

In the Name of Allah the

Most Gracious and the Most

Merciful

Isolation of Dental Pulp Stem Cells and their Ex Vivo Differentiation into Odontoblasts

THESIS

Submitted to the Faculty of Oral and Dental Medicine

Cairo University

In partial fulfillment of the requirements for the

Master's degree in Endodontics

By

Nermeen Elmotaz Bellah Ahmed, B.D.S. (2002)

Assistant Researcher (Oro-dental Genetics Department)

National Research Center

Supervisors

Prof. Dr. Siza Yacoub Zakhary

Professor of Endodontics

Department of Endodontics

Faculty of Oral and Dental Medicine

Cairo University

Prof. Dr. Eman Hassan Anwar Aboul-Ezz

Professor and head of Oro-Dental Research Division

National Research Center

To my beloved mom and dad, For you being the light of my life, My heart and soul, From you I get my strength and belief, Thank you for everything, You are my all

ACKNOWLEDGMENTS

To Dr. Misako Nakashima, thank you for accepting me in your lab in the first place. Thank you for teaching me, helping me to know better and pushing me to do my best. You are a great scientist and indeed a very kind person. Being to your lab was a dream that came true. With your knowledge, guidance and support this work became possible. I am forever grateful to you.

To my professor Dr. Eman Hassan Anwar Aboul-Ezz, this work would not have been possible without your support and encouragement. Under your supervision, I chose this topic and began the thesis. Thank you for believing in me and having faith in my abilities, even when I felt down. Thank you for always being there when I needed you. You are a real example of how a mentor should be. I am forever in your debt.

To my professor Dr. Siza Yacoub Zakhary, thank you so much for your support and encouragement. Thank you for having patience with me, supporting me in this topic and seeing me through to the very end. You are a great professor and a wonderful person. Words cannot explain my deep gratitude and respect to you.

To Dr. Tarek Hamed El Badry, Thank you for helping me to make this thesis look better with your wonderful to the point remarks and thank you for your help in writing this thesis. I send enormous thanks to the National Society of Human Genetics which I have the honor to be a member of, and to its head, the great professor Dr. Samia A. Temtamy, for their support and help. Without their financial and spiritual support, this work would have never been done. For my great professor Dr. Samia A. Temtamy, nothing can give her what she really deserves. She is and always will be my idol.

A special thanks to the entire team at the oral regeneration laboratory at the National Institute of Longevity Sciences (NILS), Obu, Japan especially Iohara Koichiro and Li Zheng for their help in the scientific execution of the experiments. Although it was difficult for them to communicate in English, they did their best to teach me, guide me and help me through my experiments. I really owe them a lot.

To all my friends, thank you for everything. Thank you for your support, trust and always being there for me, especially when I was depressed the most. Being your friend is a gift, which I should be thankful for, my entire life.

I cannot end without thanking my family, on whose constant encouragement and love I have relied throughout my time in Japan. Their patience, love, and continuous prayers were the guidance for me in my hardest times. Although it was very hard for them to have me away, they always helped me through the tough time and made it easy for me. To them I bow in respect.

VI

Table of Contents

Introduction	1
Review of Literature	5
I. Concept of Dentin Pulp Complex	5
II. Response of the Dentin-Pulp Complex to Injury	7
i. Types of Dentinogenesis	9
a. Secondary Dentinogenesis	9
b. Tertiary (reactionary) Dentinogenesis	9
c. Reparative Dentinogenesis	10
ii. Odontoblast Development and Dental Repair	12
a.Odontoblast Development	12
b.Dental Repair	13
III. Growth Factors in the Dentin Pulp Complex as Cell-Signaling Molecules	14
IV. Tooth Tissue Engineering and Stem Cells	25
i.Tooth Tissue Engineering	25
ii.Stem Cells	28
a. Self Renewal Capacity of Stem Cells	28
b. Multidifferentiation Potential of Stem Cells	29
c. Types of Stem Cells	30
1. Embryonic Stem Cells	31
2. Adult (post-natal) Stem Cells	33
a.Hematopoietic stem cells	34
b.Mesenchymal stem cells	35
V. Regenerative Endodontics	40
i. Root Canal Revascularization Via Blood Clotting	41
ii. Postnatal Stem Cell Therapy	43
iii. Pulp Implantation	44
iv. Scaffold Implantation	45
v. Injectable Scaffold Delivery	47
vi. Three-Dimensional Cell Printing	48

