

2014

Characteristics of Dental Follicle Stem Cells and Their Potential Application for Treatment of Craniofacial Defects

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CHARACTERISTICS OF DENTAL FOLLICLE STEM CELLS AND THEIR
POTENTIAL APPLICATION FOR TREATMENT OF CRANIOFACIAL
DEFECTS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Science
through
The Department of Comparative Biomedical Sciences

by
Maryam Rezai Rad
D.D.S., Tehran University of Medical Sciences, 2007
August 2014

To my beloved husband, Mahdi,
and
our lovely daughter, Rose.

ACKNOWLEDGMENTS

Thank God for giving me the opportunity to explore the world.

First and foremost, I would like to express my sincerest gratitude to my supervisor, Dr. Shaomian Yao, for his invaluable and insightful guidance throughout the course of my Ph.D. Study at Louisiana State University. He gave me the main idea of this work, and without his continuous encouragement as well as his deep knowledge of molecular biology, this dissertation could not have been accomplished. I am always thankful for his support and all I have learned from his great professional vision and scientific insight. He taught me how to face problems in my research and developed the true research spirit in me. In addition to all he has done regarding my Ph.D. studies, I will always remember his kindness concerning my life's difficulties from the day I entered his lab. What he has done to help me through this arduous task is more than I would have expected to find in an advisor, and it will never be forgotten.

I want to express my special thanks to my graduate committee members for patiently attending my general and final exams and providing me with their thoughtful comments and suggestions. Their help has always been constructive, beneficial, and very valuable to me. Thanks to Dr. Henrique Cheng for wonderful and unforgettable lectures regarding intracellular pathways and to Dr. Ji-Ming Feng for his comprehensive advice in immunocytochemistry. Also, thank you to Dr. Jeffery Gimble for his wonderful guidance through my graduate program. His deep knowledge and valuable vision of stem cell therapy greatly impacted my entire dissertation research.

I also need to thank the people who helped me with my project: Dr. Arthur Penn for his course, Critical Analysis in Molecular Biology/Medicine, Dr. Denial Hayes and Dr. Ammer Qurashi for providing the scaffolds, Dr. Xiaochu Wu for his tireless assistance in electron

microscopy, Dr. Jonathan Bova and Dr. Jenny Pepping for helping with the animal studies, Ms. Sherry Ring and Dr. Fabio Del Piero for histological analysis, and Dr. Kip Matthews and Dr. Mahdi Orooji for micro-CT analysis.

I would like to express my thanks to my parents, Dr. Gholamali Rezai Rad and Dr. Mansoureh Nikraves, who taught me to be self-motivated, have a strong work ethic, and take pride in my work. Their support and prayers have always been invaluable.

Most certainly, this dissertation would not have been possible without the love, encouragement and support of my husband, Dr. Mahdi Orooji. His sacrifice for this project was much more than anticipated and far more than fair.

I also would like to acknowledge my colleagues, Dr. Mike Flanagan and Ms. Chunhong Li for their warm friendship and the support they provided in a variety of ways.

And finally, I appreciate all my instructors in the department of Comparative Biomedical Sciences and the department of Biological Sciences at Louisiana State University. Their classes have had an immense impact on my knowledge.

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ABSTRACT

Utilization of patient-specific stem cells in regenerative medicine provides a novel treatment approach for diseases and disorders. Embryonic stem cells and induced pluripotent stem cells can differentiate into any cells found within the body; however, ethical, technical and safety concerns have to be overcome before they can be used in clinics. Patient-specific stem cells can be isolated from adult tissues with no ethical, fewer technical, and safety concerns. Obtaining tissues for stem cell isolation usually requires invasive procedures, but impacted teeth are often extracted in the clinics and can be used for isolation of dental follicle stem cells (DFSCs).

The overall goal of this dissertation is to characterize the osteogenic potential of DFSCs and to explore the possibility of using DFSCs for the treatment of craniofacial defects. In this regard, we first showed that DFSCs can be induced to differentiate primarily toward the osteoblast lineage. Our experiments showed that DFSCs at passages 3 to 5 have a strong osteogenic capability that is reduced during *in vitro* expansion. Comparing DFSCs with non-stem cell dental follicle cells (DFCs), we determined that dentin matrix protein 1 (DMP1) is highly expressed in DFSCs. Further study suggests that DMP1 is likely necessary to maintain the osteogenic differentiation capability of DFSCs via regulating expression of osteogenic genes. Given that adult stem cells exist in a quiescent state under normal physiological conditions, we attempted to activate DFSCs with heat-stress. Culturing DFSCs under mild heat-stress (39°C-40°C) could effectively promote their proliferation and osteogenic differentiation. In the final part of this project, *in vivo* transplantation experiments were conducted to evaluate the osteogenic potential of DFSCs for treatment of calvarial critical-size defects using a rat model. Bone regeneration was assessed by micro-computed tomography (micro-CT) and histological

analysis at 4 and 8 weeks post-transplantation. The results showed that transplantation of DFSCs seeded into PCL scaffold significantly improved bone regeneration. An average of 50% bone recovery was observed with treatment of PCL-DFSC transplantation at 8 weeks. In conclusion, this study found that DFSCs are valuable tissue stem cells possessing strong osteogenic potential that can be used for repairing craniofacial defects.

CHAPTER 1: STEM CELLS AND DENTAL FOLLICLE STEM CELLS IN REGENERATIVE MEDICINE

1.1 OVERVIEW

One of the most fascinating areas in regenerative medicine is the utilization of stem cells for regeneration of damaged tissues or replacement of diseased organs. Although embryonic stem cells (ESCs) are pluripotent and can differentiate into any cell types found within the body, use of ESCs raises various issues including safety and ethical concerns [1-3]. Induced pluripotent stem cells (iPSCs) also have been reported [4, 5]. They can be produced by reprogramming patients' own somatic cells into patient-specific stem cells that can be used for autogenous transplantation. However, tumorigenicity after transplantation of ESCs and iPSCs is the major threat in human therapies. Such tumorigenicity is likely due to their pluripotency [6]. Currently, it is not clear whether the obstacles to use of ESCs and iPSCs for human treatment can be overcome.

Stem cells also can be isolated from adult tissues. Due to lack of tumorigenicity after transplantation [7], they are considered safe for human therapies. Tissue-derived adult stem cells are multipotent and can differentiate into limited types of cells, within the same germ layer of tissues in which they reside. Our lab, as well as others, reported the isolation of dental follicle stem cells (DFSCs), with multipotent differentiation capabilities [8-10].

This dissertation aims to further characterize the DFSCs in Chapters 2-4. Moreover, in Chapter 5, we explored the possibility of the utilization of DFSCs for repair of craniofacial defects using rats as experimental animals. This dissertation provides important information toward the clinical applications of DFSCs.

1.2 STEM CELLS

Stem cells are clonogenic, self-renewing, and undifferentiated cells. Under the proper signals or stimulations, they can give rise (differentiate) into different cell types (lineages). In the body, tissue stem cell lineage is determined by a stem cell microenvironment or niche. A niche provides proper signals which determine the differentiation of local stem cells toward a specific lineage. However, stem cells have plasticity; by implanting them into a totally different niche, they may differentiate into cell types resembling those of their new environment [11]. This differentiation capability makes them very useful for regenerative medicine. Based on their origin, there are two main types of stem cells: Embryonic stem cells (ESCs) derived from the inner cell mass (ICM) of a blastocyst [12]; and adult stem cells (AdSCs), found within most, if not all, adult tissues. Stem cells can also be classified based on their differentiation potential: Totipotent stem cells can give rise to an entire organism, including all three embryonic germ layers (e.g. ectoderm, endoderm and mesoderm) plus placenta; pluripotent stem cells can give rise to every type of cell from all three germ layers but not to the extra-embryonic structures; and multipotent stem cells can only give rise to limited cell types, usually within the same germ layer of tissues in which they reside [13].

1.2.1 Embryonic stem cells (ESCs)

ESCs are derived from the inner cell mass (ICM) from an early developmental stage of the embryo [14]. ESCs are pluripotent, and can maintain an undifferentiated state and normal karyotype for at least 80 passages [15, 16]. However, isolation of ESCs requires the destruction of embryos which raises legal and moral controversies [1]. Another drawback to the use of ESCs is immune rejection. Because autogenous transplantation of ESCs is not feasible, transplantation between genetically unrelated individuals may generate severe immune

responses. In order to avoid graft rejection, matching alloantigens, including major histocompatible complex (MHC) antigens, is important. In addition, transplantation of ESCs has been shown to form tumors (teratomas) *in vivo* [2, 3]. Therefore, tumorigenicity of ESCs has become a major concern in the application of these cells.

1.2.2 Adult stem cells (AdSCs)

AdSCs exist in most, if not all, adult tissues functioning as a source of cells for normal tissue turnover and regeneration/repair of damaged tissues. AdSCs have multipotent differentiation capability; i.e., they can differentiate into limited types of cells [17]. AdSCs generally generate those cell types found in the tissue in which they reside. However, given proper stimulation, AdSCs can differentiate into cell types from other tissues [18-21]. AdSCs can be isolated and used in the same patient as an autograft, which eliminates the risk of graft rejection. Another advantage of AdSCs is that, in contrast to ESCs, they are generally not tumorigenic.

However, due to the limited quantity of AdSCs in given tissues, in order to have a sufficient number of cells for the clinical applications, the primary isolated AdSCs usually need to be expanded via *in vitro* culture. Studies demonstrated that *in vitro* expansion of AdSCs may influence both the proliferation and differentiation potentials of the cells [22, 23]. Study of human bone marrow stem cells (BMSCs) demonstrated decreased differentiation capability after multiple population doublings [24]. Different AdSCs lose their differentiation capability at different passage numbers. BMSCs, for instance, lose their differentiation capability around passage 6 [25, 26], while adipose-derived stem cells maintain their ability to differentiate into adipocytes until passage 10 [27]. In contrast, stem cells derived from human umbilical cord maintain their differentiation capability for a longer period of time until passage 18 [28]. Loss of

differentiation capability during *in vitro* expansion dramatically impacts the utilization of the AdSCs for regenerative medicine. As in many cases, it is impossible to continuously obtain tissues from a given patient for stem cell isolation. Efforts have been attempted to maintain AdSCs differentiation capability when cultured *in vitro*, but with limited success.

1.2.3 Induced pluripotent stem cells (iPSCs)

Another type of stem cell is iPSCs, which are produced by converting differentiated cells into pluripotent stem cells. The technique was first demonstrated in murine fibroblasts [4] and later in human [5] by reprogramming somatic cells with epigenetic expression of transcription factors, such as OCT3/4, SOX2, KLF4, and C-MYC [4]. iPSCs resemble ESCs, in that they can differentiate into all three germ layers and have a similar proliferation pattern [29]. Although use of these cells raises no ethical concerns, there is a risk of tumorigenicity, either by gene disruption due to DNA insertion or by activation of oncogenes [30]. Generation of iPSCs without introducing viral vectors has been reported [31]. It has been shown that repeated transfection with expression plasmids containing reprogramming genes results in generation of iPSCs without DNA integration [31]. A recent study showed that *in vivo* directed differentiation of iPSCs toward the specific lineage may minimize teratomas formation [32].

1.3 DENTAL STEM CELLS

AdSCs have been also isolated from dental tissues. Since the discovery of the AdSCs in dental pulp [33], several other types of dental stem cells (DSCs) were successfully isolated from various dental tissues including periodontal ligament (PDL) [34], exfoliated deciduous teeth [35], root apical papilla [36], and the dental follicle (DF) [8-10]. DSCs can be classified in two categories based on their differentiation potentials toward the dentin or periodontium tissues (i.e., tooth supporting tissues including PDL, alveolar bone, and cementum). Dental pulp stem cells

(DPSCs), stem cells from apical papilla (SCAPs) and stem cells from exfoliated deciduous teeth (SHEDs) are classified in the first group and associated with the dental pulp complex formation. The second group includes stem cells from dental follicle (DFSCs) and stem cells isolated from PDL (PDLSCs) and related to the formation of periodontium. Table 1.1 summarizes some of the differentiation properties as well as some of the surface markers that have been reported to be associated with different types of DSCs [37-41].

DSCs share some similarities with BMSCs, which are the most studied AdSCs. They possess multilineage differentiation properties, self-renewal capability, as well as colony-forming efficiency [8-10]. Obtaining BMSCs requires bone marrow aspiration, which is a painful and invasive procedure. Furthermore, the frequency and differentiation potentials of these cells are significantly age-related [42]. Therefore, considerable attention has been paid to stem cells from dental tissues that can be obtained from discarded medical wastes during dental procedures. For example, DF can be obtained from extracted impacted teeth. Moreover, DSCs are derived from the neural crest, a different origin from mesoderm-derived BMSCs [43]. This may make DSCs a better choice for repair of damages in neural crest-derived tissues, including periodontium and craniofacial defects. Several studies have also investigated the differentiation capability of DSCs toward the neuronal direction to explore their potential applications for treatment of neural diseases [44-46].

1.4 DENTAL FOLLICLE AND DENTAL FOLLICLE STEM CELLS

The dental follicle (DF) is a loose connective tissue surrounding an unerupted tooth. It plays an important role in the coordination of tooth eruption [47]. DF produces growth factors and cytokines to regulate osteoclastogenesis and osteogenesis required for tooth eruption [48].

Table 1.1 Differentiation capabilities and marker genes reported to be associated with different types of DSCs [37-41]

Cell types	<i>In vitro</i> multipotency	Ectopic tissue formation <i>in vivo</i>	Major marker genes
DPSCs			
Isolated from dental pulp	Adipogenesis Chondrogenesis Dentinogenesis Neurogenesis Myogenesis Osteogenesis	Dentin-like formation Bone-like formation	STRO-1, CD13, CD29, CD44, CD59, CD73, CD90, D105, CD146, NESTIN
SHEDs			
Isolated from exfoliated deciduous teeth	Adipogenesis Chondrogenesis Dentinogenesis Neurogenesis Myogenesis Osteogenesis	Dentin-like formation Bone-like formation	STRO-1, CD13, CD29, CD44, CD73, CD90, CD105, CD146, CD166
SCAPs			
Isolated from apical papilla	Adipogenesis Dentinogenesis Neurogenesis Osteogenesis	Dentin-like formation Bone-like formation	STRO-1, CD13, CD24, CD29, CD44, CD73, CD90, D105, CD106, CD146, NESTIN
PDLSCs			
Isolated from periodontal ligament (PDL)	Chondrogenesis Cementogenesis Dentinogenesis Neurogenesis Osteogenesis	PDL-like formation Cementum-like formation	STRO-1, CD13, CD29, CD44, CD59, CD73, CD90, CD105
DFSCs			
Isolated from dental follicle	Adipogenesis Chondrogenesis Neurogenesis Osteogenesis	PDL-like formation Bone-like formation Cementum-like formation	STRO-1, CD13, CD29, CD44, CD59, CD73, CD90, D105, CD146, NOTCH1, NESTIN

During tooth development, the DF is separated from dentin by an epithelial layer, which is called Hertwing's sheet. This sheet disintegration during tooth root development enables a contact between ectomesenchymal cells of the DF and the dentin surface [49, 50]. Such contact subsequently causes DF cells to differentiate into periodontium including the alveolar bone, cementum, and periodontal ligament (PDL) [51-53]. This biological function suggests the presence of stem cells in the DF. Several studies have reported the isolation of progenitor/stem cells from DF in different species, using an enzymatic digestion of the DF to release cells, followed by a culture of the cells in a stem cell growth medium [8-10]. The resultant cell population was identified as DFSCs as the cells possess multipotent differentiation capability [8]. Similar to other mesenchymal stem cells, DFSCs adhere to plastic and can form colonies [9]. In addition, they are characterized by a high proliferation rate, as well as expression of mesenchymal and neural stem cell markers listed in Table 1.2 [37-40].

