Dentine Sialophosphoprotein Expression and Mineralized Nodule Formation of Primary Pulpal Fibroblasts, Gingival Fibroblasts and MG63

Wisakha Paingam

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Author Miss Wisakha Paingam

Major Program Oral Health Sciences

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The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Oral Health Science

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ชื่อวิทยานิพนธ์ การแสดงออกของเด็นทินไซอาโลฟอสโฟโปรตีนและการสร้างแร่ธาตุของเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม, เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์

ผู้เขียน นางสาววิสาขา ไผ่งาม

สาขาวิชา วิทยาศาสตร์สุขภาพช่องปาก

ปีการศึกษา 2551

บทคัดย่อ

บทนำ ผลการศึกษาที่ผ่านมาพบว่าเนื้อเยื่อโพรงประสาทฟันแท้มีความสามารถในการสร้าง แร่ธาตุเมื่อทำการเลี้ยงภายใต้สภาวะที่มีการกระตุ้นให้สร้างสารเกี่ยวกับกระดูก ยิ่งไปกว่านั้นมีการศึกษาหนึ่งรายงานว่า ฟันน้ำนมของมนุษย์ที่หลุดเองนั้นเป็นแหล่งของสเต็มเซลล์จำนวนมากและยังพบอีกว่าเซลล์เหล่านี้มีความสามารถในการเจริญเติบโตได้อย่างรวดเร็วและมีอัตราการแบ่งตัวที่เหนือกว่าของสเต็มเซลล์ที่ได้จากฟันแท้ ดังนั้นเนื้อเยื่อโพรงประสาทฟันน้ำนมน่าจะสามารถเป็นแหล่งในการรักษาเนื้อเยื่อโพรงประสาทฟันแบบการสร้างเนื้อเยื่อใหม่

วัตถุประสงค์ เพื่อตรวจสอบความสามารถการสร้างแร่ธาตุและการแสดงออกของยีนเด็นทินไซอาโลฟอสโฟโปรตีนในเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์ เมื่อทำการเพาะเลี้ยงเซลล์ในน้ำเลี้ยงเซลล์ที่มีการใส่สารกระตุ้นและการศึกษายึดเกาะของเซลล์ทั้ง 3 กลุ่ม บนไคโตซานชนิดแผ่น

วัสดุและวิธีการ ใช้น้ำเลี้ยงเซลล์ที่มีการใส่สารกระตุ้นเพื่อเลี้ยงเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม ,เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือก และเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์ ในแต่ละช่วงเวลา (14, 21 และ 28 วัน) ศึกษาการสร้างแร่ธาตุโดยการย้อมสีอะลิซาลินเรดเอส และ ศึกษาการแสดงออกของยีนเด็นทินไซอาโลฟอสโฟโปรตีนโดยวิธีรีเวิส ทรานสคริปชันโพลีเมอเรสเชนรีแอคชัน ณ ช่วงเวลา 0, 7 และ 21 วัน ทำการเลี้ยงเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม, เซลล์ไฟโบรบลาสต์ จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูก ที่พัฒนามาจากเซลล์มะเร็งของมนุษย์บนไคโตซานชนิดแผ่น โดยทำการเลี้ยง เซลล์เป็นเวลา 24 ชั่วโมง และทำการยึดเซลล์ด้วยสารฟอร์มาดีไฮด์และย้อมเซลล์ด้วยสีทริปแฟนบลู 0.4 เปอร์เซนต์ ทำการวิเคราะห์รูปร่างของเซลล์ด้วยกล้องจุลทรรศน์ชนิดใช้แสง

ผลการศึกษา พบว่าเซลล์ทุกกลุ่มสามารถสร้างแร่ธาตุได้โดยมีการเพิ่มจำนวนของแร่ธาตุในแต่ละ

ช่วงเวลาในกลุ่มของเซลล์ไฟโบรบลาสต์ของเนื้อเยื่อในโพรงประสาทฟันน้ำนม และเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์ โดยเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกมีการสร้างแร่ธาตุได้น้อยในช่วงเวลา 14 และ 21 วัน เมื่อเทียบกับช่วงเวลา 28 วัน และไม่พบการแสดงออกของ ยีนเด็นทีนไซอโลฟอสโฟโปรตีนในทุกกลุ่มเซลล์ในทุกช่วงเวลา เมื่อพิจารณารูปร่างของเซลล์ด้วยพบว่า เซลล์ทุกกลุ่มที่เลี้ยงบนพื้นผิวที่เคลือบไคโตซานชนิดแผ่นมีรูปร่างกลมกว่าเซลล์ที่เลี้ยงบนพื้นผิวที่ไม่มีไคโตซานเคลือบการย้อมสีทริปแฟนบลู พบว่าเซลล์ในทุกกลุ่มบนพื้นผิวที่มีไคโตซานเคลือบมีลักษณะกลมมากกว่าบนพื้นผิวที่ไม่มีไคโตซานเคลือบ จำนวนเซลล์ของเซลล์ไฟโบรบลาสต์เนื้อเยื่อในโพรงประสาทฟันน้ำนม เมื่อทำการเลี้ยงเซลล์ ณ เวลา 24 ชั่วโมงมีจำนวนมากกว่าเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์ มะ เร็ง ของมนุษย์ทั้งบนพื้นผิวที่มีไคโตซานเคลือบและบนพื้นผิวที่ไม่มีไคโตซาน

สรุป จากการศึกษานี้แสดงให้เห็นว่าเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม เซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์ และเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกสามารถสร้างแร่ธาตุได้เมื่อทำการเลี้ยงภายใต้น้ำเลี้ยงเซลล์ที่ใส่สารกระตุ้นแต่ไม่พบการแสดงออกของยีนเด็นทีนไซอโลฟอสโฟโปรตีน นอกจากนี้เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนมมีการยึดเกาะที่สูงกว่า เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์ ทั้งบนพื้นผิวที่มีไคโตซานและพื้นผิวที่ไม่มีไคโตซาน

Thesis Title Dentine Sialophosphoprotein Expression and Mineralized Nodule Formation of Primary Pulpal Fibroblasts, Gingival Fibroblasts and MG63

Author Miss Wisakha Paingam

Major Program Oral Health Sciences

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Abstract

Introduction: Previous studies showed that dental pulp cells of permanent tooth had the ability to form mineralized nodules under osteogenic condition. Moreover, one study found that human exfoliated deciduous teeth contained a rich supply of stem cells in dental pulp tissues and also found that these cells had the ability to grow much faster, had a higher replicative rate and greater viability than that of dental pulp stem cells in permanent teeth. Therefore, dental pulp tissue can be a promising source for regenerative pulp therapy.

Objective: This study aimed to investigate the capability of primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line to form mineralized nodule and to detect the expression of dentine sialophosphoprotein under inductive medium cultivation. The cell attachment on chitosan sheet was also studied.

Materials and Methods: The inductive medium was used to induce mineralized nodule formation of primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line at three different time points (14, 21 and 28 days). The nodule formation was identified by Alizarin Red S staining. Dentine sialophosphoprotein expression was identified by reverse transcription of polymerase chain reaction at period of 0, 7 and 21 days. Primary pulpal fibroblasts, gingival fibroblasts and MG63 were seeded on chitosan sheet and cultivated for 24 hrs. The cell attached and growth of primary pulpal fibroblasts, gingival fibroblasts and MG63 were monitored and compared after 24 hrs of cell seeding. All cells were fixed with formaldehyde and stained with 0.4%Tryphan blue. The morphology of cells was analyzed under light microscope.

Result: The mineralized nodules were found in all cell groups. The amount of mineralized nodules increased with times in all groups. Dentine sialophosphoprotein expression could not be detected in all groups at any time point. The morphology of cell stained by Tryphan blue showed

that all cells plated on the chitosan coated appeared rounder than non-chitosan coated wells. The number of primary pulpal fibroblasts after 24 hours cultivated was more than gingival fibroblasts and MG63 on both chitosan and non-chitosan coated wells.

Conclusion: Our results demonstrated that primary pulpal fibroblasts, gingival fibroblasts and MG63 formed mineralized nodules under inductive condition but did not expressed dentine sialophosphoprotein. Primary pulpal fibroblasts had higher cell attachment than gingival fibroblasts and MG63 after 24 hours of cell cultivation.

Keywords: primary pulpal fibroblasts, gingival fibroblasts, MG63, mineralized nodule, dentine sialophosphoprotein, chitosan

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List of Abbreviations and Symbols

ASCs	= Adult stem cells
Bp	= base pair
β	= Beta
β-GP	= Beta glycerophosphate
BMP	= Bone morphogenic protein
BSP	= Bone sialprotein
$^{\circ}C$	= Degree Celsius
$Ca(OH)_2$	= Calcium hydroxide
Corp.	= Corporation
CS	= Chondroitin sulphate
DNA	= deoxyribonucleic acid
dNTP	= deoxynucleoside triphosphate
DMEM	= Dulbecco's Modified Eagle Medium
DPP	= Dentin phosphoprotein
DS	= Dermatan sulfate
DSP	= Dentin sialoprotein
DSPP	= Dentin sialophoshoprotein
ECM	= Extracellular matrix
EDTA	= Ethylenediaminetetraacetic acid
EGF	=Epidermal growth factor
ESCs	=Embryonic stem cells
et al.	= and other people
FACS	= fluorescence activated cell sorting
FBS	= Fetal bovine serum
FGF	= Fibroblast growth factor
G	= Gram
GAGs.	= glycosaminoglycans
GDFs	= Growth and differentiation factors

List of Abbreviations and Symbols (Continued)

GAPDH	= Glyceraldehyde-3-phosphate dehydrogenase
GLA	= Gamma Linolenic Acid
HF	= Human fibroblast cells
HP	= Human primary pulpal fibroblast cells
∝HS	= alpha heparan sulfate
IGFs	= Insulin-like growth factors
kb	= kilobase
kDa	= kilo dalton
KS	= Keratan sulphate
L	= Litre
m	= milli
M	= Molar
MEPE	= Matrix extracellular phosphoglycoprotein
MHC	= Major histocompatibility complex
MMP	= Matrix metalloproteinase
Mpa	= Mega Pascal
mg	= milligram
min	= minute (s)
m	= milli
mm^3	= cubic millimetre
mRNA	= Messenger ribonucleic acid
NaOH	= Sodium hydroxide
ng	= nanogram
nm	= nanometre
OD	= Optical density
OPG	= Osteoprotegerin
OPN	= Osteopontin
PBS	= Phosphate buffer saline

List of Abbreviations and Symbols (Continued)

PCR	= Polymerase chain reaction
PDL	= Periodontal ligament
PDGF	= Platelet-derived growth factor
PGA	= Propylene glycol alginate
PGs	= Proteoglycans
pH	= log hydrogen ion concentration
RER	= Rough endoplasmic reticulum
RNA	= Ribonucleic acid
rpm	= round per minute
RT-PCR	= Reverse transcriptase polymerase chain reaction
SD	= Standard deviation
Shh	= Sonic hedgehog
SLRPs	= Small leucine-rich proteoglycans
TIMP	= Tissue inhibitor of metalloproteinase
TGF	= Transforming growth factor–beta
U	= unit
USA	= United State of America
UV	= Ultraviolet
VEGF	= vascular endothelial growth factor
v/v	= Volume/volume
v/v	= volume/voloume
μM	= micromolar
w/v	= weight/volume
%	= percent

Chapter 1

Introduction

Vital pulp therapy is the method for the treatment of reversible pulpitis. The objective of vital pulp therapy is to maintain vitality of pulpal tissue including sealed restoration to keep tooth for function[1]. The dental structure has a complex composition of different specialized tissue and cell types consisting of dentin-producing odontoblasts, ameloblasts and periodontal structures such as cementum, periodontal ligament, gingiva, and alveolar bone[2]. The dental structures are often affected by infectious disease–for example caries that is the cause of pain and impaired function and also early tooth loss. In restorative dentistry, the therapy widely relies on the use of medicaments, capping procedures or root canal fillings[3]. In primary teeth, vital pulp therapy can save the teeth for many functions such as esthetics, phonetics, mastication and serve as natural space maintainers to maintain arch length. There are many medicaments using for vital pulp therapy in primary teeth. The most popular and widely used is formocresol. Although the success rate of formocresol pulp therapy in primary teeth is quite high[7], the properties of formocresol are mutagenicity and carcinogenicity in experimental animal models[8]. In addition, formocresol pulp therapy only eradicates and inhibits the inflammation in the coronal pulp to prevent further inflammation of the pulp tissue but not encourage the true healing and regeneration of dental pulp tissues[9].

Type of vital pulp therapy

1. Direct pulp capping: put medicament such as Ca (OH_2) on site of exposed pulp[10].
2. Coronal pulpotomy: remove coronal pulp tissue and put medicament[11].
3. Partial pulpotomy: remove outer most of coronal pulp tissue and put medicament [12, 13].