vii.Gene Therapy	49
a.In Vivo BMP Gene Therapy	49
b.Ex-vivo BMP Gene Therapy	50
VI. Isolation and Characterization of Dental Pulp Stem Cells	51
Aim of the Work	56
Materials and Methods	57
I. Isolation of human dental pulp stem cells (CD105 ⁺ ;CD146)	59
A. Collection of human teeth and removal of the pulp	59
B. Preparation of collagenase digesting solution	59
C. Isolation of primary total pulp cells	60
D. Isolation of dental pulp stem cells (CD105 ⁺ ;CD146 ⁻)	66
II. Characterization of human dental pulp stem cells (CD105 ⁺ ;CD146 ⁻)	71
III. Measuring the self-renewal capability of human dental pulp stem cells	73
(CD105 ⁺ ;CD146 ⁻)	
A. Colony formation efficiency testing	73
B. Expansion of cells	74
C. Counting of cells and drawing of the proliferation curve	74
IV. Induce multidifferentiation lineages in human dental pulp stem cells (CD105 ⁺ ;CD146 ⁻)	79
A. Adipogenic differentiation	79
B. Chondrogenic differentiation	81
V. Differentiation of human dental pulp cells (CD105 ⁺ ;CD146 ⁻) into odontoblasts using	83
BMP2	
Results	89
I. Isolation of human dental pulp stem cells (CD105 ⁺ ;CD146 ⁻)	91
II. Characterization of human dental pulp cells (CD105 ⁺ ;CD146 ⁻)	94
III. Measuring the self-renewal capability of human dental pulp stem cells	95
(CD105 ⁺ ;CD146 ⁻)	
A.Colony formation efficiency testing	95
B.Self renewal capacity and proliferation curve	96
IV. Induction of multidifferentiation lineages in human dental pulp stem cells	97
(CD105 ⁺ ;CD146 ⁻) in vitro	

A. Adipogenic differentiation	97
B. Chondrogenic differentiation	101
V. Differentiation of human dental pulp cells ($CD105^+$; $CD146$) into odontoblasts using	103
BMP2	
Discussion	107
Summary and Conclusions	122
Recommendations	126
References	128
Arabic Summary	

List of Figures

Figure (1):	The cellular events involved in reparative and reactionary dentinogenesis.	11
Figure (2):	Growth factors may act in endocrine, autocrine, paracrine, juxtacrine, and intracrine modes.	15
Figure (3):	Growth factor interaction with specific receptor on the cell surface.	16
Figure (4):	Role of growth factors in signalling odontoblast differentiation.	17
Figure (5):	Triad of tissue engineering.	27
Figure (6):	Self renewal capability of stem cells.	29
Figure (7):	Embryonic stem cells.	33
Figure (8):	Schematic presentation of the study design and stages.	58
Figure (9):	Collection of teeth.	62
Figure (10):	Preparing collagenase-digesting solution.	63
Figure (11):	Isolating primary total pulp cells.	64
Figure (12):	Continuing isolation of primary total pulp cells.	65
Figure (13):	Isolation of dental pulp stem cells by fluorescent activated cell sorting (FACS).	69
Figure (14):	Flow cytometer JSAN, Japan.	70
Figure (15):	Polymerase Chain Reaction (PCR).	72
Figure (16):	Monitoring cells.	76
Figure (17):	Using hemacytometer and inverted phase contrast light microscope for cell count.	77
Figure (18):	Cell counting.	78
Figure (19):	Cell pellets incubated in 5% CO2 at 37°c	82
Figure (20):	Homogenizer.	87

Figure (21):	Schematic presentation of the study results.	90
Figure (22):	Cultured human (CD105 ⁺ ;CD146 ⁻) cells (original magnification $x10$).	92
Figure (23):	Cultured human primary pulp cells (original magnification x10).	93
Figure (24):	Expression of Bmi1 and Stat3, stem cell markers.	94
Figure (25):	The growth of a cell colony derived from single pulp stem cell (CD105 ⁺ ;CD146 ⁻).	95
Figure (26):	Proliferation curve of human pulp stem cells (CD105 ⁺ ; 146 ⁻) showing the cumulative cell number up to 95 days.	96
Figure (27):	Adipogenic differentiation of pulp stem cells (CD105 ⁺ ;CD146 ⁻) on day 9 (original magnification x 10).	98
Figure (28):	Adipogenic differentiation of pulp stem cells (CD105 ⁺ ;CD146 ⁻) on day 20 (original magnification x 10).	98
Figure (29):	Human dental pulp stem Cells ($CD105^+$; $CD146^-$) stained with Oil Red O stain (original magnification x10).	99
Figure (30):	Expression of PPAR gamma 2, marker of adipogenic differentiation.	100
Figure (31):	Cells pellet stained with Alcian Blue (original magnification x40).	102
Figure (32):	Odontogenic differentiation of pulp stem cells ($CD105^+$; $CD146^-$) on day 7 (original magnification x10).	103
Figure (33):	Odontogenic differentiation of pulp stem cells ($CD105^+$; $CD146^-$) on day 25 (original magnification x10).	104
Figure (34):	Human dental pulp stem cells (CD105 ⁺ ;CD146 ⁻) on day 30 stained with Alizarin red stain (original magnification $x10$).	105
Figure (35):	Expression of Dentin sialophosphoprotein and Enamelysin, markers of odontogenic differentiation.	106