Multilineage differentiation capability of DFSCs has been shown *in vitro* in various studies. DFSCs have been shown to have a greater differentiation capability toward the osteogenic lineage in comparison to DPSCs which tend to differentiate toward the odontoblasts [54]. Previous studies in our laboratory revealed that rat DFSCs have the capability to differentiate into adipocytes, neuroblasts, neurosphere and osteoblasts [8, 55]. Others reported that mouse DFSCs can differentiate toward the adipocytes, chondrocytes and osteoblasts [56]. Studies on human DFSCs showed a discrepancy in the multiple differentiation potential [57-59]. Human DFSCs isolated during early root development showed differentiation toward the adipocytes, chondrocytes and osteoblasts [58]. However, DFSCs isolated from the apex of human tooth root at a later stage of root development showed adipogenesis and osteogenesis, but no chondrogenesis capability [59]. These studies suggest that the DFSCs isolated from the DF of

Table 1.2 Mesenchymal and neural stem cell markers reported to be expressed in DFSCs [37-40]

Gene	Abbreviation	Description
Aminopeptidase-N	CD13	Mesenchymal stem cell marker
Integrin beta-1	CD29	Mesenchymal and neural stem cell marker
Transmembrane receptor to hyaluronan	CD44	Mesenchymal and cancer stem-like cell marker
Protectin	CD59	Mesenchymal stem cell marker
Ecto-5-prime-nucleotidase (NT5E)	CD73	Mesenchymal stem cell marker
Thy-1 membrane glycoprotein	CD90	Mesenchymal stem cell marker
Endoglin	CD105	Mesenchymal stem cell marker
Melanoma cell adhesion molecule (MCAM)	CD146	Mesenchymal stem cell marker
Trypsin-resistant cell surface antigen	STRO-1	Mesenchymal stem cell marker
Notch homolog 1	NOTCH1	Neural stem cell marker
Nestin	NESTIN	Neural stem cell marker

different developmental stages may affect differentiation potential. Despite these discrepancies within different laboratories, they all confirmed the strong differentiation capability of DFSCs toward the osteoblast lineage. Moreover, other studies revealed that the DF contains heterogeneous stem cells consisting of different subpopulations of stem cells varying in cell morphology, gene expression pattern, as well as differentiation capability. However, heterogeneity in a DFSC population does not affect their bone formation capability [60]

Due to the small size of the DF tissue, primary isolated DFSCs need to be expanded *in vitro* until sufficient numbers of cells are obtained. Optimization of the culture conditions to

grow and maintain DFSC properties is under investigation. The role of various factors in DFSCs proliferation and differentiation has been evaluated [61]. Studies suggest that Collagen type I is effective for both growth and osteogenic differentiation of the DFSCs. Also, fibronectin has been shown to enhance cell proliferation, and therefore it can be useful for rapid proliferation of DFSCs. Elucidation of the roles of different growth factors for maintaining DFSC properties would facilitate the establishment of the optimal culture conditions for proliferation of DFSCs.

1.5 *IN VIVO* HARD TISSUE-FORMING POTENTIAL OF DFSCS

Differentiation capability of DFSCs along osteogenic pathways *in vitro* suggests that they can be used for *in vivo* bone reconstruction/regeneration. However, limited studies have been conducted to evaluate *in vivo* bone formation of DFSCs. Subcutaneous transplantation of bovine and human DFSCs mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) ceramics showed formation of mineralized structure on the border of the ceramics [62, 63]. Two other studies investigated DFSCs for bone regeneration in critical-size defects created in the calvarium of immuno-deficient rats. The first study compared the osteogenic lineage differentiation potential of porcine DFSCs, PDLSCs and alveolar BMSCs [61]. Bone formation was observed after transplantation of these stem cells showed no statistical difference. The second study evaluated the bone formation potential of the different subpopulations of human DFSCs and found that transplantation of different cell populations promoted bone formation at the same extent [60]. In both studies, the new bone formation was similar to that seen in intramembranous ossification because there was no intermediate cartilage formation, indicating no endochondral ossification. However, no scaffolds were used for those experiments, and only DFSC pellets were transplanted. Scaffolds are important components in stem cell-based tissue engineering. Particularly, they are required for regeneration of larger defects.

1.6 USE OF SCAFFOLDS IN TISSUE ENGINEERING

Scaffolds mimic extracellular matrix and provide a physical three-dimensional structure to support cell attachment, migration, and differentiation [64]. In addition, they provide an environment for vascularization. Thus, scaffolds are an important element in tissue engineering. Scaffolds suitable for stem cell-based therapies should display the following properties [65]:

- 1) Provide a porous structure for vascularization, while the porosity should be sufficient for nutrient transfer and cell attachment without compromising mechanical strength.
- 2) Be biodegradable at the same rate as the regeneration of the new tissue.
- 3) Be biocompatible to support cell attachment, growth and differentiation.
- 4) Be mechanically stable during the tissue regeneration period.

There are three major types of scaffolds for tissue engineering: (a) calcium phosphate ceramics including β -tricalcium phosphate (β -TCP) and hydroxyapatite (HA), which resemble the inorganic component of bone; (b) naturally derived scaffolds including collagen, silk, derivatives of hyaluronic acid, gelatin, chitosan and fibrin, which mimic bone organic components; and (c) synthetic polymers which can be customized to be biocompatible with different tissues. Table 1.3 summarizes the advantages and disadvantages of these scaffolds.

1.7 SPECIFIC AIMS AND SIGNIFICANCE OF THIS RESEARCH

We hypothesize that DFSCs are a valuable type of AdSCs for bone regeneration for the treatment of craniofacial defects. The overall goal of this dissertation is to further characterize DFSCs and to evaluate the DFSCs for the treatment of rat calvarial critical-size defects. The following aims have been explored.

Table 1.3 Comparison of major types of scaffolds used for tissue engineering

Scaffolds	Advantages	Disadvantages
1. Calcium phosphate ceramics: β -tricalcium phosphate (β -TCP), hydroxyapatite (HA)	Osteoinductive Osteoconductive Support the attachment, growth and differentiation of cells	Low mechanical strength
2. Naturally derived scaffolds: Collagen, silk, derivatives of hyaluronic acid, gelatin, chitosan and fibrin	Biocompatibility Biodegradability Osteoconductive Support cell infiltration and vascularization	Low biomechanical properties Relatively high degradation rate
3. Synthetic Polymers: Poly-L-lactide acid (PLLA), poly- glycolic acid (PGA), poly-lactic-co-glycolic acid (PLGA), poly-ethylene glycol (PEG), poly-ethylene glycol diacrylate (PEDGA), poly-ethylene terephthalate (PET), poly-caprolactone (PCL)	Easy to modify chemical properties	No osteoinductive property

Aim 1: Characterization of osteogenic differentiation and identification of genes highly expressed in DFSCs (Chapter 2). First, the osteogenic capabilities of different passages of DFSCs were studied, and the DFSC passages that possess strong osteogenesis were identified. Next, we compared the expression of stem cell-related genes in the DFSCs and in non-stem cell dental follicle cells (DFCs). We found that several stem cell-related genes were expressed substantially higher in the DFSCs than in non-stem cell DFCs, and maximal differential expression was seen in dentin matrix protein 1 (DMP1). The results would provide fundamental information for utilization of DFSCs in tissue engineering, particularly in bone regeneration.

Aim 2: Improvement of osteogenesis of DFSCs *in vitro* (Chapters 3, 4). In Chapter 3, the role of DMP1 in promoting osteogenic differentiation of DFSCs was evaluated. We explored whether DMP1 could be added into the induction medium to promote osteogenesis of DFSCs.

Moreover, it is known that AdSCs exist in a quiescent state under normal physiological conditions, and certain factors such as tissue injury signals can activate stem cells from quiescence. Hence, we hypothesized that some stressors can activate stem cells. In Chapter 4, we showed that DFSCs increased self-renewal (proliferation) and differentiation by heat-stress treatment, suggesting that heat-stress may activate quiescent stem cells. Further study is recommended to investigate whether heat-stress treatments of DFSCs would be beneficial for bone regeneration prior to or after *in vivo* transplantation.

Aim 3: Evaluation of DFSCs for the treatment of craniofacial defects (Chapter 5). The ultimate goal of studying DFSCs is to use them for tissue repair/regeneration. Current literature only reports successful bone regeneration by transplanting DFSC pellets in immune-deficient rats [62, 60], and the results have limited value in determining the possibility of using DFSCs for clinical treatment of bone defects. In Chapter 5, two types of scaffolds were tested for seeding DFSCs, and *in vivo* studies were performed to test whether DFSCs could effectively repair the craniofacial defects. The results obtained from this aim would be significant toward the utilization of DFSCs in regenerative medicine.

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CHAPTER 2: CHARACTERIZATION AND OSTEOGENIC DIFFERENTIATION OF DENTAL FOLLICLE STEM CELLS*

2.1 INTRODUCTION

Experimental evidence supports the existence of progenitor/stem cells in various dental tissues, including the dental follicle (DF) [1-3]. Dental follicle stem cells (DFSCs) have been isolated from different species and have been demonstrated to possess strong osteogenic capability *in vitro* [1-3]. Thus, they can be used for bone regeneration to treat bone defects, such as craniofacial defects. For isolation of human DFSCs, DFs can be obtained from extracted impacted teeth that are discarded as medical wastes. However, due to the small size of the DF, limited quantities of primary DFSCs can be isolated. Therefore, *in vitro* expansion is necessary to obtain a sufficient number of cells for therapeutic use. Previous studies revealed that adult stem cells (AdSCs) lose their differentiation capabilities during *in vitro* culture [4, 5]. There is a lack of data within the literature regarding loss of differentiation capability in DFSCs during *in vitro* culture. It was our hypothesis that similar to other AdSCs, DFSCs also gradually lose their osteogenic differentiation potential during *in vitro* proliferation. It is important to determine the passages at which DFSCs reduce/lose their osteogenic capability, so that the proper passage cells can be used for therapeutic applications.

Various studies reported an osteogenic differentiation capability of DFSCs, based on assessment of calcium deposition after osteogenic induction. It is not clear whether DFSCs specifically differentiate toward the osteoblast lineage, because cementoblasts, odontoblasts and osteoblasts are all calcium-depositing cells involved in tooth development. It is reasonable to speculate that DFSCs, as stem cells derived from dental tissue, are likely capable of

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differentiation toward the cell lineages related to tooth development. Thus, another objective of this chapter was to assess whether DFSCs can differentiate into different types of calcium-depositing cells under the osteogenic induction condition by evaluating the expression of the marker genes specific in different types of calcium-depositing cells. Accomplishing of this objective would provide fundamental information for future applications of DFSCs for bone regeneration.

Genes highly expressed in stem cells are likely to have certain functions for stem cell properties or can serve as biomarkers for identification and isolation of stem cells. CD73 (membrane-bound ecto-5'-nucleotidase), CD90 (Thy1) and CD105 (endoglin) have been used as identification markers for mesenchymal stem cells (MSCs) [6]. STRO-1, trypsin-resistance cell surface antigen, was also reported to express in MSCs playing a role in controlling stem cell migration [7], and it also has been widely used as a marker for identification and isolation of different types of MSCs, such as bone marrow stem cells and stem cells from dental tissues using cell sorting techniques [8-12]. In addition, markers are useful for evaluation of the status of growth and differentiation potential of stem cells. For example, expression of vascular cell adhesion molecule 1 (VCAM-1/CD106) and melanoma cell adhesion molecule (MCAM/CD146) has been used as an indication of MSCs' multipotency [7]. Thus, the final objective of this chapter was to identify stem cell-related genes that are highly expressed in the DFSCs.

2.2 MATERIALS AND METHODS

2.2.1 Establishment of cell cultures

Animals were handled in accordance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University (LSU). Sprague Dawley (SD) rat pups at postnatal day 6 were used for isolation of DFs. Rat pups were

ethanized by isoflurane inhalation, and then the DFs were surgically collected from the first mandibular molars. Primary cell suspensions were obtained by digestion of the DFs with 0.05% Trypsin-EDTA (Invitrogen, Grand Island, NY, USA) in a 37°C incubator for 10 minutes and centrifuged for 5 minutes at 3000 rpm to collect cell pellets. Next, trypsin was removed and cell pellets were re-suspended in the appropriate medium. The procedures for establishment of the DF derived cell cultures are illustrated in Figure 2.1.

The cell suspension was subjected to two culture systems to establish dental follicle stem cells (DFSCs) or non-stem cell dental follicle cells (DFCs). DFSCs were established by growing the primary cells in a medium containing α -MEM (Invitrogen, Grand Island, NY, USA) + 20% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA) + 1% Penicillin-Streptomycin 10,000 u/ml (Invitrogen, Grand Island, NY, USA). This medium has been used previously to establish dental stem cell cultures [1], and therefore is referred as stem cell growth medium. For DFCs, primary cells were grown in fibroblast growth medium consisting of MEM (Invitrogen, Grand Island, NY, USA) + 10% newborn calf serum (NCS, Invitrogen, Grand Island, NY, USA) + 10 mM sodium pyruvate (Invitrogen, Grand Island, NY, USA) + 1% Penicillin-Streptomycin 10,000 u/ml (Invitrogen, Grand Island, NY, USA), as previously described [13]. Cells were cultured in plastic flasks overnight at 37°C and 5% CO₂, and non-adherent cells were removed by medium change. The remaining cells were cultured until they reached 80-90% confluency. Cells were trypsinized and passaged at a ratio of 1:3 until the desired passages were reached.