Many medicaments are used in vital pulp therapy as following[14]

1. Formocresol
2. Calcium hydroxide
3. Resin modified glass ionomer cement
4. Mineral trioxide aggregate bonding agentBioactive molecules

The new treatment modality is to use the cells and tissues to build the loss or deformed organ that we called “tissue engineering”. Tissue engineering holds the promise of offering the true replacement of tooth structures[4]. Advanced knowledge of the molecular and cellular aspects in the dental field is essential for possible application in regenerative dentistry. A number of strategies have been developed to support the healing process, such as

1. Inductive dental tissue engineering strategies that activate and stimulate endogenous cells by the incorporation of demineralized dentin fragments[5, 6].
2. Use of various extracellular matrix scaffolds (fibronectin, collagen, fibrin, hydroxyapatite, etc)[5, 6].
3. Addition of cytokines such as bone morphogenic proteins and transforming growth factor[5, 6].

Tissue engineering is based on three basic components of biologic tissues [21]

1. Stem cells/ progenitor cells
2. Signaling molecules
3. Extracellular matrix/ scaffold

Dental pulp stem cells have the ability to form dentine–like structures and associated pulp tissues both *in vitro* and *in vivo*[15, 16]. In addition, human exfoliated deciduous teeth contain a rich supply of stem cells in dental pulp tissues. Moreover, pulp from primary incisor teeth had more stem cell activity than primary molar teeth[17, 18]. Therefore, dental pulp stem cells can be a promising source for regeneration pulp therapy[19]. This study aims to investigate the characteristic of primary pulpal fibroblasts in its behavior of growth and cell attached on chitosan sheet, capability of synthesizing mineralized nodule *in vitro* and dentine sialophosphoprotein (DSPP) expression.

Review of literature

1. TISSUE ENGINEERING

The human body is a complex structure. Organs deformities have an enormous impact on psychosocial interactions; therefore, many techniques have developed to rebuild them. Tissue engineering offers a new option to supplement existing treatment regimens for diseases. Moreover, compared with other treatment regimens, tissue engineering requires an interdisciplinary approach that combines with responding progenitor/stem cells, signaling molecules and scaffold. These elements must be combined to facilitate a development of tissue formation, organ establishment and the creation of organ functions (Fig.1).

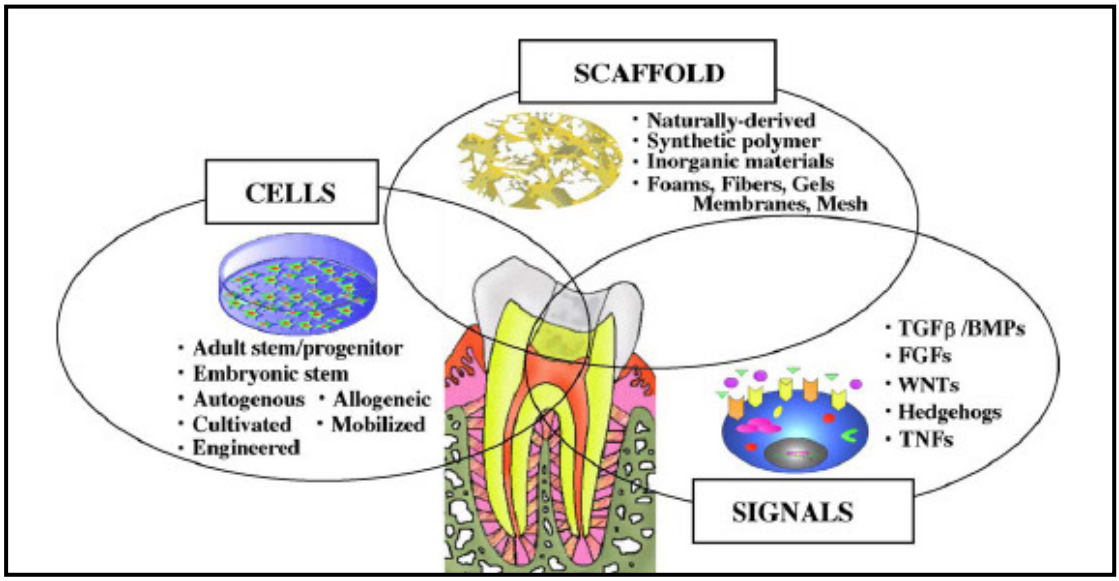


Fig. 1 The key elements of tissue engineering and dentin regeneration[26].

1.1 Stem cells

Stem cells are the unique cells that give rise to specialized tissues. Stem cells have the capacity for self-renewal and can give rise to one and sometimes many different cell types[4, 20]. Stem cells were discovered by the experimental study of Owen in 1945, when he found lifelong blood chimerism between twin cows[22]. There are two types of stem cells[20]: embryonic stem cells (ESCs) and adult stem cells (ASCs).

ESCs are found in the inner cell mass of the blastocyst (early embryo). ESCs can give rise to all tissue types and can develop into a complete organism. The ESCs may be renewed *in vitro* and expanded indefinitely by regular passage in co-culture with a wide range of feeder cells (e.g., mouse or human embryonic fibroblasts). ESCs are pluripotent and immortal, so they can potentially form large varieties of cells in the tissues of the body. They may be directed into a wide range of specific mature cell types and may be selected for specific lineages under differentiating conditions. These cells can be purified by fluorescence activated cell sorting (FACS) and shown to be functional by biological assays *in vitro* and by transplantation into preclinical animal models of human diseases[21]. There is a consider that ESCs will be used in the wide spectrum of human regenerative medicine[4] as vehicles for gene therapy, targets for drug discovery, and cells for tissue engineering.

The second type of stem cells, ASCs that are found in developed tissues of the newborn, juvenile and adult organism[4, 20]. ASCs were developmentally restricted only to differentiate into cell lineages of the specified tissue where they resided[21]. ASCs have capacity to repair and regenerate damaged and diseased tissue[4], ASCs can be found at many sites of body such as bone marrow and dental pulp[4]. ASCs play a major role in homeostasis and tissue repair (Fig. 2). Stem cells have been studied for decades, for example, it is well established that hematopoietic stem cells isolated from adult tissues can give rise to virtually all the cell-types in the blood cell lineage[23]. ASCs were originally thought to have a rather restricted potential for generating new tissues; that is, hematopoietic stem cells could only make new blood cells but recent studies have changed this perception[17]. ASCs have astonishing and unanticipated capacities to develop into diverse tissues[17, 20] for example-stem cells residing in the bone marrow called bone marrow stromal stem cells (BMSCs) are poised the occasional fractured bone and are likely

responsible for repairing microfractures that occur on a daily basis[23]. Stem cells residing in the dental pulp tissues called dental pulp stem cells (DPSCs), in addition to generating the derivatives of the dental tissues, can also give rise to adipose cells and neural cells[17] (Fig. 3). Perhaps even more astonishing, mouse central nervous system stem cells can differentiate into cells of other tissues such as muscle, blood, and heart, in addition to several types of nervous system cells[24]. ASCs had high potential in differentiate to many cell types but not the same as ESCs.

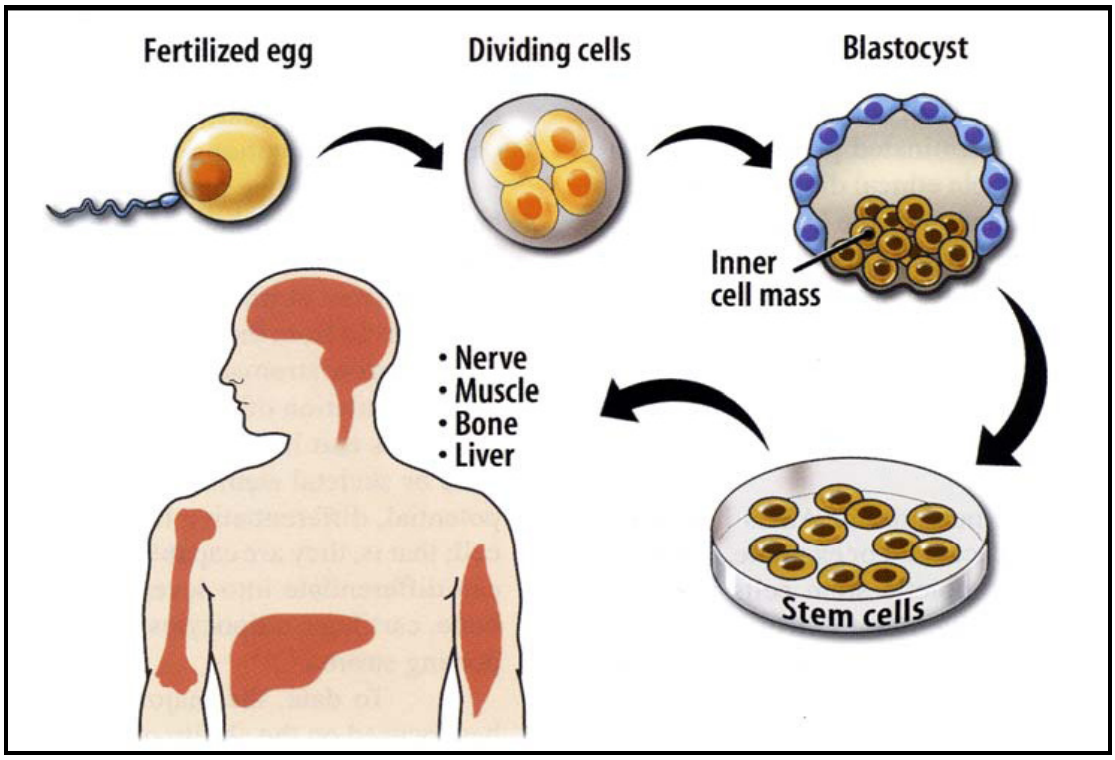


Fig. 2 ESCs have been shown to differentiate into several different cell types[4].

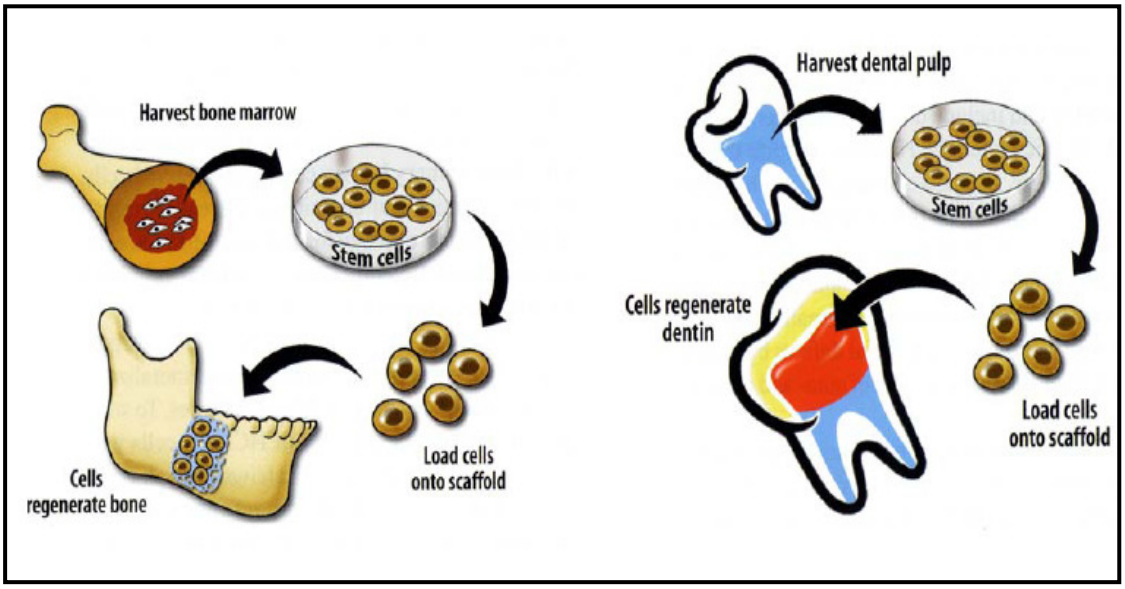


Fig. 3 ASCs have been shown to differentiate into several different cell types[4].

1.2 Signaling molecules

The signaling molecules are generated from cell-ECM and cell-cell interactions, as well as from physical-chemical and mechanical stimuli. The processes of tissue engineering involve a series of interactions of multiple signaling molecules. The various diffusible proteins induce cell and tissue interaction via complex signaling networks[29]. These signaling molecules function synergistically and/or antagonistically to organize tissue patterning during organ formation (Fig. 2). Signaling molecules function through binding to their specific cell membrane-bound receptor that trigger intracellular pathway leading to signal transduction into the nucleus where transcription factors are activated, and eventually causing change of gene expression[28, 29]. The expression of many signaling molecules during development of organs, make the result of forming a signaling network that regulate organogenesis. In dentistry, it has been studied that signaling molecules are involved in reciprocal-interaction between epithelium cells (oral epithelium) and mesenchyme cells (cranial neural crest cells) during tooth development[29]. The growth factors such as transforming growth factor beta (TGF-β), FGF and EGF (epidermal growth factor) families, have been implicated in formation of mesenchymal condensates and in

epithelial morphogenesis of many organs, including the tooth[30]. The TGF-β superfamily encompasses extracellular ligands involved in a diverse range of biological functions. Family members include TGF-β, bone morphogenetic proteins (BMPs), and growth and differentiation factors, along with activins and inhibins. The TGF-β superfamily found in almost all cell types, these proteins are involved in numerous cell processes, including bone and joint development, cell proliferation and differentiation, and dorsal/ventral patterning. Because of their ubiquitous nature, TGF-β proteins are associated with a variety of diseases ranging from skeletal abnormalities and differentiation to metabolic disorders and play critical roles in neoplastic development and stem cell differentiation. TGF-β signaling pathway is involved in many cellular processes in both the adult organism and the developing embryo including cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions. In spite of the wide range of cellular processes that the TGF-βsignaling pathway regulates, the process is relatively simple. The TGF Beta superfamily of ligands include: Bone morphogenetic proteins (BMPs), Growth and differentiation factors (GDFs), Anti-müllerian hormone (AMH), Activin, Nodal and TGF-β's[30]. The outstanding one is TGF-β 1 released from dentin matrix has target receptors both in odontoblasts and pulp tissue, indicating that signaling pathways in the cells respond to the TGF-β[31]. Specifically, TGF-β 1 has been shown to associate with reactions in response to the dental injury, by regulating tertiary dentinogenesis. Depending on the degree of injury, TGF-β1 may either stimulate pulpal cell proliferation and differentiation or may have effect on the odontoblasts and pulpal cell secretory activity and mediated reparative processes at sites of dental injury[1].

