ASSESSMENT OF THE PROLIFERATION RATE OF HUMAN DENTAL PULP STEM CELLS FROM PERMANENT AND DECIDUOUS TEETH LOADED ON GRANUMAS® SCAFFOLD

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PENGANALISAAN KADAR PROLIFERASI SEL DASAR TISU PULPA GIGI MANUSIA DARIPADA GIGI KEKAL DAN GIGI SUSU ATAS PERANCAH GRANUMAS®

ABSTRAK

Banyak sudah sel dasar gigi manusia telah diasingkan dan dibuat pencirian. Antaranya ialah sel dasar pulpa gigi (DPSCs), sel induk dari gigi susu yang telah dicabut (SHED), sel dasar ligamen periodontal, sel induk dari apikal papila dan sel progenitor folikel gigi. Populasi gigi prostnatal ini mempunyai kualiti seperti sel tunjang mesenkima di mana ia turut mempunyai keupayaan untuk memperbaharui dan berpotensi dalam menghasilkan pembezaan zuriat. Kajian ini bertujuan untuk mengasingkan dan membuat pencirian kedua-dua DPSCs dan SHED, seterusnya menilai kader proliferasi dan juga mengesan tahap ekspresi dua penanda proliferatif iaitu PCNA dan Ki-67 dengan menggunakan keadah immunositokima dan flow sitokimia. Tujuan yang seterusnya adalah untuk membezakan kadar proliferasi DPSCs dan SHED pada GranuMas® dan tanpa perencah GranuMas® dengan kaedah "Alamar Blue® assay". Empat gigi kekal daripada empat individu berumur antara 16 ke 22 tahun dan empat gigi susu daripada empat individu berumur 6-12 tahun diambil dengan kebenaran mereka semasa prosedur pencabutan gigi dibuat dan mengikut kriteria dan prosedur tertentu. Pulpa kemudiannya diasingkan daripada korona dan dicerna di dalam 3mg/ml larutan kolagenase jenis 1 dan di dalam 4 mg/ml dispase selama 1 jam pada suhu 37ºC mengikut garis panduan yang telah ditetapkan. Suspensi sel tunggal diambil dengan menyaring sel menggunakan penapis 70-µm (falcon) dan dikultur dengan medium Eagle modifikasi alfa dengan

penambahan serum Fetal Bovin sebanyak 20%, 100 μM L-asid askorbik 2- fosfate, 2mM L-glutamin, 100 unit/ml penisilin dan 100 μg/ml streptomisin. Sel yang diinkubasi dalam keadaan 5% CO_2 pada suhu 37ºC diperiksa setiap hari di bawah mikroskop. Penanda sel dasar mesenkima, Indoglin (CD105) dan Alkam (CD166) digunakan untuk membuat pencirian sel secara sitometri aliran dan sitokimia imuno dan bagi tujuan pengesanan tahap ekspresi dan keadaan DPSCs dan SHED, penanda proliferasi sel, Ki-67 dan PCNA digunakan. Kada proliferasi DPSCs dan SHED dinilai menggunakan Alamar Blue® selama 21 hari samada dengan atau tanpa perancah GranuMas®. Keputusan menunjukkan bahawa DPSCs dan SHED memberi keputusan positif terhadap penanda sel dasar CD105 dan CD166. Kedua-dua kumpulan tersebut telah menunjukkan kesan ekspresi positif dengan Ki-67 dengan DPSCs menunjukkan kesan ekspresi yang lebih besar berbanding PCNA dan SHED yang mana ia menjadi indikator untuk sintesis DNA. SHED menunjukkan kadar proliferasi yang lebih tinggi berbanding DPSCs dan HMSCs pada hari kosong, ketujuh, keempatbelas dan hari kedua puluh satu. Keputusan juga menunjukan SHED + GranuMas® menunjukan kadar proliferasi yang lebih tinggi daripada DPSCs + GranuMas® dan HMSCs + GranuMas®. SHED telah menunjukan kadar proliferasi yang paling tinggi pada GranuMas® pada hari kedua puluh satu, DPSCc pada GranuMas® pada hari ke 14 manakala HMSc pada GranuMas® pada hari ke 7. Kesimpulannya kedua-dua DPSCs dan SHED menunjukkkan kesan positif terhadap CD105 dan CD166 yang mana ianya konsisten dengan sel dasar mesenkima. SHED menunjukkan kadar proliferasi yang lebih tinggi berbanding DPSCs dalam masa 21 hari. SHED dan DPSCs menunjukan patern proliferasi pada GranuMas® tetapi pada kadar ynag lebih rendah dengan tanpa GranuMas®. DPSCs menunjukkan ekspresi yang lebih besar pada penanda proliferasi (Ki-67, PCNA) berbanding dengan SHED.