Established DFSCs and DFCs at passage 3 (P3) were evaluated for colony forming as well as osteogenic capability. DFSCs can form cell clusters (Figure 2.1; Section E) which were not seen in DFCs (Figure 2.1; Section F). DFSCs with strong osteogenic capability and DFCs

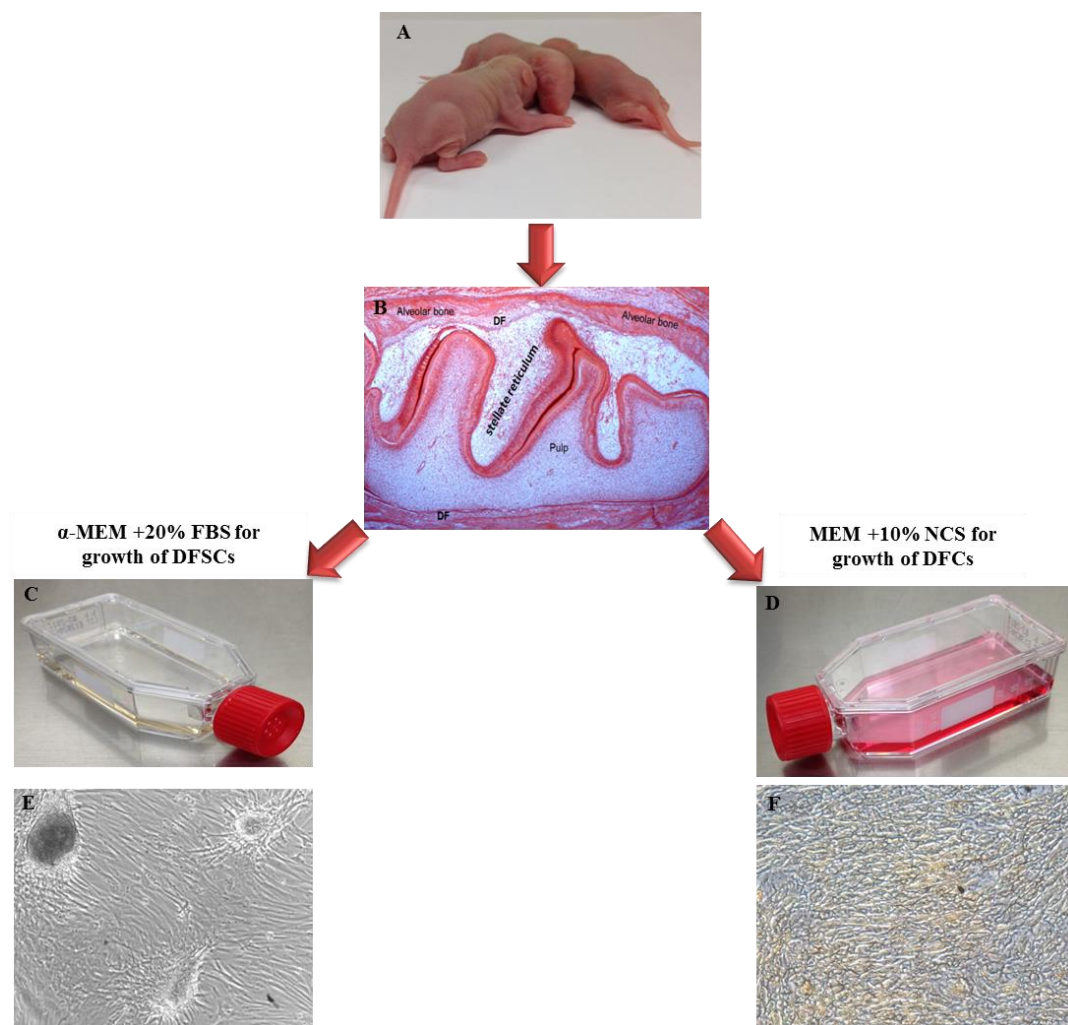


Figure 2.1 Schematic diagram of isolation of the DFSCs and DFCs from the DFs. Postnatal rat pups were used for obtaining the DFs (A). DFs were surgically removed. Note the DF shown on the histological slide (B)¹. DFs were then trypsinized, cells were collected and incubated either in stem cell growth medium for DFSCs (C) or fibroblast growth medium for DFCs (D). DFSCs were able to form cell clusters (E)²; in contrast, no cell clusters were formed in DFC cultures (F). DFs: dental follicle; DFCs: dental follicle cells; DFSCs: dental follicle stem cells; FBS: fetal bovine serum; NCS: newborn calf serum.

¹ Figure 2.1; Section B is reprinted from Chapter 16 of *Electroporation Protocols: Preclinical and Clinical Gene Medicine*, *Methods in Molecular Biology* with permission of Springer Science+Business Media New York.

² Figure 2.1; Sections E and F are reprinted from from a published paper by Yao S, Pan F, Prpic V, Wise GE. Differentiation of stem cells in the dental follicle. *J Dent Res*. 2008; 87(8): 767-71 with permission of SAGE Publications.

with no differentiation ability were transferred to cryopreservation tubes, in storage medium consisting of 50% α -MEM medium (for DFSCs) or MEM (for DFCs), 40% FBS, and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Cells were frozen at -80°C overnight in Mr. FROSTY™ Freezing container (Thermo Scientific, Waltham, MA, USA) before being transferred to liquid nitrogen for long-term storage.

2.2.2 Evaluation of osteogenic capability of the DFSCs and DFCs

To determine the osteogenic capability of DF derived cells, DFSCs and DFCs were seeded in 6-well plates at a cell density of 10^4 cells/cm². DMEM-LG (Invitrogen, Grand Island, NY, USA) containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA) and 1% Penicillin-Streptomycin 10,000 u/ml (Invitrogen, Grand Island, NY, USA) was used as basal medium, and osteogenic induction reagents consisting of 50 μ g/ml ascorbate-2 phosphate, 10^{-5} mM dexamethasone and 10 mM β -glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA) were added to the basal medium. This medium was designated as the osteogenic medium, as it has been used previously to induce osteogenesis of stem cells [1, 14-16]. DFSCs and DFCs cultured in α -MEM + 20% FBS (stem cell growth medium) without osteogenic induction reagents were included as a control. After 14 days of incubation, cells were washed with PBS and fixed with 10% Neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO, USA). Next, cells were stained with 1% Alizarin Red solution (GFS Chemicals, Inc., Columbus, OH, USA) for 5 minutes. In a separate well, osteogenic induced DFSCs were treated with 10% EDTA (AMRESCO, Solon, OH, USA) before being stained with Alizarin Red to ensure that the Alizarin Red staining was indeed calcium deposition.

2.2.3 Evaluation of osteogenic capability of different passages of DFSCs

Established DFSCs were sub-cultured until desired passages were obtained. Different passages of DFSCs were seeded in 6-well plates at a cell density of 10^4 cells/cm². Cells were then incubated in osteogenic medium. After 14 days of induction, cells were fixed and stained with Alizarin Red as described in Section 2.2.2. The staining was quantitated by Image-Pro Analyzer version 7.0 (Media Cybernetics, Rockville, MD, USA). In particular, the intensity of Alizarin Red staining was determined by segmentation, followed by counting the number of pixels in the range of bright red.

2.2.4 Expression of marker genes in the DFSCs and DFCs

In order to evaluate the expression of differentiation markers in DFSCs, the DFSCs at P3 or P5 were cultured in T-25 flasks at a cell density of 2×10^5 cells/flask and induced to differentiate, as described in Section 2.2.2. Cells were collected with RLT buffer for RNA extraction at days 7 and 14. The expression of the differentiation marker genes bone sialoprotein (BSP), osteocalcin (OCN), dentin sialophosphoprotein (DSPP) and F-spondin were determined using real-time RT-PCR. The primers used in this study are given in Table 2.1.

To assess the differential expression of stem cell-related genes in the DF derived cells, we conducted two separate experiments. In the first experiment, DFSCs and DFCs were cultured in T-75 flasks at a cell density of 10^6 cells/flask in their appropriate culture medium, as described in Section 2.2.1; i.e., DFSCs in α -MEM + 20% FBS (stem cell growth medium) and DFCs in MEM + 10% NCS (fibroblast growth medium), for 7 days. In the second experiment, we transferred established DFCs to stem cell growth medium and incubated for 7 days, so the DFSCs and DFCs were grown in the same medium condition. Cells were then collected for gene expression analysis by real-time RT-PCR.

Table 2.1 Primer pairs used for real-time RT-PCR in Chapter 2

Gene	Primer sequence	GeneBank reference #
ALP	F: 5'-GACAAGAAGCCCTTCACAGC-3' R: 5'-ACTGGGCCTGGTAGTTGTTG-3'	NM_013059.1
BMP3	F: 5'-TACTACAGTCCCTTCCGTCTCC-3' R: 5'-AAACAACCTAGCCACAGACACA-3'	NM_017105.1
BSP	F: 5'-ACGCTGGAAAGTTGGTAGCTG-3' R: 5'-TTCCTCTTCCTCGTCGCTTTCCTT-3'	NM_012587.2
BCRP	F: 5'-GTTTGGACTCAAGCACAGCA-3' R: 5'-AATACCGAGGCTGGTGAATG-3'	NM_181381.2
C-KIT	F: 5'-ACAAGAGGAGATCCGCAAGA-3' R: 5'-AGCAAATCATCCAGGTCCAG-3'	AF296696.1
DMP1	F: 5'-ACCTTTGGAGACGAAGACAATGGC -3' R: 5'-TGTCTTCACTGGACTGTGTGGTGT-3'	NM_203493.3
DSPP	F: 5'-GGGAAGCTCAGTGGAAGTAAAG -3' R: 5'-CTGCTGTGTCCCATGTTGTAT-3'	NM_012790.2
ESRRB	F: 5'-GTGCCTGAAGGGGATATCAA-3' R: 5'-AGAAACCTGGGATGTGCTTG-3'	NM_001008516.2
NOTCH1	F: 5'-CTCAACACACTGGGCTCTTTC-3' R: 5'-ACACCCTCATAACCTGGCATAC-3'	NM_001105721.1

ALP: alkaline phosphatase; BMP3: bone morphogenetic protein 3; BSP: bone sialoprotein; BCRP: breast cancer resistance protein 1; C-KIT: receptor tyrosine kinase; DMP1: dentin matrix protein1; DSPP: dentin sialophosphoprotein; ESRRB: estrogen-related receptor beta.

(Table 2.1 continued)

Gene	Primer sequence	GeneBank reference #
OCN	F: 5'-ACTGCATTCTGCCTCTCTGACCT-3' R: 5'-TATTCACCTCCTTACTGCCCTCCT-3'	NM_013414.1
F-spondin	F: 5'-GACCTACGAGTCACCAAACAA-3' R: 5'-CACCTTCCGGGTCATAGAAAG-3'	M88469.1
TERT	F: 5'-ATGTTTCCTTCTGGCTAATGG-3' R: 5'-TGCTCCACACACTCTTACGG-3'	NM_053423.1
β -actin	F: 5'-CTAAGGCCAACCGTGAAAAGAT-3' R: 5'-AGAGGCATACAGGGACAACACA-3'	NM_031144.3

NOTCH1: neurogenic locus notch homolog protein 1; OCN: osteocalcin; TERT: telomerase reverse transcriptase; β -actin: beta actin.

2.2.5 Methods for real-time RT-PCR

To extract total RNA, cells were lysed in RLT buffer (Qiagen, Valencia, CA, USA) and centrifuged at 13000 rpm for 3 minutes. RNA isolation was performed from the supernatants with the RNeasy® Mini kit, according to the manufacturer's description (Qiagen, Valencia, CA, USA). RNA Concentration was determined with a Nanodrop 8000 (Thermo Scientific, Waltham, MA, USA). RNA was reverse-transcribed into cDNA with M-MLV Reverse Transcriptase (Invitrogen, Grand Island, NY, USA). Briefly, equal amounts of RNA were mixed with 250 ng random primer (Invitrogen, Grand Island, NY, USA) and reverse-transcription buffer. Reverse transcription was performed at 37°C for 50 minutes followed by heating at 70°C for 15 minutes to inactivate the reaction. cDNA templates were used for SYBR Green real-time PCR to detect cycle threshold (C_T) values with Applied Biosystems' 7300 Real-Time PCR System. The C_T

values were normalized to β -actin to calculate ΔC_T . Relative gene expression (RGE) was calculated with the formula $2^{-\Delta\Delta C_T}$ using the control as the reference (RGE=1) [17].

2.2.6 Expression of DMP1 protein in the DFSCs and DFCs

For assessment of DMP1 expression at protein level in the DF derived cells, DFSCs and DFCs were harvested after 7 days culture in their original culture medium using CytoBuster™ protein extraction buffer (EMD Millipore, Billerica, MA, USA) supplemented with 1/50 Protease Inhibitor cocktail (Santa Cruz Biotechnology, Inc. Dallas, TX, USA). After centrifugation of the cell lysate, supernatants containing total protein were transferred to new tubes, and protein concentration was determined with the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Total protein (15 μ g) of each sample was subjected to electrophoresis on 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated with a polyclonal rabbit anti-DMP1 antibody (Abnova Corporation, Walnut, CA, USA) or rabbit anti-actin polyclonal antibody (Abcam, Cambridge, MA, USA). The membrane was subsequently incubated with goat anti-rabbit IgG and then washed with PBST buffer to remove unbound antibody. The membrane was developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology, INC. Dallas, TX, USA).

2.2.7 Statistical analysis

All experiments were repeated a minimum of N=3 times. Analysis of variance (ANOVA) and least significant difference (LSD) were used for multiple comparisons of more than two means with SAS program version 9.3 (SAS, Cary, NC, USA). A student *T*-test was used for comparison of two means to obtain the *P*-values for determination of statistical significance. $P \leq 0.05$ was considered as statistically significant.

2.3 RESULTS

2.3.1 Osteogenic differentiation of DF derived cells

Two distinct populations of cells were established from the DFs and designated as DFSCs and DFCs. Both DFSCs and DFCs were subjected to osteogenic differentiation induction, and Alizarin Red staining was conducted to assess calcium deposition after 14 days of incubation (Figure 2.2). The wells containing DFSCs showed positive staining when cultured in osteogenic induction medium (Figure 2.2; Section A). In contrast, the wells containing DFSCs cultured in stem cell growth medium (α -MEM + 20% FBS) or basal medium (DMEM-LG + 10% FBS) without the addition of osteogenic induction reagents did not show any staining (Figure 2.2; Sections B and C). No staining was seen in the DFCs in any of the treatment groups (Figure 2.2; Sections D, E and F). This differentiation assay confirmed that the DFSCs contained stem/progenitor cells capable of osteogenic differentiation, whereas the DFC population contained no stem cells/precursor cells. In addition, DFSCs did not spontaneously differentiate into calcium-depositing cells in stem cell growth medium.