In early tooth germ development, the morphogenetic signaling networks include the five major classes of evolutionarily conserved genes[25]: bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), wingless- and int-related proteins (Wnts), Hedgehog proteins (Hhs), and tumor necrotic factor (TNF) families[29]. These families exhibit redundant and reiterative signaling, each with distinct temporal and spatial expression during initiation, patterning formation, morphogenesis, and cytodifferentiation[26]. These may specify early patterning of organs through regulation of molecules at the cell surface and the extracellular matrix. Although five distinct families of morphogens are involved in embryonic tooth development, BMPs played an important role for tooth regeneration and are used sequentially and

repeatedly throughout embryonic tooth development, initiation, morphogenesis, cytodifferentiation and matrix secretion. Among BMP groups, BMP-2 and BMP-4 form a subgroup, BMP-2 represents a classical prototype of the BMPs, and it is expressed by both functional odontoblasts and human adult pulp tissue. BMPs may have a special role in repair processes in mature teeth, since it has been shown that BMP-2 may induce reparative dentin formation[26-29].

1.3 Scaffolds

The commonly used strategy for tissue engineering is involved in the seeding of cells onto porous scaffolds. The scaffolds may vary in their compositions, but the desirable properties are to have cells attach to the scaffolds, increase cells growth, differentiate and act as template for tissue formation that are the major goal for using scaffolds[48, 49]. Scaffolds are either made of natural or synthetic material. Natural derived, such as collagen, chitosan, fibrin, and alginates, have the advantage of source as natural substances so that they are inherently compatible to a living body. However, there are disadvantages such as relatively high tissue rejection reactivity and infection rate, and it is difficult to control their decomposition properties. The second type of scaffold is synthetic polymers such as polyglycolide, polylactide, and lactidelglycolide copolymer. The synthetic polymers have limited physical properties such as easily breakable, are very soluble in water which puts great limitations on use for regeneration materials for human tissue. In this study we will emphasize on chitosan scaffold. Chitosan is a derivative of chitin, a natural biopolymer that is biodegradable. The chemical composition of chitosan is a binary polyheterosaccharide of N- acetylglucosamine and glucosamine[57, 58]. The properties of chitosan is non-toxic and can be formulated in a variety of forms including powders, gels and films with an array of biological properties and applications. The versatility of chitosan offers a wide range of applications. There are many forms of chitosan such as in the form of a viscous gel in periodontal regeneration or as a solid form applicable in alveolar and craniofacial regeneration[58]. The porous property of chitosan allow a wide range of molecules to be attached and can deliver growth factors that will help optimize the physical and biological properties of this material as a matrix scaffold and vehicle system in tissue regeneration[59].

2. STEM CELLS AND DENTAL TISSUE ENGINEERING

Regenerative dentistry, including periodontics, endodontics and maxillofacial surgery, is a new field that seeks to apply the concepts of tissue engineering to the management of lost oral tissues by using various types of stem cells, growth factors, and scaffolds. Tissue engineering strategies are also being applied in an attempt to regenerate the dentin–pulp complex[4]. Stem cells, signaling molecules and different physical matrices have been used in conjunction with human or animal dental pulp cells both in vitro and in vivo[15, 16]. The promising results reported in animal models by several researchers together with studies demonstrating the regenerative capacity of human dental pulp, are positive steps toward successful regenerative treatment in humans[37]. Transplantation of dental pulp stem cells may be used to repair craniofacial bone or even repair or regenerate teeth[4]. At the present date, most studies have shown the effectiveness of stem cell regenerating therapy in experimental animal models with the successful results [16, 52]. The mineralized tissue in the body apart from bone is dentine. The precursor cells maintaining repair process of dentine is the pulp tissues, which has ability to differentiate to odontoblasts. The specialized cells in dental pulp tissues called DPSCs, in experimental animal model, generated a dentine –like structure with collagen fibers and contained DSPP[16, 52]. Based on the fact that we have populations of stem cells that regenerate bone and its marrow, dentine, cementum, it is possible to envision restoration of hard tissues in the oral cavity by using the dental pulp stem cells[4]. Dental tissues have the capability to repair itself after injury. The dentine that formed after injury is called tertiary dentinogenesis. Tertiary dentinogenesis responses may be sub-divided into reactionary and reparative dentinogenesis depending on the fate of the odontoblasts underlying the injury. Unlike bone, dentin and cementum have no physiological turnover[44]. Indeed it has been indicated that these structures have only a limited reparative capacity to form tertiary dentin and new cementum. In the process of dentine synthesis, odontoblasts secret a unique set of ECM comprised of type I collagen and three non-collagenous proteins: dentine matrix protein (DMP), DPP and DSP. Type I collagen is the major component of the dentin matrix and provides the framework for the deposition of apatite crystals. DPP is the most abundant non-collagenous protein. The DPP has been shown to initiate the formation of hydroxyapatite crystals within collagen fibrils and to control collagen fibril formation[47]. DSP is a

sialic acid–rich glycoprotein, which has a similar amino acid composition to other sialoproteins. The role of DSP in dentin mineralization is still unclearOne of the major non-collagenous proteins that play the important role in dentin matrix formation is DSPP, a single transcript encoding both DPP and DSP[45-47]. DSPP is predominantly expressed by odontoblasts and widely accepted to be an important phenotypic marker for odontoblasts, although it is transiently expressed in preameloblasts[46] and bone at very low levels[49]. The teeth of the DSPP gene knockout mouse showed defective dentin mineralization[47]. Recently, stem cells have been identified and isolated from dental pulp. Their cluster-forming and differentiation abilities, as well as their cell surface markers, were described by Gronthos and co-workers in 2002[16]. The dental pulp stem cells represent a clonogenic and highly proliferative cell population generating densely calcified nodules in vitro. After transplantation in vivo dentin-like and dentin sialophosphoprotein- rich mineralization products were found.

3. CELLS IN DENTAL PULP

Dental pulp cell is a connective tissue that comprise of blood, lymph vessels, nerves interstitial fluid and varieties of cells[33]. Cells in dental pulp are function together for synthesizing dentine. There are many cells found in pulp such as odontoblasts, pulp fibroblasts, macrophage, dendritic cell, lymphocyte and mast cells (table 1).

3.1 Odontoblasts

Odontoblasts is responsible for dentinogenesis both development and in the mature tooth. The odontoblast is the most characteristic cell of the dentin-pulp complex. The ultrastructural features of the odontoblast have been the subject of numerous investigations. The cell body of the active odontoblast has a large nucleus that may contain up to four nucleoli[27]. The nucleus is situated at the basal end of the cell and is contained within a nuclear envelope. Apparently the odontoblast synthesizes mainly type I collagen, although small amounts of typeV collagen have been found in the ECM. In addition to proteoglycans and collagen, the odontoblast

secretes dentine sialoprotein and phosphophoryn, a highly phosphophorylated phosphoprotein involved in extracellular mineralization. Phosphophoryn is unique to dentine and is not found in any other mesenchymal cell lines. The odontoblast also secretes both acid phosphatase, an enzyme that closely link to mineralization[29, 35].

3.2 Pulp fibroblasts

Fibroblasts are the most numerous cells of the pulp. They appear to be tissue-specific cells and are capable to giving rise to the specific cells that are committed to differentiate when they are induced by the proper signal. These cells synthesize types I and III collagen, as well as proteoglycans and glycosaminoglycans (GAGs). Thus they produce and maintain the matrix proteins of the ECM and are responsible for collagen turnover in the pulp. Many pulpal cells do seem to remain in a relatively undifferentiated modality. This character has been supported by the observation of large numbers of reticulin -like fibers in the pulp. Reticulin fibers have an affinity for silver stains and are similar to the argyrophilic fibers of the pulp. Many experimental models have been developed to study wound healing in the pulp, particularly dentinal bridge formation after pulp exposure or pulpotomy. One study demonstrated that mitotic activity preceding the differentiation of replacement odontoblasts appears to occur primarily among perivascular fibroblasts[35]. The dental pulp contains cells that are responsible for the formation and turnover of a complex non-mineralized extracellular matrix. Most of the cells are fibroblast-like cells, macrophages, nerves, and capillaries. Division of these cells within the crown of the adult pulp appears to be limited, although cell renewal probably occurs. While, superficially, all the fibroblasts appear morphologically similar, variations in their proliferative activity suggest that they represent a heterogeneous cell population[37]. Such heterogeneity is further suggested from studies in human deciduous teeth where 183 fibroblast strains were isolated, with only 6 of these strains being capable of forming mineralization nodules[38]. These results indicated that some groups of cells in dental pulp have the properties of forming the mineralization-representing the renewal of destructive hard tissue (dentin). Pulp fibroblasts are elongated, with a large nucleus and well-developed Rough endoplasmic reticulum (RER). The golgi apparatus located near the nucleus and the presences of secretory vesicles reflect the

synthetic capacity of these cells. Pulp fibroblasts produce a complex ECM that is different in ratio from dentin. The ECM of pulp fibroblasts contain type I and type III collagen fibrils (56 and 41%, respectively), in contrast to the predominantly type I collagen of dentin. Many studies tested the ability of pulp fibroblast in producing the mineralized matrix. The condition for inducing pulp fibroblasts to differentiate and produce the mineralized matrix comprised of many reagents such as β-glycerophosphate, L-glutamine, L-ascorbic acid and dexamethasone[39-41].

3.3 Macrophage

Macrophages are monocytes that have left the bloodstream, entered the tissues, and differentiated into various subpopulations. Many are found in close proximity to blood vessels. A major subpopulation of macrophages is quite active in endocytosis and phagocytosis. Because of their mobility and phagocytic activity, they are able to act as scavengers, removing extravasated red blood cells, dead cells, and foreign bodies from the tissue. Ingested material is destroyed by the action of lysosomal enzymes. Another subset of macrophages participates in immune reactions by processing antigen and presenting it to memory T cells. The processed antigen is bound to class II major histocompatibility complex (MHC) molecules on the macrophage, where it can interact with specific receptors present on naive or meomory T cells. Such interaction is essential for T cell-dependent immunity. When activated by the appropriate inflammatory stimuli, macrophages are capable of producing a large variety of soluble factors, including interleukin-1, tumor necrosis factors, growth factors, and other cytokines[34].

3.4 Dendritic cell

Dendritic cells are accessory cells of the immune system. Similar cells are found in the epidermis and mucous membranes, where they are called *Langerhans' cells.* Dendritic cells are primarily found in lymphoid tissues, but they are also widely distributed in connective tissues, including the pulp. These cells are termed *antigen-presenting* cells and are characterized by dendritic cytoplasmic processes and the presence of cell surface class II antigens. They are known to play a central role in the induction of T cell-dependent immunity[34].

3.5 Lymphocyte

Some studies reported finding T lymphocytes in normal pulps from human teeth. T8 (suppressor) lymphocytes were the predominant T-lymphocyte subset present in the pulp[43]. Lymphocytes have also been observed in the pulps of impacted teeth. The presence of macrophages, dendritic cells, and T lymphocytes indicates that the pulp is well equipped with cells required for the initiation of immune responses. B lymphocytes are scarcely found in the normal pulp.

3.6 Mast cell

Mast cells are widely distributed in connective tissues, where they occur in small groups in relation to blood vessels. Mast cells are seldom found in the normal pulp tissue, although they are routinely found in chronically inflamed pulps. The mast cell has been the subject of considerable attention because of its dramatic role in inflammatory reactions. The granules of mast cells contain heparin, an anticoagulant, and histamine, an important inflammatory mediator, as well as many other chemical factors[36].