ASSESSMENT OF THE PROLIFERATION RATE OF HUMAN DENTAL PULP STEM CELLS FROM PERMANENT AND DECIDUOUS TEETH LOADED ON GRANUMAS® SCAFFOLD

ABSTRACT

Many different human dental stem/progenitor cells have been isolated and characterized such as dental pulp stem cells (DPSCs), stem cells from extracted deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), and dental follicle progenitor cells (DFPCs). These post-natal populations have mesenchymal-stem-cell-like (MSCs) qualities, including the capacity for self-renewal and multilineage differentiation potential. The aims of this study were to isolate and characterize both DPSCs and SHED and to determine the expression level of PCNA and Ki-67 by using immunocytochemistry and flow cytometery. And to compare the proliferation rate of DPSCs and SHED on GranuMas® and without GranuMas® scaffold, by using Alamar Blue® assay. Four permanent teeth from four individuals aged 16 to 22 years old and four deciduous teeth from four individuals aged 6 to 12 years old were obtained with informed consent during tooth extraction procedure based on selected criteria and under standardized procedures. According to the standard protocol the pulp then was separated from the crown then digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 hour at 37°C. Single-cell suspensions were obtained by passing the cells through a 70-μm strainer (Falcon) and cultured in alpha modification of Eagle's medium supplemented with 20% fetal bovine serum, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 units/ml penicillin and100

μg/ml streptomycin. The cells were incubated at 37°C in 5% CO_2 and observed daily under microscope. The cells were characterized by immunocytochemistry and flow cytometry using mesenchymal stem cells markers, Endoglin (CD105) and Alcam (CD166) and determination the expression level of (Ki-67 and PCNA) on DPSCs and SHED. The proliferation rate of DPSCs and SHED was assessed using Alamar Blue® assay with and without GranuMas® scaffold for 21 days. The results showed that DPSCs and SHED were positive to stem cells markers CD105 and CD 166. Both cell types showed positive expression to Ki-67 and PCNA, DPSCs showed greater expression in PCNA than SHED, which is an indicator to DNA synthesis. SHED showed higher proliferation rate as compared to DPSCs and HMSCs at day zero, seven, 14 and 21. The Results showed that SHED + GranuMas® showed higher proliferation rate than DPSCs + GranuMas® and HMSCs + GranuMas®. SHED showed the peak of proliferation rate on GranuMas® on day 21, DPSCs on GranuMas® on day 14, while HMSCs on GranuMas® on day seven. As conclusion, both DPSCs and SHED were positive to CD105 and CD166 which are consistent to mesenchymal stem cells. SHED showed higher proliferation rate than DPSCs during 21 days. SHED and DPSCs showed a pattern of proliferation on GranuMas® but with lower values than that without GranuMas®. DPSCs showed greater expression in proliferative markers (Ki-67, PCNA) as compared to SHED.

CHAPTER ONE
INTRODUCTION

INTRODUCTION

1.1 Introduction

Stem cells are clonogenic cells that have the capacity for self-renewal and multilineage differentiation. The microenvironment in which stem cells resides is called a stem cell niche and is composed of heterologous cell types, extracellular matrix and soluble factors to support the maintenance and self-renewal of the stem cells. Stem cells can be divided into two main types' embryonic stem cells and adult stem cells.

The adult stem cells also named as tissue stem cells, somatic stem cells or postnatal stem cells are found in all adult tissues and have physiological and pathological functions according to a very dynamic and differentiative plasticity. Adult stem cells have been isolated from bone marrow, peripheral blood umbilical cord blood, muscles, dental pulp, brain and pancreas (Pessina and Gribaldo, 2006, Yen and Sharpe, 2008).

An adult stem cell is an undifferentiated cell that can differentiate into all the specialized cell types of the tissue from which it originated. The multilineage differentiation potential of adult stem cells has been extensively studied not only in basic research but also for therapeutic use. The most studied type of adult stem cell is hematopoietic stem cells and mesenchymal stem cells in bone marrow, and multilineage cells were actually isolated from adipose tissue, artery wall, and umbilical cord blood. The dental pulp is also thought to be a source of adult stem

cells. Multilineage populations of cells were isolated from the dental pulp of human permanent teeth and exfoliated deciduous teeth (Abe *et al.*, 2008).

Multi-lineage differentiation refers to the capacity of a single population of stem cells to differentiate into at least two distinctively different cell types. For example, a single population of MSCs can differentiate into both osteoblasts and chondrocytes. Pre-osteoblasts can differentiate into osteoblasts, but are incapable of differentiating into other mesenchymal lineages, such as chondrocytes or adipocytes. It is convenient and often necessary to define a progenitor cell as one that is between a stem cell and an end-stage cell, a pre-osteoblast is a progenitor cell between an MSC and an osteoblast or osteocyte (Mao *et al.*, 2006).

Adult /progenitor stem cells reside in varieties of tissues, and have unique characteristics, they exist as undifferentiated cells and maintain this phenotype by the environment and /or adjacent cell population until they are exposed or respond to signals, they also have the ability for self replication in prolonged periods, and they maintain their multiple differentiation potential throughout the living of organism. Stem cells plasticity may be caused by the fusion of stem cells with endogenous tissue-specific cells (Nakashima and Akamine, 2005).