To ensure that the Alizarin Red staining seen was calcium deposition, some wells were treated with 10% EDTA prior to staining. We found that the tentative calcium deposition could be completely removed by EDTA, such that no Alizarin Red staining could be seen in EDTA-treated wells (Figure 2.3; Section C), indicating that these deposits were calcium.

2.3.2 Expression of differentiation marker genes in DFSCs in response to osteogenic induction

Calcium can be deposited by a variety of cells, including cementoblast, odontoblasts and osteoblasts. Alizarin Red staining detects only overall calcium deposition. To determine whether DFSCs can differentiate into different calcium-depositing cells, we further evaluated the expression of marker genes specific for osteoblasts (BSO, OCN), odontoblasts (DSPP), and

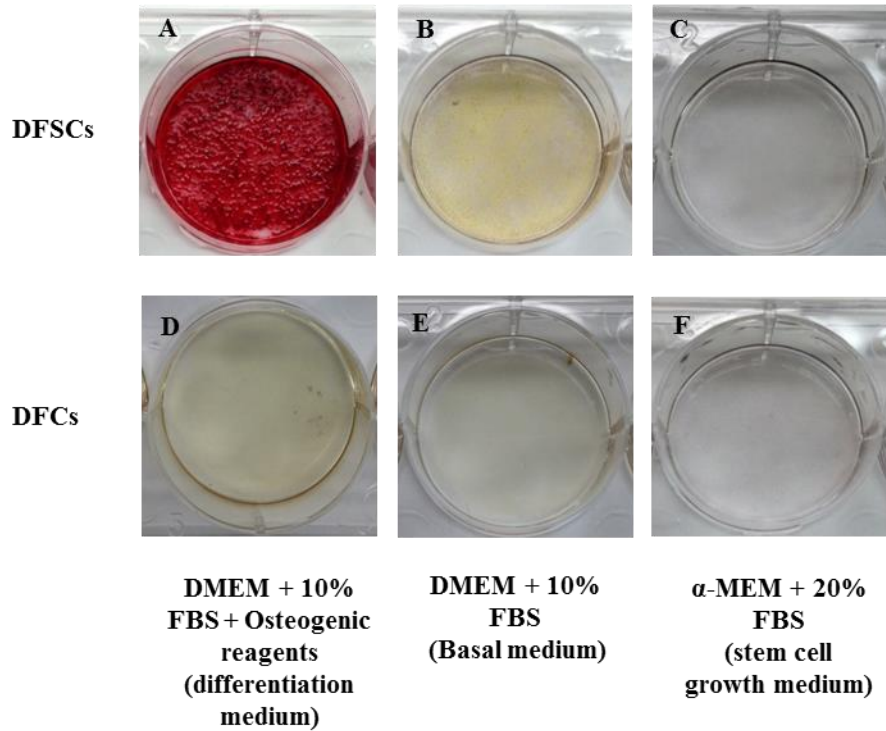


Figure 2.2 Evaluation of calcium deposition in the DFSCs and DFCs cultured in different medium as shown by Alizarin Red staining. Note that Alizarin Red staining was detected only in the DFSCs incubated in medium containing osteogenic induction reagents (A), but not in either basal medium (B) or stem cell growth medium (D) without the induction reagents. In contrast, DFCs did not display any calcium deposition in all treatment groups (D-F). DFCs: dental follicle cells; DFSCs: dental follicle stem cells; FBS: fetal bovine serum.

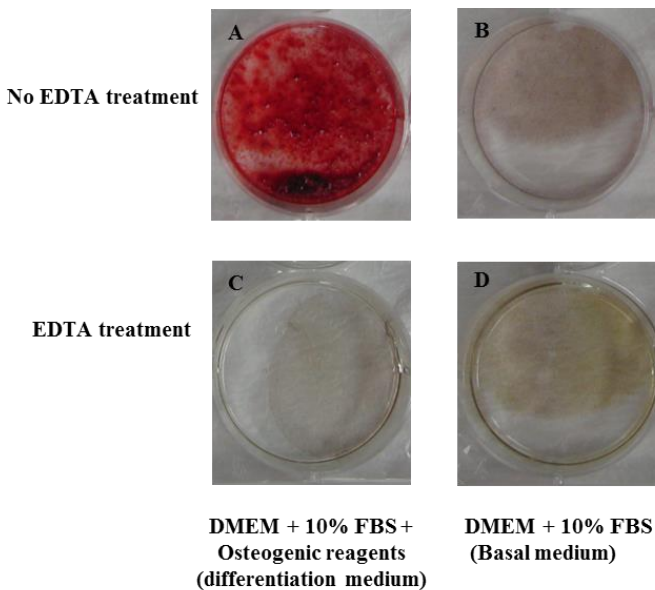


Figure 2.3 EDTA treatment to ensure calcium deposition in Alizarin Red staining. Alizarin Red positive staining was seen only when cells were cultured in the differentiation medium (A); no Alizarin Red staining was seen in the DFSCs cultured in basal medium without induction reagents (B); and 10% EDTA treatment of the cultures prior to Alizarin Red staining completely removed the calcium depositions and resulted in no staining in the induced cells (C), similar to the non-induced cells (D). DFSCs: dental follicle stem cells; EDTA: Ethylenediaminetetraacetic acid; FBS: fetal bovine serum.

cementoblast (F-spondin) before and after induction. The results are presented in Figure 2.4. After 7 days of induction, the expression of BSP and OCN was significantly increased in DFSCs, with the RGE of 59.69 ± 6.84 for BSP and 307.12 ± 70.64 for OCN compared to undifferentiated control (RGE=1), indicating that the DFSCs underwent osteogenic differentiation. Expression of these two markers continued to increase significantly until day 14 (Figure 2.4; Sections A and B). In contrast to BSP and OCN, we observed minimal changes in expression of the odontoblast (DSPP) and cementoblast markers (F-spondin). DSPP expression was decreased after 7 days of induction when compared to controls. Although the average RGE of 5.48 ± 2.61 was seen after 14 days of induction, the increase was not statistically significant compared to undifferentiated controls (Figure 2.4; Section C). The increase in expression of F-spondin was not statistically significant after 7 days of induction, and expression was decreased about 90% (i.e., RGE of 0.1) after 14 days of induction (Figure 2.4; Section D). The increased expression of the osteoblast markers and decreased expression of the cementoblast and odontoblast markers suggested that the DFSCs differentiated toward the osteoblast lineage under the osteogenic induction conditions.

2.3.3 Osteogenic capability of DFSCs during *in vitro* expansion

When different passages of DFSCs were subjected to osteogenic induction for 14 days (Figure 2.5), the maximum osteogenesis was observed in the DFSCs at P3 and P5, as revealed by Alizarin Red staining. A reduction in staining was noted by P7. The calcium deposition was reduced dramatically by P9. By P11, DFSCs lost their osteogenic differentiation capability completely (Figure 2.5; Section A). The staining was measured quantitatively with Image-Pro Analyzer, and data were analyzed statistically. Significant reduction in staining was seen in P7 and P9 (Figure 2.5; Section B). These results indicated that the DFSCs gradually reduced their

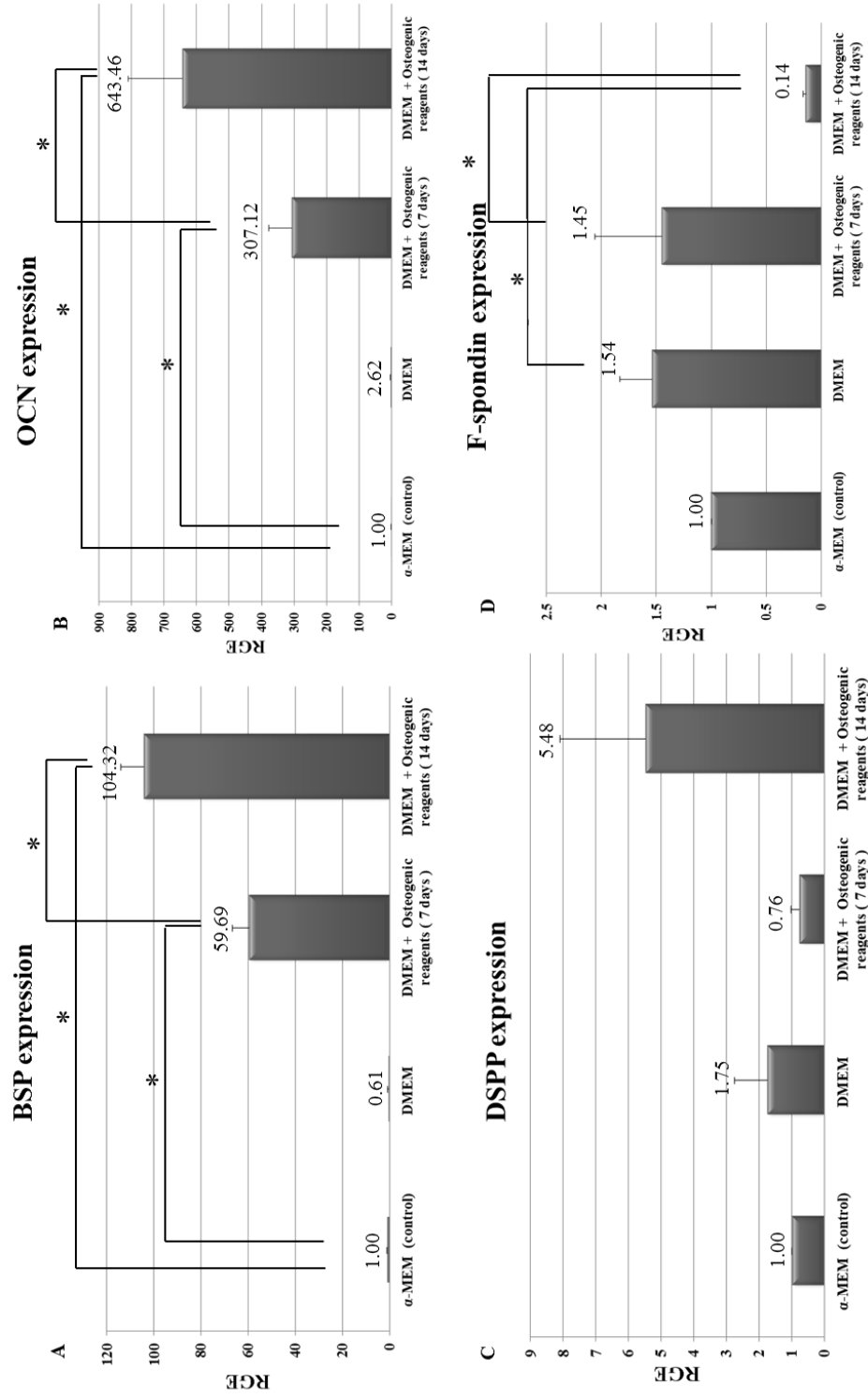
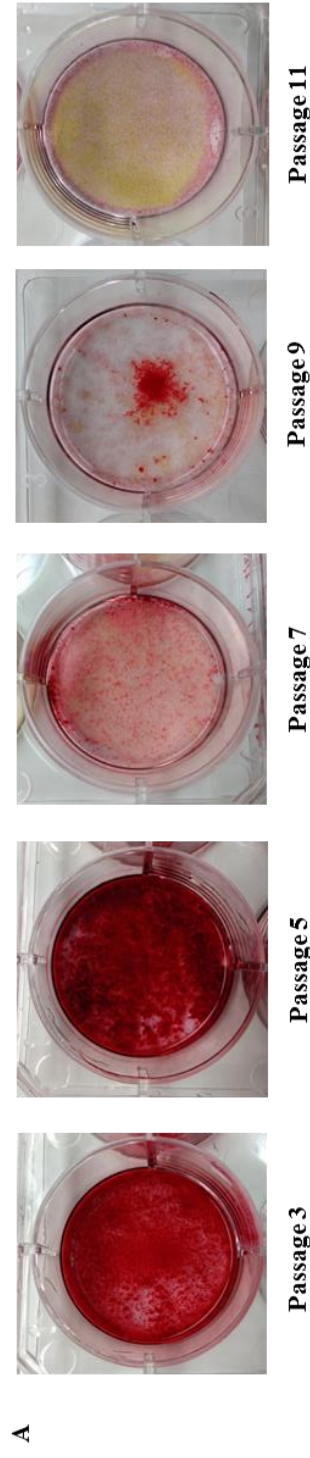
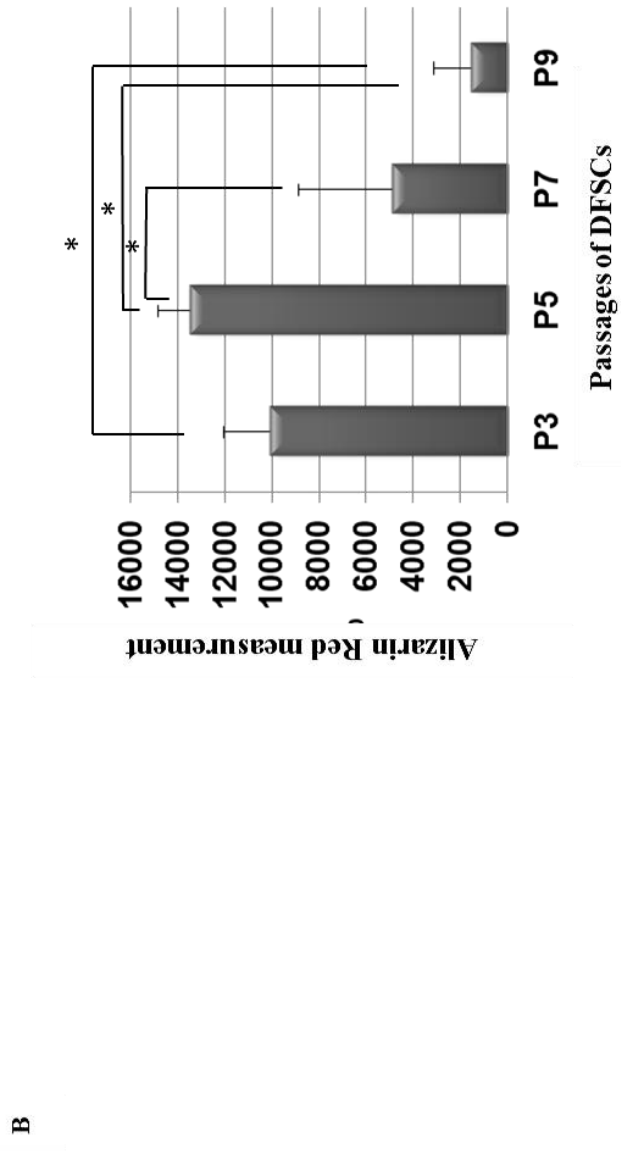


Figure 2.4 Expression of cell differentiation markers in the DFSCs before and after osteogenic induction as determined by real-time RT-PCR. After 7 and 14 days of incubation, the expression of osteoblast markers, BSP (A) and OCN (B), was significantly increased in differentiated DFSCs (DMEM + osteogenic reagents) when compared with undifferentiated cells (α-MEM) or undifferentiated DFSCs (DMEM), whereas minimal changes in expression of the odontoblast marker (DSPP) (C) and cementoblast marker (F-spondin) (D) were observed during differentiation induction of DFSCs. * indicates significant difference from undifferentiated cells at $P \leq 0.05$ (N=3). BSP: bone sialoprotein; DSPP: dentin sialophosphoprotein; FBS: fetal bovine serum; OCN: osteocalcin; RGE: relative gene expression.