Table 1 Cells and Extracellular Matrix Components found in Dentin and Pulp [33]

Component	Dentin	Pulp
Cells	Odontoblasts exclusively	Fibroblasts (pulpoblasts), vascular cells, pericytes, neural cells, histiocytes/macrophages, dendritic cells, lymphocytes, mast cells
Collagens	Types I and I trimer (98%) Types III (1-2%) and V (1%) (90% of the dentin extracellular matrix)	Type I (56%) Types III (41%) and V (2%); Type VI (0.5%) associated with microfibrillin
Non-collagenous	(10% of the dentin extracellular matrix) Phosphorylated matrix proteins (SIBLINGs) :DSPP > DSP and DPP DMP-1, BSP, Osteopontin (OPN), Matrix extracellular phosphoglycoprotein (MEPE) Non-phosphorylated matrix proteins: Matrix GLA protein, osteocalcin, osteonectin Small leucine-rich Proteoglycans (SLRPs) Chondroitin sulphate (CS)/ Dermatan sulfate (DS) Proteoglycans (PGs):	 none BSP, OPN Fibronectin Osteonectin (in tooth germs) Versican

Table 1 (Continued)

Component	Dentin	Pulp
Non-collagenous	(CS-4 81%, CS-6 14%, CS/DS 2%) Keratan sulphate (KS) PGs: lumican, fibromodulin, Osteoadherin Amelogenin 5-7 kDa Growth factors: TGF-β, Insulin-like growth factors (ILGF) -I and -II, FGF-2, vascular endothelial growth factor (VEGF), Platelet-derived growth factor (PDGF) Metalloproteinases: collagenase (MMP-1), gelatinases (MMP-2 and -9), stromelysin-1 (MMP-3), enamelysin (MMP-20), metalloproteinases (MT) 1-MMP, Tissue inhibitor of metalloproteinase (TIMP)-1 to -3 Alkaline phosphatase, phospholipids Serum-derived proteins: alpha heparan sulfate (∝HS) 2-glycoprotein, albumin, lipoproteins	CS -4 and -6, 60%; DS, 34%; KS, 2% Hyaluronic acid BMPs Types IA and II receptors for TGF- β, activin, and BMPs Metalloproteinases (MMPs) : collagenases, gelatinases, stromelysin-1 TIMPs Fibronectin of serum origin

Table 1 (Continued)

Component	Dentin	Pulp
Non-collagenous	Membrane phospholipids (66%) Extracellular-mineral-associated phospholipids (33%)	Membrane and ECM phospholipids

Aim of the study

This study aimed to investigate the capability of primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line to form mineralized nodule and expression of dentine sialophosphoprotein under inductive medium cultivation. The attachment of these cells on chitosan sheet was also studied.

Chapter 2

Materials and Methods

1. Subject and cell culture

Normal exfoliated, caries free human deciduous teeth were collected from 6 to 12 –year-old children under the approved guideline by the Ethics Committee at Prince of Songkla University. Consents were obtained from the parents. The pulp was gently separated from the crown and root. Dental pulp tissue was soaked in tincture iodine solution for 30 seconds (s) to prevent contamination and then rinsed twice in PBS for 30 s. Tissue was dissected into 1 mm^3 with dental surgical blades No.11[57], then seeded on 60-mm plastic Petri dish (3-6 pieces/dish) containing Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10%heat inactivated fetal bovine serum (FBS, Biochrom KG, Germany), 2 mM L-glutamine (Gibco, USA), 100 IU Penicillin G (M&H, Thailand), 100 μg Streptomycin (M&H, Thailand), 100 μg Kanamycin (M&H, Thailand), 50 U Mycostatin (Bristol, Mayer Squib; Thailand) under lamina flow hood. The cultures were maintained at 37 $^{\circ}C$ in a humidified atmosphere containing 5%CO_2[17].The media were changed every other day until the cells grown out from the explants. Cells were collected by trypsinization and transferred to T-25 flasks. When they reached 70% confluence, cells were trypsinized and keep in liquid nitrogen tank until later used[42]. For all sets of experiment, primary pulpal fibroblast cells were used at 3^{rd} -6^{th} passage. Data presented herein are representative of experiments with pulp from 6 different donors[73]. Gingival fibroblasts passage 3^{rd} -6^{th}, MG63 osteoblast cell line passage 14^{th}-16^{th} ATCC number CRL-1427 were used as control.

Groups of study

The cells were divided into 3 categories.

Group A: primary pulpal fibroblasts (HP)

Anterior teeth = 71, 73 (HP1) and Posterior teeth = 74, 64, 84 (HP2)

Group B: gingival fibroblasts (HF)

Group C: MG63 osteoblast cell line

2. Mineralized nodule formation assay

Primary pulpal fibroblasts, gingival fibroblasts and MG63 were seeded at a density of 30,000 cells per well in 24 well plate in 1 ml of DMEM with culture condition as previously mentioned until they reached 70% confluence. In order to provide the conditions for promoting mineralization, the medium was supplemented with 10 mM β glycerophosphate (β-GP), 100 μg/ml L-ascorbic acid 2-phosphate and 10^{-8} M dexamethasone. The inductive medium was changed every other day[42, 43]. The plates were divided to 3 sets along the 3 periods (14, 21 and 28 days) Mineralized nodule formation was detected by Alizarin Red S staining of calcium deposits[15]. Briefly, cells were fixed with 4 % formaldehyde in PBS for 10 min and rinsed with PBS twice followed by Alizarin Red S staining for 5 min and washed with double distilled water twice. Mineralized nodule formations were viewed with a light microscope (Nikon, Japan).

Alizarin Red S staining preparation

The Alizarin red stain is an indicator of calcium phosphate which appears when mineralized nodules are formed. If the samples stain red, calcium phosphate is present. Two percents Alizarin red was prepared by mixing 2 grams of Alizarin red powder (Sigma, USA) with 100 ml of sterile distilled water, adjust pH to 4.2 with 10% ammonium hydroxide.

3. Identification of DSPP expression by reverse transcription of polymerase chain reaction

Total RNA isolation

Primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line seeded in 60-mm culture dish and were cultivated with standard medium as previously mentioned. The dishes were divided to 3 sets along the 3 periods (0, 7 and 21 days). Day 0, cells were cultivated in 2.5 ml of standard medium without the addition of the mineralization

promoting supplement until they reached 100% confluence then RNA was extracted. After 70% confluence, the cells were further cultured for 7 and 21 days with the inductive medium of the mineralization promoting supplement (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU Penicillin G, 100 μg Streptomycin, 100 μg Kanamycin, 50 U Mycostatin with additional of 10 mM β-GP, 100 μg/ml, L-ascorbic acid 2-phosphate and 10^{-8} M dexamethasone). The cells were grown at 37 $^{\circ}$C in humidified atmosphere of 5% CO_2 until day 7 and 21. After the stated time points of cell cultivation, total RNA were isolated from primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line using RNeasy Mini Kit (Qiagen®, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, cells were lysed in Buffer RLT, 70% ethanol was added to the homogenized lysate and mixed well**.** The sample was then passed through an RNeasy mini column and centrifuged for 15 s at 10,000 rpm. The column was then washed with 700 μl of Buffer RW1 and centrifuged at 10,000 rpm for 15 s. RNeasy mini column was transferred to a new collection tube and the column was washed twice with 500 μl of Buffer RPE. The RNA was eluted with 50 μl of RNase-free water. The RNA concentration was determined by UV/visible spectrophotometer (spectrophotometer Oultrospec 3300 Pro, Amershampharmacia®, USA).

: Primer for RT-PCR

Primers sequences for dentin sialophosphoprotein and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and annealing temperatures were described in table 2

Table 2 Polymerase chain reaction (PCR) amplification primer sets

Protein	Sequence (5'-3')	Amplicon size (bp)	Tm (°C)
DSPP	Forward: AATGGGACTAAGGAAGCTG Reward: AAGAAGCATCTCCTCGGC	814	54
GAPDH	Forward: GAAGTGGAAGGTCGGAGTC Reward: GAAGATGGTGATGGGATTTC	313	55

Table 3 Components of master mix preparation

Components	Volume /reaction
Rnase free water	variable
5x QIAGEN OneStep RT PCR buffer	10 µl
dNTP Mix QIAGEN	2.0 µl
Forward Primer (0.6 µM)	3 µl
Reward Primer (0.6 µM)	3 µl
OneStep RT-PCR Enzyme Mix	2.0 µl
Rnase inhibitor	0.5 µl
RNA	2 µ g
Total volume	50 µl

Table 4 RT-PCR parameters for automated DNA thermal cycler

Segment	Step	Temperature (°C)	Time (minutes)	Number of cycles
1	Reverse transcription	50	30	1
2	Initial PCR activation	95	15	1

Table 4 (Continued)

Segment	Step	Temperature (°C)	Time (minutes)	Number of cycles
3	Denaturing Annealing Extension	94 55 72	0.5 0.5 2	35
4	Final extension	72	10	1
5	Holding	4	∞	-

Reverse transcription of polymerase chain reaction (RT-PCR)

RT-PCR was performed to assess the expression of DSPP. GAPDH was used as the internal control in this study. Two micrograms of total RNA from primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line along the 3 periods (0, 7 and 21 days) were used for complementary deoxyribonucleic acid (cDNA) synthesis by QIAGEN®. OneStep RT-PCR (Qiagen®, Valencia, CA, USA). The DSPP primers were designed according to Papagerakis et al., 2002 (Table2)[62]. The GAPDH primers were designed according to Maddi et al., 2006 (Table2)[63]. OneStep RT-PCR was performed by cDNA synthesis and PCR amplification according to the manufacturer's instructions, two μg of total RNA was used per RT-PCR reaction in a total volume of 50 μl master mix preparation (Table 3). A typical thermal cycler program, including steps for both reverse transcription and PCR, was designed under the following conditions of the OneStep® RT-PCR Kit protocol: The reactions were incubated in a PCR thermal cycler at 95 °C for 15 minutes (min) for one cycle and then 94 °C (30 s), 54 °C (30 s), 72°C (120 s) for 35 cycles, with a final 10 min extension at 72 °C (Table 4). RT-PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and analyzed by Gel Doc (model 1000, BIORAD, USA). The products were quantified by Scion Image Analysis Program (version Beta 4.0.3 of Scion Image, Scion Corporation, Frederick, Maryland (USA). The expression level of DSPP was normalized by GAPDH

4. Cell attached on chitosan sheet

Chitosan sheet preparation

Chitosan solution was prepared by dissolving 2 grams of shrimp chitosan in 2% acetic acid[61] under magnetic stirring and filtered with cheesecloth then poured into the 24 well plates for 1ml per well. Allowing the solution to settle and solidify for 72 hours to form a thin sheet and neutralized with 1 N NaOH, wash 3 times with double distilled water and air dry. The 24 well plate with chitosan coated was sterilized by ultraviolet light for 1 hour. Primary pulpal fibroblasts, gingival fibroblasts and MG63 were seeded at a density of 30,000 cells per well in 1 ml of DMEM, supplemented with cultured condition as previously described and stored at 37 $^{\circ}$C in a humidified atmosphere of 5%CO_2. Non-chitosan coated plate with primary pulpal fibroblasts, gingival fibroblasts and MG63 were used as controls. Cells were grown for a period of 24 hours. The attached cell and cell morphology were qualitatively analyzed by staining with 0.4% Tryphan blue. Briefly, cells were fixed with 4% formaldehyde in PBS for 10 min and rinsed with PBS twice followed by staining with 0.4% Tryphan blue for 30 min and washed with double distilled water twice. The attachment was viewed with light microscope magnification B×10 (Nikon, Japan). Measurement of attached cells was carried out by counting absolute cell numbers using three random sites per well.

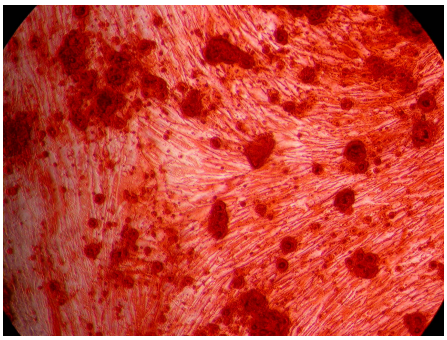
Chapter 3

Results

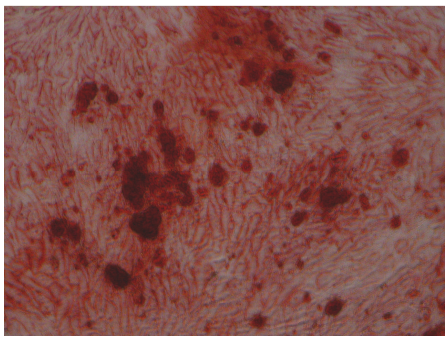
1. Mineralized nodule formation assay

: Identification of mineralized nodule formation by Alizarin Red S staining

Mineralized nodule formation was detected by Alizarin Red S staining of calcium deposits. At day 14, mineralized matrix of the newly-formed tissue was markedly positive for alizarin red (stained in red or orange) as shown in Fig. 4A-4D. Multicellular foci or nodules were observed, all cells were arranged in parallel, aligned paralleled to the cell nodule with elongated cell process. Anterior primary pulpal fibroblasts (HP1) and posterior primary pulpal fibroblasts (HP2) had round, oval and bright redden nodule scattered around the well (Fig. 4A, 4B). MG63 had round dark red nodule spreaded (Fig. 4D). Gingival fibroblasts had fewer and smaller nodules than the others. There was sparse mineral deposition on gingival fibroblasts group (Fig. 4C).