The primary roles of adult stem cells are the maintenance and repair of tissues in which the cells are found as well as maintaining the stem cell population. The cells must go through the process of differentiation in order acquire the capability to function as a specialised cell to generate tissues. These are partly differentiated progenitors or precursors that are capable of undergoing cell division and further

specialisation. The differentiation potential and self-renewal potential of precursors are limited compared to their stem cell forebears. There is evidence that some adult stem cell types are pluripotent, and able to differentiate into multiple cell types (Reali *et al.*, 2006).

The embryonic stem cells (ESCs) are derived from the inner cell mass of developing blastocysts. An ESCs is self-replicative, pluripotent and, in theory, immortal (Carpenter *et al.*, 2003). However, the application of these cells in scientific research is limited primarily due to ethical concerns and tight regulations. Pluripotent embryonic stem cells are derived from the inner cell mass of mammalian blastocysts and can be maintained indefinitely in culture. Human embryonic cells can be stimulated to differentiate into precursors of any cell type.

Cells from many other tissues have been isolated with each showing great similarities in their phenotypes but each is unique in their properties and it is maybe a surface antigen expression, inability to differentiate into one of the mesenchymal lineages or capable of unlimited proliferation by telomerase expression. Each of these differences may be related to the local microenvironment from which they have been isolated from and therefore gives each of them a unique ability, which can be clinically applied. Cells from osteophyte tissues have never been isolated and characterized. It is possible that these cells may have some unique characteristics that could be clinically applied, may be in cartilage regeneration (Singh *et al.*, 2009).

Tissue engineering is a new highly promising field of reconstructive that draws in recent advances in medicine and surgery, molecular and cellular biology, polymer chemistry and physiology. Dental tissue engineering requires three basic elements: an appropriate cell source, a biodegradable scaffold to serve as a temporaryextracellular matrix (ECM) and optimal signals for cell functioning. Regarding the cell source, dental pulp-derived adult stem cells (DPSCs) are especially attractive because they showed potential for odontogenic differentiation and the ability to form a regular-shaped dentine-pulp complex. Matrices for tissue engineering in bone and soft connective tissues have included synthetic and natural calcium phosphates and myriad synthetic (polylactic acid, polyglyclic acid), and natural polymers (collagen, fibrin), these material can be used for the fabrication of matrices to engineer tissue in-vitro or to facilitate regeneration in-vivo, must have micro structure and chemical composition required for normal cell growth and function (Samuel E.Lynch, 1999, Zhang *et al.*, 2006, Ando *et al.*, 2009).

Stem cells represent a particularly attractive cell type for tissue-engineering applications; they are characterized by two unique properties in one cell: their high self-renewal activity and their multilineage differentiation potential, which make them an ideal source for cellular therapy and regenerative medicine. These cells can be expanded in-vitro and differentiated into diverse cell types, processes that can be supported or induced by biomaterials (Neuss *et al.*, 2008).

Stem cells therapy involves the transplantation of autologous or allogeneic stem cells into patients, either through local delivery or systemic infusion. There is a precedent in hematopoietic stem cell transplantation, which has been used for some years in

treatment of leukemia and other cancers. Some striking examples of the therapeutic use of marrow-derived mesenchymal stem cells, including cardiovascular repair, treatment of lung fibrosis, spinal cord injury and bone and cartilage repair. The widespread use of stem cells therapy also depend upon the availability of validated methods for large scale culture, storage and distribution, in addition there is a need for novel engineered devices for tissue-specific delivery of cells, such as cell-coated stents and catheter-based delivery in cardiovascular application, and arthroscopic delivery in the treatment of joint disease (Barry and Murphy, 2004).

The greatest advantage of using the patient's own stem cells therapeutically, are the absence of immunogenicity of these cells. Adult stem cells from a patient could be expanded in-vitro, differentiated into the required cell type by a differentiation cocktail, and then reintroduced into the patient. There are however, several disadvantages with this approach, not the least being that adult stem cells are extremely rare and difficult to isolate from mature tissues, and it is difficult to expand their numbers in-vitro compared to ESCs.

1.2 Problem statement

The study is conducted with the hope to understand the problems that are currently present.

Tissue engineering depends on undifferentiated mesenchymal stem cells. In dental tissue engineering, dental pulp stem cells were isolated and compared with bone marrow stromal stem cells. Thus there is a need to assess and compare the proliferation rate of the DPSCs and SHED.

The assessment of proliferation rate of dental pulp stem cells were previously done using many methods including the colony forming unit fibroblast (CFU-F), Bromodeoxy Uridine (Brd-Urd), and thymidine assay. In addition 3H-thymidine labelling, bromodeoxyuridine incorporation, mitotic count, and DNA flow cytometry, they are either too cumbersome or relatively inaccurate for clinical application (Steck and el-Naggar, 1994). Thus the use of Alamar Blue® in this study may offer some advantages.

