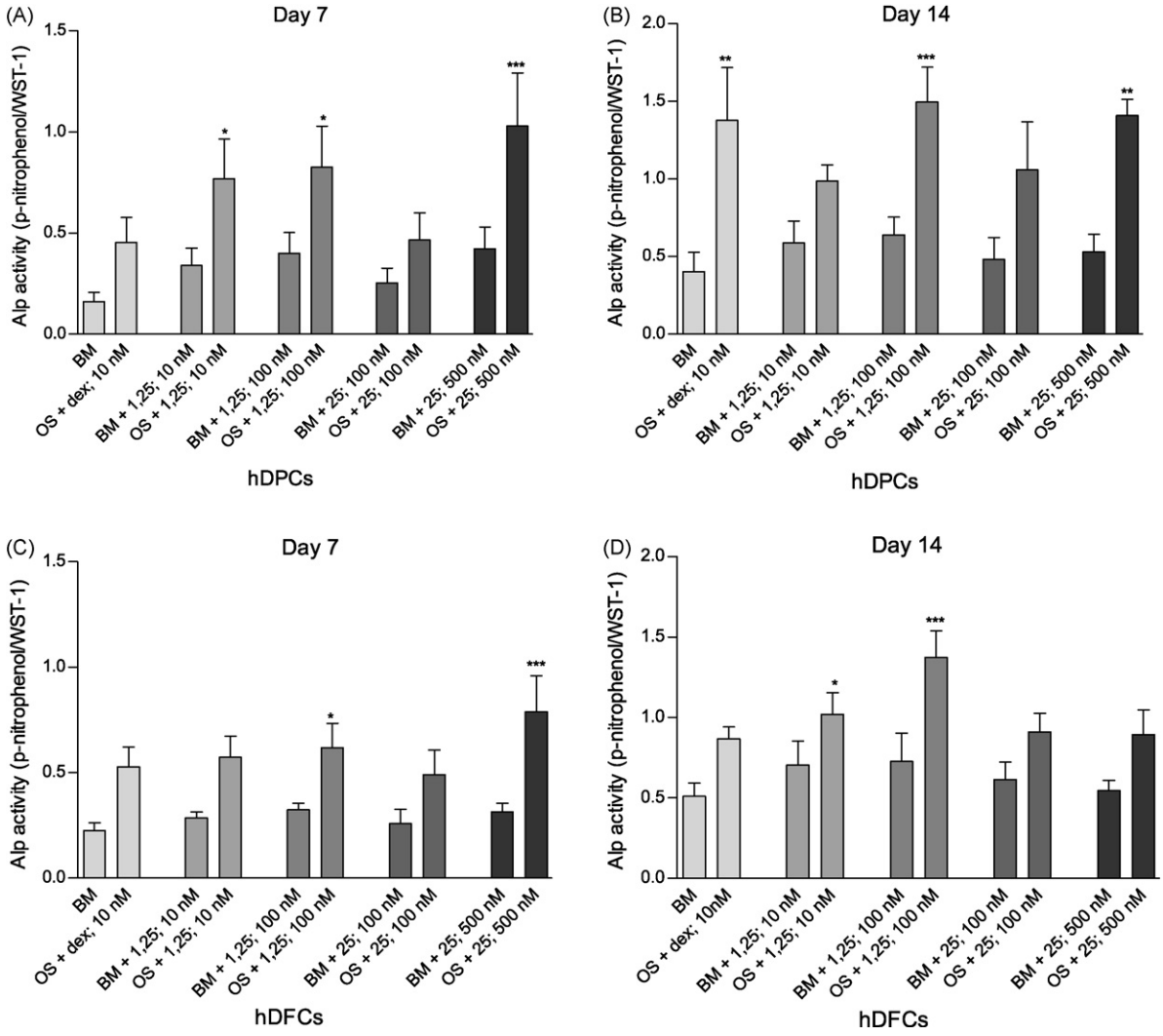

Fig. 3. ALP activity of cells cultured in $1\alpha,25(OH)_2D_3$ and $25OHD_3$ in the presence of osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) in comparison to cells cultured in Dex. ALP activity of hDPCs (A and B) and hDFCs (C and D) was assessed after 7 and 14 days of differentiation. Columns represent mean ± SEM ($n = 3$). Statistically significant difference when treated samples were compared to BM of days 7 and 14, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

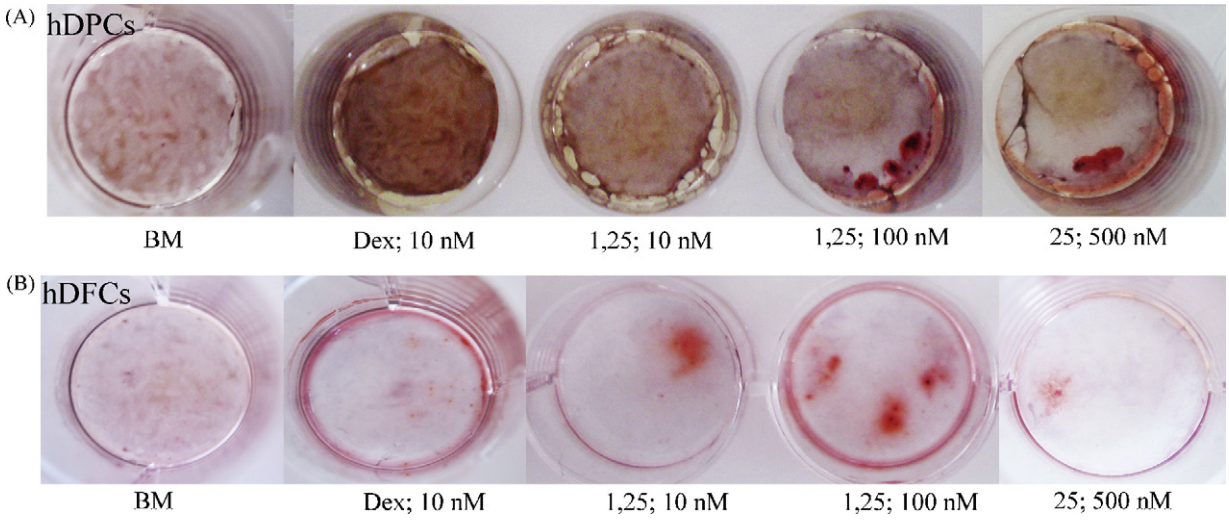

Fig. 4. Alizarin red staining of mineralized deposits after exposure to vitamin D_3 metabolites and Dex. Human DPCs (A) and hDFCs (B) were cultured in (1,25) 10 and 100 nM, (25) 500 nM and (Dex) 10 nM with osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) for 3 weeks. Data are representative of one cell culture ($n = 3$).

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

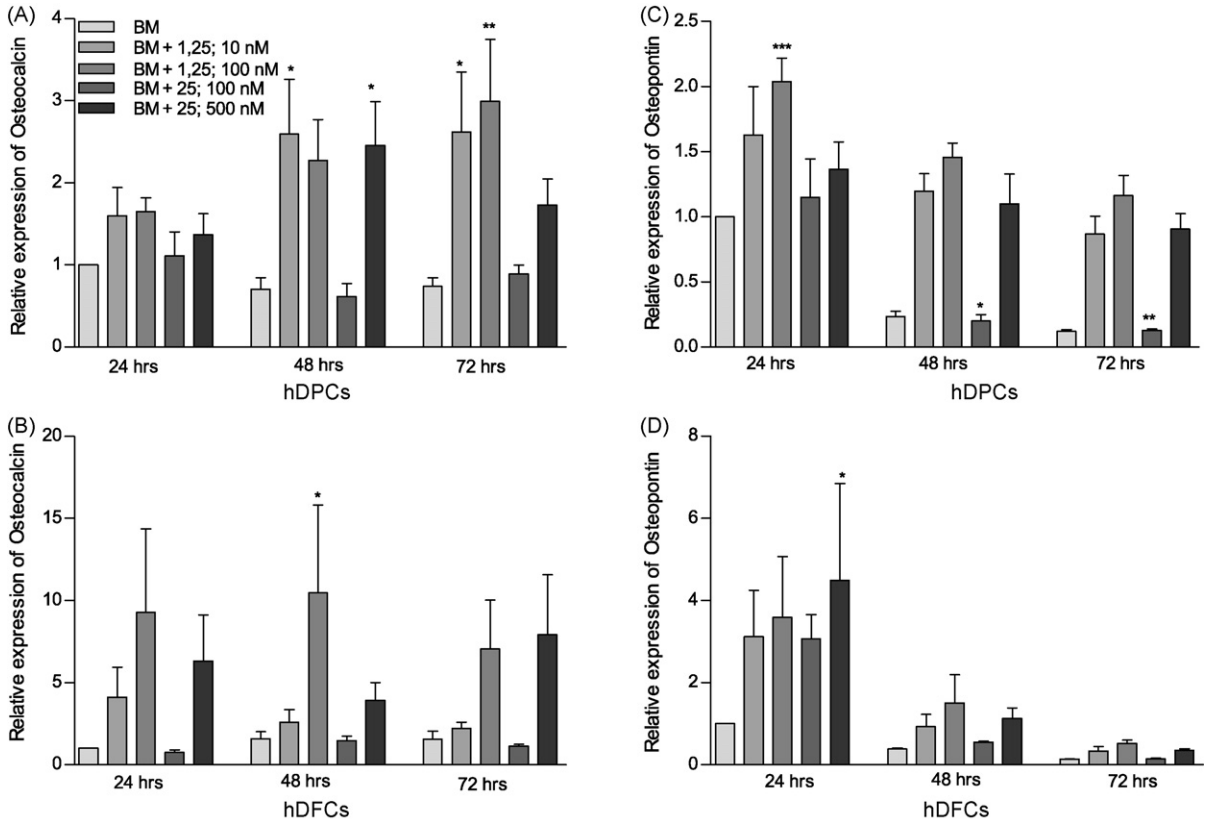

Fig. 5. Expression of bone markers were quantified by qRT-PCR. Relative mRNA expression of OC in hDPCs and hDFCs (A and B) and OPN in hDPCs and hDFCs (C and D) at 24, 48 and 72 h were analyzed. Data are normalized to housekeeping gene RPLP0. Results are reported as change in gene expression relative to untreated control (basic medium; BM) at 24 h time point. Columns represent mean ± SEM ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

tions of vitamin D_3 metabolites used except for $25OHD_3$ (100 nM) between 48 and 72 h time course in hDPCs (Fig. 5A), wherein, $1\alpha,25(OH)_2D_3$ (100 nM) alone significantly increased the expression of OC at 48 h time point in hDFCs (Fig. 5B). OPN expression was significantly upregulated by $1\alpha,25(OH)_2D_3$ (100 nM) in hDPCs and $25OHD_3$ (500 nM) in hDFCs at 24 h. Thereafter, downregulation in the expression was seen from 48 to 72 h in both hDPCs (Fig. 5C) and hDFCs (Fig. 5D).

3.4. Expression of VDR, CYP24 and $1\alpha,25(OH)_2D_3$ production

Vitamin D_3 regulating genes especially CYP24 expression was significantly upregulated by $1\alpha,25(OH)_2D_3$ (100 nM) and $25OHD_3$ (500 nM) but there was down regulation seen by $25OHD_3$ (100 nM) in hDPCs (Fig. 6A) and hDFCs (Fig. 6B). In addition, the expression of VDR was low at 24 h time point for both hDPCs and hDFCs. The VDR mRNA expression increased significantly following 48–72 h time point in the cells treated with $1\alpha,25(OH)_2D_3$ (10 and 100 nM) in both hDPCs (Fig. 6C) and hDFCs (Fig. 6D). The VDR mRNA expression was significantly upregulated by $25OHD_3$ (500 nM) concentration in hDPCs while expression was minimally regulated by $25OHD_3$ (100 nM) in both hDPCs and hDFCs. The activity of the CYP27B1 enzyme was confirmed by evaluating the conversion of $25OHD_3$ into $1\alpha,25(OH)_2D_3$ in hDPCs and hDFCs by enzyme immunoassay. $25OHD_3$ (500 nM) was converted into $1\alpha,25(OH)_2D_3$ at pM concentration by both hDPCs (Fig. 7A) and hDFCs (Fig. 7B). The conversion was significantly inhibited by cells cultured in the presence of the inhibitor. Subsequently, the mRNA expression of CYB27B1 was analyzed which was upregulated only by the higher concentration of $25OHD_3$ in both hDPCs (Fig. 7C) and hDFCs (Fig. 7D).

4. Discussion

Our study shows that osteoblast differentiation in hDPCs and hDFCs was stimulated by both $1\alpha,25(OH)_2D_3$ and $25OHD_3$. In addition, the current study provides evidence that $25OHD_3$ can be converted into $1\alpha,25(OH)_2D_3$ *in vitro* by hDPCs and hDFCs. Differentiation of MSCs such as those derived from adipose tissue, bone marrow tissue or from dental tissue into osteoblast like cells is induced *in vitro* by treating the cells with Dex, ascorbic acid and β-glycerophosphate as reported in many studies [19,30]. Interestingly, it has been reported that $1\alpha,25(OH)_2D_3$ may be superior to dexamethasone as an agent that induces osteogenic differentiation in human adipose derived cells [23,24,31]. Considering the previous reports it was reasonable to confirm the effects of vitamin D_3 metabolites such as $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) with or without the addition of osteogenic supplements (OS; L-ascorbic acid 2-phosphate and β-glycerophosphate) on proliferation and osteogenic differentiation of hDPCs and hDFCs. Osteogenic differentiation of MSCs *in vitro* is normally characterized by early expression of ALP activity, extracellular matrix mineralization and expression of osteoblasts associated genes.

Vitamin D_3 is also reported to function locally by binding with the VDR to inhibit proliferation of certain cell types such as osteoblasts and osteoclasts [32]. Our study revealed that, the addition of vitamin D_3 metabolites to hDPCs and hDFCs did not increase cell proliferation. Moreover, $1\alpha,25(OH)_2D_3$ (100 nM) inhibited cell proliferation in hDFCs. Our results are consistent with a recent study conducted on hMSCs, wherein, $1\alpha,25(OH)_2D_3$ inhibited cell proliferation of hMSCs [22]. These results also suggest that when

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

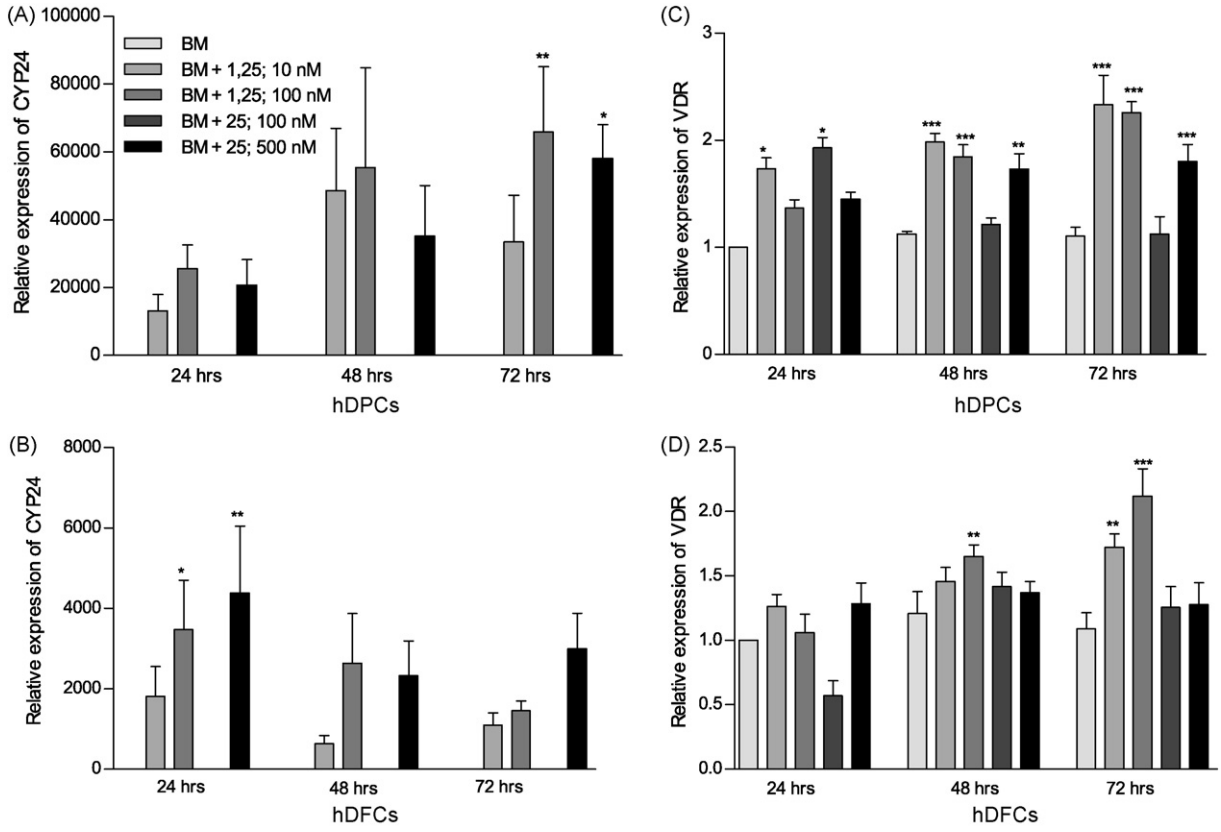

Fig. 6. Expression of vitamin D_3 regulating genes were quantified by qRT-PCR. Relative mRNA expression of CYP24 in hDPCs and hDFCs (A and B) and VDR in hDPCs and hDFCs (C and D) at 24, 48 and 72 h were analyzed. Data are normalized to housekeeping gene RPLP0. Results are reported as change in gene expression relative to untreated control (basic medium; BM) at 24 h time point. Columns represent mean ± SEM ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cells are differentiating osteogenically, cell proliferation will be inevitably suppressed.

So far studies towards the direct effects of vitamin D_3 on osteogenic differentiation of enzymatically isolated hDPCs and hDFCs have been limited. To confirm osteogenic capacity of the hDPCs and hDFCs, ALP expression was studied, which is membrane bound enzyme and is an early marker of osteogenic differentiation [33]. It was reported that the addition of $1\alpha,25(OH)_2D_3$ metabolite to OS + Dex significantly increased Alp activity of hDPCs [34]. Here, we show that $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and Dex in addition with OS increased the ALP activity in hDPCs. Additionally, for the first time we have shown that $25OHD_3$ (500 nM) also increased the ALP activity in hDPCs and hDFCs. In correlation with the ALP expression, the initiation of osteogenesis resulted in progression of mineralized matrix formation when cells were cultured in $1\alpha,25(OH)_2D_3$ (100 nM) and $25OHD_3$ (500 nM) in hDPCs. Furthermore, we observed difference in response to the treatments between patient samples in forming calcified nodules *in vitro*. The most likely reason for this could be associated with donor variability in response to various treatments. Whereas for hDFCs, previous reports suggest that, Dex + OS stimulated ALP activity and mineralization [19,35] when cells were cultured for 4 weeks [20]. However, we could not observe increase in ALP activity when cells were cultured with Dex for 2 weeks and weak response to mineralization was seen when hDFCs were cultured for 3 weeks, contrary to what is known. This discrepancy in our results can be explained by referring to the long term cultures made to observe increase in ALP activity and mineralization in the reported literature and variability between patient samples. Moreover, effect of the vitamin D_3 metabolites on ALP activity and mineralization of hDFCs has not been yet elucidated. Here we report that, hDFCs formed mineralized matrix when treated with vitamin D_3 metabolites in the presence of OS. Interestingly, vitamin D_3 metabolites had better effect on mineralization than Dex (10 nM) in combination with OS in hDPCs and hDFCs. These results highlight that vitamin D_3 could be used as an alternative to Dex for *in vitro* mineralization considering the potential catabolic effects of Dex when used *in vivo* [21].