A



B

Figure 2.5 Alizarin Red staining to determine calcium deposition in different passages of DFSCs after 14 days incubation in osteogenic medium. (A) a typical staining pattern at indicated passages of DFSCs. (B) Alizarin Red staining intensity was measured by Image-Pro Analyzer (N=3). Note that that maximum staining was seen in P3 and P5. Substantial reduction of staining was observed at P9. * indicates significant difference at $P \leq 0.05$ (N=3). DFSCs: dental follicle stem cells; P: passage.

osteogenic capability during *in vitro* culture with complete loss of the ability occurring around P11.

2.3.4 Expression of stem cell-related genes in the DFSCs and DFCs

Using real-time PCR, we evaluated the expression of 40 stem cell-related genes in DFSCs vs. DFCs. The result revealed that 14 genes were expressed higher in the DFSCs than in DFCs, when the cells were grown in the original medium for establishing the cultures described in Section 2.2.1. Of the 14 genes, 8 genes showed statistically higher in the DFSCs than in the DFCs (Figure 2.6), and no statistical significance was determined in other 6 genes (data not shown). The maximum difference was seen in dentin matrix protein 1 (DMP1), with RGE of 856 ± 152.94 , indicating an average of more than 850-fold higher in DFSCs compared to DFCs (RGE=1). In addition to DMP1, the genes that expressed over 10-fold higher in DFSCs were telomerase reverse transcriptase (TERT), estrogen-related receptor beta (ESRRB), bone morphogenetic protein 3 (BMP3), receptor tyrosine kinase (C-KIT) and neurogenic locus notch homolog protein 1 (NOTCH1). The differences were statistically different in these genes. To determine the possible medium effect, both DFSCs and DFCs were incubated in α -MEM + 20% FBS (used for growth of stem cells) for 7 days before collecting the cells for real-time RT-PCR analysis. A similar trend of RGE was observed for these genes; however, a huge increase of the average RGE for DMP1 was observed (Figure 2.7). To confirm the DMP1 expression at the protein level, western blotting was conducted. The result of western blotting was generally consistent with RT-PCR result. A clear DMP1 protein band was seen for the DFSCs, whereas almost no DMP1 protein was detected for the DFCs (Figure 2.8).

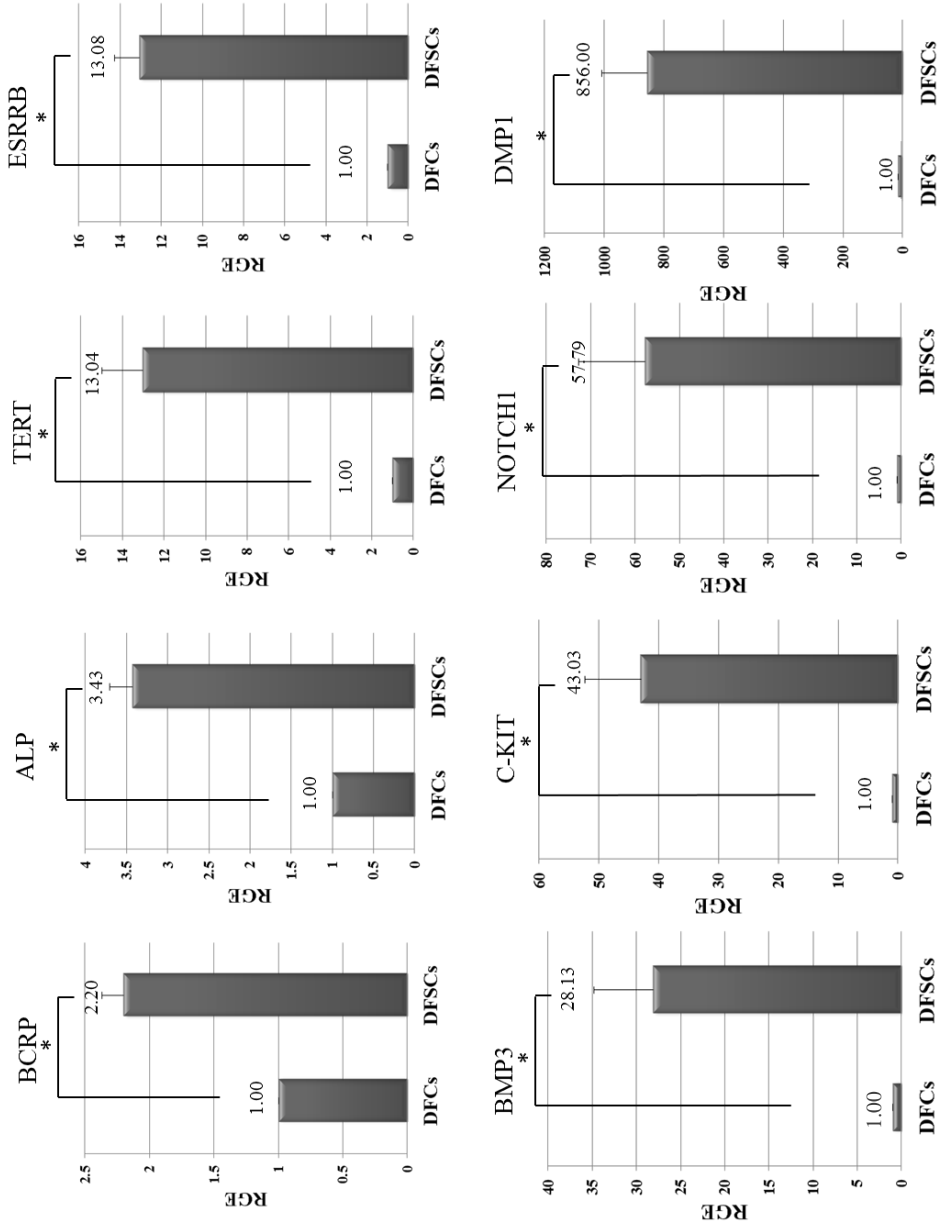


Figure 2.6 Comparison of expression of stem cell-related genes in the DFSCs and DFSCs grown in the original medium for establishing the cultures. RGE was calculated with ΔC_T method by normalizing to β -actin and using DFSCs as the reference control (RGE=1). * indicates significant difference at $P \leq 0.05$ (N=3). ALP: alkaline phosphatase; BMP3: bone morphogenetic protein 3; BCRP: breast cancer resistance protein 1; C-KIT: receptor tyrosine kinase; DMP1: dentin matrix protein1; DFSCs: dental follicle stem cells; DFSCs: dental follicle cells; ESRRB: estrogen-related receptor beta; NOTCH1: neurogenic locus notch homolog protein 1; RGE: relative gene expression; TERT: telomerase reverse transcriptase.

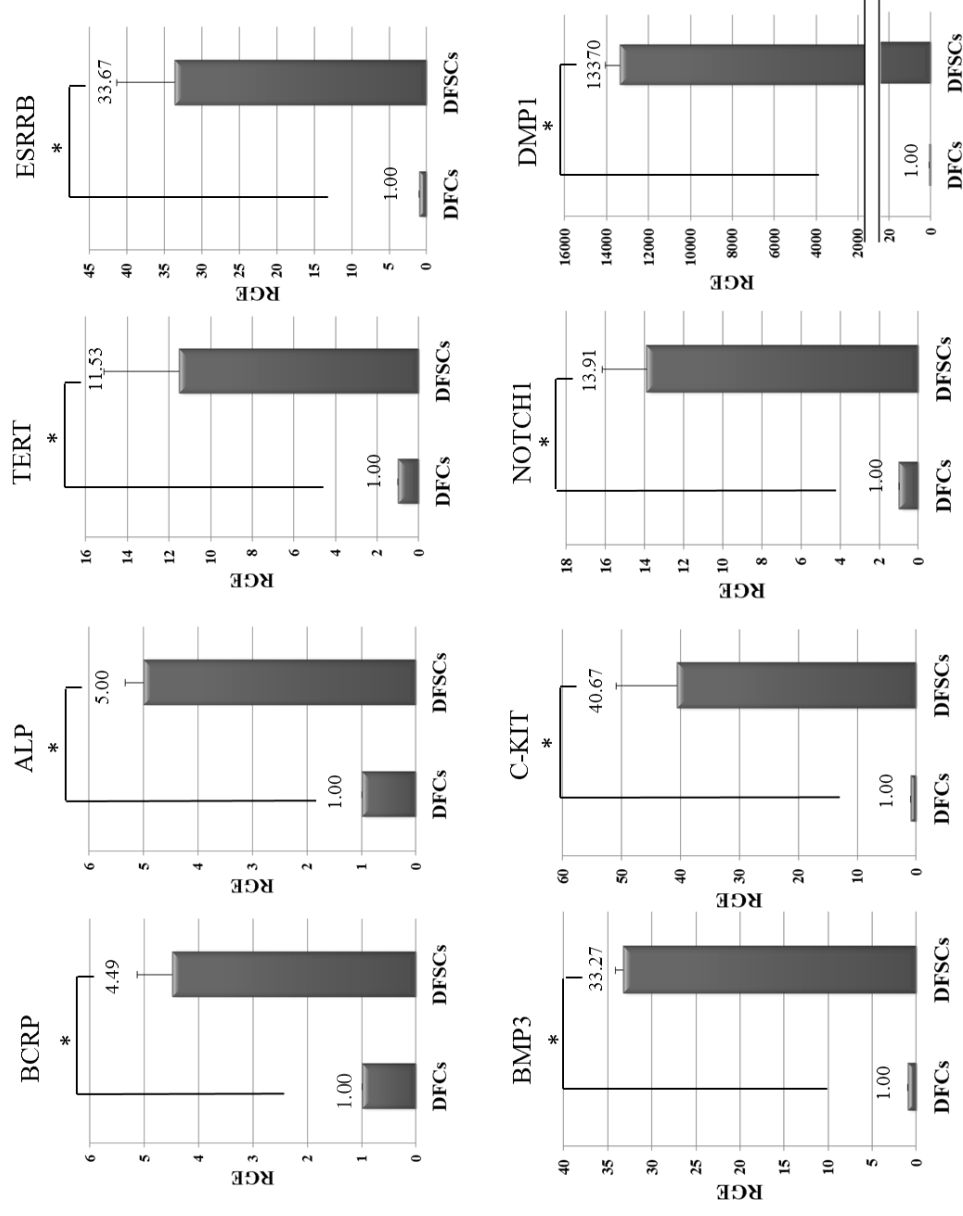


Figure 2.7 Comparison of expression of stem cell-related genes in the DFSCs and DFSCs grown in stem cell growth medium. RGE was calculated with ΔC_T method by normalizing to β -actin and using DFSCs as the reference control (RGE=1). * indicates significant difference from the control at $P \leq 0.05$ (N=3). ALP: alkaline phosphatase; BMP3: bone morphogenetic protein 3; BCRP: breast cancer resistance protein 1; C-KIT: receptor tyrosine kinase; DMP1: dentin matrix protein1; DFSCs: dental follicle stem cells; DFSCs: dental follicle cells; ESRRB: estrogen-related receptor beta; NOTCH1: neurogenic locus notch homolog protein 1; RGE: relative gene expression; TERT: telomerase reverse transcriptase.

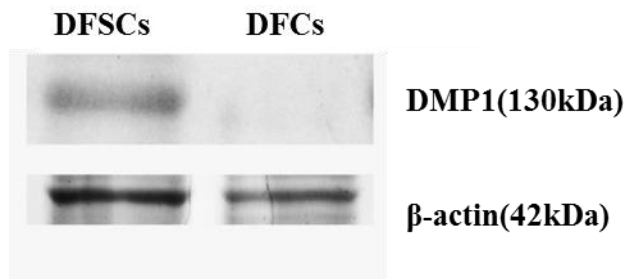


Figure 2.8 Comparison of DMP1 expression in the DFSCs and DFCs at protein level. Western blotting analysis indicated that the DMP1 protein band was seen in the DFSCs, but not clearly seen in the DFCs. DFCs: dental follicle cells; DFSCs: dental follicle stem cells; DMP1: dentin matrix protein 1.

2.4 DISCUSSION

Stem cells have been demonstrated to exist in the DFs of different species [1-3]. This study demonstrated that the DFSCs isolated from rats can be induced to differentiate into calcium-depositing cells. Because cementoblasts, odontoblasts and osteoblasts are capable of depositing calcium and are involved in tooth development, it is interesting to determine whether the DFSCs can differentiate into these cells under the induction condition used in this study. We examined the expression of marker genes specific for these calcium-depositing cells in DFSCs before and after induction. Based on the differentiation marker expression results, we conclude that DFSCs can be induced into calcium-depositing cells primarily toward the osteoblast lineage. The marker genes used for this assessment are specific for given lineages of differentiation as discussed below.

BSP has been suggested as a marker of osteoblast differentiation [18]. Studies suggested that a decrease in the level of BSP expression has a negative effect on osteoblast differentiation [19, 20]. OCN is secreted by the osteoblasts [21] and has been used as a marker for bone formation in various studies [22, 23]. DSPP is a specific marker for odontoblast differentiation [24, 25], and mutations in this gene have been associated with impaired dentinogenesis [26]. F-spondin expression was observed in cementoblasts derived from the periodontal ligament [27]. It has been identified as a promoting factor for cementoblast differentiation [28]. Evaluating the expression of these differentiation markers, we found that when subjecting DFSCs to induction

medium, more than 100-fold increased expression of the BSP and OCN (osteogenic markers) was observed. In contrast, the expression of an odontoblast marker, DSPP, did not change significantly after osteogenic induction. No significant increased expression of F-spondin (cementoblast marker) was observed. In fact, decreased expression of F-spondin was seen after induction. These results suggested that DFSCs were induced mainly to differentiate into the osteoblast lineage, but not cementoblast or odontoblast lineage. Differentiation into odontoblasts and cementoblasts, if any, would be very minimal under the induction conditions used in this study. A similar study on mouse immortalized DFSCs demonstrated the absence of amelogenin (ameloblast marker) and DSPP after 21 days of induction [29].