1.3 The justification of the study

In order to achieve proper dental tissue engineering we should isolate and characterize DPSCs and SHED and assessment their proliferation on GranuMas® scaffold after passage three.

There are many methods available to assess the proliferation rate of the cells. The use of Alamar Blue® assay as a non-radioactive, non-toxic, easy and fast method in this study in order to assess the proliferation rate of dental pulp stem cells, after some advantages since the cells can be maintained throughout the study period without sacrificing the cells or killing the cells. Some other methods of cell proliferation

assessment such as MTT assay or MTS assay may kill the cells during the study period. The quantification of the expression level of specific cell cycle antigens such as the proliferating cell nuclear antigen (PCNA) and Ki-67 as the proliferating markers especially in DPSCs and SHED will help to provide an insight on how these cells respond to these markers since these markers are correlated with cell proliferation activity. It is important to understand the response of these markers to DPSCs and SHED, So that it can provide to us the percentage of proliferating and quiescent cells after passage three.

1.4 The objective of the study

The general objective of the study is to assess the proliferation rate of human dental pulp stem cells from permanent and deciduous teeth with and without GranuMas® scaffold.

1.5 Specific objectives

1. To characterize the DPSCs and SHED by using flow cytometry and immunohistochemistry.
2. To determine the expression level of PCNA and Ki67 by using flow cytometry and immunohistochemistry.
3. To compare the proliferation rate of DPSCs and SHED by using Alamar Blue® assay.
4. To compare the proliferation rate of DPSCs and SHED on GranuMas® scaffold, by using Alamar Blue® assay.

1.6 Working hypotheses

1. The proliferation rate cell of SHED is higher than DPSCs and bone marrow stromal stem cells.
2. The higher level of specific cell cycle antigens such as proliferating cell nuclear antigen PCNA and Ki-67 is directly proportional with the higher level of proliferation rate.
3. The use of GranuMas® scaffold may improve the proliferation rate of DPSCs and SHED for tissue engineering purposes.

CHAPTER TWO
LITREATURE REVIEW

CHAPTER TWO

LITERATURE REVIEW

2.1 The biology of dental pulp

Teeth consist of two major parts, namely the crown and roots where the visible part is shrouded in layers of crown enamel while the roots are covered with layers of cementum. The two layers are met at the cemento-enamel junction of teeth on the neckline. In the teeth of healthy adults, these lines are under or the area covered by the attachment of the gums. Thus, clinically, or that appear to the eye, tooth crown is the tooth above the gum attachment area. While anatomically or actual limits crown was passed under the area of attachment of the gums.

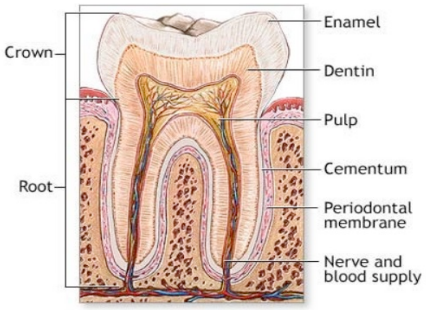

Figure 2.1: tooth anatomy

Dentin is the creamy white to yellow mineralized substance, which makes up the bulk of teeth. It is a dense matrix of minerals, primarily calcium, and it serves to protect the sensitive pulp of the tooth and create a base under the enamel, or outer coating of the tooth. It is extremely important to take care of dentin, because although it is dense and hard, it is susceptible to rot and infection, which can lead to oral pain and expensive dental treatments. Dentin, one of the main mineralized tissue components of teeth, is a hard tissue with dentinal tubules penetrating throughout the

entire thickness of the teeth. The dental pulp is a heterogeneous soft tissue located in the center of teeth, which contains a variety of cell types and extracellular matrix molecules. Both dentin and the pulp are derived from neural crest cells. Because of their close relationship, especially during embryonic stages of tooth development, it is difficult to discuss these two types of tissues separately.

The primary function of pulp is to produce dentin, including primary dentin during early tooth development, secondary dentin throughout the entire life span of the tooth, and tertiary dentin under pathogenic stimuli. Odontoblasts, a layer of cells lining the periphery of the pulp at the inner dentin surface, are the specialized cell type capable of synthesizing dentin. The dental pulp is a highly vascularized tissue with abundant myelinated and unmyelinated nerves. This property correlates with the other two main functions of the dental pulp, which are to provide nutrition to dentin, and to function as a biosensor to detect unhealthy stimuli (Nanci, 2007).

Anatomically, the dental pulp is almost fully encapsulated by hard dentin. The only connection between the dental pulp and the surrounding tissue is through the tiny root apexes. All of the main blood vessels and lymph drainages of dental pulp pass though the tooth root apexes, which make the apex the main pathway for tooth nutrition and waste exchange. In some teeth, there are also much smaller openings of lateral canals, located near the apical foramen. This limited accessibility and unyielding environment of the dental pulp makes it difficult to eliminate inflammation, once it has occurred (Zhang *et al.*, 2009).