The studies on osteoblastic cells *in vitro* have shown that $1,25(OH)_2D_3$ increases alkaline phosphatase activity [36] and stimulates the expression of some of the non-collagenous proteins of bone such as OC, which is a small protein found in abundance in bone, cementum, and dentin [36,37]. $1,25(OH)_2D_3$ has been observed to play an essential role in synthesizing OC in hDPCs and bone cells *in vitro* [37,38]. The correlation between decreased OC production and hypocalcified dentin formation due to vitamin D deficiency has also been reported in hDPCs [37]. Our results verify that OC expression was upregulated by $1\alpha,25(OH)_2D_3$ in hDFCs and hDPCs. The expression of other non-collagenous protein such as OPN, which promotes bone resorption and stimulation of bone deposition [39], is also regulated by $1,25(OH)_2D_3$ in osteoblast cells [40]. It has been shown in clonal rat dental pulp cells that OPN produced by pulp cells by action of $1\alpha,25(OH)_2D_3$ causes mineralization to form reparative dentin and pulp stones [41]. Our data shows that $1\alpha,25(OH)_2D_3$ (100 nM) upregulated expression of OPN in hDPCs at 24 h and following 72 h time course showed gradual down regulation. Moreover, the induction of OPN expression in hDFCs by $25OHD_3$ has not been reported previously.

From *in vivo* observation it is concluded that $1\alpha,25(OH)_2D_3$ is important in mineralization of dental tissues, as shown by the presence of VDR in human dental tissues such as ameloblasts and odontoblasts in mouse model [7,42]. We report here that addition of $1\alpha,25(OH)_2D_3$ (10 and 100 nM) upregulated VDR expression in hDPCs and hDFCs. Moreover, $25OHD_3$ has also been

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

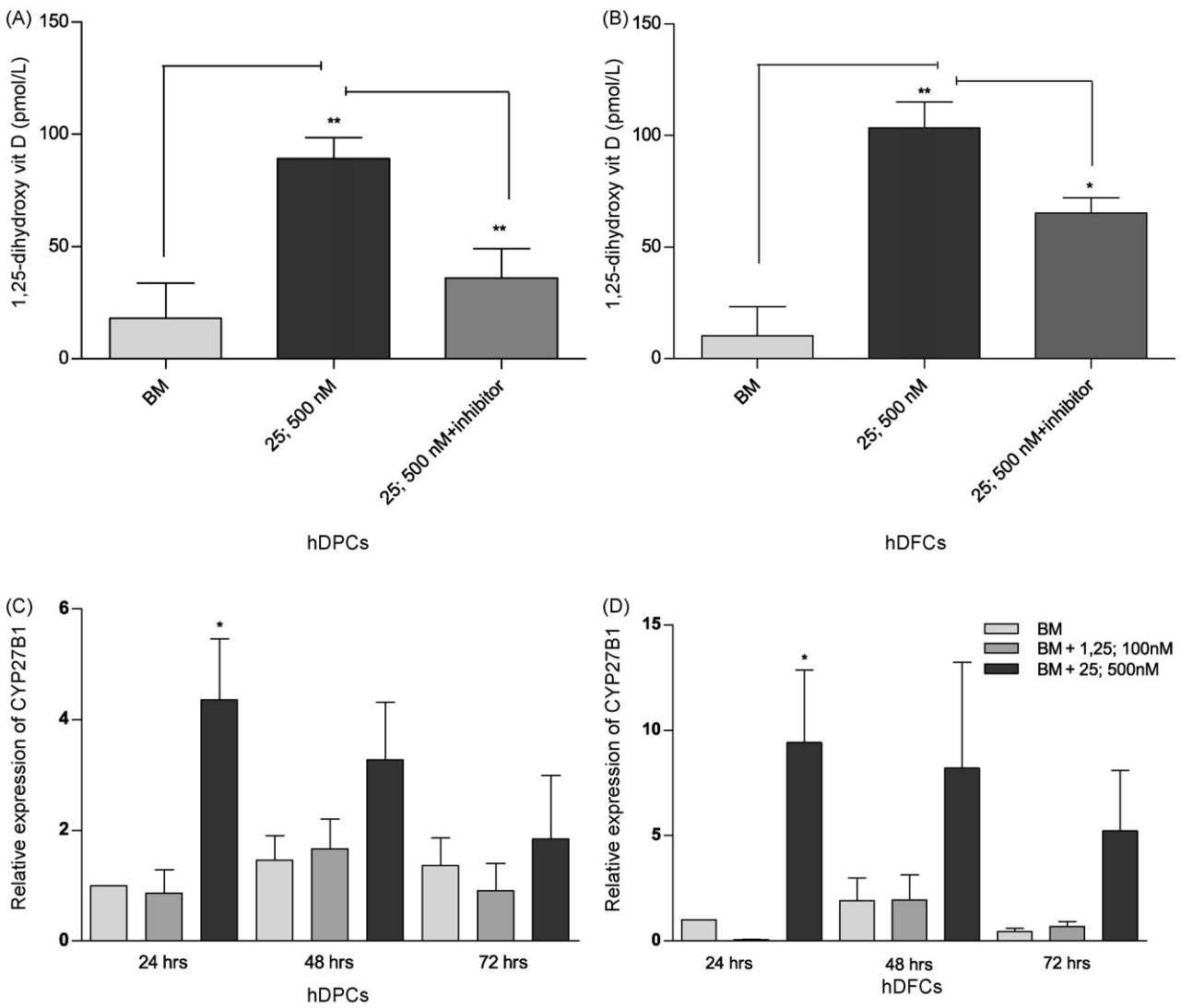

Fig. 7. Enzymatic activity and expression of CYP27B1 in hDFCs and hDPCs. Enzyme immunoassay showed the conversion of 25; 500 nM into $1\alpha,25(OH)_2D_3$ in (A) hDFCs and (B) hDPCs. The conversion was evaluated as $1\alpha,25(OH)_2D_3$ (pM) concentration. The results were considered significant when BM was compared to 25; 500 nM treated sample and 25; 500 nM treated sample was compared with 25; 500 + inhibitor. Relative mRNA expression of CYP27B1 in hDPCs and hDFCs (C and D) was analyzed. Results are reported as change in gene expression relative to untreated control (basic medium; BM) at 24 h time point. Columns represent mean ± SEM ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

reported to show biological activity through VDR, but the response is 500–1000 fold lower than $1\alpha,25(OH)_2D_3$ [43]. In this study, higher concentration of $25OHD_3$ upregulated VDR expression in hDPCs. Furthermore, the metabolic conversion of $25OHD_3$ into $1\alpha,25(OH)_2D_3$ by hDPCs and hDFCs suggested that both of these cell types express functional CYP27B1 enzyme. Further evidence of the effect of vitamin D_3 metabolites on hDPCs and hDFCs, can be described by the upregulation of CYP24, which metabolizes vitamin D_3 and thereby inactivates the conversion $25OHD_3$ into $1\alpha,25(OH)_2D_3$ [44]. The present study shows that higher concentrations of $1\alpha,25(OH)_2D_3$ (100 nM) and $25OHD_3$ (500 nM) upregulated CYP24 gene expression in hDPCs and hDFCs. These results confirm the regulation of synthesis and inactivation of $1\alpha,25(OH)_2D_3$ in hDPCs and hDFCs is similar to as shown in cells derived from bone marrow and bone cells [45,46].

A recent study reported that vitamin D_3 deficiency was treated with $25OHD_3$ which resulted in effective bone turnover in haemodialysis patients [3]. It is worthwhile to note that our studies were performed in cultures treated with $25OHD_3$ (500 nM), which resulted in osteogenic differentiation *in vitro*. Considering the report and our results we can conclude that $25OHD_3$ metabolite could be considered as a potential clinical osteogenic inducer for bone tissue engineering. This research also shows the potential of hDPCs and hDFCs as an alternative to other MSCs, obtained from extracted human third molars (i.e. wisdom teeth) with no tissue site morbidity, often discarded tissue that may be valuable source of cells for future research.

In summary, vitamin D_3 metabolites regulated the expression of vitamin D_3 regulating and bone marker genes at mRNA level in the hDPC and hDFC cultures, while increase in ALP enzyme activity was mediated by the presence of L-ascorbic acid-2-phosphate or β-glycerophosphate. This research also indicates the essentiality of the synergists to achieve osteoblastic differentiation in addition to vitamin D_3 and thereby assisting mineralization *in vitro*.

Acknowledgments

The authors thank Minna Salomäki, Anna Maija-Honkala, Miia Juntunen and Sari Sarkaniemi for their excellent technical assistance. This work was supported by Finnish Funding Agency for Technology and Innovation (TEKES), Competitive Research Funding of Pirkanmaa hospital district and University of Tampere.

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.08.001.

References

[1] M. van Driel, H.A. Pols, J.P. van, Leeuwen, Osteoblast differentiation and control by vitamin D and vitamin D metabolites, Curr. Pharm. Des. 10 (21) (2004) 2535–2555.
[2] S. Christakos, P. Dhawan, Y. Liu, X. Peng, A. Porta, New insights into the mechanisms of vitamin D action, J. Cell Biochem. 88 (4) (2003) 695–705.
[3] G. Jean, J.C. Terrat, T. Vanel, J.M. Hurot, C. Lorriaux, B. Mayor, C. Chazot, Daily oral 25-hydroxycholecalciferol supplementation for vitamin D deficiency in haemodialysis patients: effects on mineral metabolism and bone markers, Nephrol. Dial. Transplant. 23 (11) (2008) 3670–3676.
[4] M.F. Holick, Resurrection of vitamin D deficiency and rickets, J. Clin. Invest. 116 (8) (2006) 2062–2072.
[5] X. Zhang, F. Rahemtulla, P. Zhang, P. Beck, H.F. Thomas, Different enamel and dentin mineralization observed in VDR deficient mouse model, Arch. Oral Biol. 54 (4) (2009) 299–305.
[6] M.C. Farach-Carson, I. Nemere, Membrane receptors for vitamin D steroid hormones: potential new drug targets, Curr. Drug Targets 4 (1) (2003) 67–76.
[7] M. Mesbah, I. Nemere, P. Papagerakis, J.R. Nefussi, S. Orestes-Cardoso, C. Nessmann, A. Berdal, Expression of a 1,25-dihydroxyvitamin D_3 membrane-associated rapid-response steroid binding protein during human tooth and bone development and biomineralization, J. Bone Miner. Res. 17 (9) (2002) 1588–1596.
[8] K. Takeyama, S. Kitanaka, T. Sato, M. Kobori, J. Yanagisawa, S. Kato, 25-Hydroxyvitamin D_3 1alpha-hydroxylase and vitamin D synthesis, Science 277 (5333) (1997) 1827–1830.
[9] P. Anderson, B. May, H. Morris, Vitamin d metabolism: new concepts and clinical implications, Clin. Biochem. Rev. 24 (1) (2003) 13–26.
[10] G.T. Huang, S. Gronthos, S. Shi, Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine, J. Dent. Res. 88 (9) (2009) 792–806.
[11] B. Lindroos, K. Maenpaa, T. Ylikomi, H. Oja, R. Suuronen, S. Miettinen, Characterisation of human dental stem cells and buccal mucosa fibroblasts, Biochem. Biophys. Res. Commun. 368 (2) (2008) 329–335.
[12] S. Gronthos, J. Brahim, W. Li, L.W. Fisher, N. Cherman, A. Boyde, P. DenBesten, P.G. Robey, S. Shi, Stem cell properties of human dental pulp stem cells, J. Dent. Res. 81 (8) (2002) 531–535.
[13] G. Laino, A. Graziano, R. d'Aquino, G. Pirozzi, V. Lanza, S. Valiante, A. De Rosa, F. Naro, E. Vivarelli, G. Papaccio, An approachable human adult stem cell source for hard-tissue engineering, J. Cell Physiol. 206 (3) (2006) 693–701.
[14] G. Laino, R. d'Aquino, A. Graziano, V. Lanza, F. Carinci, F. Naro, G. Pirozzi, G. Papaccio, A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB), J. Bone Miner. Res. 20 (8) (2005) 1394–1402.
[15] A.R. Ten Cate, The development of the periodontium—a largely ectomesenchymally derived unit, Periodontology 2000 13 (1997) 9–19.
[16] C. Morsczeck, W. Gotz, J. Schierholz, F. Zeilhofer, U. Kuhn, C. Mohl, C. Sippel, K.H. Hoffmann, Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth, Matrix Biol. 24 (2) (2005) 155–165.
[17] S.Y. Lee, J. Lim, G. Khang, Y. Son, P.H. Choung, S.S. Kang, S.Y. Chun, H.I. Shin, S.Y. Kim, E.K. Park, Enhanced ex vivo expansion of human adipose tissue-derived mesenchymal stromal cells by fibroblast growth factor-2 and dexamethasone, Tissue Eng. A 15 (9) (2009) 2491–2499.
[18] M.M. Beloti, A.L. Rosa, Osteoblast differentiation of human bone marrow cells under continuous and discontinuous treatment with dexamethasone, Braz. Dent. J. 16 (2) (2005) 156–161.
[19] C. Morsczeck, C. Moehl, W. Gotz, A. Heredia, T.E. Schaffer, N. Eckstein, C. Sippel, K.H. Hoffmann, In vitro differentiation of human dental follicle cells with dexamethasone and insulin, Cell Biol. Int. 29 (7) (2005) 567–575.
[20] C. Morsczeck, Gene expression of runx2, osterix, c-fos, DLX-3, DLX-5, and MSX-2 in dental follicle cells during osteogenic differentiation in vitro, Calcif. Tissue Int. 78 (2) (2006) 98–102.
[21] S. Pierotti, L. Gandini, A. Lenzi, A.M. Isidori, Pre-receptorial regulation of steroid hormones in bone cells: insights on glucocorticoid-induced osteoporosis, J. Steroid Biochem. Mol. Biol. 108 (3–5) (2008) 292–299.
[22] Y. Feng, Y. Sun, W. Jia, C. Zhang, Platelet-rich plasma and 1,25(OH)(2) vitamin D (3) synergistically stimulate osteogenic differentiation of adult human mesenchymal stem cells, Biotechnol. Lett. (2010).
[23] Y.S. Zhou, Y.S. Liu, J.G. Tan, Is 1,25-dihydroxyvitamin D_3 an ideal substitute for dexamethasone for inducing osteogenic differentiation of human adipose tissue-derived stromal cells in vitro? Chin. Med. J. (Engl.) 119 (15) (2006) 1278–1286.
[24] A. Gupta, D.T. Leong, H.F. Bai, S.B. Singh, T.C. Lim, D.W. Hutmacher, Osteomaturation of adipose-derived stem cells required the combined action of vitamin D_3, beta-glycerophosphate, and ascorbic acid, Biochem. Biophys. Res. Commun. 362 (1) (2007) 17–24.
[25] M.J. Barron, S.T. McDonnell, I. Mackie, M.J. Dixon, Hereditary dentine disorders: dentinogenesis imperfecta and dentine dysplasia, Orphanet J. Rare Dis. 3 (2008) 31.
[26] J.L. Giunta, Dental changes in hypervitaminosis D, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 85 (4) (1998) 410–413.
[27] X. Tang, H. Meng, Osteogenic induction and 1,25-dihydroxyvitamin D_3 oppositely regulate the proliferation and expression of RANKL and the vitamin D receptor of human periodontal ligament cells, Arch. Oral Biol. 54 (7) (2009) 625–633.
[28] H. Agata, H. Kagami, N. Watanabe, M. Ueda, Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells, Differentiation 76 (9) (2008) 981–993.
[29] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (9) (2001) e45.
[30] G.T. Huang, K. Shagramanova, S.W. Chan, Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro, J. Endod. 32 (11) (2006) 1066–1073.
[31] Y.D. Halvorsen, D. Franklin, A.L. Bond, D.C. Hitt, C. Auchter, A.L. Boskey, E.P. Paschalis, W.O. Wilkison, J.M. Gimble, Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells, Tissue Eng. 7 (6) (2001) 729–741.
[32] J. Reichrath, Vitamin D and the skin: an ancient friend, revisited, Exp. Dermatol. 16 (7) (2007) 618–625.
[33] C. Wennberg, L. Hessle, P. Lundberg, S. Mauro, S. Narisawa, U.H. Lerner, J.L. Millan, Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice, J. Bone Miner. Res. 15 (10) (2000) 1879–1888.
[34] A. Tonomura, Y. Sumita, Y. Ando, D. Iejima, H. Kagami, M.J. Honda, M. Ueda, Differential inducibility of human and porcine dental pulp-derived cells into odontoblasts, Connect. Tissue Res. 48 (5) (2007) 229–238.
[35] Z.L. Jin, Y.K. Zhang, H.Y. Sun, Z. Lin, Y.C. Bi, Y.Z. Duan, Y. Ding, Osteogenic-related gene expression profiles of human dental follicle cells induced by dexamethasone, Acta Pharmacol. Sin. 29 (9) (2008) 1013–1020.
[36] M. van Driel, M. Koedam, C.J. Buurman, M. Roelse, F. Weyts, H. Chiba, A.G. Uitterlinden, H.A. Pols, J.P. van, Leeuwen, Evidence that both 1alpha,25-dihydroxyvitamin D_3 and 24-hydroxylated D_3 enhance human osteoblast differentiation and mineralization, J. Cell Biochem. 99 (3) (2006) 922–935.
[37] H. Shiba, Y. Uchida, K. Kamihagi, M. Sakata, T. Fujita, S. Nakamura, T. Takemoto, Y. Kato, H. Kurihara, Transforming growth factor-beta1 and basic fibroblast growth factor modulate osteocalcin and osteonectin/SPARC syntheses in vitamin-D-activated pulp cells, J. Dent. Res. 80 (7) (2001) 1653–1659.
[38] I. Titorencu, V.V. Jinga, E. Constantinescu, A.V. Gafencu, C. Ciohodaru, I. Manolescu, C. Zaharia, M. Simionescu, Proliferation, differentiation and characterization of osteoblasts from human BM mesenchymal cells, Cytotherapy 9 (7) (2007) 682–696.
[39] J. Sodek, B. Ganss, M.D. McKee, Osteopontin, Crit. Rev. Oral Biol. Med. 11 (3) (2000) 279–303.
[40] G.J. Atkins, P.H. Anderson, D.M. Findlay, K.J. Welldon, C. Vincent, A.C. Zannettino, P.D. O'Loughlin, H.A. Morris, Metabolism of vitamin D_3 in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D_3, Bone 40 (6) (2007) 1517–1528.
[41] P. Pavasant, T. Yongchaitrakul, K. Pattamapun, M. Arksornnukit, The synergistic effect of TGF-beta and 1,25-dihydroxyvitamin D_3 on SPARC synthesis and alkaline phosphatase activity in human pulp fibroblasts, Arch. Oral Biol. 48 (10) (2003) 717–722.
[42] X. Zhang, P. Beck, F. Rahemtulla, H.F. Thomas, Regulation of Enamel and Dentin Mineralization by Vitamin D Receptor, Front Oral Biol. 13 (2009) 102–109.
[43] H. Reichel, H.P. Koeffler, A.W. Norman, The role of the vitamin D endocrine system in health and disease, N. Engl. J. Med. 320 (15) (1989) 980–991.
[44] M.F. Holick, Defects in the synthesis and metabolism of vitamin D, Exp. Clin. Endocrinol. Diabetes 103 (4) (1995) 219–227.
[45] S. Zhou, M.S. LeBoff, J. Glowacki, Vitamin, D metabolism and action in human bone marrow stromal cells, Endocrinology 151 (1) (2010) 14–22.
[46] M. van Driel, M. Koedam, C.J. Buurman, M. Hewison, H. Chiba, A.G. Uitterlinden, H.A. Pols, J.P. van Leeuwen, Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells, FASEB J. 20 (13) (2006) 2417–2419.