List of Tables

(Table 1):	Superfamilies and families of the more commonly recognized growth	14
	factor.	
(Table 2):	List of Bone Morphogenic Proteins (BMPs).	19
(Table 3):	Biological action of BMP.	20
(Table 4):	Types of stem cells.	31
(Table 5):	Human and porcine primers used for real-time reverse transcription-	88
	polymerase chain reaction.	

List of Abbreviations

BM:	Basement membrane
BMP:	Bone morphogenic protein
BMSSC:	Bone marrow stromal stem cells
β actin:	Beta actin
CD:	Clusters of differentiation
cDNA:	Complementary Deoxyribonucleic acid
DEJ:	Dentino-enamel junction
DEPC:	Diethyl procarbonate
DMEM:	Dulbecco modified eagle's media
DNA	Deoxyribonucleic acid
DP:	Dental papilla
DPSC:	Dental pulp stem cell
DSPP:	Dentin sialophosphoprotein
EBM-2:	Endothelial basal cell medium-2
ECM:	Extracellular matrix
EDTA:	Ethylenediamine tetraacetic acid
ESC:	Embryonic stem cell
FACS:	Florescence-activated cell sorting
FBS:	Fetal bovine serum
GAG:	Glycosaminoglycans
Gdf:	Growth differentiation factor
GFAP:	Glial fibrillary acid protein
HEPS:	1-(2-Hydroxyethyl)-1 piperazineethanesulfonic acid buffer solution
HSC:	Hematopoietic stem cell
IDE:	Inner dental epithelium

MACS:	Magnetic-activated cell sorting
MC:	Mesenchymal cell
MgCl ₂ :	Magnesium chloride
mRNA:	Messenger ribonucleic acid
MSC:	Mesenchymal stem cell
PBS:	Phosphate buffered solution
PCR:	Polymerase chain reaction
PDL:	Periodontal ligament
PDLSC:	Periodontal ligament stem cell
PI:	Propidium iodide
PPARY2:	Peroxisome proliferating activated receptor gamma 2
rh-BMP2:	Recombinant human bone morphogenic protein 2
rh-EGF:	Recombinant human epidermal growth factor
rh-IGF-1:	Recombinant human insulin growth factor-1
RNA:	Ribonucleic acid
Rt-PCR:	Reverse transcriptase polymerase chain reaction
SCAP:	Stem cells from root apical papilla
SHED:	Stem cells from human exfoliated deciduous teeth
SP:	Side population
STAT3:	Signal transducer and activator of transcription 3
TGF:	Transforming growth factor
UV:	Ultraviolet
VCAM-1:	Vascular adhesion molecule-1
VEGF:	Vascular endothelial growth factor

Introduction

Despite our extensive knowledge regarding the pathology of dental disease, restoration of diseased dental tissue to date remains fairly empirical. However, our increasing understanding of the exquisite regenerative potential of the dentine- pulp complex highlights the importance of characterizing fully the cellular and molecular processes under-pining dentin regeneration.

Pulpal exposure, due to caries shows very limited potential for pulp recovery due to bacterial infection of the pulp for substantial period of time, which compromises the defense reaction.

Vital pulp therapy aims to treat reversible pulpal injury and maintain pulp vitality and function. It includes two therapeutic approaches: indirect pulp capping in cases of deep dentinal cavities and direct pulp capping in cases of pulp exposures. Successful outcome for vital pulp therapy is very dependent on the type and location of injury, age of the tooth, treatment modality (capping material) and integrity of the cavity restoration.

Whilst the biological processes directed by the treatment strategy have received much attention, controversy still exists regarding the biological basis of the mechanism by which the capping material regulates healing and repair of the pulp in vital pulp therapy. Even if reparative dentin is formed, its orientation

1