Injured dental pulp has limited potential for self-recovery. If the stimuli are mild or progress slowly, such as occur in the cases of mild caries, moderate attrition, erosion, or superficial fracture, odontoblasts can usually survive and continue to produce the dentin barrier beneath the injury, allowing the underlying soft pulp tissue to retain its function. The essential strategy under these situations is to protect the remaining odontoblasts. When the stimuli are strong and/or rapidly progressing, such as occur in deep dentin caries, severe abrasion, and fracture, the primary odontoblasts will be destroyed. In these cases, the post mitotic terminally differentiated odontoblasts lack the ability to proliferate to replace injured odontoblasts, or to produce new dentin. Under these circumstances, undifferentiated mesenchymal cells within the dental pulp can differentiate into odontoblasts and secrete reparative dentin (Fitzgerald *et al.*, 1990). These descriptions fit the profile of stem cells.

Undifferentiated mesenchymal cells within the pulp also have the potential to differentiate into other cell types, including fibroblasts, to repair the damaged soft pulp tissue. The ability to stimulate the stem cell differentiate into odontoblasts-like cells, rather than fibroblasts, is critical in dentin repair (Zhang *et al.*, 2009).

2.2 Stem cells from dental tissues

2.2.1 Dental pulp stem cells (DPSCs)

Dental pulp, a soft tissue of mesenchymal origin of neural crest cells, occupies the central chamber of the tooth. Structurally, the dental pulp is a loose connective tissue with fibroblast, odontoblast, collagen fibers, extracellular glycosaminoglycans, nerves and blood vessels. Its function is protective and reparative. The coronal pulp of young human teeth has three layers; the odontoblastic layer, the cell free zone (the zone of Weil), the cell rich zone at the periphery and the centre of pulp. When odontoblasts are irreversibly damaged, they are replaced by a second generation of newly differentiated odontoblasts that give rise to a reparative dentin matrix. This suggests the presence of resting progenitor or stem cell of odontoblast in dental pulp (Abe S *et al.*, 2007, Abe S *et al.*, 2008).

The use of tritiated thymidine to study cell division in the pulp by autoradiography following damage has revealed differences in labelling depending on the location related to trauma site. No labelling is observed in the existing odontoblast layer or in any specific pulp location whereas perivascular labelling suggests that progenitor cells are located around the vessels. The number of labeled cells in the pulp increases over time suggesting the existence of a continuous source of cells for replacement; the shift of labeled cells is consistent with the influx from deep tissue in the pulp to the periphery. These results support the theory that undifferentiated mesenchymal cells exist in the pulp and have the ability to differentiate into odontoblast-like cells, which are responsible for new dentin formation following dental injury (Yen and Sharpe, 2008).

Gronthos *et al*., (2000) isolated the dental pulp stem cells (DPSCs) from extracted human third molar and they had determine the existence of stem cells in the dental pulp by applying a methodology that had been developed for the isolation and characterization of bone marrow stem cells (BMSSCs) by using colony forming unit fibroblast assay (CFU-F).

Gronthos *et al*., (2000) and Shi. *et al.,* (2001) found that the DPSCs showed higher proliferation rate than BMSSCs under the same culture conditions attributable to the strong expression of cyclin-dependent kinase 6, a cell cycle activator.

Recently mesenchymal stem cell populations derived from adult third molars dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), and adult periodontal ligament periodontal ligament stem cells (PDLSCs) were identified, by their ability to generate clonogenic adherent cell clusters when plated under the same growth conditions as described for bone marrow stromal stem cells (BMSSCs). However, since dental pulp and periodontal ligament are solid tissues, colony efficiency assays were performed using single cell suspensions prepared by collagenase/dispase digestion followed by filtration through fine mesh strainers. The incidence in the number of CFU-F colonies (aggregates of fifty cells or more) formed at day 10 to 12 of culture was then evaluated for all unfractionated cell preparations. Approximately, 14 BMSSCs, 400 DPSCs, 200 SHED and 170 PDLSCs, derived CFU-F generated per 10^5 cells plated. The higher frequency of CFU-F in the dental tissues was most likely due to their fibrous nature in contrast to fluid bone marrow aspirates, which are comprised predominantly of bone marrow haematopoietic cells

including varying proportions of contaminating peripheral blood cells (Shi *et al*., 2005).

The dental pulp contains progenitor/stem cells, which can proliferate and differentiate into dentin-forming odontoblasts. Damaged odontoblasts can be replaced by newly generated populations of odontoblasts derived from stem cells from pulp. Following physiological stimulation or injury, such as caries and operative procedures, stem cells in pulp may be mobilized to proliferate and differentiate into odontoblasts by morphogens released from the surrounding dentin matrix (lohara *et al.,* 2005).

Tran-Hung *et al.,* (2008) quantified the angiogenic growth factors released after human dental pulp cells injury and demonstrate that demonstrated that human pulp cells secrete platelet-derived growth factor (PDGF-AB), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2), particularly after injury. The release of these factors was very rapid (5 h after injury) and corresponds well to the pathological changes in the pulp following injury. The level of these factors returned to initial values after one day. It has been shown that human perivascular progenitor/stem cells can be activated and proliferated in 1 day in response to pulp injury. After this proliferation, these cells migrate to the injury site in two weeks.