Please cite this article in press as: R. Khanna-Jain, et al., Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells, J. Steroid Biochem. Mol. Biol. (2010), doi:10.1016/j.jsbmb.2010.08.001

Stem Cell
Research & Therapy

Khanna-Jain et al., J Stem Cell Res Ther 2012, 2:4
http://dx.doi.org/10.4172/2157-7633.1000126

Research Article Open Access

Growth and Differentiation of Human Dental Pulp Stem Cells Maintained in Fetal Bovine Serum, Human Serum and Serum-free/Xeno-free Culture Media

Rashi Khanna-Jain[1,2,3]*, Sari Vanhatupa[1,2,3], Annukka Vuorinen[1,2,3,4], George K.B. Sandor[1,2,3,7], Riitta Suuronen[1,2,3,4,5,6], Bettina Mannerstrom[1,2,3] and Susanna Miettinen[1,2,3]

[1]*Adult Stem Cells, Institute of Biomedical Technology, University of Tampere, Finland*
[2]*BioMediTech, Tampere, Finland*
[3]*Science centre, Tampere University Hospital, Tampere, Finland*
[4]*Finnish Student Health Service, Tampere, Finland*
[5]*Department of Eye, Ear and Oral Diseases, Tampere University Hospital, Finland*
[6]*Department of Biomedical Engineering, Tampere University of Technology, Finland*
[7]*Oral and Maxillofacial Surgery, University of Oulu, Oulu, Finland*

Abstract

Introduction: Dental pulp stem cells (DPSCs) are an accessible cell source with therapeutic applicability in regeneration of damaged tissues. Current techniques for expansion of DPSCs require the use of Fetal Bovine Serum (FBS). However, animal-derived reagents stage safety issues in clinical therapy. By expanding DPSCs in serumfree/xenofree medium (SF/XF-M) or in medium containing human serum (HS-M), the problems can be eliminated. Therefore, the aim of our study was to identify suitable cell culture media alternatives for DPSCs.

Methods: We studied the isolation, proliferation, morphology, cell surface markers (CD29, CD44, CD90, CD105, CD31, CD45 and CD146), stemness markers expression (Oct3/4, Sox2, Nanog and SSEA-4) and *in vitro* multilineage differentiation of DPSCs in HS-M or SF/XF-M in comparison to FBS-M.

Results: DPSCs expressed the cell surface and stemness markers in all studied conditions. The proliferation analysis of cells cultured in different HS concentrations revealed that cells isolated in 20% HS-M and passaged in 10% or 15% HS-M supported the cell growth. Direct isolation of cells in SF/XF-M did not support cell proliferation. Therefore, cells cultured in 20% HS-M were used for further SF/XF-M studies. However, proliferation of DPSCs was significantly lower in SF/XF-M when compared with cells cultured in FBS-M and HS-M. In addition, proliferation of DPSCs in SF/XF-M could be enhanced by addition of 1% HS in cell culture medium. There were differences in osteogenic, chondrogenic and adipogenic differentiation efficacy between cells cultured in FBS, HS and SF/XF differentation media. More pronounced adipogenic and osteogenic differentiation was observed in HS differentiation medium, however, in FBS-M cultured cells more effective chondrogenic differentiation was detected.

Conclusions: Our results indicate that HS is a suitable alternative to FBS for the expansion of DPSCs. The composition of SF/XF-M needs to be further optimized in terms of cell expandability and differentiation efficiency to reach clinical applicability.

Keywords: Dental pulp stem cells (DPSCs); Human serum (HS); Serumfree/xenofree (SF/XF); Cell isolation; Expansion and differentiation

Introduction

Adult mesenchymal stem cells (MSCs) isolated from bone marrow (BM) have been an important source of stem cells for stem cell based therapies for the past several years [1,2]. Besides their regenerative capacity, there are certain limitations associated with BM-MSCs such as tissue site morbidity, low cell numbers and painful procedure for procuring the tissue [3,4]; therefore, several alternative sources of MSCs have been sought. MSCs have been expanded from adipose tissue [5], skeletal muscle [6], umbilical cord [7], amniotic fluid [8], dental pulp tissue [9] and numerous other tissues [10].

Dental pulp stem cells (DPSCs) have been reported to exhibit multipotent differentiation capacity into various cell lineages such as adipocytes, osteocytes, chondrocytes, and myocytes *in vitro* [11]; including *in vivo* studies showing differentiation of DPSCs into odontoblasts [12], neural cells [13], and in cardiac repair by improving angiogenesis [14]. Moreover, there are several animal studies reporting the potential of DPSCs in regenerating bone [15,16,17] and one clinical study showing the successful use of DPSCs in bone augmentation in tooth extraction sockets [18]. Apart from their osteogenic regenerative potential, it has been reported that DPSCs display increased immunosuppressive activity when compared with BM-MSCs [19]. Because of the multipotent nature and immunomodulatory properties of DPSCs [20], they may be an important source of MSCs for stem cell based therapies.

Furthermore, for cell based therapies, experimental concerns caused due to current cell culture protocols comprising of animal derived components needs to be eliminated. There are several

***Corresponding author:** Rashi Khanna-Jain, Adult Stem Cells, Institute of Biomedical Technology, University of Tampere, Biokatu-12, 33520 Tampere, Finland, Tel: +358-4-1901789; Fax: +358-3-35518498; E-mail: rashi.khanna-jain@uta.fi

Received July 21, 2012; **Accepted** August 16, 2012; **Published** August 18, 2012

Citation: Khanna-Jain R, Vanhatupa S, Vuorinen A, Sandor GKB, Suuronen R, et al. (2012) Growth and Differentiation of Human Dental Pulp Stem Cells Maintained in Fetal Bovine Serum, Human Serum and Serum-free/Xeno-free Culture Media. J Stem Cell Res Ther 2:126. doi:10.4172/2157-7633.1000126

problems encountered by expanding and differentiating cells in Fetal Bovine Serum (FBS) which are associated with possible allergic reactions caused by FBS proteins internalized in the stem cells and risks of transmitting viruses, prions, bacteria, yeast or endotoxins upon transplantation [21,22,23]. Also, the concentration of growth factors or bulk proteins in complex FBS may vary between lot to lot [24], hence leading to difficulty in maintaining a consistent cell culture protocol. To address these problems, various alternatives have been explored by several investigators to maintain proliferation and differentiation of MSCs. Among these are human blood derived alternatives such as autologous human serum (autoHS), allogenic Human Serum (alloHS) [25], human platelet lysates [26], umbilical cord blood serum [27] and autologous plasma derived from bone marrow (AP) [28]. There are several investigations on the efficacy of alloHS, autoHS and AP as an option to FBS for BM-MSCs culture [29,30] but none for DPSCs. However, conflicting data on the use autoHS and alloHS has been reported, where the BM-MSCs proliferated at a slower rate and diminished differentiation capability was observed [31,32]. Conversely, autoHS and alloHS have been reported to maintain the proliferation and differentiation of MSCs as effectively as FBS [33,34,35]. Most recently, a study conducted *in vitro* and *in vivo* showed that HS was as efficient as FBS in supporting proliferation and differentiation of BM-MSCs [30]. In order to overcome the inconsistent performance associated with HS, a robust serumfree/ xenofree medium (SF/XF-M) for MSCs culture has to be developed. The use of chemically defined SF/XF-M could result in eliminating lot to lot variability issues, possible immune reactions and associated complications [36]. Interestingly, a study from our group has shown the ability of adipose tissue derived mesenchymal stem cells (AD-MSCs) to maintain the multipotent differentiation capacity and to proliferate better in SF/XF conditions in comparison to HS and FBS culture conditions [37], whereas, there are lack of reports related to the response of SF/XF medium on DPSCs. However, the effect of different serum free media comprising of xenogenic growth factors on DPSCs proliferation or colony formation have been reported [38].

Taken together, it is important to elucidate the effect of HS and SF/XF media on DPSCs isolation and expandability before using them for clinical therapies. In this study, DPSCs were expanded in HS or SF/XF media by using xenofree supplements to limit the possibility of xenogenic contaminations. We studied the morphology, cell surface marker expression and proliferation rate of the DPSCs cultured in FBS, HS or SF/XF media. Subsequently, we studied expression of cell surface stage-specific embryonic antigen (SSEA)-4 as well as intracellular stemness markers octamer-binding transcription factor (Oct3/4), SRY (sex determining region Y) box-2 (Sox2) and Nanog to further analyze the stemness of DPSCs cultured in different media. Moreover, we investigated the multilineage differentiation potential of DPSCs into osteogenic, adipogenic and chondrogenic lineages in different culture conditions.

Material and Methods

Cell isolation and culture

Human impacted third molars were obtained with informed written consent from Finnish Student Health Services, Tampere, Finland. The Ethics Committee, of the Pirkanmaa Hospital District, Tampere, Finland (R06009), approved the collection of stem cells from tooth samples specifically for this study. Human dental pulp explants were obtained from partially or completely impacted third molar teeth of 4 patients, aged 21-26 years (23 ± 2.5 years). The pulp tissue explants were brought from the health centre to the laboratory in Dulbecco's Phosphate buffered saline (PBS; BioWhittaker Lonza, Verviers, Belgium) containing 2% antibiotics/antimycotics (a/a; 100 U/ml penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B; Life Technologies, Paisley, Scotland, UK). The dental pulp tissue fragments were minced by using sterilized scalpels and digested in collagenase type I 3 mg/ml (Invitrogen) and dispose 4 mg/ml (Invitrogen) for 1 hour at 37°C. Once digestion was completed the obtained cell pellet was suspended in 600 µl of PBS and was passed through a 100 µm cell strainer (Falcon, BD Labware, Franklin lakes, NJ, USA). The isolated dental pulp stem cells (DPSCs) were cultured in two different media (1) Dulbecco's modified Eagle medium (DMEM)/F-12 1:1 (Gibco Life Technologies, Paisley, UK) supplemented with 1% l-analyl-l-glutamine (Gluta-MAX I; Life Technologies), 1% a/a and 10% fetal bovine serum (FBS; Invitrogen, Paisley, UK) (FBS-M) and (2) (DMEM)/F-12 1:1 supplemented with Gluta-MAX I, 1% a/a and 20% allogenic Human Serum (HS; PAA Laboratories GmbH, Pasching, Austria) (HS-M).

Further, DPSCs expanded in HS-M were used for testing StemPro® MSC xenofree, serumfree/xenofree medium (SF/XF-M; Life Technologies), where culture wells were coated with CELLstart (Life Technologies) to assist in cell attachment (Table 1). Initially, DPSCs were directly cultured in SF/XF-M on carboxyl, amine (BD Biosciences) or CELLstart coated culture plates but cells did not proliferate; therefore, cells cultured in HS-M were later cultured in SF/

Medium	Abbreviation	Basal medium	Serum	Xenofree	Coating	Supplementation
Fetal Bovine Serum-Medium	FBS-M	DMEM/F-12	10% FBS	No	No	1% GlutaMAX, 1% a/a
Human Serum-Medium	HS-M	DMEM/F-12	20% HS	Yes	No	1% GlutaMAX, 1% a/a
Serum Free/ XenoFree-Medium (Stem Pro® MSC SFM XenoFree)	SF/XF-M	Stem Pro® MSC SFM xenofree	No	Yes	CELLstart (Life Technologies)	Stem Pro® MSC SFM Xeno Free supplement, 1% a/a

Table 1: Different culture media to test the growth of DPSCs. Dulbecco's modified eagle medium (DMEM/F-12) containing fetal bovine serum (FBS-M), human serum (HS-M) and serumfree/xenofree (SF/XF-M).

XF medium for all the experiments, as described in (Figure 1). DPSCs isolated and expanded in FBS-M were harvested using 1% trypsin (Lonza/BioWhittaker, Verviers, Belgium). The DPSCs isolated and cultured in HS-M and SF/XF-M were harvested using TrypLE Select (Life Technologies) for XF detachment of cells. Cell culture plates and T-75 culture flasks (Thermo Fischer, Nunc; Roskilde, Denmark) were monitored daily for cell growth, with medium changes taking place three times per week (Table 1). All assays were performed using cells between passage 3-4 and experiments were repeated using DPSCs derived from 4 different donors.

Immunocytochemistry

For immunocytochemistry, 2500 cells/ well were plated on 48 well plates. After 3 days of culturing, cells were fixed with 4% paraformaldehyde (Fluka, Italy) containing 0.2% of TritonX-100. After fixation, cells were stained with stemness markers. Briefly, unspecific staining was blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 45 min at room temperature. The cells were washed 2-3 times with PBS. Thereafter, the primary antibodies; goat anti-octamer-binding transcription factor (Oct) 3/4, mouse anti-SRY (sex determining region Y) box-2 (Sox2) and goat anti–Nanog (all: R&D Systems) as well as mouse anti-stage specific embryonic antigen (SSEA)-4 (Santa Cruz) in 1% BSA-PBS solution were incubated with cells at +4°C, overnight. Next day, the cells were washed three times with PBS and were incubated in secondary antibodies, Alexa Fluor 488 and 568 conjugated to anti-goat and anti-mouse (Molecular Probes, Invitrogen) in 1% BSA-PBS for 1 h at room temperature. Finally, cells were washed three times with PBS, twice with water and mounted with Vectashield containing 4', 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, England) to identify nuclei. Cell samples were analyzed by using an Olympus IX51 phase-contrast microscope equipped with fluorescence unit and an Olympus DP30BW camera (Olympus).

Flow cytometric surface marker expression analysis

DPSCs cultured in FBS-M, HS-M and SF/XF-M and were analyzed for cell surface antigen expression by flow cytometry (FACSAria®; BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies (MAb) against CD29-Allophycocyanin (APC), CD44-Phycoerythrin (PE), CD90-APC, CD45-APC, CD146-PE (BD Biosciences), CD105-PE (R&D Systems Inc., Minneapolis, MN, USA), CD31-fluorescein isothiocyanate (FITC) (Immunotools GmbH, Friesoythe, Germany), and major histocompatibility class II antigen (HLA-DR)-PE (Immunotools) were used. FACS analysis was performed on 100,000 cells/sample and the positive expression was defined as the level of fluorescence greater than 99% of the corresponding unstained cell sample.

Cell proliferation assay

This assay was done to measure the viability and induction of DPSCs proliferation when cultured in FBS-M, HS-M in different serum conditions (5%, 10%, 15%, 20%), SF/XF-M or SF/XF-M with 1 or 5% of HS. The DPSCs (n=4) were seeded on a 48 or 24-well plate at a density of 2500 or 5000 cells/well, depending on well format. The SF/XF-M culture wells were pre-coated with CELLstart. Cell proliferation was quantified at 1, 4, 7 and 14 days using the colorimetric reagent WST-1 (Takara Bio Inc, Otsu, Japan) for comparing the effect of FBS-M, 15% HS-M and SF/XF-M cultured cells. Briefly, WST-1 reagent was added to each well containing fresh medium (50 µl of WST-1/ 500 µl of medium in each well of 24-well plate), incubated for 60 min. For HS serum concentration gradient (5%, 10%, 15%, 20%) growth assay (Figure 5B) and for comparative SF/XF and SF/XF+HS (1% and 5%) growth assay (Figure 5C) cells were washed with PBS and 20 µl of WST-1 reagent in addition to 200 µl PBS was added to each well. The plate was incubated for 4 hours at 37°C prior to the measurement. The absorbance was measured at 450 nm using a microplate reader Victor 1420 (Perkin Elmer life Sciences, Turku, Finland).

In vitro multilineage differentiation analysis

DPSCs (n = 4) were examined for their ability to differentiate toward the adipogenic, osteogenic and chondrogenic lineages by quantitative real time-polymerase chain reaction (qRT-PCR) and lineage specific stainings. Briefly, for osteogenic and adipogenic differentiation analysis cells were seeded at a density of 5000 cells/well on a 24 well plate in FBS-M, HS-M or SF/XF-M. After 24 hours, osteogenic differentiation medium (OM) and adipogenic differentiation medium (AM) were added for each serum culture condition as stated in Table 2. The chondrogenic differentiation of DPSCs was assessed by using micromass cell culture method. Briefly, 100,000 cells were seeded on a 24 well plate in a 10 µl volume of FBS-M, HS-M or SF/XF-M, that were let to adhere for 3 hours in an incubator prior to the addition of chondrogenic differentiation medium (CM) as described in Table 2. For all the analyses the control cultures were maintained in FBS-M, HS-M or SF/XF-M. The SF/XF-M culture wells were pre-coated with CELLstart for osteogenic and adipogenic differentiation while for chondrogenic micro mass aggregate formation, the culture wells were not coated. All cultures were maintained for 21 days for the differentiation analysis.

Alizarin red staining

In vitro mineralization was induced by FBS-OM, HS-OM or SF/XF-OM and was analyzed by alizarin red staining after 21 days. Briefly, cells were fixed in ice-cold 70% ethanol for 60 min at -20°C. Then, cells were washed twice with distilled water and stained with 40 mM Alizarin red S solution (Sigma) for 10 min at room temperature. The pH value of the solution was adjusted to 4.2 with 25% ammonium hydroxide prior to staining. After staining, excess dye was washed with distilled water and digital images of stained mineral deposits were taken.

Alcian blue staining

After 21 days of culture, the chondrogenically induced micro

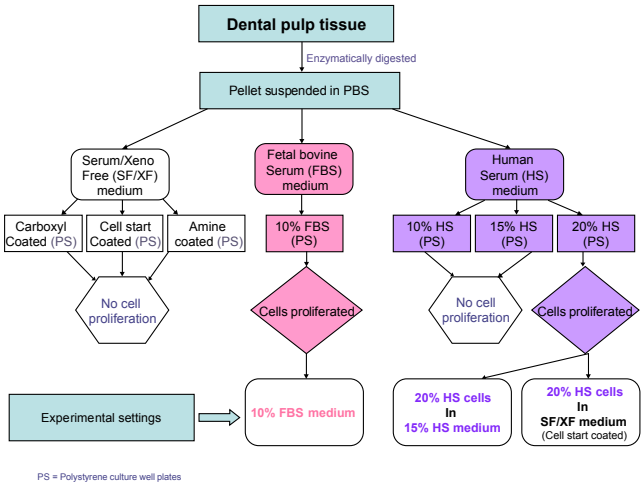

Figure 1: Flowchart representing effects of different cell culture media on isolation of dental pulp stem cells (DPSCs) and showing the final experimental settings.

masses were fixed in 4% paraformaldehyde (PFA) for 60 mins. The micro masses were then embedded in paraffin, and sectioned at a thickness of 5 µm for histological evaluation. The undifferentiated and differentiated micro masses sections were stained with 0.5% Alcian blue stain and counterstained with Nuclear Fast Red solution (Biocare Medical, Concord, MA, USA). The stained micromass sections were viewed under the microscope to evaluate the proteoglycan content.

Oil O Red staining

DPSCs were stained with 0.3% Oil Red O-solution to detect the accumulation of extracellular lipid droplets after 21 days of culture in FBS-AM, HS-AM and SF/XF-AM. Briefly, cells were fixed with 4% PFA for 60 mins. Further the cells were rinsed with distilled water and incubated in 60% isopropanol for 5 mins. Thereafter, the cells were stained with Oil O red solution for 15 mins at room temperature. Following the staining the wells were washed thoroughly to remove the excess stain and microscopic images were taken.

QRT-PCR

The cell culture conditions were same as described above in *in vitro* multilineage differentiation analysis. The total RNA was extracted at 21 days time point by Eurozol (Euroclone S.p.A, Pero, Italy). First-strand cDNA syntheses were performed by a High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Quantitative RT-PCR was conducted using RPLP0 (human acidic ribosomal phosphoprotein) as the house keeping gene and lineage specific primers such as for osteogenic differentiation: osteocalcin (OCN) and osteopontin (OPN), chondrogenic differentiation; SRY (Sex determining Region Y)-box 9 (SOX9) and Type X collagen alpha-1 (COL10A1) and adipogenic differentiation; fatty acid binding protein4 (aP2) and human peroxisome proliferator-activated receptor gamma (hPPARG) and stemness markers; Oct3/4, Sox2 and Nanog (Table 3). To exclude signals from contaminating DNA, the forward and reverse sequence of each primer were designed on different exons. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for quantitative PCR reactions according to the manufacturer's instructions. The reactions were performed with AbiPrism 7300 Sequence Detection System (Applied Biosystems) at 95°C 10 min, and then 45 cycles at 95°C /15 s and 60°C /60 s. The Ct values for OCN, OPN, SOX9, COL10A1, AP2 PPARG, Oct3/4, Sox2 and Nanog were normalized to that of the housekeeping gene RPLP0, as described elsewhere [39].