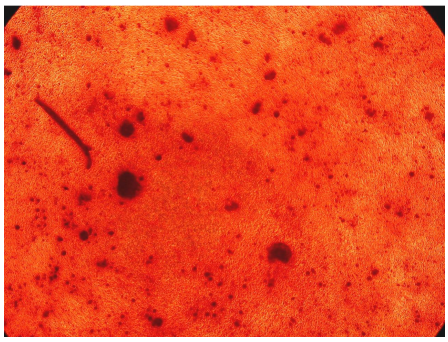


(A)



(B)

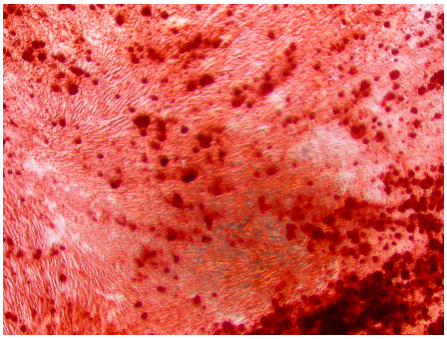
(C)



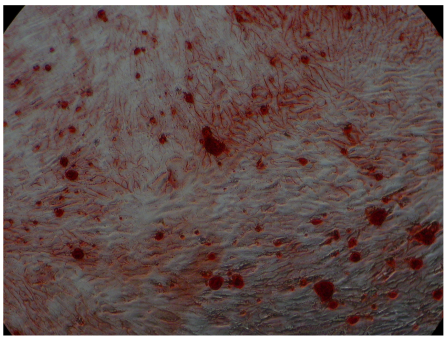
(D)

Fig. 4 Morphology of cell culture staining with Alizarin Red S at 14 days (A) HP1 (B) HP2 (C) HF and (D) MG63 (original magnification×10).

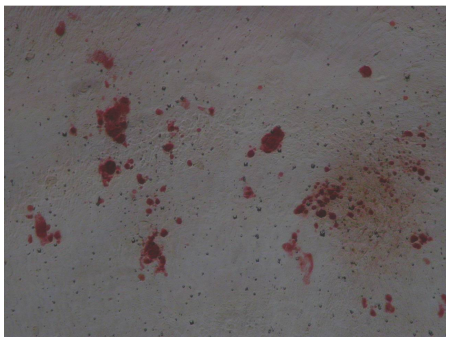
Nodule formation was more evident and mineral deposition becoming increasingly apparent after 21 day cultivation. As demonstrated in Fig. 5A-5D, HP1, HP2 and MG63 show much more mineral deposition. HP1 and HP2 have similar morphology. Their nodules were round, oval and bright redden scattered around the well (Fig. 5A, 5B). For MG63 osteoblast cell line, the nodule morphology was rounder and darker than the other groups (Fig. 5D). The nodule of gingival fibroblasts did not develop well and had the same morphology as the period of 14 days (Fig. 5C).



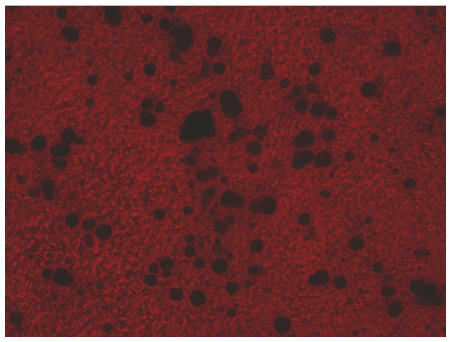
(A)



(B)



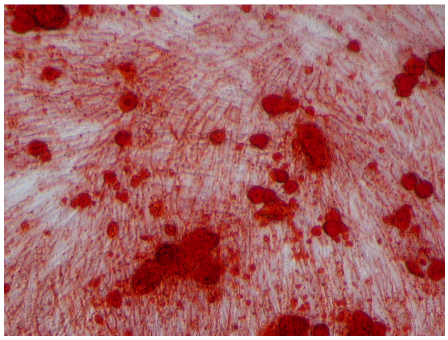
(C)



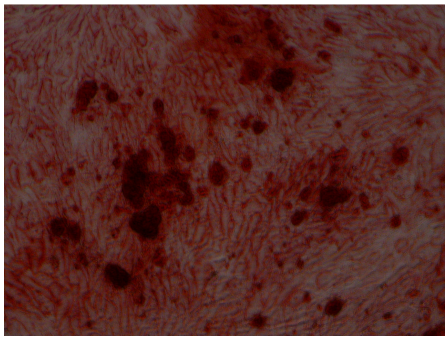
(D)

Fig. 5 Morphology of cell culture staining with Alizarin Red S at 21 days (A) HP1 (B) HP2 (C) HF and (D) MG63 (original magnification×10).

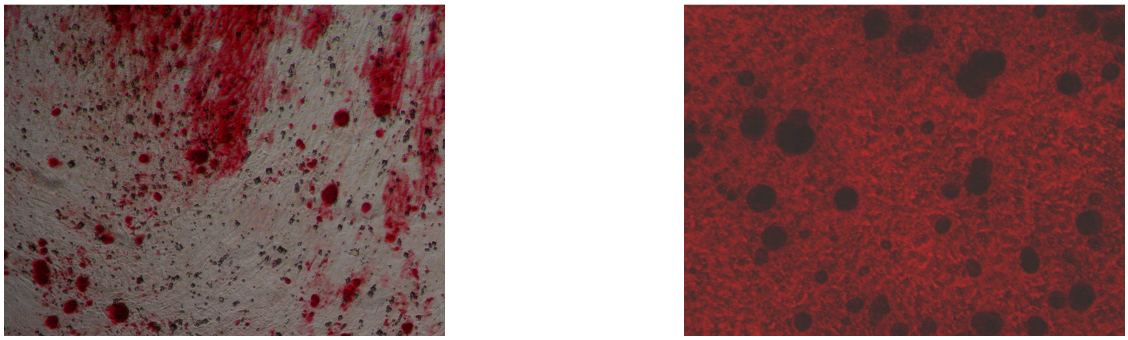
The last period of cultivation is day 28th. Nodule formations of primary pulpal fibroblasts and MG63 did not appear much more evident than the period of 21 days. The nodule morphology is rounder in all groups and darker in MG63. The nodules of HP1 and HP2 were similar to the nodules of their groups at the period of 21 days in term of size and color (Fig. 6A, 6B). However, MG63 osteoblast cells nodule increased in size and the mineral deposition became more apparent than day 21 (Fig. 6D). The gingival fibroblasts created much more nodules than the previous period. The nodule size are small (bead-like) scattered on the well.



(A)



(B)



(C) **(D)**

Fig. 6: Morphology of cell culture staining with Alizarin Red S at 28 days (A) HP1 (B) HP2 (C) HF and (D) MG63 (original magnification×10).

2. Identification of DSPP expression by reverse transcription of polymerase chain reaction

To investigate the expression of DSPP, we performed RT-PCR on primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line after the 3 periods of culture (Day 0, 7 and 21). The results showed that DSPP was not expressed in any types of the cells used and was not expressed in any culture periods. We did find a smear band at about 1,200 bp (Fig. 6) which did not correspond to the size of DSPP (814 bp). In contrast, The GAPDH was found in all groups and every time point. To confirm the RT-PCR results, we varied the annealing temperatures from 50 °C to 55 °C and 58 °C in tested sample (primary pulpal fibroblasts day 7). Even though we varied the annealing temperature to either 55 °C (Fig. 7A) or 58 °C (Fig. 7B), we still did not find the band size corresponding to the DSPP.

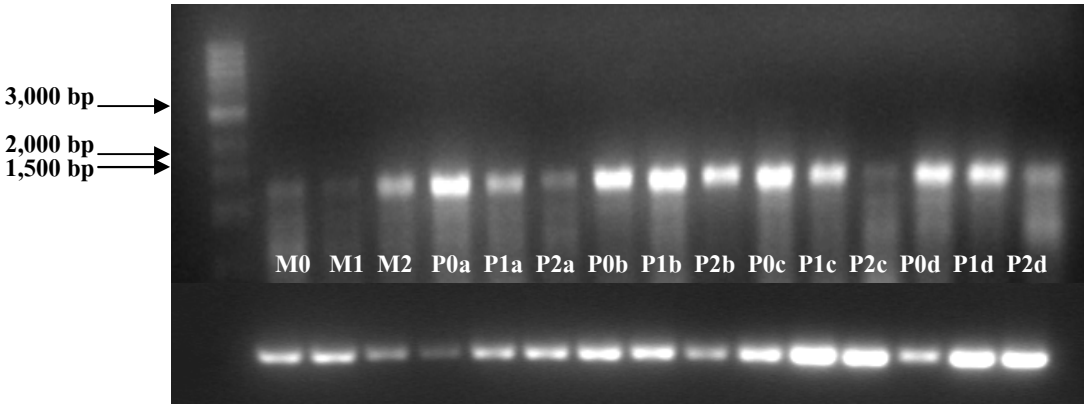
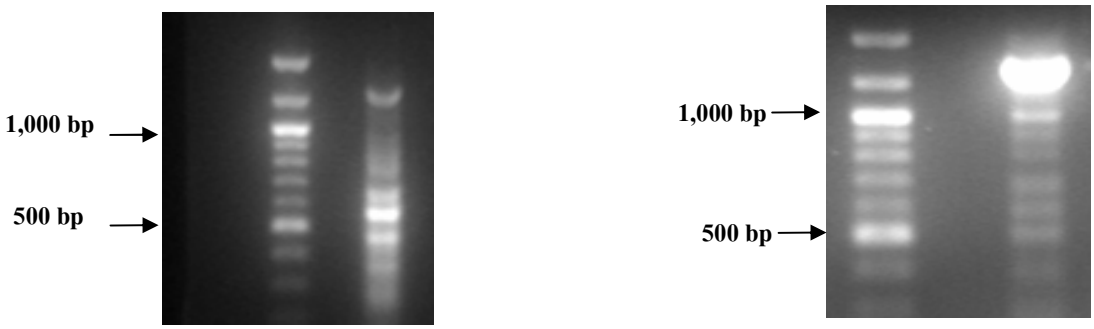


Fig. 7 **Expression of the DSPP by RT-PCR from human primary pulpal fibroblasts culture and MG63. M0-M2 were MG63 culture at day 0, 7, and 21, respectively. P0-P2 were primary pulpal fibroblasts at day 0, 7 and 21, respectively, a-d represented cells collected from different individual. GAPDH was used as internal control. The first lane was 1 Kb DNA marker.**



(A) **(B)**

Fig. 8 **RT-PCR of DSPP from RNA extracted from 7 day primary pulpal fibroblasts culture using 55 °C (A) and 58 °C (B) annealing temperature. The first lane is 100 bp DNA marker.**

We continued solving the problem by changing a new set of DSPP primers, both forward and reverse primers and also varied the annealing temperatures to 45°C, 48°C and 55°C. The new primer sets gave a larger product size, 1403 bp.

New primers sequences for DSPP

Forward: 5'-GGTCACAATGAGGATGTCGCTG-3'

Reward: 5'- CTGTCACTATCACTGCTGTCGCTC-3'

We calculated the optimal of annealing temperature by the following formulation[56]

$$Tm = \frac{64.9 + 41^{\circ}C \times (\text{number of G's and C's in primer} - 16.4)}{N \text{ (length of primer)}}$$

The calculated Tm of new primer set was 45°C. The RT-PCR was performed as previously described on Table2, but changing the annealing temperature for the new DSPP primer set to 45°C, 48°C, and 55°C. Unfortunately, we did not find the band at expected size (Fig. 8 and picture not shown). The results demonstrated that we were unable to detect DSPP expression by the primer we used of any cells and in any RT-PCR conditions used.

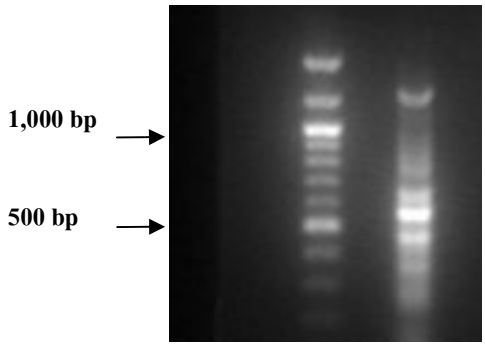


Fig. 9 RT-PCR of DSPP from RNA extracted from 7 day primary pulpal fibroblasts culture using 48°C. The first lane is 100 bp DNA marker.

3. Cell attached on chitosan sheet

: Identification of cell attached by Tryphan blue staining

Quantitative analysis of cell attached at 24 hours showed that the numbers of primary pulpal fibroblasts, gingival fibroblasts and MG63 cells on chitosan coated and non-chitosan coated wells were different. The numbers of all cell groups on chitosan coated wells were less than the numbers of all cell groups on non-chitosan coated wells (detail in Table 5 and Fig. 10). Primary pulpal fibroblasts show the highest ability of cell attachment at 24 hours followed by gingival fibroblasts and MG63 on non-chitosan coated wells. The same results occurred on chitosan coated wells, MG63 illustrated the lowest attachment ability compare to the other cells.