AdSCs lose their differentiation capabilities over time during *in vitro* culture [4, 5]. Different types of AdSCs lose their differentiation capability at different passages. Study of bone marrow stem cells (BMSCs) has demonstrated decreased differentiation capability along with morphological changes after multiple population doublings [30]. It has been reported that BMSCs lose their differentiation capability around P6 [31, 32], and adipose-derived stem cells maintain their ability to differentiate into adipocytes until P10 [33]. In contrast, stem cells derived from human umbilical cord maintain their differentiation capability for longer periods of time (P18) [34]. Since transplantation of high-differentiation potential stem cells is desired for maximal tissue regeneration, knowing the differentiation capability of *in vitro* cultured stem cells is essential before their transplantation. In this regard, we evaluated the osteogenic capability of DFSCs from early to late passages. We found that DFSCs at P3 or P5 have maximal osteogenic differentiation potential, thus they are suitable for *in vivo* transplantation for bone regeneration. However, limitation may exist regarding comparing cell passages due to variation in handling cell sub-cultures in different laboratories. We passage the cells at a ratio of 1 to 3 (i.e., 1 flask to

3 flasks) when the cultures reach 90% confluency, this protocol is commonly practiced for mammalian cell cultures [35].

Extensive studies have been carried out to identify specific marker genes for stem cells. However, no single definitive marker has been discovered specific for MSCs. The International Society for Cellular Therapy (ISCT) has proposed several markers as minimal criteria for identification of MSCs: MSCs must express CD73 (membrane-bound ecto-5'-nucleotidase), CD90 (Thy1) and CD105 (endoglin), and they must be negative for haematopoietic markers including CD34 and CD45 [6]. Other studies have reported the expression of mesenchymal and neural stem cell markers in DFSCs, including STRO-1 (trypsin-resistant cell surface antigen), CD13 (aminopeptidase-N), CD29 (Integrin beta-1), CD44 (Transmembrane receptor to hyaluronan), CD59 (protectin), CD146 (Melanoma cell adhesion molecule), NOTCH1 and NESTIN [36-38]. Markers are also useful for evaluation of the status of growth and differentiation potential of stem cells. For example, expression of vascular cell adhesion molecule 1 (VCAM-1/CD106) and melanoma cell adhesion molecule (MCAM/CD146) has been used as an indication of MSCs' multipotency [7]. This study compared expression of 40 stem cell-related genes in DFSCs vs. non-stem cell DFCs, both of which were established from the DF. We found that DFSCs express more than 10-fold of TERT, ESRRB, BMP3, C-KIT, NOTCH1 and DMP1 than do DFCs in the same or different culture medium. These genes may be considered to serve as potential makers for identification and assessment of DFSCs. It would be interesting to determine whether these genes play any regulatory roles in proliferation and differentiation of DFSCs.

In conclusion, we demonstrated the differentiation of DFSCs toward the osteoblast lineage, which makes them suitable for repair of skeletal defects. We also showed that DFSCs

reduced their osteogenic capability during *in vitro* expansion, and DFSCs at P3-P5 should be used for *in vivo* transplantation studies. Furthermore, we determined that DFSCs appear to express a high level of DMP1.

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CHAPTER 3: THE ROLE OF DENTIN MATRIX PROTEIN 1 (DMP1) IN REGULATION OF OSTEOGENIC DIFFERENTIATION OF DENTAL FOLLICLE STEM CELLS (DFSCS) *

3.1 INTRODUCTION

In Chapter 2, we reported obtaining two distinct cell populations from the dental follicle (DF), a loose connective tissue sac surrounding the unerupted tooth, using different cell culture systems. The cells derived from α -MEM + 20% Fetal Bovine Serum (FBS) were capable of multipotent differentiation and were designated as dental follicle stem cells (DFSCs). In contrast, cells grown in MEM + 10% Newborn Calf Serum (NCS) had no differentiation capability [1], but possessed the characteristics of fibroblasts. These latter cells were designated as dental follicle cells (DFCs) [2]. Others have reported the isolation of progenitor or precursor cells from the DF that could be induced to differentiate into calcium-depositing cells [3, 4]. Thus, like other adult tissues, the DF contains adult stem cells (AdSCs) that would be valuable for tissue regeneration, such as for bone and craniofacial tissue reconstruction/regeneration [5]. Elucidation of the genes and factors involved in regulating osteogenic capability of DFSCs could facilitate the development of cell-based therapies using DFSCs.

Dentin matrix protein 1 (DMP1) is present in mineralized tissues [6] and is highly expressed in osteoblasts [7] and odontoblasts [8]. Several studies have confirmed that DMP1 can participate in both the intra- and extracellular biomineralization process. The acidic domains of DMP1 can function as a nucleator for hydroxyapatite formation in the extracellular matrix [9]. DMP1 protein was also found in the nucleus during early differentiation of odontoblasts and osteoblasts [10, 11]. In addition, it is believed that in undifferentiated preosteoblasts, DMP1

* A manuscript developed from this chapter has been submitted to the Archive of Oral Biology. The manuscript is currently under revision. Authors: Maryam Rezai Rad, Dawen Liu, Hongzhi He, Hunter Brooks, Gary E. Wise, Shaomian Yao.

functions as a transcriptional factor to activate osteoblast-specific genes for osteoblast differentiation [10].

The expression of DMP1 in all non-mineralized structures surrounding developing teeth has been documented [12]. One such tissue is the DF. Because the DF appears to regulate the osteogenesis required for tooth eruption [13], and in Chapter 2, we found that DMP1 is highly expressed in the DFSCs, but not expressed in the non-stem cell DFCs, we hypothesized that DMP1 plays an important role in regulating the osteogenic differentiation of DFSCs. The objectives of this chapter were to determine (a) the correlation between DMP1 expression and the osteogenic capability of different passages of DFSCs; and (b) if DMP1 can function to promote osteogenic differentiation of DFSCs.

3.2 MATERIALS AND METHODS

3.2.1 Cell cultures

Animals were handled in accordance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University (LSU). Rat pups at day 6 were euthanized by isoflurane inhalation, and DFs were surgically isolated from the first mandibular molars. Primary cells were obtained by trypsinization of the DFs. DFSCs and homogeneous non-stem cell DFCs were established by growing the primary cells in either a stem cell growth medium or a fibroblast growth medium as described in Chapter 2 (Section 2.2.1). After establishment of the primary cultures of DFCs and DFSCs, the cultures were trypsinized at 80-90% confluency and passaged until the desired passages were reached. Cells used for all experiments were cultured at 37°C and 5% CO₂ in this study.

3.2.2 Correlation of DMP1 expression and osteogenic differentiation in different passages of DFSCs

DFSCs of passages 3, 5, 7 and 9 were collected with RLT buffer (Qiagen, Valencia, CA, USA) for total RNA extraction, and real-time RT-PCR was conducted to determine the relative gene expression (RGE). The C_T values were normalized using β -actin as the internal control to calculate ΔC_T . RGE was calculated via the formula $2^{-\Delta\Delta C_T}$ using the passage 3 as the reference (RGE=1) [14]. Different passages of DFSCs were also seeded in 6-well plates at a cell density of 10^4 cells/cm². Cells were then incubated in osteogenic medium. After 14 days of induction, the cells were fixed and stained with Alizarin Red using the method stated in Chapter 2 (Section 2.2.2) and the staining was quantitated by Image-Pro Analyzer version 7.0 (Media Cybernetics, Rockville, MD, USA) as described in Chapter 2 (Section 2.2.3). Next, the correlation between DMP1 expression and osteogenesis in different passages of DFSCs was analyzed using SAS program version 9.3 (SAS, Cary, NC, USA).

3.2.3 Transfection of DFSCs with DMP1-siRNA

A Dicer substrate siRNA was obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA) by annealing two single stranded RNA: sense strand 5'-rCrGrArCrCrArCrArGrUrGrArUrGrArGrGrCrArGrArCrAGC-3' and anti-sense strand 5'-rGrCrUrGrUrCrUrGrCrCrUrCrArUrCrArCrUrGrUrGrGrUrGrGrUrC-3'. The siRNA was designed to target the rat DMP1 mRNA. To knock down gene expression, the DFSCs at passages 3 and 5 were seeded in 6-well plates at a cell density of 10^4 cells/cm² and cultured until 70% confluency at the time of transfection. Transfection was carried out using LipofectamineTM RNAiMAX per manufacturer's instructions (Invitrogen, Grand Island, NY, USA). Briefly, the siRNA and transfection reagent were diluted to 100 nM with Opti MEM I reduced serum medium (Invitrogen, Grand Island, NY, USA). After 5 minutes of incubation at room

temperature, the siRNA and transfection reagent were mixed and incubated at room temperature for another 25 minutes to allow the formation of a siRNA- RNAiMAX complex. The resultant complex was added to the DFSC culture to bring the final siRNA concentration to 10 nM. A scrambled siRNA was transfected into the cells as the control. Knockdown of DMP1 expression was confirmed by real-time RT-PCR. Transfected DFSCs were cultured in the osteogenic differentiation medium for 14 days. The siRNA transfection was repeated at day 7 during the 14 days of osteogenic induction to ensure continuous knockdown of DMP1 expression. The cells were then stained with Alizarin Red to evaluate the calcium deposition.

To assess the effect of DMP1 knockdown on expression of osteogenic-related genes, transfected DFSCs were collected after 7 and 14 days of induction for analysis of the expression of selected genes using real-time RT-PCR with the primers listed in Table 3.1. Briefly, total RNA was extracted from the cells and 1 µg total RNA was reverse-transcribed into 20 µl cDNA. SYBR green real-time PCR was conducted with 0.5 µl cDNA from each sample in each PCR reaction to obtain the C_T values. The C_T values were normalized to β -actin for calculation of ΔC_T . RGE was calculated using $\Delta\Delta C_T$ method with untransfected DFSCs as the reference (RGE=1) [14].

3.2.4 Effect of exogenous DMP1 on osteogenic differentiation of DFSCs

The recombinant mouse DMP1 (rmDMP1) was purchased from R & D system (Minneapolis, MN, USA). DFSCs at passage 9 that had reduced endogenous DMP1 expression and reduced differentiation capability were incubated with osteogenic medium plus different concentrations (0-250 ng/ml) of rmDMP1. After 14 days of incubation, Alizarin Red staining was performed to evaluate calcium deposition.

Table 3.1 Primer pairs used for real-time RT-PCR in Chapter 3

Gene	Primer sequence	GeneBank reference#
BGN	F: 5'-AGAATGGGAGCCTGAGTTTCT-3' R: 5'-ACCTTGGTGATGTTGTTGGAGT-3'	NM_017087.1
BMP2	F: 5'-CTCAGCGAGTTTGAGTTGAGG-3' R: 5'-GGTACAGGTCGAGCATATAGGG-3'	NM_017178.1
BMP5	F: 5'-AATTTGGGCTTACAGCTCTGC-3' R: 5'-AGAAGAACCTCACTTGCCTTGA-3'	NM_001108168.1
BMP6	F: 5'-CTTACAGGAGCATCAGCACAGA-3' R: 5'-GTCACCACCCACAGATTGCTA-3'	NM_013107.1
DMP1	F: 5'-ACCTTTGGAGACGAAGACAATGGC -3' R: 5'-TGTCTTCACTGGACTGTGTGGTGT-3'	NM_203493.3
FLT1	F: 5'-ACAGAAGAGGATGAGGGTGTCT-3' R: 5'-ATCAGCTCCAGGTTTGACTTGT-3'	NM_019306.1
MMP13	F: 5'-TTTATTGTTGCTGCCCATGA-3' R: 5'-GAGAGACTGGATTCCTTGAACG-3'	NM_133530.1
RUNX2	F: 5'-TACTTCGTCAGCGTCCTATCAG-3' R: 5'-ATCAGCGTCAACACCATCATT-3'	NM_053470.2
β -actin	F: 5'-CTAAGGCCAACCGTGAAAAGAT-3' R: 5'-AGAGGCATACAGGGACAACACA-3'	NM_031144.3

BGN: biglycan; BMP2: bone morphogenetic protein 2; BMP5: bone morphogenetic protein 5; BMP6: bone morphogenetic protein 6; DMP1: dentin matrix protein1; FLT1: fms-related tyrosine kinase 1; MMP13: matrix metalloproteinase 13 (collagenase 3); RUNX2: runt-related transcription factor 2; β -actin: beta actin.

3.2.5 Statistical analysis

All experiments were repeated a minimum of N=3 times. Analysis of variance and least significant difference (LSD) were used for multiple comparisons of more than two means. A student *T*-test was used to compare the expression of osteogenic marker genes in DMP1-siRNA transfected DFSCs versus the control transfection. Pearson correlation analysis was performed to determine the correlation between DMP1 expression and osteogenesis in different passages of DFSCs. All of the statistical analyses were performed with SAS program version 9.3 (SAS, Cary, NC, USA). Statistical difference was determined at $P \leq 0.05$ being significant.

3.3 RESULTS

3.3.1 Reduction in DMP1 expression during *in vitro* culture of DFSCs

Expression of DMP1 in different passages of DFSCs was determined using real-time RT-PCR. The results revealed an overall trend of decreased expression of DMP1 with progression of cell passaging (Figure 3.1; Section A). The expression started to decline at passage 7, and by passage 9 there was greater than a 50% reduction in expression as compared to passage 3. This reduction was statistically significant (Figure 3.1; Section A).

3.3.2 Correlation between DMP1 expression and osteogenesis of DFSCs

To determine the correlation between DMP1 expression and osteogenic capability of DFSCs, different passages of DFSCs were induced for osteogenesis for 2 weeks before being stained by Alizarin Red. Osteogenesis of different passage DFSCs was quantitatively measured as shown in Figure 3.1; Section B. Pearson correlation analysis indicated a strong correlation between DMP1 expression and osteogenesis in different passages of DFSCs with a correlation coefficient of 0.92503 (Figure 3.1; Section C).