Statistical analysis

The statistical analyses of the results were performed with GraphPad Prism 5.01. The data is presented as mean ± standard deviation (SD) for all quantitative assays and experiments were carried out in triplicate for cells derived from three donor samples. One-way analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons was used for the statistical analysis. All statistical analyses were performed at the significance level $p < 0.05$.

Results

Derivation of DPSCs in different culture conditions

DPSCs were enzymatically isolated from dental pulp tissue of healthy individuals and suspended in PBS. The isolated dental pulp cells suspension in PBS was used to test the effect of different media on cell culture. We found that the cells directly isolated on carboxyl, CELLstart and amine coated culture wells in SF/XF medium, did not proliferate. Additionally, cells directly isolated in 10% or 15% HS-M did not proliferate; therefore, cells were isolated in 20% HS-M. However, after first passage, 15% HS-M supported DPSCs expansion. In addition, cells isolated in 20% HS-M were able to proliferate in SF/XF-M medium, thus, we were able to maintain xenofree conditions for cell culture. DPSCs directly isolated in 10% FBS-M proliferated. Based on these findings, isolated dental pulp cells suspended in PBS were directly divided and cultured in 1) 10% FBS and 20% HS-M. Further, 20% HS-M cultured cells were expanded in 2) 15% HS-M and in 3) SF/XF-M constituting of our final experimental settings as described in (Figure 1).

Medium	Basal Media	Serum	Supplementation
Control (FBS-M, HS-M, SF/XF-M)	DMEM/F-12, SF/XF-M	10% FBS or 15% HS or no serum	None
Osteogenic Medium (OM; FBS-OM, HS-OM, SF/XF-OM)	DMEM/F-12, SF/XF-M	10% FBS or 15% HS or no serum	50 µM L- ascorbic acid (Sigma), 10 mM beta glycerophosphate (Sigma), 100nM 1,25 hydroxy Vitamin D_3 (VD; Sigma), 1% a/a, 1% GlutaMAX
Adipogenic Medium (AM; FBS-AM, HS-AM, SF/XF-AM)	DMEM/F-12, SF/XF-M	10% FBS or 15% HS or no serum	33 µM biotin (Sigma) 1µM dexamethasone (Sigma), 100 nM insulin (life technologies), 17 µM pantothenate (Fluka), 1% GlutaMAX, and 1% a/a. Upon seeding of cells, 250 µM of isobutylmethylxanthine (IBMX; Sigma) was added for 72 hours
Chondrogenic Medium (CM)	DMEM/F-12, SF/XF-M	No serum	Insulin Transferrin-Selenium+1 (Sigma), 50 µM L- ascorbic acid, 55µM sodium pyruvate (Life Technologies), 23 µM L-proline (Sigma), 1% GlutaMAX and 1% a/a. TGF-β1 (Sigma)

Table 2: Lineage specific differentiation induced by media supplements.

Gene Name	5'-sequence-3'	Product size	Accession number
RPLP0	Forward AATCTCCAGGGGCACCATT Reverse CGTTGGCTCCCACTTTGT	70	NM_001002
OCN	Forward AGCAAAGGTGCAGCCTTTGT Reverse GCGCCTGGGTCTCTTCACT	63	NM_000711
OPN	Forward GCCGACCAAGGAAAACTCACT Reverse GGCACAGGTGATGCCTAGGA	71	J04765
SOX9	Forward AAAGGCAACTCGTACCCAAATTT Reverse TGATTGGCCACAAGTGGGTAA	75	NM_000346
COL10A1	Forward CACGCAGAATCCATCTGAGAATAT Reverse GTTCAGCGTAAAACACTCCATGAA	92	NM_000493
PPARG	Forward CAGTGTGAATTACAGCAAACC Reverse ACAGTGTATCAGTGAAGGAAT	100	NM_015869
AP2	Forward GGTGGTGGAATGCGTCATG Reverse CAACGTCCCTTGGCTTATGC	71	NM_001442
OCT3/4	Forward GACAGGGGGAGGGGAGGAGCTAGG Reverse CTTCCCTCCAACCAGTTGCCCAAAC	118	NM_002701
SOX2	Forward GGGAAATGGGAGGGGTGCAAAAGAGG Reverse TTGCGTGAGTGTGGATTGGTG	125	NM_003106
NANOG	Forward AAAGAATCTTCACCTATGCC Reverse GAAGGAAGAGGAGAGACAGT	111	NM_024865

Table 3: Primers sequence for quantitative RT-PCR.

Stemness marker expression

DPSCs isolated in 20% HS culture medium were analyzed by immunocytochemical staining with Oct3/4 and Sox2 markers (Figure 2). Oct3/4 and Sox2 were equally expressed in all samples depicting the stemness potential of DPSCs cultured in HS-M. Additionally, when nuclear (Dapi) and stemness markers (Oct3/4, Sox2) stainings were merged they resulted in nearly complete overlap, indicating that majority of DPSCs are Oct3/4 and Sox2 positive (Figure 2A-F). The expression of stemness markers Nanog and SSEA-4 was also studied in SF/XF, HS and FBS culture conditions by immunostaining and results indicate that DPSCs also express Nanog and SSEA-4 (Supplementary Figure 1).

The mRNA expression of Oct3/4, Sox2 and Nanog were analysed in cells cultured in SF/XF, HS and FBS media by qRT-PCR. Results shown in Figure 2G suggest that stemness markers were expressed at mRNA level; however, Oct3/4 was significantly upregulated ($p < 0.05$) in FBS and HS media cultured cells when compared with SF/XF-M cultured cells. Moreover, no significant differences in Sox2 and Nanog expression were observed between different cell culture conditions.

Morphological differences

The morphology of human DPSCs expanded in FBS-M, HS-M or SF/XF-M was compared by using phase contrast microscopy. Cells cultured in FBS appeared broader and flattened in shape, whereas cells cultured in HS-M were more fibroblastic and appeared more homogenous. Moreover, cells expanded in SF/XF-M exhibited a more flattened fibroblastic like morphology (Figure 3). Similar morphological differences were observed by FACS analysis, DPSCs cultured in FBS-M, HS-M or SF/XF-M differed in cell size and granularity as assessed by the forward and side scatter (Figure 4A). The cells cultured in FBS-M displayed larger cell size and greater heterogeneity. On the other hand, cells cultured in HS-M and SF/XF-M were more homogenous and smaller in cell size.

Cell surface marker expression

Furthermore, DPSCs expanded in different culture media were analyzed using flow cytometry for mesenchymal markers CD29, CD44, CD90 and CD105; hematopoietic and angiogeneic markers CD31, CD45, CD146 and for HLA-DR. FACS analysis displayed that DPSCs cultured in different media showed positive expression for the mesenchymal marker (>50%) and for CD146 perivascular marker (Figure 4A and 4B). However, results related to CD146 marker expression varied between patient samples. Moderate expression (<30%) of CD45 was observed but DPSCs lacked the expression for CD31 hematopoietic marker. In addition, DPSCs cultured in FBS-M, HS-M and SF/XF-M lacked the expression of HLA-DR (Figure 4C and 4D). Also, there were no statistical significant differences (Figure 4B and 4D) observed between the expression profile of cells cultured in FBS-M, HS-M and SF/XF-M due to variability between patient samples.

Cell proliferation

The effects of FBS-M, HS-M and SF/XF-M on DPSCs growth were analyzed following days 1, 4, 7 and 14. The cells cultured in SF/XF-M proliferated slowly in comparison to the cells cultured in FBS and HS medium, which was observed from day 4. Statistical analysis revealed that cells cultured in FBS-M and HS-M proliferated significantly faster than cells cultured in SF/XF-M at day 7 to day 14 ($p < 0.001$) (Figure 5A). Moreover, no significant differences were observed between cells cultured in FBS-M and HS-M.

In Figure 5B, we have shown the effect of 5%, 10%, 15% and 20% HS concentrations on passaged DPSCs proliferation after initial isolation of cells in 20% HS. At day 7, the cells cultured in 10%, 15% and 20% showed significant increase ($p < 0.001$) in cell numbers when compared with 5% HS cultured cells. Interestingly, on day 14 there was significant increase in cell proliferation in cells cultured in 15% HS ($p < 0.001$) when compared with 10% HS, and even higher cell proliferation was observed in 20% HS cultured cells ($p < 0.001$). Moreover, proliferation of cells cultured in 5% HS concentration was significantly slower in comparison to 10%, 15% and 20% ($p < 0.001$).

Furthermore, as shown in Figure 5A, SF/XF-M resulted in slower cell proliferation in comparison to the FBS-M and HS-M. Therefore, we speculated to see better cell proliferation by the addition of HS in the SF/XF-M. The results in Figure 5C, show that at day 4 the cells cultured in SF/XF-M + 5% HS significantly increased proliferation ($p < 0.001$) in comparison to other two conditions. However, SF/XF-M + 1% HS significantly increased cell proliferation following day 7 ($p < 0.001$) and 14 ($p < 0.05$), in comparison to SF/XF-M alone and SF/XF-M + 5% HS.

Osteogenic differentiation

The DPSCs were induced to differentiate towards osteogenic lineage with the addition of vitamin D_3 in the osteogenic medium for 21 days. The alizarin red staining results showed that DPSCs cultured in FBS-OM and HS-OM formed mineralized matrix, however, the calcified matrix staining was more pronounced in cells cultured in HS-OM. On the other hand, cells cultured in SF/XF-OM did not mineralize and the cell proliferation was also very slow as observed upon microscopical analysis (Figure 6A). Afterwards, the osteoblast genes expression pattern for OCN and OPN in DPSCs cultured in FBS-OM, HS-OM and SF/XF-OM were analyzed at mRNA level (Figure 6B and 6C). Similar to the staining results, the cells cultured in FBS-OM ($p < 0.05$) and HS-OM ($p < 0.05$) upregulated OCN levels when compared with cells cultured in FBS-M. Cells cultured in SF/XF-OM regulated OCN expression at a very low level non-significantly. Moreover, OPN expression was upregulated by cells cultured in FBS-OM ($p < 0.01$). Even though OPN levels were upregulated by cells cultured in HS-OM, the results were non-significant due to variability in expression levels between patient samples.

Chondrogenic differentiation

Chondrogenesis was estimated after staining the micro masses cultured in CM after 21 days, with Alcian blue stain, which stains the proteoglycan rich extracellular matrix. Cells isolated in FBS, HS or SF/XF media differentiated into chondrocytes-like cells when they were cultured in chondrogenic medium, however, more pronounced proteoglycan rich matrix was produced by cells isolated in FBS-M (Figure 7A). Following the staining, the mRNA expression of cells cultured in CM was analyzed. DPSCs isolated in FBS significantly upregulated SOX9 expression ($p < 0.001$), when the aggregates were differentiated in CM in comparison to undifferentiated aggregates cultured in FBS-M (Figure 7B). Moreover, expression of hypertrophic cartilage marker; Type X collagen was significantly increased in cells cultured in FBS ($p < 0.001$) and HS ($p < 0.05$) when they were induced with chondrogenic medium (Figure 7C).

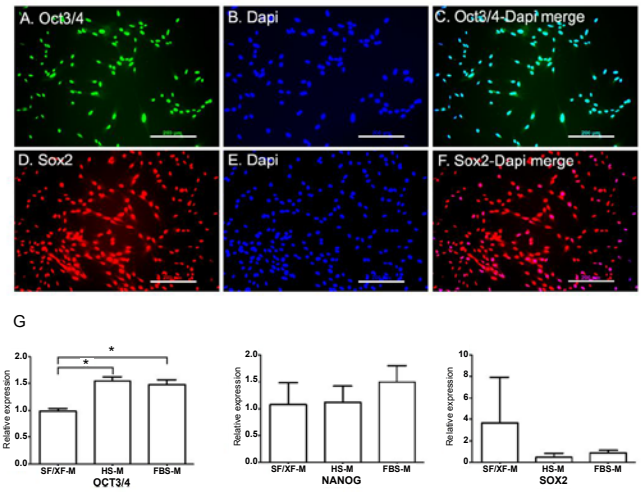

Figure 2: Stemness markers Oct3/4, Sox2 and Nanog are expressed in DPSCs. Immunostainings of intracellular stemness markers Oct3/4 (A), Sox2 (D) indicator of nuclei DAPI (B, E) and merged images of Oct3/4 and Dapi (C) and Sox2 and Dapi (F). Scale bar =200 µm. Gene expression of stemness markers were analyzed by qRT-PCR in different media, SF/XF-M, HS-M and FBS-M (G). Data represents the mean ± SD and are normalized to housekeeping gene RPLP0. (n = 4, *p < 0.05, **p< 0.01 ***p< 0.001).

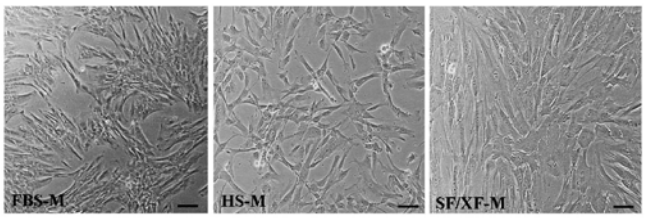

Figure 3: Morphology of DPSCs culture in (A) FBS-M were broader and flattened in shape (B) in HS-M cells appeared spindle-shaped and (C) in SF/XF-M cells appeared flattened fibroblastic. Scale bar =100 µm. DPSCs: dental pulp stem cells; FBS-M: fetal bovine serum medium; HS-M: human serum medium; SF/XF-M: serumfree/xenofree medium.

Adipogenic differentiation

Differentiation into adipocytes was analyzed after 21 days of culturing the cells in AM by Oil Red O staining. DPSCs cultured in HS-AM had stronger capacity to differentiate into adipocytes than cells cultured in FBS-AM and SF/XF-AM. These results were assessed based on the higher number of accumulated lipid droplets. The potential of DPSCs to differentiate into adipocytes was also observed when the cells were cultured in HS-M without the addition of adipogenic differentiation supplements; however, DPSCs formed very few lipid droplets. Evident morphological differences were observed in cells differentiated in SF/XF-AM showing lipid vacuoles and rounded cell shape in comparison to cells cultured in control SF/XF-M (Figure 8A). Subsequently, the mRNA expression of adipogenic markers AP2 and hPPARG were analyzed and the results showed that although expression of both the markers were upregulated by cells cultured in FBS-AM, no statistically significant difference was found in comparison to cells cultured in FBS-M (Figure 8B and 8C). Moreover, DPSCs cultured in HS-AM significantly up regulated the expression of both AP2 and hPPARG ($p < 0.001$, $p < 0.01$) in comparison to cells cultured in FBS-M. On the other hand, cells differentiated in SF/XF-AM and undifferentiated cells in SF/XF-M showed similar expression of both the markers despite of morphological differences.

Discussion

DPSCs obtained from impacted third molar teeth have been studied extensively for their excellent proliferation and multipotentional differentiation capacity [13-15,40,41]. There are several promising investigations describing the role of human DPSCs for mineralized tissue regeneration, advancing their therapeutic relevance as a valuable stem cell source [42-44]. However, in order to facilitate the translation of DPSCs from basic biology to clinical application, the development of appropriate cell culture protocols is a relevant and critical factor. Most commonly, DPSCs are cultured in FBS, which poses risk of transferring infections and induction of immune reactions upon transplantation [23,24]. With respect to the immuno pathogenic risks posed due to addition of FBS in cell culture, HS has been considered to be a safer alternative excluding the transfer of animal derived infections and related immunogenic reactions. But results related to MSCs cultured in HS are contradictory, some studies have reported successful isolation and differentiation of MSCs [30,35], whereas, others have observed slower cell proliferation or even growth arrest [22,31]. However, there is lack of information on the effect of HS in cultivation of DPSCs. In addition, other culture conditions comprising of chemically defined SF/XF media have been researched that might serve as a better alternative and would bring about effective proliferation without

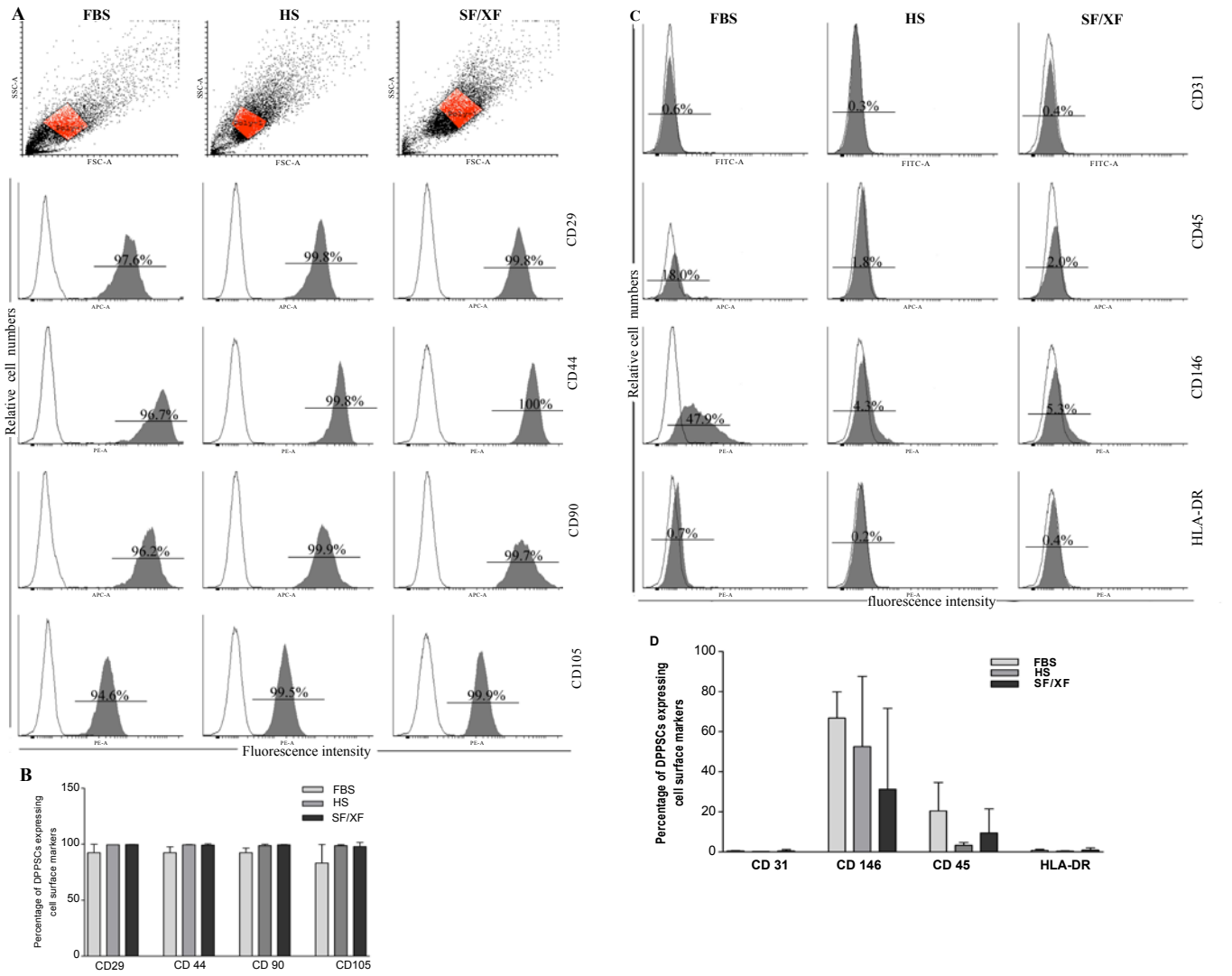

Figure 4: Surface marker expression of DPSCs cultured in FBS-M, HS-M and SF/XF-M was analyzed by flow cytometry. (A,C) Histograms demonstrating the forward and side scatter, relative cell count (y-axis) and fluorescence intensity (x-axis), with unstained control cells (empty histogram) and cells stained with antibodies against the surface proteins (filled histogram). (B,D) Column graph of flow cytometry data of surface marker expression levels, the bars represent the mean ± SD (n = 4). DPSCs: dental pulp stem cells; FBS-M: fetal bovine serum medium; HS-M: human serum medium; SF/XF-M: serumfree/xenofree medium.

altering the cellular phenotypic features. There are reports showing the effects of serum free or low serum containing media on DPSCs cultures [38,45]. Nevertheless, DPSCs cultured in SF/XF medium, has not been reported, so far. Therefore, to safely produce DPSCs for clinical applications, in this present work we evaluated the response of FBS, HS or SF/XF media on isolation, expansion, morphology, phenotype, growth and multilineage differentiation potential of DPSCs.