Cell Type	Cell number (cell/well)	
	chitosan coated	non-chitosan coated
MG63 osteoblast cell line	27.50 ± 3.54	100.00 ± 7.07
Gingival fibroblast cells (HF)	360.00 ± 52.92	966.67 ± 133.17
Primary pulpal fibroblast cells (HP)	482.67 ± 102.87	1,010.00 ± 151.88

Table5 Number of cell attached of primary pulpal fibroblasts, gingival fibroblasts and MG63 on chitosan coated and non-chitosan coated wells (cell/well)

For qualitative analysis, the tryphan blue staining under light microscope showed that the morphology of primary pulpal fibroblasts and gingival fibroblasts on chitosan coated and non-chitosan coated wells after cultivation for 24 hours were quite different. Primary pulpal fibroblasts, gingival fibroblasts plated on chitosan were attached and began to spread; as compared to MG63 that appeared rounder. Morphologically, tryphan blue staining showed that all cells plated on the chitosan coated appeared rounder than non-chitosan coated wells. MG63 morphology on chitosan coated wells were round, had short process and had less cell attachment

(Fig.11A), whereas on non-chitosan coated well, the cells were more spindle-like and had larger size (Fig.11B). The primary pulpal fibroblasts (HP) on chitosan coated wells (Fig.11E) had morphology resembled to that of gingival fibroblasts which is rounded and had short process (Fig.11C). In contrast, HP on non-chitosan coated wells demonstrated spindle shape cell with long process (Fig.11F) resembled to that of gingival fibroblasts on non-chitosan coated wells (Fig.11D). Gingival fibroblasts (HF) on chitosan coated wells (Fig.11C) showed rounded and short process of cells similar to MG63 and HP on non-chitosan coated wells. From photomicrographs of cells under cultivation for 24 hours on chitosan and non-chitosan coated wells, we can conclude that the cells morphology on chitosan coated wells were rounded and had short process. On the other hand, the cell morphology on non-chitosan coated wells was spindle shape and had long process. Primary pulpal fibroblasts, gingival fibroblasts and MG63 cells attached and spreaded better on non-chitosan coated wells than chitosan coated wells.

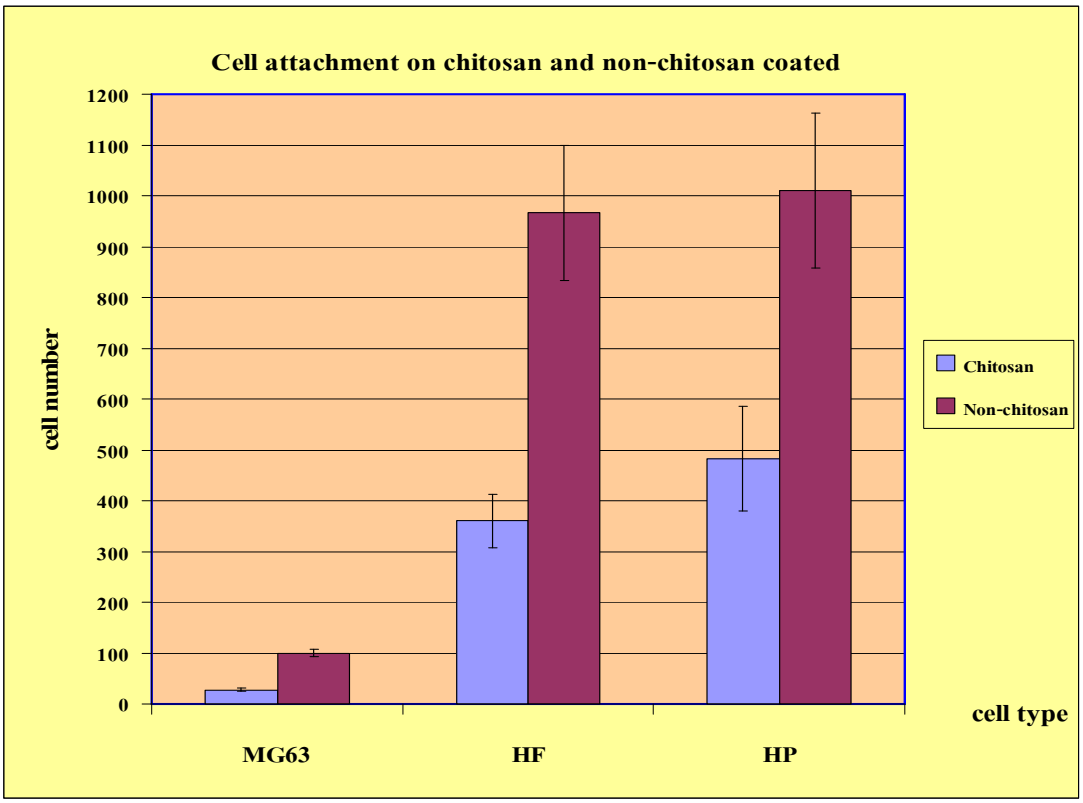
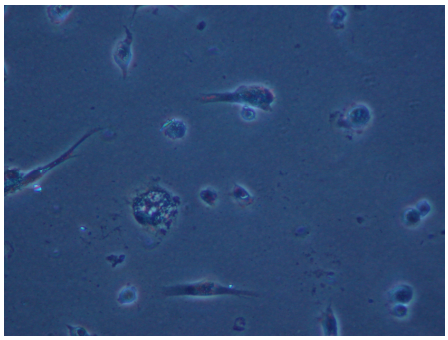
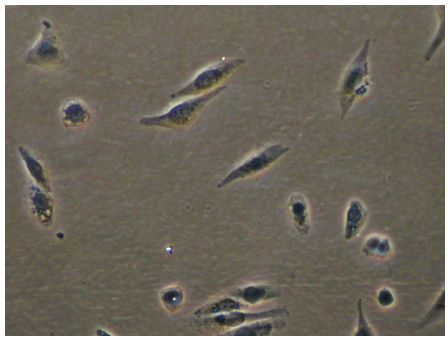


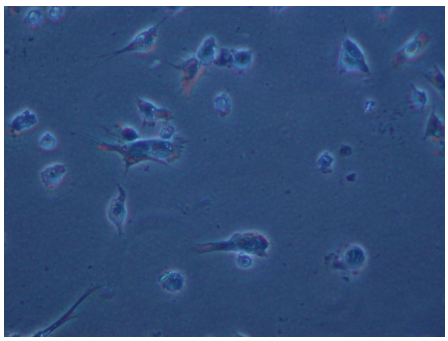
Fig. 10 Cell attachment on chitosan coated and non-chitosan coated well



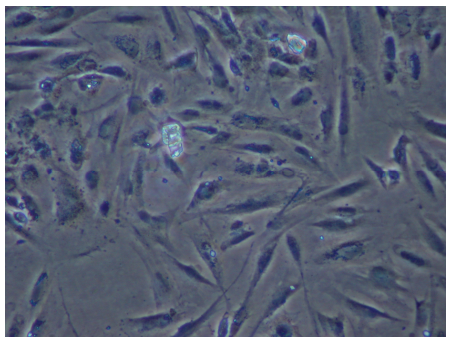
A. MG63 with chitosan coated



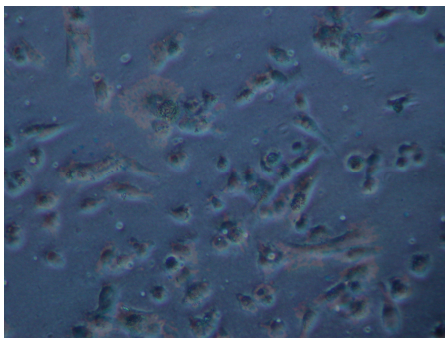
B. MG63 with non chitosan coated



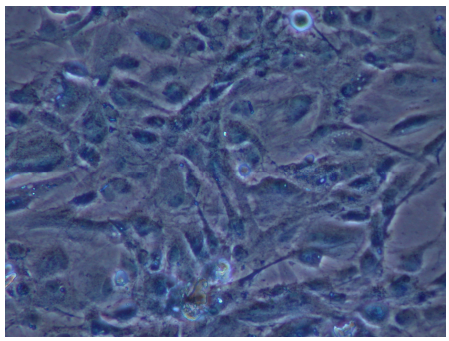
C. HF with chitosan coated



D. HF without chitosan coated



E. HP with chitosan coated



F. HP without chitosan coated

Fig. 11 **Photomicrograph at 24 hrs displaying cell attachment of primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblasts on chitosan and non-chitosan (original magnification×10).**

Chapter 4

Discussion

4.1 Mineralized nodule formation of primary pulpal fibroblasts, gingival fibroblasts and MG63

The mineralized nodule formation of BMSCs and DPSCs had been shown in previous data when these cells were long-term cultured (5-6 weeks) in the presence of L-ascorbate-2-phosphate, the glucocorticoid, and dexamethasone[15]. The other experiment treated DPSCs with a soluble extract of dentin containing a cocktail of growth factors and bioactive molecules[45]. The result showed that mineralized cell nodule formation occurred as early as 7 day after confluency, following treatment with supplemented medium. After 14 days, the cells were well organized and the cell nodules enlarged with apparent mineral deposition. These morphological observations indicated that supplemented medium themselves or together with dentin extract induced odontoblast-like cell differentiation and multilayered cell nodule formation. The others experiment was also observed in mouse MDPC-23 cells[68] and mineralized cell nodule induction in human dental papilla cells[44] on supplemented medium addition. The β-GP has also been reported to induce mineralized nodule formation in human dental pulp explants[42, 69]. Dexamethasone is a synthetic glucocorticoid employed to induce osteogenic differentiation in vitro. Dexamethasone stimulates the differentiation of human dental pulp cells into odontoblast-like cells in vitro[42]. In this study, we cultured primary pulpal fibroblasts, gingival fibroblasts and osteoblast cell line MG63 with L-ascorbic acid, β-GP and dexamethasone for 14, 21 and 28 days. We found that there was mineralized nodule formation in all cell groups. Gingival fibroblasts produced least numbers with slowest rate of mineralized nodule formation. Prominent of mineralized nodules formation were found in primary pulpal fibroblasts. The size and appearance of nodules in primary pulpal fibroblasts, MG63 cells and gingival fibroblasts are

quite different. In primary pulpal fibroblasts; there were round, oval and bright redden nodule scattered around the well, whereas in MG63 osteoblast cell line, there were dark redden and round nodules spreaded around. This different could be due to the source of cell origin. Gingival fibroblasts produced fewer and smaller nodules. Gingival fibroblasts can also produce the nodule although at a slower rate and fewer in quantity. Gingival fibroblasts are the soft tissue cells that cultivated from gingiva. They usually can not produce the matrix for hard tissue, unlike bone cells –MG63 osteoblast cell line and dental pulp cells-primary pulpal fibroblasts that can form hard tissues. Gingival fibroblasts produce the mineralized nodule at the same amount to the other cell groups after cultured in the presence of osteopromotive medium for 28 days. This result may be due to the effect of mineralized promoting medium in the long culture period that could stimulate gingival fibroblasts to differentiate. In conclusion, primary pulpal fibroblasts had a higher capability to produce mineralized nodule better than MG63 and gingival fibroblasts. Therefore, the primary pulpal fibroblast might be a suitable source for mineralized tissue regeneration.

4.2 Identification of DSPP expression by reverse transcription of polymerase chain reaction

During physiological tooth development, epithelial signals are essential to induce the dental papilla ectomesenchymal cells to terminally differentiate into odontoblasts. After tooth formation, under certain physical and pathological stimuli, bioactive molecules (including growth factors and some noncollagenous proteins) may be released from the dentin matrix and recapitulate developmental events allowing reparative dentin to form[45]. Collagenous and noncollagenous proteins are detected in the dentin. The main collagen found in dentin is the type I collagen (86%), whereas type III, IV, and V collagens are detected in fewer quantities. Dentin noncollagenous proteins such as osteonectin, osteocalcin, osteopontin, and bone sialoprotein have also been localized in bone. However, two proteins have been shown to be tooth-specific: the DSP and the DPP. Both DSP and DPP proteins are cleavage products of a single transcript coded by the DSPP gene located on the human chromosome 4[42].

Dental pulp tissues had ability to form dentin-pulp like structures and expressed DSPP both in *vivo* and *in vitro* studies[15, 16]. The potential for dental pulp tissues to generate mineralized tissue formation maybe influenced by its ability to modulate the

important regulatory proteins related to odontogenesis at the mRNA level or at the protein level. Previous study demonstrated that culture odontoblast expressed DSPP using PCR analysis of the substraction cDNA library of dental pulp tissues[70]. Cultured human dental pulp cells are representative of the human odontoblast phenotype; however, they are more variable and dependent upon many factors i.e. stage of cultured cells, culture condition, the method of culture and cells separation. A high correlation in expression profile between the human dental pulp cells and the odontoblast cell lines indicates that human dental pulp cells can be used as model systems for the molecular analysis of the regulation of genes associated with odontoblast function. Molecular analysis of the regulation of genes associated with odontoblast function, DSPP, was mostly performed in the dental pulp tissues from 3rd permanent molar teeth and few studies investigated the potential of the deciduous pulp cells[17, 71]. Deciduous teeth are significantly different from permanent teeth with regards to their developmental processes, tissue structure, and function. Previous report found that deciduous pulp cells had higher proliferation rate than pulp cells from permanent teeth and expressed DSPP detected by Western blot analysis[17].