The DPSCs cultures were also found to be negative for the odontoblast-specific marker, dentinsialophosphoprotein (DSPP), by Northern blot analysis, which is suggestive of an undifferentiated phenotype. The expression of both dentin sialoprotein and dentin phosphoprotein occurs after the formation a collagenous

predentin matrix and is associated with the process of dentinogenesis. They had also demonstrated that human DPSCs do share a similar pattern of protein expression with BMSSCs in-vitro, several subsets of cells expressing markers of bone (alkaline phosphatase, osteopontin, and bone sialoprotein), smooth muscle (a-smooth muscle actin), and endothelial cells (MUC-18) were represented in both DPSCs and BMSSCs (Gronthos *et al.,* 2000).

After BrdU immunolabeling, the result demonstrated that, the pulpal injury stimulated the proliferation of progenitor/stem cells localized in the perivascular area. These progenitor/stem cells proliferated in their tissue of origin and responded by migration to the pulpal injury site. Mild injuries stimulate the surviving post-mitotic odontoblasts at the site of injury to up-regulate their synthetic and secretory activities to secrete a reactionary dentine matrix. On the other hand, in pathological conditions that cause odontoblast death, the dentine regeneration occurs through reparative dentine secretion by a new generation of odontoblast-like cells. Therefore, unlike reactionary responses, reparative dentinogenesis is amore complex sequence of biological process, which is dependent on multiple factors, including the presence of responsive progenitor cells as well as the appropriate inductive molecular signals for induction of proliferation, migration and differentiation of the new generation of odontoblast-like cells (Tecles *et al.,* 2005).

2.2.2 DPSCs niche

The DPSCs niche in human dental pulp was identified by antibodies against STRO-1, CD146, and pericyte-associated antigen (3G5) and was found to be localized in the perivascular and perineural sheath regions (Shi and Gronthos, 2003).

These STRO-1, CD146, DPSCs form a dentin-pulp-like complex in-vivo, similar to the multiple-colony-derived DPSCs. The STRO-1- positive region in the pulp of deciduous teeth is similar to that of permanent teeth, also in the perivascular regions. STRO-1, CD146, CD44 staining of the periodontal ligament has shown that it is located mainly in the perivascular region, with small clusters of cells in the extravascular region (Chen *et al.*, 2006).

Suggesting that these are the niches of PDLSCs. STRO-1 staining of apical papilla has shown that the positive stain is located in the perivascular region as well as other regions scattered in the tissue (Sonoyama *et al.*, 2006). Thus, it appears that dental stem cells and Bone marrow stromal stem cells (BMSSCs) secure at least one niche in the perivascular region. It is speculated that the Mesenchymal stem cells (MSCs) compartment extends through the whole post-natal organism as a result of its perivascular location. Currently, it is not known if tissue-specific MSCs originate from the local mesenchymal tissues and later migrate toward the ingrown vasculature, or if they are derived from the vasculature and then influenced by the local signals to acquire their tissue specificity (da Silva Meirelles *et al.*, 2006).

2.2.3 Stem cells from human extracted deciduous teeth (SHED)

The remnant pulp of functionally exfoliated deciduous teeth occupy colonies of mesenchymal stem cells and considered postnatal mesenchymal stem cells, that differ than adult pulp stem cells, SHED proliferate faster with greater population doublings than DPSCs and BMSSCs (SHED > DPSCs > BMSSCs). SHED form sphere-like clusters when cultured in neurogenic medium. This is due to the highly proliferative cells, which aggregate in clusters that either adhere to the culture dish or

float freely in the culture medium. The sphere-like clusters can be dissociated by passage through needles and subsequently grown on 0.1% gelatin-coated dishes as individual fibroblastic cells. This phenomenon suggests a high proliferative capacity analogous to that of neural stem cells (Miura *et al.*, 2003, Peneva M., 2008).

Investigators subsequently also isolated SHED and termed the cells 'immature DPSCs' (IDPSCs) (Kerkis *et al.*, 2006). They found that IDPSCs express the embryonic stem (ES) cell markers Oct4, Nanog, stage specific embryonic antigens, and tumor recognition antigens (Kerkis *et al.*, 2006).

As reported for DPSCs, SHED showed the capacity to undergo osteogenic and adipogenic differentiation (Miura *et al.*, 2003). Furthermore, cultured SHED readily express a variety of neural cell markers. Under neurogenic conditions, SHED also exhibit multicytoplasmic processes instead of the typical fibroblastic morphology (Miura *et al.*, 2003). Myogenic and chondrogenic potentials have also been demonstrated (Kerkis *et al.*, 2006).