In this study, we first sought to investigate the influence of FBS, HS and SF/XF media directly on cell isolation and proliferation. The cells isolated and cultured in FBS showed consistent proliferation, as reported in several publications [46,47]. Here, we have shown for the first time that 10% HS did not support the isolation of DPSCs, whereas, 20% HS supported the direct isolation and whereas, further expansion of the DPSCs was possible in lower HS concentrations 10% and 15%. Intriguingly, in our study, DPSCs directly isolated in SF/XF-M and cultured on coated plates did not proliferate. Hence, cells isolated in 20% HS-M were used to expand the cells in SF/XF-M which resulted in cell proliferation but at a very slow rate. However, in our study we elucidated the role of HS in SF/XF-M for increasing the cell proliferation. Interestingly, SF/XF-M + 1% HS showed increased DPSCs proliferation in comparison to SF/XF-M alone or SF/XF-M + 5% HS. Therefore, its important to note that SF/XF-M alone may not be sufficient for DPSCs growth, however, AD-MSCs have been reported to proliferate better in the same SF/XF-M [37], suggesting variability in responsiveness to SF/XF-M between different sources of MSCs. To our knowledge, this response to SF/XF-M and addition of HS has not been reported, however, other SF/XF-M compositions remains to be tested. Nonetheless, FBS and HS media cultivated DPSCs showed increased proliferation. Thus, these findings propose that HS was equally effective as FBS in supporting DPSCs proliferation, similar response has been successfully reported in other studies with BM-MSCs and AD-MSCs [30,48,49].

Further, after assessing the adherence and proliferation, DPSCs cultured in different media were evaluated for cell surface markers

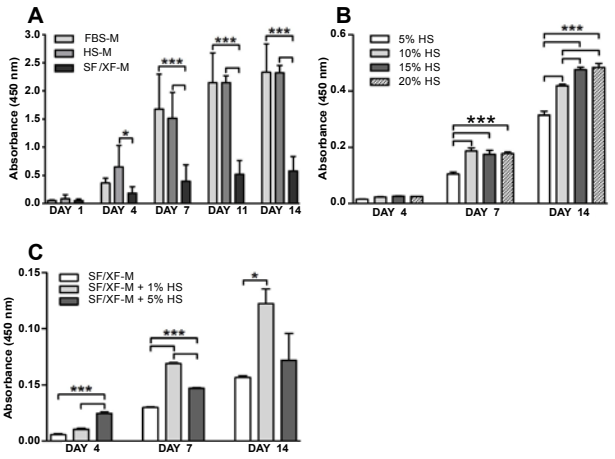

Figure 5: Response of FBS-M, HS-M and SF/XF-M on DPSCs proliferation assessed by WST-1 proliferation assay at day 1, 4, 7, 11 and 14. Cell proliferation in FBS-M, HS-M increased significantly from day 7 to 14 in comparison to cells cultured in SF/XF-M (A). Comparative analysis of DPSCs proliferation in varying concentrations of HS serum 5%-20%.The significant increase in proliferation was observed in serum concentration 10%, 15%, 20% at day 7 and 14 in comparison to 5%HS-M (B). Cell proliferation in SF/XF-M+1%HS increased significantly from day 4 to 7 in comparison to SF/XF-M (C). The bars represent the mean ± SD (n = 4, asterisks indicate significant differences, *p < 0.05, **p< 0.01, ***p< 0.001). DPSCs: dental pulp stem cells; FBS-M: fetal bovine serum medium; HS-M: human serum medium; SF/XF-M: serumfree/xenofree medium.

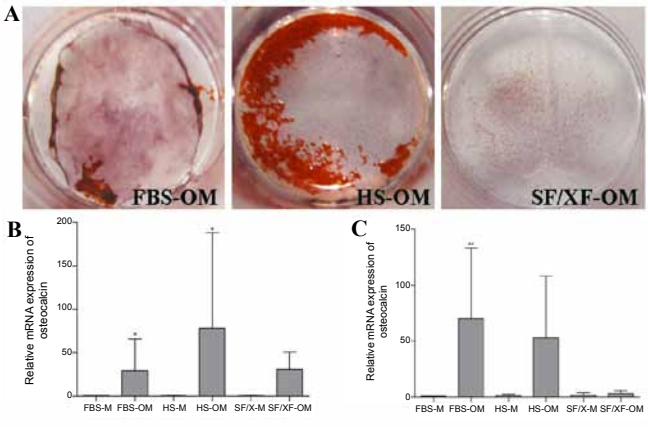

Figure 6: The osteogenic differentiation analysis for DPSCs cultured in FBS-OM, HS-OM and SF/XF-OM. Alizarin red stained the calcified mineralized matrix (A) and qRT-PCR analysis of osteogenic differentiation related gene expression for OCN (B) and OPN (C). Data represents the mean ± SD and are normalized to housekeeping gene RPLP0. (n = 4, *p < 0.05, **p< 0.01 ***p< 0.001). (Online version in colour).

expression established to define them as MSCs [50]. DPSCs expressed CD29, CD44, CD90 and CD105 mesenchymal markers which are involved in MSCs migration, cell-cell matrix interaction and cell adhesion [37,51] and moderately expressed CD45 but lacked expression of CD31 hematopoietic markers and HLA-DR. Strikingly, we did not observe high variation in the expression of mesenchymal markers in DPSCs cultured in FBS, HS and SF/XF media conditions. This is especially important, since we have seen that cells cultured in SF/XF-M showed slower cell proliferation in comparison to FBS and HS. In view of the result, it is reasonable to speculate that serum is essential for cell attachment and proliferation of DPSCs, as suggested in a report [52]. However, the effect of other serum free/ xenofree media alternative needs to be elucidated further to delineate a definitive response on DPSCs. Furthermore, it is previously suggested that DPSCs originate from perivascular niche [53]. In view of the perivascular marker CD146 expression, we observed highest variability between four patient DPSCs samples tested which were cultured in the FBS-M, HS-M and SF/XF-M.

Recent findings have proposed that cell surface antigen SSEA-4 as well as intracellular stemness markers Oct3/4, Sox2 and Nanog can be used as specific markers to detect DPSCs with high multipotent differentiation potential [13,54,55]. Studies in hESCs has revealed that the regulatory loop between Oct3/4, Sox2 and Nanog genes are uncoupled, allowing the expression of Sox2 in the absence of Nanog and Oct3/4; and similarly, expression of Oct3/4 in the absence of Sox2 and Nanog. It has been proposed that each factor controls specific cell fate and lineage commitment [56]. Nevertheless, the expressions of these genes are indicative of indefinite stem cell division, with unaffected differentiation potential or the capacity for self-renewal [57]. Our immunostaining and QRT-PCR results indicated, Oct3/4, Nanog, Sox2 and SSEA-4 markers to be expressed in cells cultured in all media conditions (SF/XF, HS and FBS). This suggests that cells in our study did retain the stemness and multilineage differentiation potential and maintained their self-renewability regardless of serum conditons.

Eventually we investigated the last criteria to define DPSCs as MSCs [50]; cells cultured in FBS, HS or SF/XF differentiation media were assessed for osteogenic, adipogenic and chondrogenic differentiation

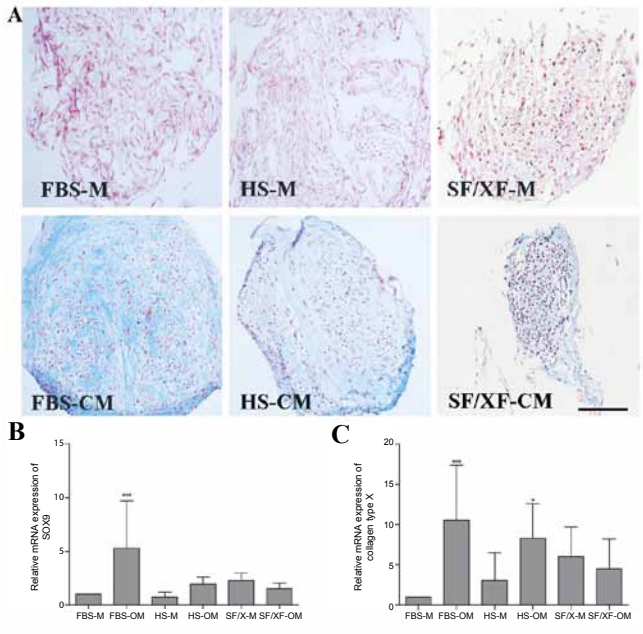

Figure 7: Chondrogenic differentiation analysis for DPSCs cultured in chondrogenic differentiation media in comparison to cells cultured in FBS, HS and SF/XF media. Histological staining by Alcian blue stain of DPSCs micromasses showing chondrogenic differentiation with more proteoglycan content in FBS isolated cells (A) (Scale Bar=100 µm) and qRT-PCR analysis of chondrogenic differentiation related gene expression for SOX9 (B) and COL10A1 (C). Data represents the mean ± SD and are normalized to housekeeping gene RPLP0. (n = 4, *p < 0.05, **p< 0.01 ***p< 0.001). (Online version in colour).

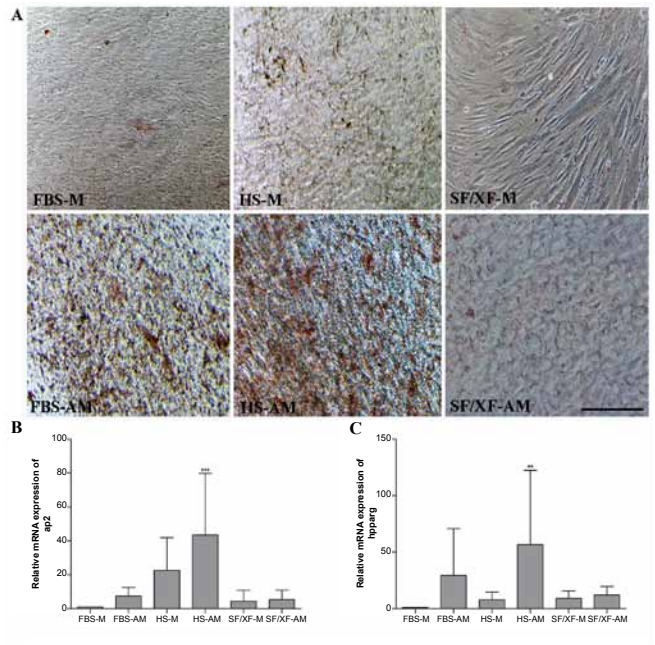

Figure 8: Adipogenic differentiation for DPSCs cultured in FBS-AM, HS-AM and SF/XF-AM in comparison to cells cultured in FBS, HS and SF/XF media. Oil O red staining showing lipid accumulation in adipogenically induced cultures (A) (Scale bar = 100 µm) and qRT-PCR analysis of adipogenic differentiation related gene expression for hPPARG (B) and AP2 (C). Data represents the mean ± SD and are normalized to housekeeping gene RPLP0. (n = 4, *p < 0.05, **p< 0.01 ***p< 0.001). (Online version in colour).

potential *in vitro*. There are several studies reporting the mineralization potential of DPSCs induced by osteogenic medium supplemented with FBS [11,19,58,59], as also shown in our previously published data [46]. However, in our previous study, DPSCs were induced to differentiate osteogenically in FBS medium containing 1α25-dihydroxyvitamin D_3 (VD) instead of dexamethasone as an osteogenic inducer. Similarly, here we have shown that mineralized tissue formation was induced by HS-OM supplemented with VD which differentiated DPSCs towards mineral nodule formation. To ensure the osteogenic differentiation, mineralized matrix was stained with alizarin red, which is a specific stain to qualitatively detect calcification *in vitro* [60]. Additionally, upregulation of OPN (intermediate osteogenic differentiation marker) and OCN (late maker of osteogenic differentiation) expressions were observed which are associated with matrix synthesis and mineralization [61,62] by DPSCs cultivated in FBS-OM and HS-OM at mRNA level. Furthermore, DPSCs cultured in FBS exhibited the capacity to differentiate towards chondrocytes-like cells, as shown in previous studies [20,58]. Here, for the first time we have reported that cells cultured in HS and SF/XF also have the ability to form chondrocyte-like cells. However, in our study SF/XF-OM failed to induce any osteogenic differentiation of DPSCs. As reported in the literature, StemPro® SF/XF medium supported the multipotent differentiation of AD-MSCs [37], the reason for the discrepancy in our results could be attributed to the absence of unknown growth factors in the SF/XF medium, essential for DPSCs differentiation. However, SF/XF-AM did result in lipid accumulation, but adipogenic differentiation was more pronounced in cells cultured in FBS or HS as observed by oil O red staining, which revealed intracellular fat droplets [63]. In addition, adipogenic specific markers AP2 and PPARG which are mainly expressed in fat tissue [64] were upregulated in FBS and HS supplemented cultures. Moreover, it is widely known that DPSCs differentiate into adipocytes in FBS adipogenic medium [9,11], but to our knowledge for the first time our investigation has reported adipogenic capacity of DPSCs in HS and SF/XF supplemented medium.

In summary, our results showed that HS-M supported isolation, expansion, expression of cell surface markers and stemness markers and retained multipotent differentiation capacity of DPSCs similar to FBS-M. Therefore, the use of pooled HS may serve as a safer alternative to FBS for cell therapies. However, the variability in results due to less number of patient samples is the limitation of this study. Nevertheless, these findings are essential for the future clinical studies of DPSCs for their use in stem cell based therapies to bioengineering tissues. Additionally, the composition of SF/XF medium needs to be further optimized for DPSCs culture in terms of cell isolation, expandability and differentiation efficiency to reach clinical applicability.

Acknowledgement

The authors thank Miia Juntunen, Minna Salomaki, Anna Maija-Honkala, and Sari Kalliokoski for their excellent technical assistance. This work was supported by Tekes-Finnish Funding Agency for Technology and Innovation and Competitive Research Funding of Pirkanmaa hospital district (9L057, 9M058).

References

1. Jager M, Hernigou P, Zilkens C, Herten M, Li X, et al. (2010) Cell therapy in bone healing disorders. Orthop Rev (Pavia) 2: e20.
2. Friedenstein AJ, Piatetzky S, II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16: 381-390.
3. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, et al. (2005) Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol 129: 118-129.
4. Miller JP, Perry EH, Price TH, Bolan CD Jr, Karanes C, et al. (2008) Recovery and safety profiles of marrow and PBSC donors: experience of the National Marrow Donor Program. Biol Blood Marrow Transplant 14: 29-36.
5. Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. Circ Res 100: 1249-1260.
6. Wei Y, Li Y, Chen C, Stoelzel K, Kaufmann AM, et al. (2011) Human skeletal muscle-derived stem cells retain stem cell properties after expansion in myosphere culture. Exp Cell Res 317: 1016-1027.
7. Carvalho MM, Teixeira FG, Reis RL, Sousa N, Salgado AJ (2011) Mesenchymal stem cells in the umbilical cord: phenotypic characterization, secretome and applications in central nervous system regenerative medicine. Curr Stem Cell Res Ther 6: 221-228.
8. Klemmt PA, Vafaizadeh V, Groner B (2011) The potential of amniotic fluid stem cells for cellular therapy and tissue engineering. Expert Opin Biol Ther 11:1297-1314.
9. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 97: 13625-13630.
10. Huang GT, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88: 792-806.
11. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA (2006) Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue Eng 12: 2813-2823.
12. Huang GT, Yamaza T, Shea LD, Djouad F, Kuhn NZ, et al. (2010) Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. Tissue Eng Part A 16: 605-615.
13. Huang AH, Snyder BR, Cheng PH, Chan AW (2008) Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. Stem Cells 26: 2654-2663.

14. Gandia C, Arminan A, Garcia-Verdugo JM, Lledo E, Ruiz A, et al. (2008) Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. Stem Cells 26: 638-645.

15. Papaccio G, Graziano A, d'Aquino R, Graziano MF, Pirozzi G, et al. (2006) Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. J Cell Physiol 208: 319-325.

16. d'Aquino R, Graziano A, Sampaolesi M, Laino G, Pirozzi G, et al. (2007) Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. Cell Death Differ 14: 1162-1171.

17. Laino G, d'Aquino R, Graziano A, Lanza V, Carinci F, et al. (2005) A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res 20: 1394-1402.

18. d'Aquino R, De Rosa A, Lanza V, Tirino V, Laino L, et al. (2009) Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. Eur Cell Mater 18: 75-83.

19. Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, et al. (2005) Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 80: 836-842.

20. Tomic S, Djokic J, Vasilijic S, Vucevic D, Todorovic V, et al. (2011) Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. Stem Cells Dev 20: 695-708.

21. Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, et al. (2007) N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells 25: 197-202.

22. Shahdadfar A, Fronsdal K, Haug T, Reinholt FP, Brinchmann JE (2005) In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem Cells 23: 1357-1366.

23. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, et al. (2004) Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 9: 747-756.

24. Dimarakis I, Levicar N (2006) Cell culture medium composition and translational adult bone marrow-derived stem cell research. Stem Cells 24: 1407-1408.

25. Aghayan HR, Arjmand B, Norouzi-Javidan A, Saberi H, Soleimani M, et al. (2011) Clinical grade cultivation of human Schwann cell, by the using of human autologous serum instead of fetal bovine serum and without growth factors. Cell Tissue Bank 13: 281-285.

26. Naveau A, Lataillade JJ, Fournier BP, Couty L, Prat M, et al. (2011) Phenotypic study of human gingival fibroblasts in a medium enriched with platelet lysate. J Periodontol 82: 632-641.

27. Ang LP, Do TP, Thein ZM, Reza HM, Tan XW, et al. (2011) Ex vivo expansion of conjunctival and limbal epithelial cells using cord blood serum-supplemented culture medium. Invest Ophthalmol Vis Sci 52: 6138-6147.

28. Lin HT, Tarng YW, Chen YC, Kao CL, Hsu CJ, et al. (2005) Using human plasma supplemented medium to cultivate human bone marrow-derived mesenchymal stem cell and evaluation of its multiple-lineage potential. Transplant Proc 37: 4504-4505.

29. Ichiyanagi T, Anabuki K, Nishijima Y, Ono H (2010) Isolation of mesenchymal stem cells from bone marrow wastes of spinal fusion procedure (TLIF) for low back pain patients and preparation of bone dusts for transplantable autologous bone graft with a serum glue. Biosci Trends 4: 110-118.

30. Aldahmash A, Haack-Sorensen M, Al-Nbaheen M, Harkness L, Abdallah BM, et al. (2011) Human Serum is as Efficient as Fetal Bovine Serum in Supporting Proliferation and Differentiation of Human Multipotent Stromal (Mesenchymal) Stem Cells In Vitro and In Vivo. Stem Cell Rev 7: 860-868.

31. Kuznetsov SA, Mankani MH, Robey PG (2000) Effect of serum on human bone marrow stromal cells: ex vivo expansion and in vivo bone formation. Transplantation 70: 1780-1787.

32. Bieback K, Hecker A, Kocaomer A, Lannert H, Schallmoser K, et al. (2009) Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells 27: 2331-2341.

33. Kobayashi T, Watanabe H, Yanagawa T, Tsutsumi S, Kayakabe M, et al. (2005) Motility and growth of human bone-marrow mesenchymal stem cells during ex vivo expansion in autologous serum. J Bone Joint Surg Br 87: 1426-1433.

34. Tateishi K, Ando W, Higuchi C, Hart DA, Hashimoto J, et al. (2008) Comparison of human serum with fetal bovine serum for expansion and differentiation of human synovial MSC: potential feasibility for clinical applications. Cell Transplant 17: 549-557.