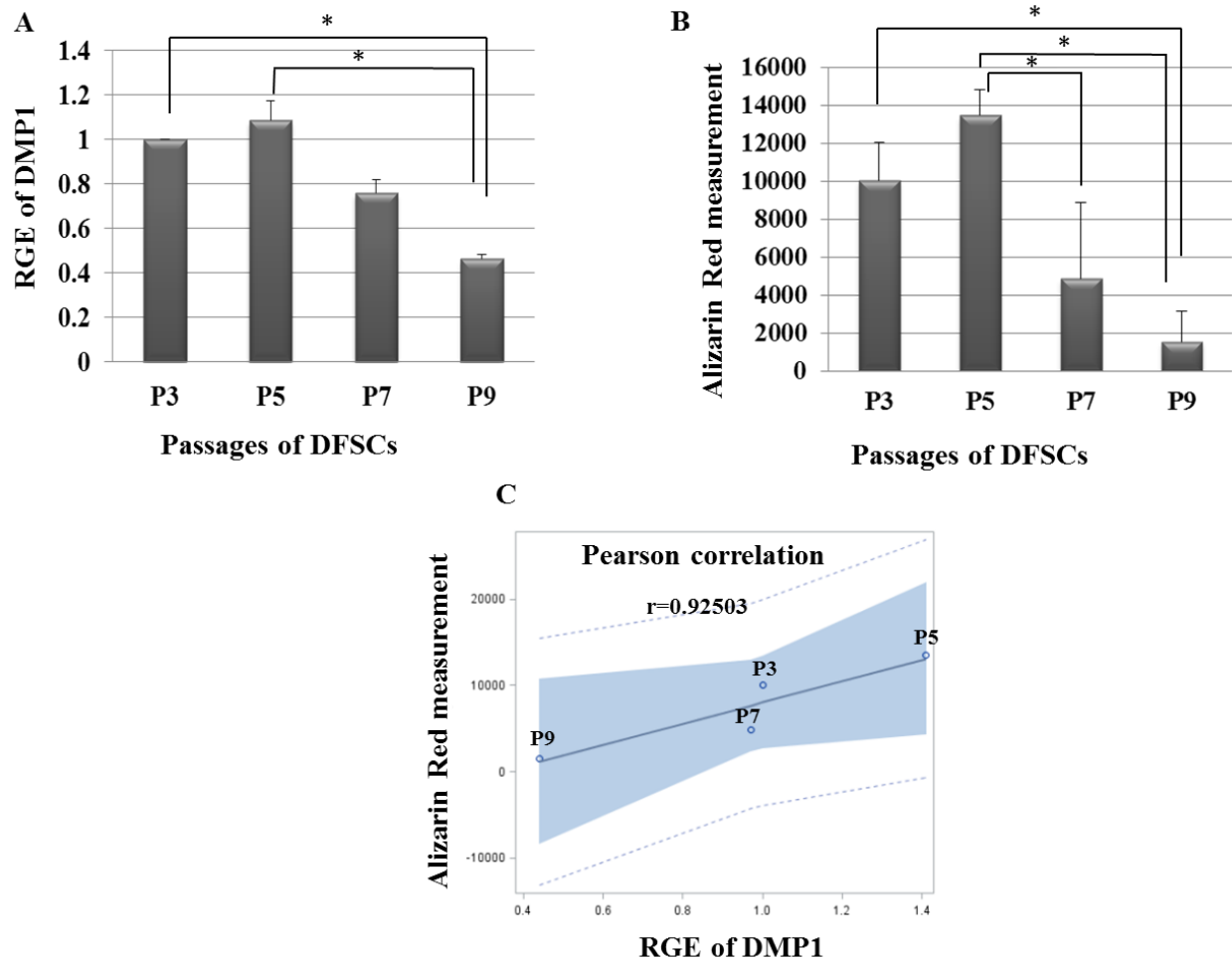


Figure 3.1 Correlation between DMP1 expression and osteogenic capability in the different passages of DFSCs. (A) DMP1 expression was significantly reduced at P9 as determined by real-time RT-PCR. (B) Significant reduction of osteogenic differentiation in the DFSCs at passages 7 and 9 determined by Alizarin Red staining measured with Image-Pro Analyzer. (C) Pearson correlation analysis showed a strong correlation between DMP1 expression and osteogenesis in different passages of DFSCs with correlation coefficient $r=0.92503$. * indicates significant difference at $P \leq 0.05$ ($N=4$). DFSCs: dental follicle stem cells; DMP1: dentin matrix protein 1; RGE: relative gene expression; P: passage.

3.3.3 Inhibition of osteogenesis by knockdown of DMP1 expression in DFSCs

Since expression of DMP1 appeared to correlate to osteogenic potential of DFSCs, it was important to determine whether there was a cause and effect relationship between DMP1 expression and osteogenesis. In this regard, early passages (P3 and P5) of DFSCs that expressed high levels of DMP1 were transfected with DMP1-siRNA. With real-time RT-PCR, we checked the knockdown efficiency and found that about 90% knockdown could be achieved 3 days after DMP1-siRNA transfection and 85% knockdown was still seen after 5 days of transfection. After subjecting the transfected DFSCs to osteogenic induction, significant reduction of osteogenesis was observed in the DMP1-siRNA transfected cells, whereas no noticeable difference was seen in cells transfected with the scrambled siRNA as compared to the mock transfection control (Figure 3.2; Section A). Knockdown of DMP1 also significantly reduced expression of selected osteogenic genes in transfected DFSCs when subjected to osteogenesis induction (Figure 3.2; Section B). In particular, the reduction was more than 40% for bone morphogenetic protein 6 (BMP6) and bone morphogenetic protein 5 (BMP5); the reduction was about 30% for bone morphogenetic protein 2 (BMP2), matrix metalloproteinase 13 (MMP13) and runt-related transcription factor 2 (RUNX2). Slight reduction of fms-related tyrosine kinase 1 (FLT1) and biglycan (BGN) expression was also detected. Statistical analysis determined that the reduction of the expression of these genes was significant as compared to the untransfected control (Figure 3.2; Section B).

3.3.4 Enhancement of osteogenesis of DFSCs by DMP1 protein

When passage 9 DFSCs that had reduced osteogenesis and expression of DMP1 were incubated in the osteogenic medium containing mrDMP1 protein for 14 days, we observed an increase of osteogenesis when mrDMP1 was added to the induction medium. This DMP1 effect

was concentration-dependent. As shown in Figure 3.3, a dramatic increase of osteogenesis was seen at concentrations of 200 and 250 ng/ml.

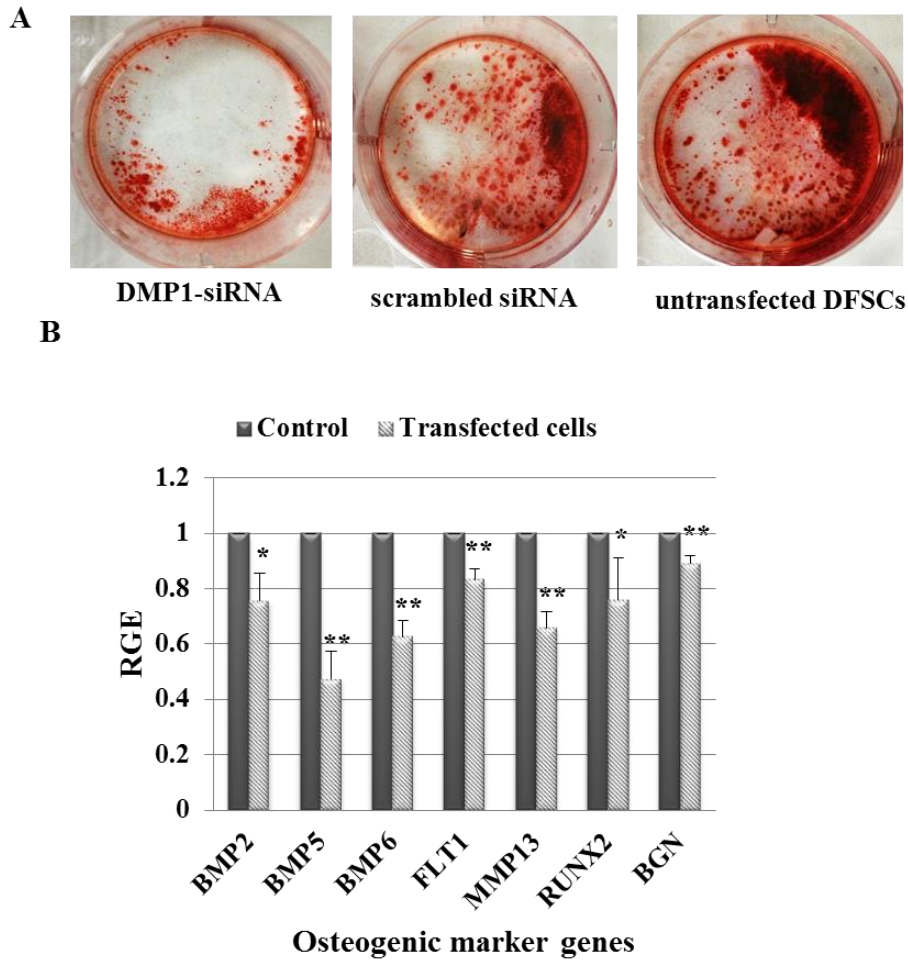


Figure 3.2 Effect of DMP1 knockdown on osteogenesis and osteogenic gene expression. (A) Alizarin Red staining revealed that knockdown of DMP1 expression in early passages of DFSCs resulted in a notable reduction of calcium deposition after 14 days of osteogenic induction when compared to the mock transfection and scrambled siRNA transfection controls. (B) Real-time RT-PCR determined the decreased RGE of selective osteogenic genes in DFSCs after DMP1 knockdown as compared to the control after 7 days of osteogenic induction. * and ** indicate significant reduction at $P \leq 0.05$ and $P \leq 0.001$ ($N=3$), respectively, as compared to the control for the given genes. BGN: biglycan; BMP2: bone morphogenetic protein 2; BMP5: bone morphogenetic protein 5; BMP6: bone morphogenetic protein 6; DMP1: dentin matrix protein 1; DFSCs: dental follicle stem cells; FLT1: fms-related tyrosine kinase 1; MMP13: matrix metalloproteinase 13 (collagenase 3); RUNX2: runt-related transcription factor 2; RGE: relative gene expression.

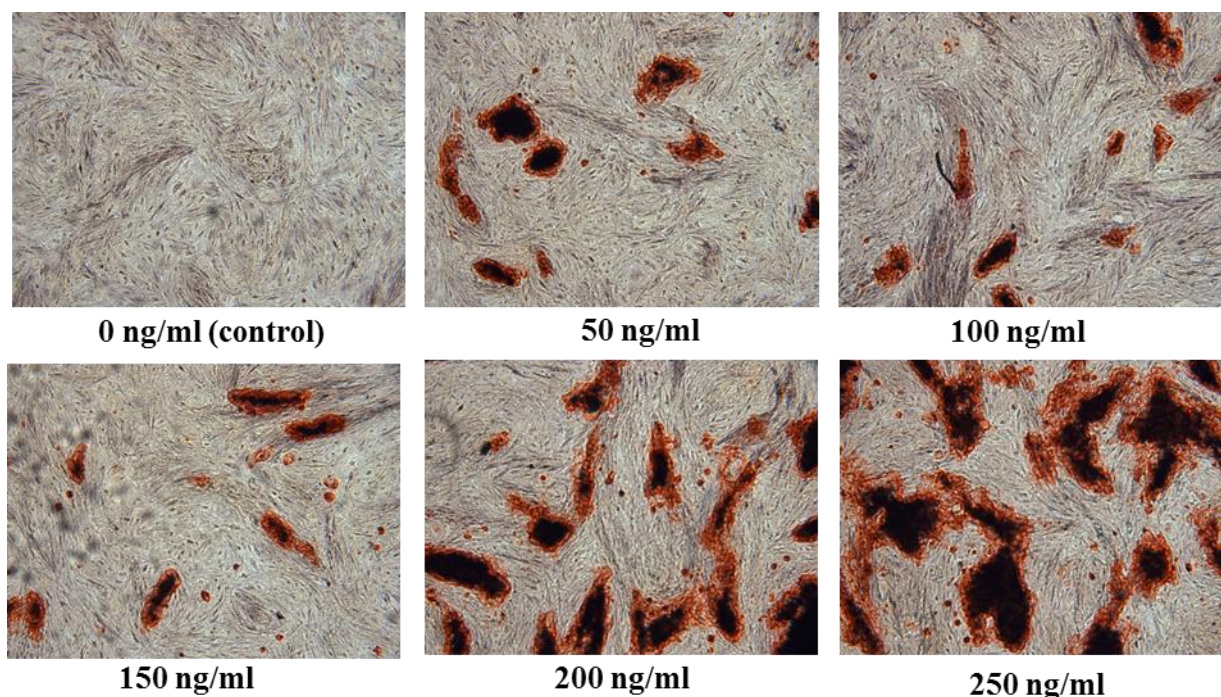


Figure 3.3 Effect of exogenous DMP1 on osteogenesis of the late passage DFSCs. Note that addition of mrDMP1 to the osteogenic induction medium increased calcium deposition in P9 DFSCs after 14 days of induction as shown by Alizarin Red staining. No staining was seen in the control without addition of mrDMP1, whereas adding mrDMP1 to the osteogenic medium resulted in increased Alizarin Red staining in a concentration-dependent manner. DFSCs: dental follicle stem cells; mrDMP1: mouse recombinant dentin matrix protein 1; P: passage.

3.4 DISCUSSION

The DF is a loose connective tissue sac consisting of fibroblast-like non-stem cell DFCs that are not capable of differentiation, and DFSCs that are capable of osteogenic differentiation [1, 2]. In Chapter 2, we showed that DMP1 was highly expressed in the DFSCs, but not expressed in DFCs. We sought to elucidate the function of DMP1 in the DFSCs in this chapter. Our results indicate that the high level of expression of DMP1 in the DFSCs is critical for maintaining the differentiation potential of DFSCs.

It has been reported that DMP1 is expressed in differentiated calcium-depositing cells, including osteoblasts, osteocytes and odontoblasts [6, 7, 8, 15]. Undifferentiated preosteoblasts

also have been shown to express DMP1 [10]. Given that the DFSCs express high levels of DMP1 as revealed in this study, it is possible that the population of DFSCs contains osteoblasts and other progenitor cells. However, our previous studies demonstrated that the DFSCs were capable of multipotent differentiation into osteoblasts, adipocytes and neurospheres [1, 16]. It is unclear whether the DFSCs are composed of different subpopulations of progenitor cells or only multipotent stem cells. Further studies are needed to clarify this and to determine whether the DMP1 is expressed in certain subpopulations of cells in the DFSCs. For example, fluorescence activated cell sorting (FACS) may be employed to obtain different cell subpopulations from the DFSCs, followed by real-time RT-PCR and western blotting to determine DMP1 expression in each subpopulation.

It is well known that DMP1 plays an important role for matrix biomineralization of bone and dentin. DMP1 is essential in the maturation of odontoblasts and osteoblasts [11, 12, 17]. In this study, we determined the strong correlation between DMP1 expression and osteogenic capability of DFSCs. DMP1 expression was reduced in long-term cultured DFSCs, and when the DFSCs with reduced DMP1 expression were subjected to osteogenic induction, their osteogenic capability was reduced. DMP1 knockdown study indicated that the DMP1 expression and osteogenic differentiation was not merely correlation, but there was a cause-and-effect relationship. This is supported by our observation in the cell culture experiment where addition of mrDMP1 could partially restore osteogenesis in the late passage DFSCs. Thus, together, the results of this study suggest that DMP1 may function to maintain osteogenic capability of the DFSCs.