SHED are easy expanded in-vitro and having high plasticity as they can differentiate into neurons, adipocytes, osteoblasts and odontoblast, readily accessible in young patients, especially suitable in mixed dentition patients, even SHEDs are potential and have high plasticity and high osteoinductive ability but they are not differentiate towards osteoblast directly they do, but did induce bone formation by forming template to recruit morine host osteogenic cells (Miura *et al.*, 2003).

Previous study by (Cordeiro *et al.*, 2008) isolated SHED and seeded them with human dermal micro vascular endothelial cells (HDMEC) to increase the cellularity of SHED and they observed that the ability of these cells to differentiate into the various populations in comparison to the control (empty) scaffolds. The result showed that the infiltration of the host cells had different cellular structure to the tissues resulting from implantation of dental pulp stem (SHED) cells. They showed low cellularity, poor spatial organization, and low micro vessel density. In the SHED cell seeded tissue constructs, the cells at the periphery of the tissue showed characteristics of active dentin-secreting odontoblasts, including expression of Dental sialoprotein (DSP) and ultra structural characteristics of nuclear polarization, the presence and position of cell-cell gap junctions, and a well-developed endoplasmic reticulum. It is known that DSP is not a specific marker for odontoblast cells, because it can also be expressed by osteoblasts. However, Dental sialoprotein (DSP) expression level is approximately 400 fold higher in dentin as compared with bone, suggesting that it is highly expressed in odontoblast cells. Together, the DSP expression assays and the anatomic analysis performed led to conclude that SHED seeded in tooth slice/scaffolds were capable of differentiating into odontoblast-like cells. Importantly, the SHED cells appeared capable of forming a microvascular network, which is a pre-requisite for the successful engineering of most tissues and organs.

One striking feature of SHED is that they are capable of inducing recipient murine cells to differentiate into bone-forming cells, which is not a property attributed to DPSCs following transplantation in-vivo. When single-colony-derived SHED clones were transplanted into immunocompromised mice, only one-fourth of the clones had

the potential to generate ectopic dentin-like tissue equivalent to that generated by multicolony-derived SHED (Miura *et al.*, 2003). However, all single-colony-derived SHED clones tested are capable of inducing bone formation in immunocompromised mice. While SHED could not differentiate directly into osteoblasts, they appeared to induce new bone formation by forming an osteoinductive template to recruit murine host osteogenic cells (Miura *et al.*, 2003).

With the osteo-inductive potential, SHED can repair critical sized calvarial defects in mice with substantial bone formation (Seo *et al.*, 2008). These findings imply that deciduous teeth may not only provide guidance for the eruption of permanent teeth, as generally assumed, but may also be involved in inducing bone formation during the eruption of permanent teeth.

2.2.4 Periodontal ligament stem cells (PDLSCs)

Tooth has mainly two parts the one appeared intraorally is known as the crown and embedded in the alveolar bone is known as root. The root surface harbors the periodontal ligament, and recent reports identified stem cells in human periodontal ligament called periodontal ligament stem cells (PDLSCs), and when the periodontal ligament stem cells were implanted in nude mice, they generated cementum /periodontal ligament-like structures that resemble the native periodontal ligament as a thin layer of cementum interfaced dense collagen fibers, similar to sharpeys fibers. In order to identify putative stem cells in the periodontal ligament, single cell suspension derived from PDL were collected to form adherent clusters and had attach to plastic surfaces and they showed fibroblast morphology, and PDLSCs can differentiate to osteoblast in certain condition with respect to their lower potentiality

in comparison to DPSCs and BMSSCs. Since PDL is similar to tendon with the respect to the density of collagen fiber structure and its ability to absorb mechanical stress during normal physiologic activity, PDLSCs have high expression level of scleraxis a tendon specific transcription factor than DPSCs and BMSSCs, suggesting that the PDLSCs might belong to unique population of postnatal mesenchymal stem cells (Seo *et al.*, 2004, Nagatomo *et al.*, 2006).

The use of processed cellular allogenic bone grafts to facilitate the repair of periodontal defects has been a common clinical practice. The beneficial effects of PDGF (platelet-derived growth factor) and IGF (insulin-derived growth factor) on periodontal repair were evidenced from animal studies as well as in human clinical trials (Lynch *et al.*, 1989, Giannobile *et al.*, 1994, Howell *et al.*, 1997, Nevins *et al.*, 2003, Nevins *et al.*, 2005, McGuire *et al.*, 2006). Platelet-rich plasma (PRP) has also been shown to improve periodontal healing and promote bone regeneration, and the preparation of PRP from patients has become part of the periodontal practice (Tozum and Demiralp, 2003).

Cell-based regenerative periodontal therapy has gained attention since the isolation of mesenchymal stem cells from various tissues. A clinical case report has demonstrated that using ex-vivo expanded autologous BMSSCs facilitated the repair of periodontal defects (Yamada *et al.*, 2006).