35. Lindroos B, Aho KL, Kuokkanen H, Raty S, Huhtala H, et al. (2010) Differential gene expression in adipose stem cells cultured in allogeneic human serum versus fetal bovine serum. Tissue Eng Part A 16: 2281-2294.

36. Chase LG, Lakshmipathy U, Solchaga LA, Rao MS, Vemuri MC (2010) A novel serum-free medium for the expansion of human mesenchymal stem cells. Stem Cell Res Ther 1: 8.

37. Lindroos B, Boucher S, Chase L, Kuokkanen H, Huhtala H, et al. (2009) Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy 11: 958-972.

38. Hirata TM, Ishkitiev N, Yaegaki K, Calenic B, Ishikawa H, et al. (2010) Expression of multiple stem cell markers in dental pulp cells cultured in serum-free media. J Endod 36: 1139-1144.

39. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

40. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, et al. (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81: 531-535.

41. Laino G, Carinci F, Graziano A, d'Aquino R, Lanza V, et al. (2006) In vitro bone production using stem cells derived from human dental pulp. J Craniofac Surg 17: 511-515.

42. Chadipiralla K, Yochim JM, Bahuleyan B, Huang CY, Garcia-Godoy F, et al. (2010) Osteogenic differentiation of stem cells derived from human periodontal ligaments and pulp of human exfoliated deciduous teeth. Cell Tissue Res 340: 323-333.

43. d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, et al. (2009) Human dental pulp stem cells: from biology to clinical applications. J Exp Zool B Mol Dev Evol 312B: 408-415.

44. Mori G, Centonze M, Brunetti G, Ballini A, Oranger A, et al. (2010) Osteogenic properties of human dental pulp stem cells. J Biol Regul Homeost Agents 24: 167-175.

45. Karbanova J, Soukup T, Suchanek J, Pytlik R, Corbeil D, et al. (2010) Characterization of Dental Pulp Stem Cells from Impacted Third Molars Cultured in Low Serum-Containing Medium. Cells Tissues Organs 193: 344-365.

46. Khanna-Jain R, Vuorinen A, Sandor GK, Suuronen R, Miettinen S (2010) Vitamin D(3) metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells. J Steroid Biochem Mol Biol 122: 133-141.

47. Graziano A, d'Aquino R, Laino G, Papaccio G (2008) Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Rev 4: 21-26.

48. Komoda H, Okura H, Lee CM, Sougawa N, Iwayama T, et al. (2010) Reduction of N-glycolylneuraminic acid xenoantigen on human adipose tissue-derived stromal cells/mesenchymal stem cells leads to safer and more useful cell sources for various stem cell therapies. Tissue Eng Part A 16: 1143-1155.

49. Yilmaz M, Ovali E, Akdogan E, Durmus A, Sonmez M, et al. (2008) Autologous serum is more effective than fetal bovine serum on proliferation of bone marrow derived human mesenchymal stem cells. Saudi Med J 29: 306-309.

50. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317.

51. Goodison S, Urquidi V, Tarin D (1999) CD44 cell adhesion molecules. Mol Pathol 52: 189-196.

52. Foreman MA, Smith J, Publicover SJ (2006) Characterisation of serum-induced intracellular Ca2+ oscillations in primary bone marrow stromal cells. J Cell Physiol 206: 664-671.

53. Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18: 696-704.

54. Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez DA, Barajas M, et al.

(2012) Dental Pulp of the Third Molar: A New Source of Pluripotent-like Stem Cells. J Cell Sci [Epub ahead of print].

55. Kawanabe N, Murata S, Fukushima H, Ishihara Y, Yanagita T, et al. (2012) Stage-specific embryonic antigen-4 identifies human dental pulp stem cells. Exp Cell Res 318: 453-463.

56. Wang Z, Oron E, Nelson B, Razis S, Ivanova N (2012) Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell 10: 440-454.

57. Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, et al. (2006) Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells Tissues Organs 184: 105-116.

58. Alge DL, Zhou D, Adams LL, Wyss BK, Shadday MD, et al. (2010) Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. J Tissue Eng Regen Med 4: 73-81.

59. Lindroos B, Maenpaa K, Ylikomi T, Oja H, Suuronen R, et al. (2008) Characterisation of human dental stem cells and buccal mucosa fibroblasts. Biochem Biophys Res Commun 368: 329-335.

60. Lazcano O, Li CY, Pierre RV, O'Duffy JD, Beissner RS, et al. (1993) Clinical utility of the alizarin red S stain on permanent preparations to detect calcium-containing compounds in synovial fluid. Am J Clin Pathol 99: 90-96.

61. Viereck V, Siggelkow H, Tauber S, Raddatz D, Schutze N, et al. (2002) Differential regulation of Cbfa1/Runx2 and osteocalcin gene expression by vitamin-D3, dexamethasone, and local growth factors in primary human osteoblasts. J Cell Biochem 86: 348-356.

62. McKee MD, Nanci A (1996) Osteopontin at mineralized tissue interfaces in bone, teeth, and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover, and repair. Microsc Res Tech 33: 141-164.

63. Hopkins PM, Kermeen F, Duhig E, Fletcher L, Gradwell J, et al. (2010) Oil red O stain of alveolar macrophages is an effective screening test for gastroesophageal reflux disease in lung transplant recipients. J Heart Lung Transplant 29: 859-864.

64. Farmer SR (2006) Transcriptional control of adipocyte formation. Cell Metab 4: 263-273.

Journal of Tissue Engineering
0(0) 1–11

DOI: 10.1177/2041731412467998
tej.sagepub.com
SAGE

Osteogenic differentiation of human dental pulp stem cells on β-tricalcium phosphate/poly (L-lactic acid/caprolactone) three-dimensional scaffolds [AQ: 1]

Rashi Khanna-Jain[1,2,3], Bettina Mannerström[1,2,3], Annukka Vuorinen[1,2,3,4], George KB Sándor[1,2,3,5], Riitta Suuronen[1,2,3,4,6,7] and Susanna Miettinen[1,2,3]

Abstract

Functional tissue engineering for bone augmentation requires the appropriate combination of biomaterials, mesenchymal stem cells, and specific differentiation factors. Therefore, we investigated the morphology, attachment, viability, and proliferation of human dental pulp stem cells cultured in xeno-free conditions in human serum medium seeded on β-tricalcium phosphate/poly(L-lactic acid/caprolactone) three-dimensional biomaterial scaffold. Additionally, osteogenic inducers dexamethasone and vitamin D_3 were compared to achieve osteogenic differentiation. Dental pulp stem cells cultured in human serum medium maintained their morphology; furthermore, cells attached, remained viable, and increased in cell number within the scaffold. Alkaline phosphatase staining showed the osteogenic potential of dental pulp stem cells under the influence of osteogenic medium containing vitamin D_3 or dexamethasone within the scaffolds.[AQ: 4] Maintenance of dental pulp stem cells for 14 days in osteogenic medium containing vitamin D_3 resulted in significant increase in osteogenic markers as shown at mRNA level in comparison to osteogenic medium containing dexamethasone. The results of this study show that osteogenic medium containing vitamin D_3 osteo-induced dental pulp stem cells cultured in human serum medium within β-tricalcium phosphate/poly(L-lactic acid/caprolactone) three-dimensional biomaterial, which could be directly translated clinically.

Keywords

dental pulp stem cells, human serum, β-tricalcium phosphate/poly(l-lactic acid/caprolactone), vitamin D_3, osteogenic differentiation

Introduction

Considerable bone loss due to trauma, periodontitis, resorption of edentulous maxillary ridge, and delayed healing of the extraction sockets compromises placement of dental implants.[1,2] It is known that sufficient bone augmentation is a prerequisite for placement of dental implants to achieve functionality and long-term treatment outcomes.[3] Traditionally, for repairing such defects, autogenous or allogenic bone grafts are harvested and implanted into the affected areas.[4] However, due to the clinical drawbacks including donor site morbidity and risk of transmitted diseases, there is a need for alternative solutions such as tissue-engineered bone grafts.[5] Tissue engineering is an interdisciplinary approach to repair damaged tissue. This approach regenerates the tissue through the use of biodegradable implants combined with in vitro cultured stem cells and differentiation factors.[6,7] Mesenchymal stem cells (MSCs) are a unique and easily isolated source of cells for bone tissue engineering. Various sources for MSCs have been reported, including the bone marrow (BM), cord blood (CD), and adipose tissue (AD), and their potential in

[1]Adult Stem Cells Group, Institute of Biomedical Technology, University of Tampere, Tampere, Finland
[2]BioMediTech, Tampere, Finland[AQ: 2]
[3]Science Centre, Tampere University Hospital, Tampere, Finland
[4]Finnish Student Health Service, Tampere, Finland
[5]Department of Oral and Maxillofacial Surgery, University of Oulu, Oulu, Finland
[6]Department of Eye, Ear and Oral Diseases, Tampere University Hospital, Tampere, Finland
[7]Department of Biomedical Engineering, Tampere University of Technology, Tampere, Finland

Corresponding author:
Rashi Khanna-Jain, Adult Stem Cells Group, Institute of Biomedical Technology, University of Tampere, Biokatu-12, Finn medi 5, 33520 Tampere, Finland.
Email: rashi.khanna-jain@uta.fi[AQ: 3]

bone regeneration has been studied extensively in vitro[8–10] and in clinical applications.[2,6]

Recently, there has been emerging inclination toward studying another source of MSCs obtained from teeth such as dental pulp tissue for its ability to regenerate bone.[11] Dental pulp stem cells (DPSCs) can be isolated from the dental pulp tissue, which is obtained from the impacted third molar teeth with ease and minimal tissue site morbidity. The dental pulp tissue is a loose connective tissue that provides nutritional and sensory properties to dentin and has reparative capacity to form tertiary dentin; therefore, it is believed to possess stem/progenitor cells.[12] Several research reports have shown clonogenic ability, rapid proliferative rate, and multiple differentiation ability of DPSCs including two studies from our group.[13–15] Most commonly, osteogenic differentiation potential of DPSCs has been induced by dexamethasone (DEX), which is a synthetic glucocorticosteroid.[16] Another osteogenic inducer, 1α,25-dihydroxyvitamin D_3 (vitamin D_3 (VD)), in combination with β-glycerophosphate and ascorbic acid has been reported as more potent than DEX for DPSC differentiation in our previous study[14] and in another study for AD-MSCs differentiation.[17] Apart from the osteogenic inducers, high fetal bovine serum (FBS) concentration has been shown to induce osteogenesis, wherein a subpopulation of DPSCs were capable of forming woven bone in vitro.[18] Intriguingly, in a clinical study, it was shown that DPSCs isolated in FBS containing medium seeded onto collagen sponge scaffolds and implanted in the tooth extraction socket hastened the process of bone regeneration.[19] Nevertheless, there are concerns regarding the use of FBS and other animal-derived supplements in culturing cells for clinical therapy due to risk of transmitting zoonoses in humans.[20,21] Thus, alternative serum supplementation is needed; the possible choice would be autologous or pooled allogenic human serum (HS), which can be tested for human pathogens before use.[22,23]

A range of biomaterials have been studied to investigate the MSC attachment, growth, and osteogenic differentiation.[24,25] Collagen sponge in combination with DPSCs has been successfully used to regenerate bone in tooth extraction sockets.[19] However, it has been shown that collagen sponge as a scaffold has poor mechanical strength and high dimensional changes.[26] On the other hand, osteoconductive, bioresorbable, bioceramic material β-tricalcium phosphate (β-TCP) has been used for bone tissue engineering applications in clinics for years but this material has poor mechanical strength.[27,28] To overcome the mechanical strength drawback of bioceramics, combination of β-TCP and poly(L-lactic acid) (PLLA) or polycaprolactone (PCL) has been fabricated, which allows defining the shape and maintenance of the structural integrity of the tissue-engineered bone graft in load-bearing applications.[25,29] PLLA and PCL are polymeric synthetic composite materials, they are biocompatible and degradable by the process of hydrolysis.[30] As a prerequisite, scaffold should degrade at the same time as bone regenerates; however, PCL has an extended degradation rate[31] and PLLA has higher degradation rate.[32] In order to tune the degradation rate, a novel PCL/PLLA composite scaffold was devised for bone tissue engineering.[30] Taken together, we hypothesized that the combination of osteoconductive properties of β-TCP and the mechanical strength of PLLA and PCL fabricated medical-grade biomaterial by Synthes® may mimic the characteristics of a natural bone extracellular matrix (ECM).[25,29,30,33]

Thus, the aim of this study was to assess DPSC adhesion, proliferation, and osteogenic differentiation within β-TCP/P(LLA/CL) three-dimensional (3D) scaffolds in clinically applicable conditions by using HS as a medium supplement to replace FBS. Furthermore, the osteogenic differentiation of the DPSCs within the β-TCP/P(LLA/CL) biomaterial scaffold induced by VD or DEX was compared under xeno-free conditions.

Material and methods

Cells isolation and culture

Human-impacted third molars were obtained with informed consent from Finnish Student Health Services, Tampere, Finland. The collection of stem cells from tooth samples was approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R06009). Human dental pulp explants were obtained from partially impacted third molar teeth (Figure 1(a)) of three patients aged 21–26 years (23 ± 2.5 years). The pulp tissue explants (Figure 1(b)) were transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS; BioWhittaker; Lonza, Verviers, Belgium) containing 2% antibiotics/antimycotics (a/a; 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B; Life Technologies, Paisley, Scotland). The dental pulp tissue fragments were minced by using scalpels and were then digested in collagenase type I (3 mg/mL; Invitrogen) and dispase (4 mg/mL; Invitrogen) for 1 h at 37°C. [AQ: 5] Once digestion was completed, the obtained cell pellet was suspended in 500 µL of DPBS and was passed through a 100-µm cell strainer (Falcon; BD Labware, Franklin lakes, NJ, USA). The isolated DPSCs were cultured and expanded in Dulbecco's modified Eagle's medium (DMEM)/F-12, 1:1 mixture (Gibco Life Technologies, Paisley, UK) supplemented with 1% L-alanyl-L-glutamine (GlutaMAX I; Life Technologies), 1% a/a, and 20% allogenic HS (PAA Laboratories GmbH, Pasching, Austria) (human serum medium (HS-M)). DPSCs isolated and expanded in HS-M were harvested using TrypLE Select (Life Technologies) for xeno-free detachment of cells.

After initial passaging, the concentration of HS was reduced to 15% in the culture medium. Cell culture plates and flasks were monitored daily for cell growth, with

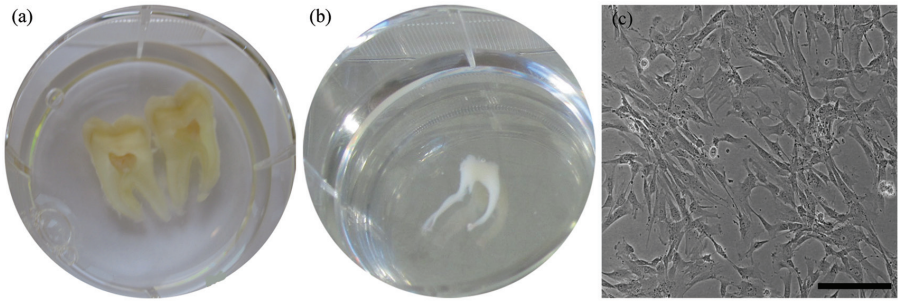

Figure 1. (a) Representative pictures of extracted human-impacted third molar tooth to obtain (b) dental pulp tissue. (c) After 14 days, the cell morphology appeared spindle shaped as observed under phase contrast microscope, scale bar = 100 µm.

medium changes taking place three times per week. All assays were performed using cells between passages 2 and 4, and all experiments were repeated using DPSCs derived from three different donors ($n = 3$).

Flow cytometric surface marker expression analysis

DPSCs cultured in HS-M were analyzed for cell surface antigen expression by flow cytometry (fluorescence-activated cell sorting (FACS); FACSAria®; BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies (MAb) against CD90-Allophycocyanin (APC), CD105-phycoerythrin (PE) (R&D Systems Inc., Minneapolis, MN, USA), CD31-fluorescein isothiocyanate (FITC) (Immunotools GmbH, Friesoythe, Germany), and CD45-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) were used. Antibodies were added to 100,000 cells/sample and then incubated for 30 min at 4°C in the dark. After incubation, cells were washed and then analyzed by flow cytometry.

Preparation and evaluation of the biomaterial by scanning electron microscope

β-TCP/P(LLA/CL) (ChronOS™) was provided by Synthes (Oberdorf, Switzerland); the material is accepted for clinical use as a bone graft substitute. The biomaterial was supplied in sterile strip form with a size of 2.5 cm × 5 cm (Figure 2(a)): two 3-mm-thick strips and three 6-mm-thick strips. For the experiments, the strips were cut into 1 cm × 0.8 cm pieces (Figure 2(a)) with scalpels in sterile conditions under the laminar flow hood.

Subsequently, for scanning electron microscope (SEM) analysis, the biomaterial samples were rinsed with DPBS and dehydrated through a series of ascending concentration of ethanol (30%, 50%, 70%, 90%, and 100%). The samples were then incubated in hexamethyldisilazane (HMDS) for 10 min and dried overnight in a dessicator. The dried biomaterial scaffolds were cut into half and mounted on a double-sided carbon tape. A platinum coating was sputtered on the samples before SEM observation.

Cell seeding and treatment conditions

The biomaterial scaffold pieces were transferred into 24-well plates, washed with DPBS, and incubated in HS-M at 37°C in 5% CO_2 for assisting in attachment of cells before cell seeding. After 48 h of incubation, the scaffolds were seeded with 300 cells/mm^3, and 150 µL of cell suspension was added onto each biomaterial scaffold. Cells were allowed to attach within the porous scaffold for 2 h before adding 500 µL of the culture or differentiation medium. Osteogenic medium (OM), containing HS and either of the hormones dexamethasone (OM-DEX; 10 nM) or 1α,25-dihydroxyvitamin D_3 (OM-VD; 100 nM) in addition to 50 µM L-ascorbic acid 2-phosphate (AA; Sigma) and 10 mM β-glycerophosphate (Sigma) were added for inducing osteogenic differentiation in the cell-seeded scaffold.[AQ: 6]DPSC-seeded biomaterials in HS-M were used as control.

Cell attachment and viability

Cell attachment and viability of DPSCs in biomaterial scaffolds cultured in HS-M were evaluated at days 1, 7, and 14 using live/dead-cell staining kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. In brief, cell-seeded scaffolds were incubated in DPBS-based dye solution, containing 0.5 µM of calcein AM (green fluorescence; Molecular Probes) (4 mmol/L) and 0.5 µM of ethidium homodimer-1 (EthD-1; red fluorescence; Molecular Probes) (2 mmol/L) for 45 min at room temperature (RT). The dye solution was replaced by fresh DPBS solution. The viable cells (green fluorescence) and necrotic cells (red fluorescence) were examined using a fluorescence microscope.

CyQUANT® cell proliferation assay

CyQUANT® Cell Proliferation Assay Kit (CyQUANT; Molecular Probes, Invitrogen) was used according to the manufacturer's protocols to assess the cell numbers at 1, 7, and 14 days. Cell-seeded scaffolds were cultured in OM-DEX, OM-VD, and HS-M. In brief, 500 µL of 0.1%

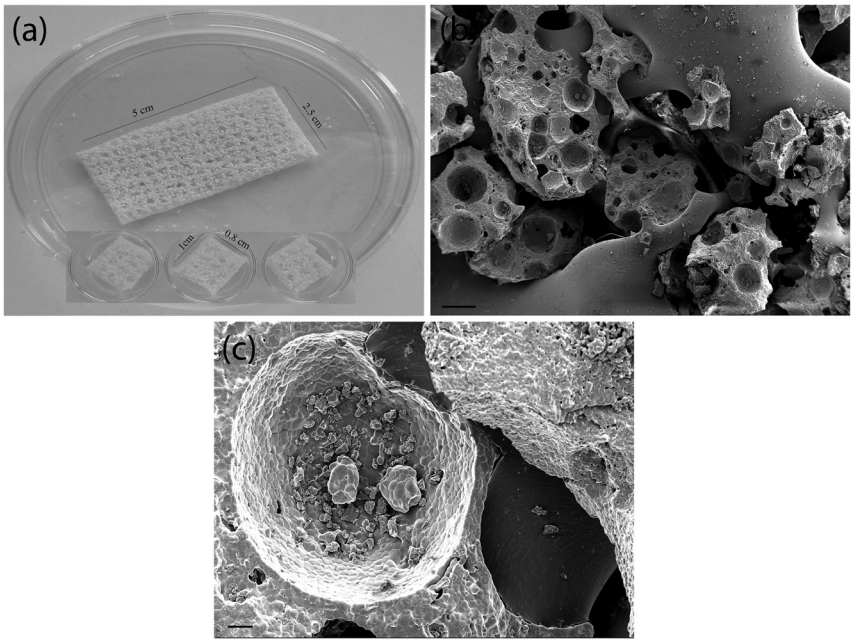

Figure 2. (a) Representative pictures of the β-TCP/P(LLA/CL) (ChronOS) biomaterial strip and the biomaterial pieces cut into 1 cm × 0.8 cm dimension for in vitro analysis. (b) Scanning electron microscope analysis showing β-TCP particles embedded in P(LLA/CL) and (c) the fine structure of β-TCP particle, scale bar = 20 and 100 µm. **[AQ: 18]**
β-TCP/P(LLA/CL): β-tricalcium phosphate/poly(L-lactic acid/caprolactone).