The cellular model used in the present study was based on dental pulp tissue from primary teeth under condition of primary cell culture system. Dental pulp tissues from caries free of primary teeth were chosen and cultured under similar inductive medium used as demonstrated by previous studies[42, 70, 72]. However, we did not find evidence of DSPP expression in all cell types used. Our studies differ from previous reports under the same culture condition. The first reason to explain our result is the different culturing technique. Many investigators used enzyme for digesting the dental pulp tissue which then passed through the strainer to separate to single cell suspension and cultivate for further experiment[15, 17, 71]. However, we cultured the whole dental pulp tissues that contained different population of cells and would have less mesenchymal stem cells. The second explanation is the cells used in this study came from exfoliated deciduous teeth which differed from others' reports that used unerupted 3rd molar. The unerupted 3rd molars are in the developing stage, therefore, it might contain more undifferentiated mesenchymal stem cells comparing to exfoliated deciduous pulp. Although in our study, deciduous pulpal fibroblast formed the large amount of mineralization nodules under the inductive condition, contrast to the result of dentin differentiation marker expression (DSPP). From this aspect, most of cells populations in deciduous pulp tissue are fibroblast-like cells that its character is

heterogeneity. Alhough primary pulpal fibroblasts can form mineralization nodule but it may not be able to differentiate to specialized cells that expressed DSPP.

4.3 Cell attached on chitosan sheet of primary pulpal fibroblasts, gingival fibroblasts and MG63

Previous data showed contradictory results on the effect of chitosan on fibroblasts[66, 67]. Chitosan has been shown to have stimulatory effect on fibroblasts and osteoblasts, on the other hand it also show inhibitory effects on fibroblast cells growth. These conflicting results may be due to differences in cells population used and variation in the properties of chitosan used. Our findings give the new information about the capability of primary pulpal fibroblasts to attach and proliferate on the presence chitosan. An interesting outcome of our study was the finding that human deciduous pulp cells have capability to attach and proliferate well on chitosan than the other cell groups. The results of this in *vitro* study suggest that chitosan supports the initial attachment and spreading of primary pulpal fibroblasts and gingival fibroblasts better than osteoblast cell lines MG63. Our findings are in accordance with previous reports showing the osteoconductive potential of chitosan in vitro[66, 67]. Microscopic observations at 24 hours revealed that primary pulpal fibroblasts and gingival fibroblasts attached and spreaded on chitosan sheet better than osteoblast cell lines MG63. All cells in chitosan coated wells showed rounded morphology with markedly reduced cell attached levels. This might be due to the following factors. Firstly, the improper pH of chitosan used, after fabrication of chitosan sheet, we did not calibrate pH before culturing the cells. This could result in the improper pH of chitosan sheet. Secondly, too thick of chitosan and softness surface of chitosan in the culture medium might not support cell attached. Finally, the shrinkage at the center of chitosan sheet on 24 well plate did not support the cell spreaded. There were the cluster of cells at center of well. Our findings confirm the ability of both primary pulpal fibroblasts and gingival fibroblasts to attach and proliferate on chitosan sheet better than osteoblast cell lines MG63. An interesting outcome of our study was the findings that chitosan support the initial cell attached and spreaded of primary cells (pulpal fibroblasts and gingival fibroblasts) better than cell lines (osteoblast cell lines MG63).

Therefore, in this study we found that primary pulpal fibroblasts had much more capability to form mineralized nodule and higher cell attachment than gingival fibroblasts and

osteoblast cell lines MG63, although DSPP expression could not be detected. Our results may be helpful for further investigations on the characteristics of deciduous dental pulp tissues.

Chapter 5

Conclusion

Identification of mineralized nodule formation by Alizarin Red S staining

Primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line had capability to form mineralized nodule. Gingival fibroblasts can form mineralized nodule at the slowest rate compare to primary pulpal fibroblasts, gingival fibroblasts and MG63 osteoblast cell line.

Cell attached on chitosan sheet by Tryphan blue staining

Primary pulpal fibroblast cells had the highest capability to attach on chitosan and non-chitosan coated well compared to gingival fibroblasts and MG63 osteoblast cell lines.

Identification of DSPP expression by Reverse transcription of polymerase chain reaction

In this study, we did not find the expression of DSPP on primary pulpal fibroblasts. Further study should be investigated on the technique for stem cells separation from primary pulpal fibroblasts.

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Appendices

Appendix 1

แบบเสนอโครงการวิจัย
เพื่อการพิจารณารับรองจากคณะกรรมการจริยธรรมในการวิจัย
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

1. ชื่อโครงการวิจัย

   การแสดงออกของเด็นทินไซอาโลฟอสโฟโปรตีนและการสร้างแร่ธาตุของเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม ,เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์

   Dentine Sialophosphoprotein Expression and Mineralized Nodule Formation of Primary Pulpal fibroblasts, Gingival Fibroblasts and MG63

   1.1 ประเภทของโครงการวิจัย

   Non-Drug trial (experimental study design)

   1.2 จำนวนสถานพยาบาลที่ร่วมวิจัย

   ☐ Multicenters (ในประเทศ) ☐ Multicenters (ร่วมกับต่างประเทศ)

   ☑ Single center

2. ชื่อหัวหน้าโครงการวิจัยและที่อยู่ที่สามารถติดต่อได้สะดวก

   ชื่อหัวหน้าโครงการ

   ทันตแพทย์หญิง วิสาขา ไผ่งาม

   คุณวุฒิ ทันตแพทยศาสตรบัณฑิต

   ภาควิชา ทันตกรรมป้องกัน โทรศัพท์ 074-429875

3. แหล่งทุนสนับสนุนการวิจัย

   : บัณฑิตวิทยาลัย มหาวิทยาลัยสงขลานครินทร์

4. วัตถุประสงค์ของการวิจัย

   4.1 เพื่อศึกษาลักษณะของการเจริญเติบโตของ เนื้อเยื่อในโพรงฟันน้ำนม

   4.2 เพื่อศึกษาการแสดงออก (expression) ของDSPP และการสะสมของสารประกอบแคลเซียมเนื้อเยื่อในโพรงฟันน้ำนม

5. การดำเนินการวิจัย

   5.1 ประชากรที่เข้ารับการศึกษา

เชิญชวนกลุ่มตัวอย่างอายุ 6-12 ปี เข้าร่วมการวิจัย โดยผู้ปกครองเซ็นใบยินยอมให้เก็บเนื้อเยื่อโพรงประสาทฟันน้ำนมที่จำเป็นต้องได้รับการถอนเนื่องจากหลุดช้ากว่ากำหนดของกลุ่มตัวอย่าง โดยทำการเก็บฟันน้ำนมที่ได้จากการถอนฟันเมื่อฟันแท้ขึ้นมาแทนที่ ณ.คลินิกทันตกรรมสำหรับเด็ก คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

5.2 นำฟันที่ได้ไปแยกเอาส่วนของเนื้อเยื่อโพรงประสาทฟัน และไปเพาะเลี้ยงเนื้อเยื่อ จนมีเซลล์เจริญออกมาและนำเซลล์ที่ได้ไปทำการศึกษาวิจัย

5.3 นำเซลล์เนื้อเยื่อในโพรงฟันน้ำนมที่ได้แบ่งเป็นกลุ่มทดลอง และ กลุ่มควบคุมโดย

- กลุ่มควบคุมมี 2 กลุ่มคือ กลุ่ม1 เป็นกลุ่ม เซลล์เนื้อเยื่อเซลล์สร้างกระดูก (osteoblast cell line MG63) และ กลุ่มที่ 2 คือ gingival fibroblasts
- กลุ่มทดลองมี 1 กลุ่มคือ เป็น กลุ่ม เซลล์เนื้อเยื่อในโพรงฟันน้ำนม
- ศึกษาการแสดงออกของเด็นทินไซอาโลฟอสโฟโปรตีนโดยวิธีการใช้ RT-PCR และศึกษาการสะสมของสารประกอบแคลเซียมโดยการย้อมสี Alizarin Red S

เกณฑ์การคัดเลือกประชากร (Inclusion Criteria)

- ฟันน้ำนมที่ได้จากการถอนฟันเมื่อฟันแท้ขึ้นมาแทนที่ ณ.คลินิกทันตกรรมสำหรับเด็ก คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

เกณฑ์การคัดออก (Exclusion Criteria) ประชากร

- ฟันน้ำนมที่ได้จากการถอนฟันเมื่อฟันแท้ขึ้นมาแทนที่และผุ

5.4 สถานที่ทำการวิจัย

- คลินิกทันตกรรมสำหรับเด็ก ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

5.5 สิ่งที่ผู้วิจัยต้องการศึกษา (Intervention) และจะให้ (Administer) กับประชากรที่เข้าร่วมการศึกษาต้องระบุรายละเอียดดังนี้

- ไม่มี intervention เพื่อการวิจัย แต่เป็นการรักษาตามปกติโดยให้ยาชาเฉพาะที่และถอนฟันน้ำนม

5.6 ระยะเวลาการวิจัย

: 17 เดือน

6. ข้อพิจารณาด้านจริยธรรม

6.1 เหตุผลและความจำเป็นที่ต้องวิจัยในคน

ในปัจจุบันนี้มีการพัฒนาความรู้และวิทยาการใหม่ๆในด้านการนำเทคโนโลยีชีวภาพ

มาใช้ในด้านการแพทย์และทันตกรรมซึ่งการสร้างเซลล์เนื้อเยื่อทดแทน (tissue engineering) ก็เป็นอีกวิธีหนึ่งในการรักษาโพรงประสาทฟันที่ทำให้เกิดการสร้างเนื้อเยื่อโพรงประสาทฟันขึ้นมาใหม่และเกิดการหายอย่างแท้จริง

6.2 ประโยชน์ที่จะได้รับจากการวิจัยนี้ รวมทั้งประโยชน์ต่อประชากรที่เข้าร่วมในโครงการวิจัย

- พัฒนาทางเลือกในการรักษาโพรงประสาทฟันสำหรับฟันที่มีชีวิตโดยทำให้เกิดการสร้างเนื้อเยื่อโพรงประสาทฟันขึ้นมาใหม่และเกิดการหายอย่างแท้จริง
- ได้ข้อมูลพื้นฐานเพื่อการศึกษาประสิทธิภาพของการใช้ สเต็มเซลล์จากฟันน้ำนมต่อไป
- ได้ข้อมูลพื้นฐานเพื่อการศึกษาเทคนิคใหม่ในการสร้างฟันต่อไป

6.3 ความเสี่ยงที่ประชากรที่เข้าร่วมการศึกษาจะได้รับ

: เป็นความเสี่ยงจากการถอนฟันเพื่อการรักษาตามปกติ เช่น ความเสี่ยงจากการฉีดยาชา, ถอนฟันซึ่งผู้ทำการรักษาได้มีการป้องกันความเสี่ยงเหล่านี้ตามมาตรฐานของการรักษาในผู้ป่วยทุกราย และไม่มีความเสี่ยงแก่ผู้ป่วยเพิ่มเติมจากการเก็บเนื้อเยื่อโพรงประสาทฟันน้ำนมเพื่อทำการวิจัย

7. รายชื่อ ที่อยู่และคุณวุฒิของผู้ร่วมวิจัยทุกคน

7.1 ดร. ทพญ. สุทธาทิพย์ กมลมาตยากุล ปริญญาดุษฎีบัณฑิต
ตำแหน่งทางวิชาการ ผู้ช่วยศาสตราจารย์ ภาควิชาทันตกรรมป้องกัน
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ โทรศัพท์429875-074

7.2 ดร .ทพญ. สุวรรณา จิตภักดีบดินทร์ ปริญญาดุษฎีบัณฑิต
ตำแหน่งทางวิชาการ ผู้ช่วยศาสตราจารย์ ภาควิชาชีววิทยาช่องปาก
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ โทรศัพท์429875-074

7.3 ทพญ. ดวงธิดา ไพบูลย์วรชาติ วิทยาศาสตร์มหาบัณฑิต
ตำแหน่งทางวิชาการ อาจารย์ ภาควิชาทันตกรรมป้องกัน
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ โทรศัพท์429875-074

7.4 ทพญ. วิสาขา ไผ่งาม ปริญญาตรี ทันตแพทยศาสตร์บัณฑิต
ตำแหน่ง นักศึกษาปริญญาโท ภาควิชาทันตกรรมป้องกัน
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ โทรศัพท์429875-074

ผู้เสนอโครงการวิจัยสัญญาว่าคณะผู้วิจัยจะดำเนินการวิจัยตามรายละเอียดที่ระบุไว้ในข้อเสนอโครงการวิจัยอย่างเคร่งครัด หากมีการแก้ไขข้อเสนอโครงการวิจัย ผู้เสนอโครงการ

จะแจ้งให้คณะกรรมการฯ ทราบโดยเร็ว เพื่อการพิจารณาอนุมัติ และจะส่งรายงานการวิจัยจำนวน 1 ชุด ให้คณะกรรมการฯ ภายใน 6 เดือน เมื่อการวิจัยสิ้นสุดลงหรือเมื่อการวิจัยถูกยกเลิก