In repairing periodontal defects, it has been considered that regenerating the PDL is as important as repairing the bone. Focusing only on bone regeneration using the recombinant human bone morphogenetic protein-2 (rhBMP-2) can stimulate

clinically significant regeneration of alveolar bone and cementum, but not a functionally oriented PDL, which frequently results in ankylosis between the teeth and the newly formed bone in the coronal aspect of the supra-alveolar defect (Selvig *et al.*, 2002). PDLSCs may be an ideal cellular source for regeneration of the PDL. A recent report on a mini pig model has shown that periodontal defects may be repaired by the application of PDLSCs (Liu *et al.*, 2008). This PDLSC-mediated treatment resulted in a regeneration of PDL and the recovery of the heights of alveolar bone. This is the first report demonstrating the application of autologous PDLSCs to regenerate PDL and alveolar bone heights in a large animal model.

2.2.5 Dental Follicle Precursor Cells (DFPCs)

Dental follicle is an ectomesenchymal tissue surrounding the enamel organ and the dental papilla of the developing tooth germ prior to eruption. This tissue contains progenitor cells that form the periodontium, *i.e.*, cementum, PDL, and alveolar bone. Precursor cells have been isolated from human dental follicles of impacted third molars. Similar to other dental stem cells, these cells form low numbers of adherent clonogenic colonies when released from the tissue following enzymatic digestion (Morsczeck *et al.*, 2005, Yen and Sharpe, 2008).

Cells in dental follicles express markers such as Notch-1 and Nestin, suggesting the presence of undifferentiated cells. After cells are released from the tissue, only a small number of single dental follicle cells are attached onto the plastic surface and form CFU-F. DFPCs show a typical fibroblast-like morphology and express Nestin, Notch-1, collagen type I, bone sialoprotein (BSP), osteocalcin (OCN), and fibroblast growth factor receptor (FGFR) 1-IIIC (Morsczeck *et al.*, 2005, Morsczeck *et al.*,

2008).

DFPCs demonstrate osteogenic differentiation capacity in-vitro after induction. A membrane-like structure forms in DFPCs cultures after five weeks of stimulation with dexamethasone. It has been reported that STRO-1 and BMP receptors (BMPR) are expressed in dental follicles in-vivo (Kemoun *et al.*, 2007). Incubation with rhBMP-2 and rhBMP-7 or enamel matrix derivatives (EMD) for 24 hours increases the expression of BMP-2 and BMP-7 by DFPCs. Expression of cementum attachment protein and cementum protein-23 (CP-23), two putative cementoblast markers, has been detected in EMD-stimulated whole dental follicle and in cultured DFPCs stimulated with EMD or BMP-2 and BMP-7 (Kemoun *et al.*, 2007).

2.2.6 Stem Cells from Apical Papilla (SCAP)

Apical papilla refers to the soft tissue at the apices of developing permanent teeth (Sonoyama *et al.*, 2006, Sonoyama *et al.*, 2008). Apical papilla is more apical to the epithelial diaphragm, and there is an apical cell-rich zone lying between the apical papilla and the pulp (Rubio *et al.*, 2005).

Similar to DPSCs and SHED, ex-vivo expanded SCAP can undergo odontogenic differentiation in-vitro. SCAP express lower levels of DSP, matrix extracellular phosphoglycoprotein (MEPE), transforming growth factor-receptor II (TGF-RII), FGFR3, Flt-1 (VEGF receptor 1), Flg (FGFR1), and melanoma-associated glycoprotein (MUC18) in comparison with DPSCs. Significantly, CD24 is expressed by SCAP, which is not detected, on DPSCs or BMMSCs. The expression of CD24 by SCAP is down regulated in response to osteogenic stimulation. However, the biological significance of this finding requires further investigation (Sonoyama *et al.*,

2006, Sonoyama *et al.*, 2008, Abe S *et al.*, 2007).

SCAP also demonstrated the capacity to undergo adipogenic differentiation following induction in-vitro (Sonoyama *et al.*, 2006). Interestingly, without neurogenic stimulation, cultured SCAP show positive staining for several neural markers (Abe *et al.*, 2007). After stimulation, additional neural markers are also expressed by SCAP, including glutamic acid decarboxylase (GAD), neuronal nuclear antigen (NeuN), neuro filament M (NFM), neuron-specific enolase (NSE), and glial markers 2, 3-cyclic nucleotide 3- phosphodiesterase (CNPas) (Sonoyama *et al.*, 2008, Yen and Sharpe, 2008, Morsczeck *et al.*, 2008).

The distinction between dental pulp and apical papilla is that apical papilla is the precursor tissue of the radicular pulp. From this perspective, it may be speculated that SCAP are similar to stem cells residing in the dental papilla that gives rise to the coronal dentin- producing odontoblasts. Once the apical papilla turns into pulp, whether the SCAP convert into DPSCs or the latter are derived from a different stem cell pool is currently unclear. When SCAP and DPSCs are compared in-vitro, there are some differences. Overall, SCAP are derived from a developing tissue that may represent a population of early stem/progenitor cells, which may be a superior cell source for tissue regeneration. Additionally, these cells also highlight an important fact that developing tissues may contain stem cells distinctive from those of mature tissue (Abe S *et al.*, 2007, Abe S *et al.*, 2008).