Triton X-100 (Sigma) was pipetted through the cell-seeded scaffolds and the lysed cell suspensions were frozen until analysis. The CyQUANT cell proliferation assay is based on the green fluorescence dye, CyQUANT GR dye, which intensifies when it binds to the nucleic acid of DNA. The fluorescence, which is directly proportional to the number of cells in the sample, was measured at 480/530 nm using a microplate reader (Victor 1420 Multilabel Counter; Wallac, Turku, Finland).

Alkaline phosphatase staining

In vitro osteogenic differentiation capacity of the DPSCs induced by OM-DEX and OM-VD was determined at day 14 by alkaline phosphatase (ALP) staining. The control cell-seeded scaffolds were cultured in HS-M. Cell-seeded scaffolds were stained by using a leukocyte ALP kit according to Sigma procedure 86 (cat. no. 86R-1KT). In brief, cell-seeded scaffolds were fixed with 4% paraformaldehyde (PFA) solution for 2 min. ALP staining solution was added to the scaffolds following the fixation. After 15 min of incubation in the dark, the ALP staining solution was removed and the scaffolds were washed to remove excess stain. Thereafter, digital images were taken of the ALP stained and unstained scaffolds.

Immunostaining

After 14 days of inducing osteogenic differentiation in the cell-seeded scaffolds using OM-DEX and OM-VD, they were immunostained with primary antibody antihuman osteocalcin (OCN) (AbD Serotec, Immunodiagnostics Oy, Finland). The cell-seeded scaffolds cultured in HS-M were used as controls. In brief, the cell-seeded scaffolds were fixed with 4% PFA for 10 min and then blocked against nonspecific antigen binding with 10% normal donkey serum (NDS), 0.1% Triton X-100, and 1% bovine serum albumin (BSA) in DPBS. After 45 min of blocking, the cell-seeded scaffolds were washed with 1% NDS, 0.1% Triton X-100, and 1% BSA in DPBS (washing solution). The primary antibody antihuman OCN was diluted to 1 : 50 in the washing solution. The cells were incubated overnight at +4°C with the primary antibody. The next day, the cells were washed with 1% BSA in DPBS and incubated for 1 h at RT. Thereafter, cells were incubated for 1 h in Alexa Fluor-488 (1 : 1000; Invitrogen) conjugated anti-mouse secondary antibody, diluted in 1% BSA in DPBS. Then, cells were sequentially washed with PBS and phosphate buffer and mounted with Vectashield (4′,6-diamidino-2-phenylindole (DAPI); Vector Laboratories, Peterborough, UK). For negative controls, primary antibody was omitted. Stained DPSCs within the scaffolds were imaged using a microscope equipped with a fluorescence unit and camera.

Quantitative real-time polymerase chain reaction

The cell culture conditions were same as described for ALP staining. The control samples were maintained in the HS-M. The total RNA was extracted at 7 and 14 days using Eurozol

Table 1. Primer sequences for quantitative RT-PCR

Name	5′-sequence-3′	Product size	Accession number
RPLP0	Forward: AATCTCCAGGGGCACCATT Reverse: CGTTGGCTCCCACTTTGT	70	NM_001002
Osteocalcin	Forward: AGCAAAGGTGCAGCCTTTGT Reverse: GCGCCTGGGTCTCTTCACT	63	NM_000711
Osteopontin	Forward: GCCGACCAAGGAAAACTCACT Reverse: GGCACAGGTGATGCCTAGGA	71	J04765
Alkaline phosphatase	Forward: CCCCGTGGCAACTCTATCT Reverse: GATGGCAGTGAAGGGCTTCTT	73	NM_000478.4

RT-PCR: real-time polymerase chain reaction.

(Euroclone S.p.A, Pero, Italy). First-strand complementary DNA (cDNA) syntheses were performed by a High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using primers for human acidic ribosomal phosphoprotein (RPLP0), OCN, osteopontin (OPN), and ALP (Table 1). To exclude signals from contaminating DNA, the forward and reverse sequences of each primer were designed on different exons. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for quantitative PCR reactions according to the manufacturer's instructions. The reactions were performed with ABI Prism 7300 Sequence Detection System (Applied Biosystems) at 95°C/10 min, and then 45 cycles at 95°C/15 s and 60°C/60 s were performed. The Ct values for OCN, OPN, and ALP were normalized to that of the housekeeping gene *RPLP0*, as described elsewhere.[34] **[AQ: 7]**

Statistical analysis

The statistical analyses of the results were performed with GraphPad Prism 5.01. The data are presented as mean ± standard deviation (SD) for all quantitative assays, and experiments were carried out in triplicate for cells derived from three donor samples. All statistical analyses were performed at the significance level $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons.

Results

Cell isolation and morphology

From day 1 to day 9, DPSCs cultured in HS-M proliferated in colonies and spherical clusters. Morphologically, cells mostly appeared spindle shaped and comprised a homogenous cell population when viewed under the phase contrast microscope (Figure 1(c)). After first passage, the cells did not proliferate in clusters; rather, cells were more spread out and proliferated uniformly.

Flow cytometric surface marker expression analysis for human DPSCs

Prior to seeding the cells into the biomaterial scaffolds, flow cytometric assay was done to define the mesenchymal surface marker expression of the cells. The DPSCs were strongly positive for MSC markers CD90 and CD105 and were negative for hematopoietic lineage markers CD31 and CD45 (Figure 3).

Cell attachment and viability

The cell-seeded scaffolds cultured in HS-M were analyzed for cell viability. The results revealed that DPSCs were viable, attached, and migrated into the pores of the biomaterial scaffold following day 1, day 7, and day 14 time course, with very few dead cells. Visually, upon viewing microscopically, more cells were observed at day 7 and day 14 in comparison to day 1 (Figure 4).

Cell proliferation

The cell-seeded scaffolds cultured in HS-M, OM-DEX, and OM-VD were assessed for increase in cell numbers from day 1 and day 7 to day 14. There was significant increase in cell numbers at day 7 ($p < 0.01$) and day 14 ($p < 0.001$) time points, when cells cultured in OM-VD were compared to the control HS-M at days 7 and 14 (Figure 5). Though there was no significant increase in cell numbers when cells were cultured in OM-DEX, the cells numbers increased nonsignificantly. Moreover, there was significant increase ($p < 0.001$) in cell numbers cultured in OM-VD when compared to OM-DEX at day 14.

ALP staining and messenger RNA expression of ALP

ALP is an early marker that indicates differentiation of cells toward osteogenic lineages.[35] The ALP activity of cells cultured in HS-M, OM-DEX, and OM-VD is depicted in (Figure 6(a) to (c)). Interestingly, ALP activity expressed

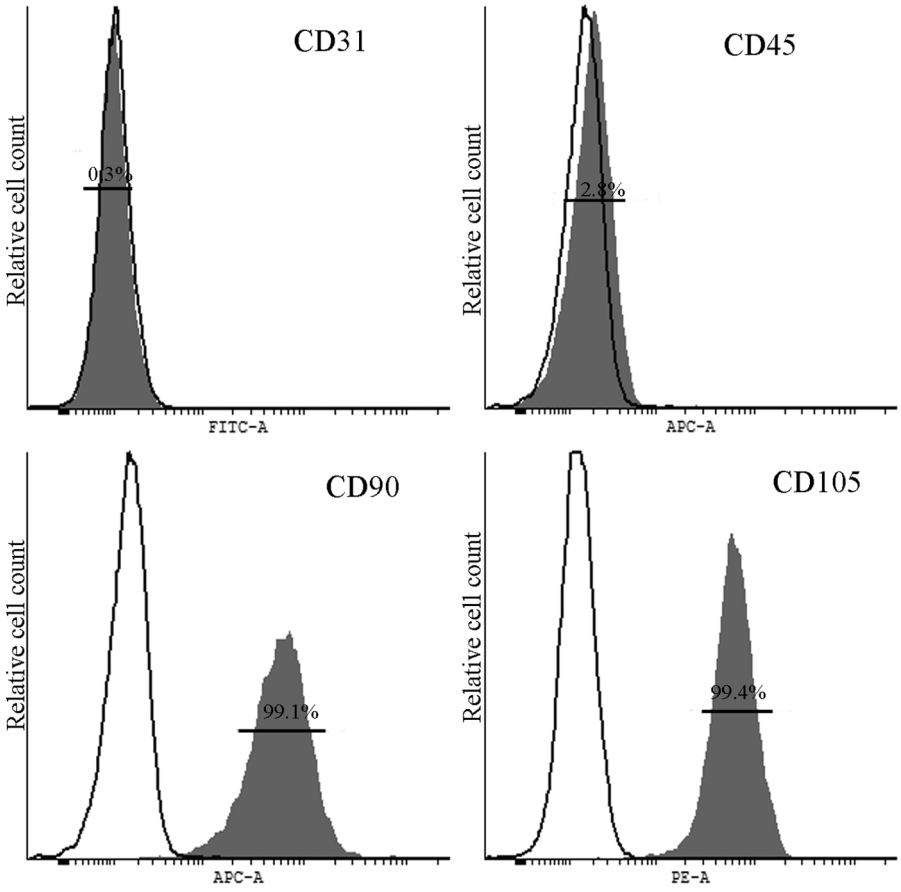

Figure 3. Surface marker expression of dental pulp stem cells cultured in human serum medium as analyzed by flow cytometry. Histograms demonstrating the relative cell count (*y*-axis) and fluorescence intensity (*x*-axis), with unstained control cells (empty histogram) and cells stained with antibodies against the surface proteins CD90, CD105, CD31, and CD45 (filled histogram).
FITC: fluorescein isothiocyanate; APC: allophycocyanin; PE: phycoerythrin.

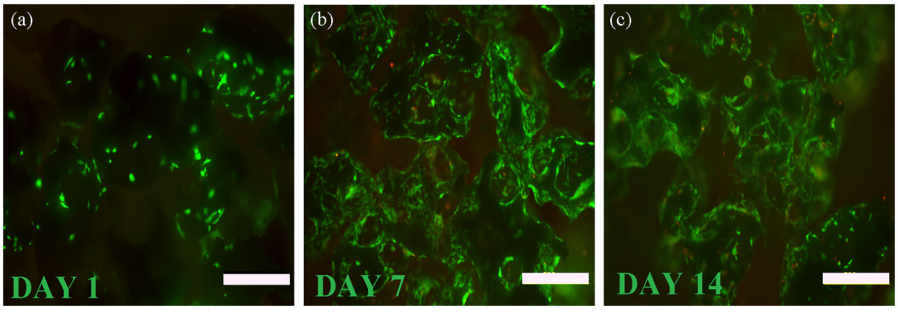

Figure 4. Representative images of viable and dead DPSCs seeded on β-TCP/P(LLA/CL) 3D scaffolds at (a) day 1, (b) day 7, and (c) day 14 (scale bar = 500 µm). Viable cells stained green and dead cells stained red after calcein AM/EthD-1 staining. The cells were seen to remain viable after day 7 and day 14 of culture in β-TCP/P(LLA/CL) as observed under the fluorescence microscope ($n = 3$) (online version in color).
DPSCs: dental pulp stem cells; β-TCP/P(LLA/CL): β-tricalcium phosphate/poly(L-lactic acid/caprolactone); 3D: three-dimensional.

by cells cultured in OM-VD was stronger than the cells cultured in OM-DEX. The control cells were cultured in HS-M and no ALP staining could be detected. Subsequently, similar findings were observed when ALP expression was analyzed at mRNA level. ALP expression was significantly induced at day 7 ($p < 0.05$) and day 14 ($p < 0.05$) when the DPSCs treated with OM-VD were compared with control sample. However, no significant differences were observed when cells treated with OM-DEX and OM-VD were compared, as shown in (Figure 6(d)).

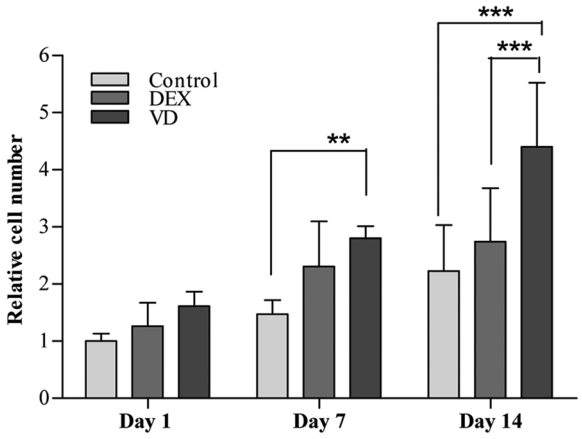

Figure 5. Cell growth characteristics of DPSCs seeded on β-TCP/P(LLA/CL) 3D scaffolds at days 1, 7, and 14 assessed by measuring the DNA content.
DPSCs: dental pulp stem cells; β-TCP/P(LLA/CL): β-tricalcium phosphate/poly(L-lactic acid/caprolactone); 3D: three-dimensional; SD: standard deviation; DEX: dexamethasone; VD: vitamin D_3.
The bars represent the mean ± SD and data are presented as relative cell numbers ($n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

mRNA expression and immunostaining for OCN

The deposition of OCN in the ECM was used as a marker of osteogenic differentiation of DPSCs within the biomaterial scaffold. OCN protein expression was assessed by immunostaining (Figure 7(a) to (c)), the cells cultured in OM-VD expressed OCN slightly more in comparison to OM-DEX. Moreover, cells cultured in HS-M did not express OCN. When OCN mRNA expression was assessed, the OCN levels were significantly higher ($p < 0.001$) in cells cultured in OM-VD at day 14 in comparison to OM-DEX (Figure 7(d)). Also, OCN mRNA expressions were significantly ($p < 0.001$, $p < 0.001$) upregulated, when the control samples at day 7 and day 14 were compared with OM-VD cultured cells at day 14.

Consequently, we studied the expression of another late osteogenic marker, OPN, at mRNA level. Similar significant OPN expressions were observed as OCN in different treatment conditions, even though the relative mRNA expression levels of OPN were weaker than relative mRNA expression levels of OCN. OPN mRNA expressions were

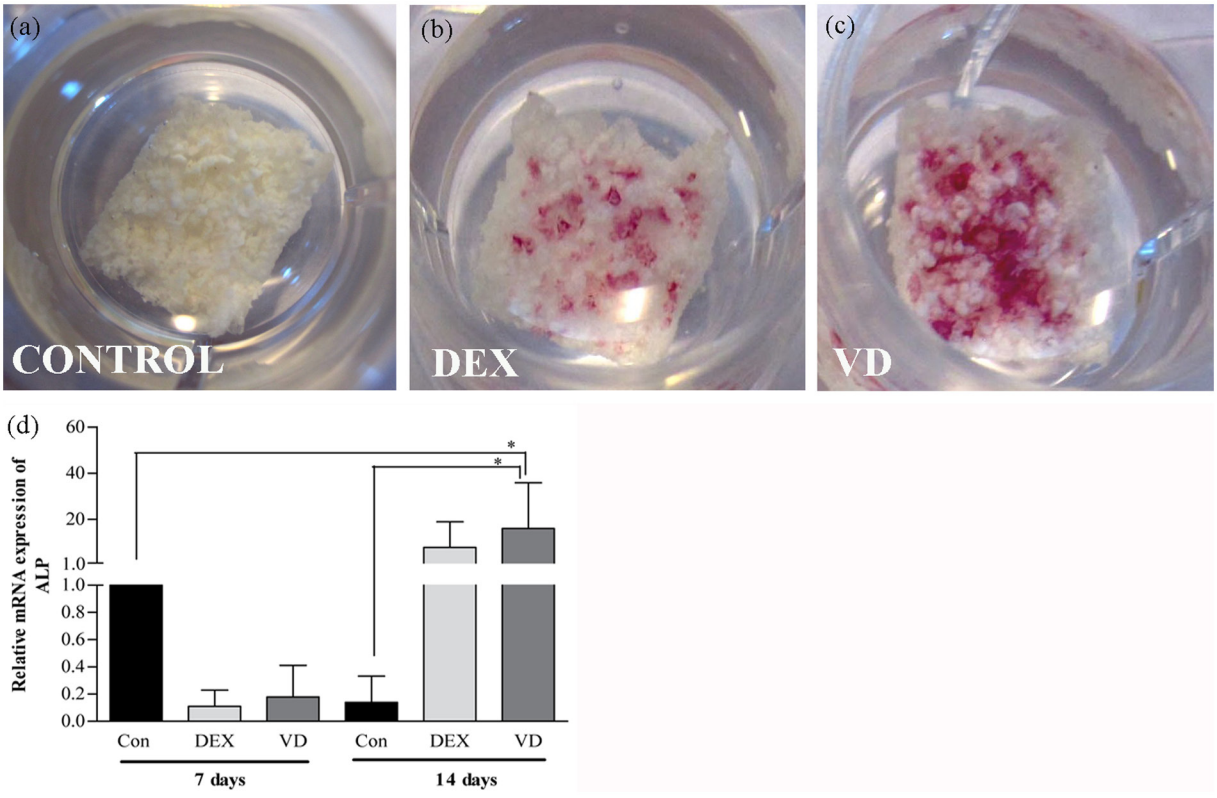

Figure 6. ALP staining and relative mRNA expression of ALP in DPSCs seeded on β-TCP/P(LLA/CL) 3D scaffolds cultured in (a) HS-M, (b) OM-DEX, and (c) OM-VD. Weaker ALP staining was observed in scaffolds cultured in (b) OM-DEX than (c) OM-VD at day 14. (d) The relative mRNA expression of ALP gene in DPSCs cultured in OM-VD was significantly higher than HS-M at days 7 and 14 time points. Results are reported as change in gene expression relative to untreated control (HS-M) set as 1 at day 7 time point.
ALP: alkaline phosphatase; mRNA: messenger RNA; DPSCs: dental pulp stem cells; β-TCP/P(LLA/CL): β-tricalcium phosphate/poly(L-lactic acid/caprolactone); 3D: three-dimensional; SD: standard deviation; DEX: dexamethasone; VD: vitamin D_3; HS-M: human serum medium; OM: osteogenic medium.
Data represent the mean ± SD and are normalized to housekeeping gene *RPLP0* ($n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) (online version in color). **[AQ: 19]**