.....	.....
ทพญ. วิสาขา ไผ่งาม	ดร.ทพญ.สุทธาทิพย์ กมลมาตยากุล
หัวหน้าโครงการ	อาจารย์ที่ปรึกษา

8. ลงลายมือชื่อของหัวหน้าหน่วยงานของหัวหน้าโครงการวิจัยที่อนุมัติให้ดำเนินการวิจัยได้ กรณีที่เป็นโครงการวิจัยที่เป็นวิทยานิพนธ์ จะต้องมีชื่อ พร้อมลายมือชื่อของผู้ควบคุมวิทยานิพนธ์ด้วย

ใบเชิญชวน

ขอเชิญเข้าร่วมโครงการวิจัยเรื่องการแสดงออกของเด็นทินไซอาโลฟอสโฟโปรตีนและการสร้างแร่ธาตุของเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม ,เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์

เรียน ท่านผู้อ่านที่นับถือ

ข้าพเจ้า ทพญ.วิสาขา ไผ่งาม ใคร่ขอเล่าถึงโครงการวิจัยที่กำลังทำอยู่ และขอเชิญชวนท่านเข้าร่วมโครงการนี้ เนื่องมาจากการรักษาโพรงประสาทฟันในฟันน้ำนมที่ยังมีชีวิตอยู่นั้นมีวัสดุที่ใช้ในการรักษาหลายชนิดด้วยกัน ซึ่งแม้ว่าจะให้ผลสำเร็จในการรักษาเป็นที่น่าพอใจแต่วัสดุเหล่านี้ไม่ได้ทำให้เกิดการสร้างเนื้อฟันขึ้นใหม่ พบว่าในฟันน้ำนมของเด็กที่หลุดเองตามธรรมชาติมีส่วนประกอบของเซลล์ ซึ่งสามารถสร้างเนื้อฟัน แต่ข้อมูลที่เกี่ยวกับเซลล์ ในฟันน้านมมีอยู่น้อยมาก ดังนั้นผู้ทำการวิจัยเห็นความสำคัญของการนำเซลล์จากโพรงประสาทฟันของฟันน้ำนมมาศึกษาเพื่อดูลักษณะเพื่อดูการเจริญเติบโตและการสร้างเนื้อฟันของเซลล์ อันจะเป็นข้อมูลพื้นฐานต่อการการรักษาโพรงประสาทฟันน้ำนมให้คงความมีชีวิตสืบต่อไปในอนาคต

โดยผู้ทำการวิจัยจะทำการศึกษาเนื้อเยื่อโพรงประสาทฟันซึ่งจำเป็นต้องได้รับการถอน เนื่องจากไม่หลุดเองตามธรรมชาติแล้วนำเนื้อเยื่อในโพรงประสาทฟันไปใช้ในงานวิจัย ซึ่งจะมีผลนำไปสู่การพัฒนาวิธีการรักษาโพรงประสาทฟันแก่ผู้ป่วยต่อไป แทนที่จะทิ้งฟันไปโดยเปล่าประโยชน์ การเข้าร่วมการศึกษาครั้งนี้ไม่มีความเสี่ยงเพิ่มขึ้นจากการรักษาตามปกติที่บุตรหรือเด็กในปกครองของท่านจะต้องเข้ารับการรักษาตามปกติอยู่แล้ว

โดยผู้รับผิดชอบโครงการวิจัยนี้คือ

1. ทพญ.วิสาขา ไผ่งาม สถานที่ติดต่อ ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ เบอร์โทรศัพท์ 429875-074

2. ทพญ.ดร.สุทธาทิพย์ กมลมาตยากุล สถานที่ติดต่อ ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ เบอร์โทรศัพท์ 429875-074 หากเมื่อมีปัญหาใดๆ เกิดขึ้นเนื่องจากการทำวิจัยในเรื่องนี้ท่านสามารถร้องเรียนไปที่คณบดีคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ.หาดใหญ่ จ.สงขลา 90112 โทรศัพท์ 074-28-7510

ถ้าท่านตัดสินใจเข้าร่วมในโครงการนี้จะมีขั้นตอนของการวิจัยที่เกี่ยวข้องกับท่าน คือ มอบฟันน้ำนมที่ถอนแล้วให้แก่ผู้ทำการวิจัย ไม่ว่าบุตรหรือเด็กในปกครองของท่านจะเข้าร่วมในโครงการวิจัยนี้หรือไม่ บุตรหรือเด็กในปกครองของท่านจะยังคงได้รับการรักษาที่ดีเช่นเดียวกับผู้ป่วยคนอื่น ๆ และถ้าท่านต้องการที่จะถอนตัวออกจากการศึกษานี้เมื่อใด ท่านก็สามารถกระทำได้

อย่างอิสระ และหากภายหลังคณะผู้ทำการวิจัยได้นำเนื้อเยื่อโพรงประสาทฟันไปใช้ในงานวิจัยอื่น ท่าน

- ○ ขัดข้อง
- ○ ไม่ขัดข้อง

หากท่านมีคำถามใด ๆ ก่อนที่จะตัดสินใจเข้าร่วมโครงการนี้ โปรดซักถามคณะผู้วิจัยได้อย่างเต็มที่

ขอขอบคุณเป็นอย่างสูง

ทพญ.วิสาขา ไผ่งาม

หมายเหตุ : - กรุณาอ่านข้อความให้เข้าใจก่อนเซ็นชื่อยินยอมเข้าร่วมโครงการ

แบบยินยอมเข้าร่วมการศึกษา

โครงการวิจัยเรื่องการแสดงออกของเด็นทินไซอาโลฟอสโฟโปรตีนและการสร้างแร่ธาตุของเซลล์ไฟโบรบลาสต์จากเนื้อเยื่อในโพรงประสาทฟันน้ำนม ,เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์สร้างกระดูกที่พัฒนามาจากเซลล์มะเร็งของมนุษย์

วันที่……..เดือน……พ.ศ…….

ข้าพเจ้า……..……..อายุ…….ปี อาศัยอยู่บ้านเลขที่……

ถนน……ตำบล……อำเภอ……..จังหวัด……

เป็นผู้ปกครองของ.....

ได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียดและมีความเข้าใจดีแล้ว โดยผู้รับผิดชอบโครงการวิจัย นี้คือ

1. ทพญ.วิสาขา ไผ่งาม สถานที่ติดต่อ ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ เบอร์โทรศัพท์429875-074
2. ทพญ. ดร.สุทธาทิพย์ กมลมาตยากุล สถานที่ติดต่อ ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ เบอร์โทรศัพท์074-429875

หากเมื่อมีปัญหาใดๆเกิดขึ้นเนื่องจากการทำวิจัยในเรื่องนี้ข้าพเจ้าสามารถร้องเรียนไปที่ คณบดีคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ.หาดใหญ่ จ. สงขลา 90112โทร . 074-287510

หากผู้วิจัยมีข้อมูลเพิ่มเติมทั้งด้านประโยชน์และโทษที่เกี่ยวข้องกับการวิจัยนี้ ผู้วิจัยจะแจ้งให้ข้าพเจ้าทราบอย่างรวดเร็ว โดยไม่ปิดบัง

ข้าพเจ้ามีสิทธิที่จะของดการเข้าร่วมโครงการวิจัยโดยมิต้องแจ้งให้ทราบล่วงหน้า โดยการงดการเข้าร่วมการวิจัยนี้ จะไม่มีผลกระทบต่อการได้รับบริการหรือการรักษาที่ข้าพเจ้าจะได้รับแต่ประการใด

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะที่เกี่ยวกับตัวข้าพเจ้าเป็นความลับจะไม่เปิดเผยข้อมูลหรือผลการวิจัยของข้าพเจ้าเป็นรายบุคคลต่อสาธารณชน จะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย หรือการเปิดเผยข้อมูลต่อผู้มีหน้าที่ที่เกี่ยวข้องกับการสนับสนุนและกำกับดูแลการวิจัย

ข้าพเจ้าได้อ่าน/ได้รับการอธิบายข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ จึงได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจโดยนักวิจัยได้ให้สำเนาแบบยินยอมที่ลงนามแล้วกับข้าพเจ้าเพื่อเก็บไว้เป็นหลักฐาน จำนวน 1 ชุด

ลงชื่อ.....ผู้ยินยอม

ลงชื่อ.....หัวหน้าโครงการ

ลงชื่อ.....พยาน

ลงชื่อ.....พยาน

Appendix 2

Materials

1. Biological Materials

Primary cell culture pulpal fibroblast from deciduous anterior and posterior teeth

Primary cell culture Gingival fibroblast cells

MG63 osteoblast cell line ATCC number CRL-1427, USA

2. Equipments and Instruments

Autoclave Hiclave™, Model HB-50, Hirayama Manufactoring Corp., Japan

Automatic pippetter, Portable Pipette-Aid Model XP, Drummond Scientific Company, USA

Biohazard Laminar Airflow Class II, Astec Microflow Model ABS1200TCN, Bioquell Lab, UK

CO_2 Incubator, Series II water jecketed CO_2 Incubator Model 3111, Thermo Forma, USA

Digital Camera, Nikon Coolpix Model 4500, NIKON, Japan

Desk Top Centrifuge, Model 5417C, Eppendorf, Germany

Electrophoresis, Model 5417C, Eppendorf, Germany

Hemocytometer Neubauer improved bright-line Dept 0.1 mm, Model 717810, BRAND, Germany

Hot Air Oven, Model UM500, Memert, USA

Inverted light microscope, Elipse Model TE2000-U, Nikon, Japan

PCR machine system 9600, Cat No. U66585E, Pipetman Gilson®, France

Pipetter 20-microlitre, Cat No. U66585E, Pipetman Gilson®, France

Pipetter 200-microlitre, Cat No.U65792E, Pipetman Gilson®, France

Pipetter 1000-microlitre, Cat No.U69088E, Pipetman Gilson®, France

pH-meter, Precisa Model pH90, Precisa Instrument, Switzerland

Refrigerated Centrifuge, Labofuge Model 400 R, Heraeus, Germany

Water Bath, Model WB22, Memmert, Germany

3. Disposable materials

Centrifuge tubes 15 ml, Cat No.430052, Corning, Corning intercorporated, USA

Centrifuge tubes 50 ml, Cat No.BCT-P50BS, Biologix, USA

Cryotube 2.0 ml, Cat No. 2008-12, Nunc™, Denmark

Culture flask 25, 75 square cm, Cat No.430641, Corning, Corning intercorporated, USA

Culture flask 24 well, Cat No.3524, Costar, Corning intercorporated, USA

Disposable Pipette tips 10 µl, Treff Lab Cat No.96.8704.9.01, Treff AG, Switzerland

Disposable Pipette tips 200 µl, Treff Lab Cat No.96.8700.4.02, Treff AG, Switzerland

Disposable Pipette tips 1000 µl, Treff Lab Cat No.96.8776.6.01, Treff AG, Switzerland

Eppendorf centrifugation tube 1.5 ml, Eppendorf, Germany

Glass cover slips, Cat No.470045, BRAND, Germany

Glass Pasteur pipettes, Cat No.747720, BRAND, Germany

Microscope glass slides, Cat No.474743, BRAND, Germany

Sterile filter 0.2 µm, Cat no.11107 47 N, Sartorius, Germany

4. Chemical and reagents

Alcohol 70%, Cat No.IA807/39, Coholsahakarn, Thailand

Alizarin Red S, CAS Number. 130-22-3, Sigma, USA

Beta glycerophosphate100 gm, Cat No.A4000, Sigma, USA

10^{-8} M dexamethasone (Sigma, USA) Dimethylsulfoxide (DMSO), Cat No,103234L, Analar®, BDH Laboratory supplies, England

Dulbecco's Phosphate buffer saline, Cat No.21600-051, Gibco, USA.

Ethanol Absolute for Analysis 500 ml, Cat No.E7023, Sigma, Germany

Fetal bovine serum, Cat No.L152, Biochrome AG, Germany

L-ascorbic acid, CAS No. 50-81-7, Sigma, USA

Mycostatin , Bristol&Mayer Squib, Thailand

QIAGEN OneStep RT-PCR, Cat No.210212, Valencia, CA, USA

Penicillin G/Streptomycin/Kanamycin , M&H, Thailand

RNeasy Mini Mini Spin Columns, Cat No. 74104, Qiagen, Valencia, CA, USA

Trypsin/EDTA (0.5%/0.2%) in (10x) PBS, CatNo. L2153, Biochom AG, Germany

5. Software

SPSS for windows, Version 11.0, Standard Software Package Inc., USA

Gel Doc model 1000, BIORAD, USA

VITAE

Name Wisakha Paingam

Student ID 4762009

Educational Attainment

Degree	Name of Institution	Year of Graduation
DDS	Faculty of dentistry, Mahidol University	2541

Work – Position and Address

Sanamjun Hospital, Nakornpathom

List of Publication and Proceeding (If Possible)

Poster presentation: Effects of chitosan on deciduous pulp cells, osteoblasts and fibroblasts.

20th International Association for Dental Research, Southeast Asian Division (IADR) Southeast Asian Division Annual Meeting.

Held on: 3-4 September, 2005, Malacca, Malaysia.