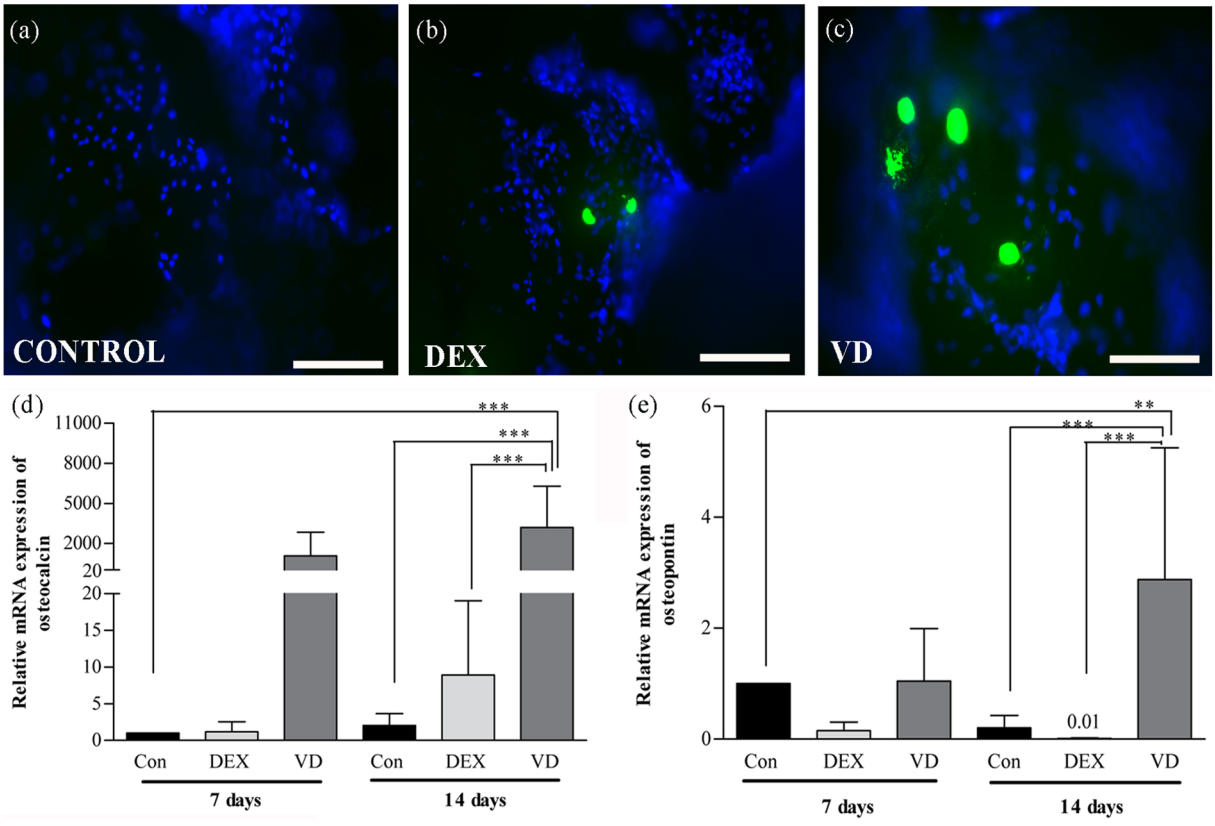

Figure 7. Representative immunofluorescence images of OCN expression in DPSCs seeded on β-TCP/P(LLA/CL) 3D scaffolds cultured in (a) HS-M, (b) OM-DEX, and (c) OM-VD (scale bar = 200 μm). The relative mRNA expressions of late osteogenic markers (d) OCN and (e) OPN in differentiated DPSCs cultured in OM-VD were significantly higher than untreated control (HS-M) and OM-DEX treated cells. Results are reported as change in gene expression relative to untreated control (HS-M) set as 1 at day 7 time point. ALP: alkaline phosphatase; mRNA: messenger RNA; DPSCs: dental pulp stem cells; β-TCP/P(LLA/CL): β-tricalcium phosphate/poly(L-lactic acid/caprolactone); 3D: three-dimensional; SD: standard deviation; DEX: dexamethasone; VD: vitamin D_3; HS-M: human serum medium; OM: osteogenic medium; OCN: osteocalcin; OPN: osteopontin.
Data are represented as mean ± SD and are normalized to housekeeping gene *RPLP0* ($n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) (online version in color). **[AQ: 20]**

significantly ($p < 0.01$, $p < 0.001$) upregulated, when the control samples at day 7 and day 14 were compared with OM-VD cultured cells at day 14 (Figure 7(e)). In addition, at day 14, cells treated with OM-VD significantly ($p < 0.001$) upregulated OPN levels when compared with cells cultured in OM-DEX.

Discussion

Reconstruction of the alveolar bone by autografts or allografts to place dental implants requires an invasive technique to harvest grafts with a number of disadvantages including donor site morbidity, compromised vascularity, or limited tissue availability.[36,37] Present treatment modalities for bone regeneration by tissue engineering necessitate temporarily substituting ECM by the use of synthetic porous, osteoconductive biomaterials that would assist in cell attachment and osteogenesis.[25,29] The natural biomaterials such as chitosan and cellulose have certain disadvantages including low mechanical stiffness, lot-to-lot variability, and may be prone to contamination.[38,39] Further considering the clinical application of tissue-engineered bone grafts, the use of animal-derived components are not recommended, because they can elicit an immune response in patients upon implantation.[21] This is because human cells are able to take up animal proteins and present them on their membranes, thereby initiating xenogenic immune response leading to failure of the tissue-engineered graft.[20,40] **[AQ: 8]** Therefore, our research concentrated on xeno-free alternatives for animal-derived culture reagents and use of medical-grade synthetic composite material for bone tissue engineering as a step toward translation to clinical application. Here, we have described the viability, cell attachment, proliferation, and osteogenic differentiation ability of the DPSCs within a synthetic biomaterial β-TCP/P(LLA/CL) 3D scaffold maintained in xeno-free conditions.

Notably, to our knowledge, this is the first study where DPSCs were maintained in xeno-free culture conditions in vitro. In the literature, there are abundant reports discussing the isolation and culture condition for DPSCs.[11,15,41] Nonetheless, in these studies, DPSCs were cultured in FBS, which adds to the risk of transferring xenogenic antibodies and transmitting viral/prion diseases upon clinical application.[19,42,43] Moreover, HS has been explored as a possible alternative to FBS for culturing MSCs.[23,44] Furthermore, it was reported that HS could support proliferation and differentiation of human MSCs as efficiently as FBS medium in vitro and could maintain their bone forming capacity in vivo.[22] In this study, DPSCs cultured in HS adhered to the cell culture plastic and expressed membrane molecules CD90 and CD105 showing the mesenchymal origin of the cells and lacked expression of the hematopoietic markers CD31 and CD45, fulfilling the minimum criteria for defining MSCs.[45]

Furthermore, the clinical outcome is influenced by many different factors. For successful initial approach toward bone tissue engineering, the interaction between potential osteogenic MSCs, osteoconductive biomaterial, and differentiation factors maintained in xeno-free conditions are the most relevant factors to be considered. Our choice of MSCs derived from dental pulp tissue was based on their ability to proliferate and differentiate osteogenically. Additionally, DPSCs are readily accessible and involve no invasive technique to obtain MSCs, which makes them a good candidate for use in bone tissue engineering. In a recent study, it was shown that canine DPSCs in combination with platelet-rich plasma formed mature bone with neovascularization and the response was similar to that of canine BM-MSCs at the dental implant site.[46] Apart from that, the ability of human DPSCs to regenerate into bone has been reported in several in vitro/in vivo studies[18,42,43] and also in a clinical study.[19]

In our study, we have shown that DPSCs are attached, remained viable, proliferated, and differentiated osteogenically within β-TCP/P(LLA/CL) scaffolds. With the purpose of distribution of cells and osteoconductivity within a biomaterial, porous β-TCP with good bone bonding properties is preferred.[27] But due to poor mechanical strength, β-TCP is used for bone regeneration at nonloading sites or to fill bone defects.[28] From a tissue engineering point of view, a biomaterial should have sufficient strength initially to withstand the stresses of mastication until the newly regenerated bone takes over.[47] Moreover, the structural integrity is also crucial for the long-term success of implants in the bone.[4] In order to achieve desirable mechanical strength for bone tissue engineering, synthetic polymer–based biomaterials such as PLLA and PCL are combined with osteoconductive bioceramics.[30,48] With respect to biomaterial properties, β-TCP/P(LLA/CL) 3D biomaterial scaffold was preferred for our study; it influenced the adhesion of DPSCs onto the biomaterial surfaces and supported osteogenic differentiation of the cells within the porous solid structures.

To our knowledge, this is a first study showing the osteogenic potential of DPSCs within β-TCP/P(LLA/CL) 3D biomaterial scaffolds induced by OM-VD or OM-DEX. Having shown in our previous study that OM-VD supported osteogenic differentiation of the DPSCs better than the OM-DEX medium in vitro, we next sought to evaluate the response of osteogenic media on DPSCs within the β-TCP/P(LLA/CL) scaffolds.[14] Consequently, to assess the differentiation of DPSCs toward osteogenic lineage, ALP staining and expression were studied. ALP is a known marker for detection of early osteogenic differentiation of cells and is an ectoenzyme involved in the degradation of inorganic pyrophosphate to release phosphate for mineralization.[35] Importantly, in our study, we observed that DPSCs seeded in the scaffolds and cultured in OM-DEX and OM-VD expressed ALP activity within the biomaterial. Nevertheless, DPSCs induced with OM-DEX showed weak ALP activity, and the pattern was quite similar in all three patient cell samples. In addition, mRNA expression of ALP was observed to be significantly increased in the cells cultured in OM-VD. At the same time, the expression of OCN which is considered a late osteogenic marker[49] was evaluated, the levels were upregulated when the cells were treated with VD OM. Moreover, it is known that OCN is an important osteogenic marker that regulates the formation of mineral nodules and hence leads to osteogenesis.[50] Therefore, the expression levels of OCN are commonly correlated with the mineralization ability of the cells. Previously, weak levels of OCN have been reported in cells treated with OM-DEX.[51,52] Similar findings were observed in our study. Another important late-stage osteoblast differentiation maker OPN[53] was expressed when cells were cultured in VD OM. Considering the mRNA expressions of the genes, our results indicate that there was osteogenic differentiation, and ALP staining results showed that there was matrix mineralization in vitro. Based on our findings, β-TCP/P(LLA/CL) scaffolds strongly supported the osteogenic differentiation of DPSCs induced by VD OM. However, weaker osteogenesis was observed by OM-DEX in comparison to VD OM.

In summary, from our results, we can conclude that human DPSCs have the ability to survive, proliferate, and differentiate into osteogenic lineage within β-TCP/P(LLA/CL) scaffolds in vitro, which is important before evaluating the efficacy in vivo. Furthermore, the animal-derived cell culture supplements such as FBS was replaced with xeno-free supplements and medical-grade synthetic composite biomaterial was used. Hence, the outcome of this study can be directly applied to perform future clinical trials.

Funding

This study was supported by Finnish Funding Agency for Technology and Innovation (TEKES) and Competitive Research Funding of Pirkanmaa hospital district (9L057, 9M058). **[AQ: 10]**

Acknowledgements

The authors thank Synthes for kindly providing the biomaterial for our study. The authors also thank Minna Salomäki, Anna Maija-Honkala, Miia Juntunen, and Sari Kalliokoski for their excellent technical assistance.[AQ: 9]

References

1. Baumer A, Pretzl B, Cosgarea R, et al. Tooth loss in aggressive periodontitis after active periodontal therapy: patient-related and tooth-related prognostic factors. *J Clin Periodontol* 2011.[AQ: 11]
2. Mendonca JJ and Juiz-Lopez P. Regenerative facial reconstruction of terminal stage osteoradionecrosis and other advanced craniofacial diseases with adult cultured stem and progenitor cells. *Plast Reconstr Surg* 2010; 126: 1699–1709.
3. Esposito M, Grusovin MG, Polyzos IP, et al. Timing of implant placement after tooth extraction: immediate, immediate-delayed or delayed implants? A Cochrane systematic review. *Eur J Oral Implantol* 2010; 3: 189–205.
4. Misch CM. Maxillary autogenous bone grafting. *Oral Maxillofac Surg Clin North Am* 2011; 23: 229–238.
5. Laurie SW, Kaban LB, Mulliken JB, et al. Donor-site morbidity after harvesting rib and iliac bone. *Plast Reconstr Surg* 1984; 73: 933–938.
6. Mesimaki K, Lindroos B, Tornwall J, et al. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. *Int J Oral Maxillofac Surg* 2009; 38: 201–209.
7. Chen F, Ouyang H, Feng X, et al. Anchoring dental implant in tissue-engineered bone using composite scaffold: a preliminary study in nude mouse model. *J Oral Maxillofac Surg* 2005; 63: 586–591.
8. Tirkkonen L, Halonen H, Hyttinen J, et al. The effects of vibration loading on adipose stem cell number, viability and differentiation towards bone-forming cells. *J R Soc Interface* 2011.[AQ: 12]
9. Hass R, Kasper C, Bohm S, et al. Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal* 2011; 9: 12.
10. Jager M, Zilkens C, Bittersohl B, et al. Cord blood—an alternative source for bone regeneration. *Stem Cell Rev* 2009; 5: 266–277.
11. Tirino V, Paino F, d'Aquino R, et al. Methods for the identification, characterization and banking of human DPSCs: current strategies and perspectives. *Stem Cell Rev* 2011.[AQ: 13]
12. Smith AJ. Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. *J Dent Educ* 2003; 67: 678–689.
13. Gronthos S, Brahim J, Li W, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002; 81: 531–535.
14. Khanna-Jain R, Vuorinen A, Sandor GK, et al. Vitamin D(3) metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells. *J Steroid Biochem Mol Biol* 2010; 122: 133–141.
15. Zhang W, Walboomers XF, Shi S, et al. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. *Tissue Eng* 2006; 12: 2813–2823.
16. Kadar K, Kiraly M, Porcsalmy B, et al. Differentiation potential of stem cells from human dental origin—promise for tissue engineering. *J Physiol Pharmacol* 2009; 60 (Suppl. 7): 167–175.
17. Zhou YS, Liu YS and Tan JG. Is 1, 25-dihydroxyvitamin D3 an ideal substitute for dexamethasone for inducing osteogenic differentiation of human adipose tissue-derived stromal cells in vitro? *Chin Med J (Engl)* 2006; 119: 1278–1286.
18. Laino G, Carinci F, Graziano A, et al. In vitro bone production using stem cells derived from human dental pulp. *J Craniofac Surg* 2006; 17: 511–515.
19. d'Aquino R, De Rosa A, Lanza V, et al. Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. *Eur Cell Mater* 2009; 18: 75–83.
20. Shahdadfar A, Fronsdal K, Haug T, et al. In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. *Stem Cells* 2005; 23: 1357–1366.
21. Heiskanen A, Satomaa T, Tiitinen S, et al. N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. *Stem Cells* 2007; 25: 197–202.
22. Aldahmash A, Haack-Sorensen M, Al-Nbaheen M, et al. Human serum is as efficient as fetal bovine serum in supporting proliferation and differentiation of human multipotent stromal (mesenchymal) stem cells in vitro and in vivo. *Stem Cell Rev* 2011.[AQ: 14]
23. Bieback K, Hecker A, Kocaomer A, et al. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem Cells* 2009; 27: 2331–2341.
24. Binulal NS, Deepthy M, Selvamurugan N, et al. Role of nanofibrous poly(caprolactone) scaffolds in human mesenchymal stem cell attachment and spreading for in vitro bone tissue engineering—response to osteogenic regulators. *Tissue Eng Part A* 2010; 16: 393–404.
25. Van der Pol U, Mathieu L, Zeiter S, et al. Augmentation of bone defect healing using a new biocomposite scaffold: an in vivo study in sheep. *Acta Biomater* 2010; 6: 3755–3762.
26. Mohajeri S, Hosseinkhani H, Ebrahimi NG, et al. Proliferation and differentiation of mesenchymal stem cell on collagen sponge reinforced with polypropylene/polyethylene terephthalate blend fibers. *Tissue Eng Part A* 2010; 16: 3821–3830.
27. Wang FM, Qiu K, Hu T, et al. Biodegradable porous calcium polyphosphate scaffolds for the three-dimensional culture of dental pulp cells. *Int Endod J* 2006; 39: 477–483.
28. Barrere F, Van Blitterswijk CA and De Groot K. .Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. *Int J Nanomedicine* 2006; 1: 317–332.
29. Rai B, Lin JL, Lim ZX, et al. Differences between in vitro viability and differentiation and in vivo bone-forming efficacy of human mesenchymal stem cells cultured on PCL-TCP scaffolds. *Biomaterials* 2010; 31: 7960–7970.
30. Guarino V, Causa F, Taddei P, et al. Polylactic acid fibre-reinforced polycaprolactone scaffolds for bone tissue engineering. *Biomaterials* 2008; 29: 3662–3670.

31. Sun H, Mei L, Song C, et al. The in vivo degradation, absorption and excretion of PCL-based implant. *Biomaterials* 2006; 27: 1735–1740.
32. Nampoothiri KM, Nair NR and John RP. An overview of the recent developments in polylactide (PLA) research. *Bioresour Technol* 2010; 101: 8493–8501.[AQ: 15]
33. Weigel T, Schinkel G and Lendlein A. Design and preparation of polymeric scaffolds for tissue engineering. *Expert Rev Med Devices* 2006; 3: 835–851.
34. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; 29: e45.
35. Stucki U, Schmid J, Hammerle CF, et al. Temporal and local appearance of alkaline phosphatase activity in early stages of guided bone regeneration. A descriptive histochemical study in humans. *Clin Oral Implants Res* 2001; 12: 121–127.
36. Nemzek JA, Arnoczky SP and Swenson CL. Retroviral transmission in bone allotransplantation. The effects of tissue processing. *Clin Orthop Relat Res* 1996; 275–282.[AQ: 16]
37. Forrest C, Boyd B, Manktelow R, et al. The free vascularised iliac crest tissue transfer: donor site complications associated with eighty-two cases. *Br J Plast Surg* 1992; 45: 89–93.
38. Willerth SM and Sakiyama-Elbert SE. Combining stem cells and biomaterial scaffolds for constructing tissues and cell delivery. 2008.[AQ: 17]
39. Mallon BS, Park KY, Chen KG, et al. Toward xeno-free culture of human embryonic stem cells. *Int J Biochem Cell Biol* 2006; 38: 1063–1075.
40. Dimarakis I and Levicar N. Cell culture medium composition and translational adult bone marrow-derived stem cell research. *Stem Cells* 2006; 24: 1407–1408.
41. Pierdomenico L, Bonsi L, Calvitti M, et al. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. *Transplantation* 2005; 80: 836–842.
42. Papaccio G, Graziano A, d'Aquino R, et al. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *J Cell Physiol* 2006; 208: 319–325.
43. d'Aquino R, Graziano A, Sampaolesi M, et al. Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. *Cell Death Differ* 2007; 14: 1162–1171.
44. Lindroos B, Boucher S, Chase L, et al. Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. *Cytotherapy* 2009; 11: 958–972.
45. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8: 315–317.
46. Yamada Y, Nakamura S, Ito K, et al. A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology. *Tissue Eng Part A* 2010; 16: 1891–1900.
47. Sittinger M, Hutmacher DW and Risbud MV. Current strategies for cell delivery in cartilage and bone regeneration. *Curr Opin Biotechnol* 2004; 15: 411–418.
48. Nof M and Shea LD. Drug-releasing scaffolds fabricated from drug-loaded microspheres. *J Biomed Mater Res* 2002; 59: 349–356.
49. Viereck V, Siggelkow H, Tauber S, et al. Differential regulation of Cbfa1/Runx2 and osteocalcin gene expression by vitamin-D3, dexamethasone, and local growth factors in primary human osteoblasts. *J Cell Biochem* 2002; 86: 348–356.
50. Shi X, Wang Y, Varshney RR, et al. In-vitro osteogenesis of synovium stem cells induced by controlled release of bisphosphate additives from microspherical mesoporous silica composite. *Biomaterials* 2009; 30: 3996–4005.
51. Shi X, Wang Y, Varshney RR, et al. Microsphere-based drug releasing scaffolds for inducing osteogenesis of human mesenchymal stem cells in vitro. *Eur J Pharm Sci* 2009; 39: 59–67.
52. Mauney JR, Sjostorm S, Blumberg J, et al. Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro. *Calcif Tissue Int* 2004; 74: 458–468.
53. McKee MD and Nanci A. Osteopontin at mineralized tissue interfaces in bone, teeth, and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover, and repair. *Microsc Res Tech* 1996; 33: 141–164.