DMP1 has been showed to bind to the promoter of dentin sialophosphoprotein (DSPP) to regulate the expression of that gene [11]. Given that DMP1 possesses a promoter binding

property and can function as a transcription factor [10], it would be interesting to determine whether DMP1 can regulate the expression of osteogenic-related genes. We found that knockdown of DMP1 resulted in reduction of the expression of osteogenic-related genes (BMP2, BMP5, BMP6, FLT1, MMP13, RUNX2 and BGN) during osteogenic differentiation of the DFSCs, suggesting that DMP1 is likely involved in up-regulating the expression of these osteogenic genes necessary for differentiation of DFSCs. This observation is supported by Narayanan *et al.*, who reported that overexpression of DMP1 in mesenchymal stem cells induces the expression of genes involved in early and proliferative stages of mineralization such as RUNX2, BMP2, BMP4, ALP, OCN, DMP2, and DSP [18]. Further studies are needed to determine whether DMP1 can bind to the promoter and enhancer regions of these osteogenic genes via bioinformatics analysis.

Exogenous DMP1 can exert its effect by entering the cells via endocytosis or by binding to its membrane receptors to activate an internal signaling pathway leading to osteogenesis [19, 20]. Recent studies revealed that $\alpha v \beta 3$ integrin is the cell surface receptor of DMP1 [19]. We found that adding mrDMP1 to osteogenic induction medium could recover/promote the osteogenesis of late passage DFSCs that had reduced osteogenic capability and decreased DMP1 expression. This finding confirms that DMP1 can also act as an extracellular factor for promoting osteogenesis in the DFSCs.

In summary, our study shows that DMP1 is highly expressed in the DFSCs derived from the DF, but not in the non-stem cell fibroblast-like DFCs of the same origin. The expression of DMP1 was not due to presence of other calcium-depositing cells, such as osteoblasts and odontoblasts, in the DFSC population. This high level expression of DMP1 is likely necessary to

maintain the osteogenic differentiation capability of DFSCs. Moreover, our results indicate that DMP1 participates in regulating the expression of osteogenic genes in the DFSCs.

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CHAPTER 4: ACTIVATION OF THE PROLIFERATION AND DIFFERENTIATION OF DENTAL FOLLICLE STEM CELLS (DFSCS) BY HEAT-STRESS*

4.1 INTRODUCTION

Stem cells residing in adult tissues are termed adult stem cells (AdSCs). AdSCs are non-differentiated cells that play important roles for maintaining tissue integrity *in vivo* through both normal tissue renewal and pathological tissue regeneration [1]. Due to therapeutic potential of the AdSCs, extensive research has been attempted to isolate and characterize the AdSCs from various tissues including dental tissues (e.g., dental follicle and dental pulp). Dental stem cells have been shown to be an optimal alternative type of stem cells in reconstructive dentistry and regeneration of craniofacial defects [2]. Studies have shown that dental follicle stem cells (DFSCs) possess typical AdSCs properties of self-renewal, colony formation and multilineage differentiation [3, 4], suggesting that DFSCs would be useful for tissue engineering and regeneration.

It is believed that the majority of AdSCs residing in tissues exists in a slow cycling and quiescent state under normal physiologic conditions [5, 6]. This quiescent property is one of the self-protection mechanisms of AdSCs to prevent the cells from malignant transformation [5, 7]. However, this quiescent state could be awakened by certain factors, such as tissue injury signals released by damaged cells [8, 9]. Once activated, AdSCs can be recruited to sites of injury to repair or regenerate the damaged tissue [10].

Besides slow cycling, another self-protection mechanism is that stem cells exhibit higher stress tolerance than differentiated cells [11]. Recent studies in our laboratory revealed that DFSCs express higher levels of certain heat shock proteins (HSPs) than do their non-stem cell

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counterparts [12]. It is well-known that HSPs can protect cells from stress damage. Thus, high level expression of HSPs would allow DFSCs to endure the stress conditions. We have shown that DFSCs appear to grow more rapidly than their non-stem cell counterparts at a heat-stress condition, suggesting that heat-stress could likely serve as a signal to activate stem cells from the quiescent state [12]. Thus, we proposed that this stress tolerant differential of stem cells and non-stem cells could be explored to develop cell culture conditions for purification and proliferation of stem cells.

Given the above background, our objectives of this study were to answer the following questions: (a) What is the optimal temperature for growth of DFSCs? (b) Can heat-stress serves as a signal to activate/ stimulate proliferation and differentiation of DFSCs? (c) What stem cell-related genes are affected by the heat-stress during proliferation and differentiation? Addressing the questions would help in understanding stem cell biology and developing methods for proliferation and differentiation of DFSCs. The results of the research also have significant implication on application of DFSCs, as well as other AdSCs.

4.2 MATERIALS AND METHODS

4.2.1 Cell cultures

Sprague Dawley rats were bred to produce pups for isolating DFSCs with a protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University (LSU). Dental follicles (DFs) were surgically isolated from the first mandibular molar of rat pups at days 6-7 postnatally. Followed by trypsinization, the primary dental follicle cell suspension was obtained, and then the cell suspension was cultured in stem cell growth medium containing α -MEM (Invitrogen, Grand Island, NY, USA) + 20% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA) + 1% Penicillin-

Streptomycin 10,000 u/ml (Invitrogen, Grand Island, NY, USA) [3] to establish the DFSC culture. To establish non-stem cell dental follicle cells (DFCs), the primary cells were cultured in fibroblast growth medium [13]. DFCs established under such conditions have been shown to contain no stem cells [3]. The cell cultures were maintained at 37°C and 5% CO₂ atmosphere. Stem cell medium was changed every 4 days, and the cells were detached with trypsin and passed into new flasks at 80-90% confluency. DFSCs at passages 3-5 were used in this study.

4.2.2 Cell proliferation assays

The equal numbers of DFSCs (10^3 cells/cm²) were seeded in 6-well plates and cultured in stem cell growth medium. The plates were incubated at 37°C (control), 38°C, 39°C, 40°C and 41°C for 1, 3 and 5 days. Cell proliferation was evaluated by Alamar blue reduction assay [12]. For Alamar blue reduction assay, the culture medium was removed and 1 ml of assay medium containing α -MEM, 10% FBS and 10% Alamar blue (Invitrogen, Grand Island, NY, USA) was added to each well. After 2 hours of incubation, 100 μ l of assay medium was loaded into 96-well plates and the optical density was read at 570 nm and 595 nm. Alamar blue reduction was calculated using the formula provided by the manufacturer (Invitrogen, Grand Island, NY, USA).

To confirm the results of Alamar blue reduction assay, a cell counting-based method was performed to obtain the number of viable dissociated cells as described in the literature [14]. To do that, DFSCs were seeded in T-25 flasks at density of 10^4 cells/flask and cultured for 5 days. Cells then were trypsinized and collected by centrifugation. After re-suspension in 5 ml fresh medium, the number of dissociated cells was counted for each treatment using CellometerTM Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA, USA). The results were reported as the number of dissociated cells/ml.

4.2.3 Osteogenic differentiation

For assessment of the effect of temperatures on osteogenic capability of DFSCs, cells proliferated at 37°C were seeded in 6-well plates at a cell density of 10^4 cells/cm² and incubated in osteogenic medium at different temperatures ranging from 37 to 41°C. The osteogenic medium consisted of DMEM-LG (Invitrogen, Grand Island, NY, USA), 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA), 1% Penicillin-Streptomycin 10,000 u/ml (Invitrogen, Grand Island, NY, USA), 50 µg/ml ascorbate-2 phosphate, 10^{-5} mM dexamethasone and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA). After 7 and 14 days of induction, mineral deposition was evaluated by Alizarin Red staining.

4.2.4 Gene expression study

To explore the effect of the elevated temperatures (i.e., heat-stress) on molecular characterization of DFSCs, the cells were collected after designated times of incubation in various heat-stress temperatures during proliferation and differentiation, respectively. For proliferation, DFSCs were collected after 7 days of incubation in stem cell growth medium. For differentiation, the cells were harvested after 7 and 14 days of osteogenic induction. We also assessed the effect of the heat-stress on expression of marker genes in the non-stem cell DFCs. To do that, the DFCs were incubated in the same heat-stress condition as for DFSCs for 7 days before they were collected for RNA isolation.

Total RNA was extracted with RNeasy® Mini kit (Qiagen, Valencia, CA, USA) from the collected cells. Gene expression of selected stem cell markers and osteogenic markers was determined using real-time RT-PCR with the primers listed in Table 4.1. Briefly, about 2 µg RNA was reverse-transcribed into 20 µl cDNA using random primers and MLV reverse transcriptase (Invitrogen, Grand Island, NY, USA). SYBR green real-time PCR was conducted

Table 4.1 Primer pairs used for real-time RT-PCR in Chapter 4

Gene	Primer sequence	GeneBank reference #
ALP	F: 5'-GACAAGAAGCCCTTCACAGC-3' R: 5'-ACTGGGCCTGGTAGTTGTTG-3'	NM_013059.1
BCRP	F: 5'-GTTTGGACTCAAGCACAGCA-3' R: 5'-AATACCGAGGCTGGTGAATG-3'	NM_181381.2
BGN	F: 5'-AGAATGGGAGCCTGAGTTTTCT-3' R: 5'-ACCTTGGTGATGTTGTTGGAGT-3'	NM_017087.1
BMP2	F: 5'-CTCAGCGAGTTTGAGTTGAGG-3' R: 5'-GGTACAGGTCGAGCATATAGGG-3'	NM_017178.1
BMP3	F: 5'-TACTACAGTCCCTTCCGTCTCC-3' R: 5'-AAACAACCTAGCCACAGACACA-3'	NM_017105.1
BMP6	F: 5'-CTTACAGGAGCATCAGCACAGA-3' R: 5'-GTCACCACCCACAGATTGCTA-3'	NM_013107.1
COL3A1	F: 5'-GAAAGAATGGGGAGACTGGAC-3' R: 5'-TATGCCTTGTAATCCTTGTGGA-3'	NM_032085.1
COL9A1	F: 5'-TGAGAGTTGTGCAAGAGCATTT-3' R: 5'-ATCTGACCAGGGAAACCATTC-3'	NM_001100842.1
C-KIT	F: 5'-ACAAGAGGAGATCCGCAAGA-3' R: 5'-AGCAAATCATCCAGGTCCAG-3'	AF296696.1

ALP: alkaline phosphatase; BCRP: breast cancer resistance protein; BGN: biglycan; BMP2: bone morphogenetic protein 2; BMP3: bone morphogenetic protein 3; BMP6: bone morphogenetic protein 6; COL3A1: Collagen Type III A1; COL9A1: collagen Type IX A1; C-KIT: receptor tyrosine kinases.

(Table 4.1 continued)

Gene	Primer sequence	GeneBank reference #
CTSK	F: 5'-TCTCACATTTCCTTCCTCAACAG-3'	NM_031560.2
	R: 5'-GACTCCAGCGTCTATCAGCAC-3'	
FLT1	F: 5'-ACAGAAGAGGATGAGGGTGTCT-3'	NM_019306.1
	R: 5'-ATCAGCTCCAGGTTTGACTTGT-3'	
ESRRB	F: 5'-GTGCCTGAAGGGGATATCAA-3'	NM_001008516.2
	R: 5'-AGAAACCTGGGATGTGCTTG-3'	
RUNX2	F: 5'-TACTTCGTCAGCGTCCTATCAG-3'	NM_053470.2
	R: 5'-ATCAGCGTCAACACCATCATT-3'	
SPP1	F: 5'-TACTACAGTCCCTTCCGTCTCC-3'	NM_017105.1
	R: 5'-AAACAACCTAGCCACAGACACA-3'	
β -actin	F: 5'-CTAAGGCCAACCGTGAAAAGAT-3'	NM_031144.3
	R: 5'-AGAGGCATACAGGGACAACACA-3'	

CTSK: cathepsin K; ESRRB: estrogen-related receptor beta; FLT1: fms-related tyrosine kinase 1; RUNX2: runt-related transcription factor 2; SPP1: Secreted phosphoprotein 1; β -actin: beta actin.

with 0.5 μ l cDNA for each sample. Relative gene expression (RGE) was calculated with the ΔC_T method using temperature of 37°C as the control.

4.2.5 Alkaline phosphatase (ALP) staining

Cells were seeded in 6-well plates at a cell density of 10^3 cells/cm². After 7 days of incubation at designed temperatures, cells were stained for cell membrane ALP using a Stem TAG™ alkaline phosphatase staining kit (Cell Biolabs, INC, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, cells were fixed with fixing solution provided by the kit and then incubated in staining solution for 20 minutes without exposure to light. The staining

solution was removed and the plates were washed with PBS. ALP staining was observed with an inverted microscope. Cells cultured at normal temperature (37°C) were used as the control.

4.2.6 Statistical analysis

Each of the experiments was repeated a minimum of N=3 times. The treatment effect was compared with analysis of variance (ANOVA) using the SAS version 9.3 (SAS, Cary, NC, USA). The means were separated with least significant difference (LSD) at $P \leq 0.05$.

4.3 RESULTS

4.3.1 Optimal temperature for growth of DFSCs

Alamar blue reduction assay was conducted to monitor cell proliferation. An increase in Alamar blue reduction indicates an increase of cell growth in the culture. Significantly higher Alamar blue reduction was seen when cells were incubated at 38 to 40°C as compared to the 37°C control during 3 days of incubation (Figure 4.1; Section A). In contrast, cells incubated at 41°C gave the lowest Alamar blue reduction, which was lower than cells cultured at 37°C and other heat-stress temperatures (Figure 4.1; Section A).

Alamar blue is a non-toxic dye used for continuously monitoring cell growth in cultures. Its sensitivity reduces once cultures reach certain cell densities. Thus, a more accurate and labor-intensive cell-counting based method was used to obtain the mean number of dissociated cells/ml after 5 days of incubation as shown in Figure 4.1; Section B. The number of dissociated cells was $2.1 \times 10^3/\text{ml}$ when cells were incubated at 37°C (control), whereas a continuous increase in the number of cells was observed when cells were cultured at elevated temperatures with a maximum cell number of $5.45 \times 10^3/\text{ml}$ at 40°C (Figure 4.1; Section B). Cell number increase was statistically significant at 38 to 40°C when compared to the 37°C control. In contrast to that increase, a significant reduction of cell number was seen when cells were cultured at 41°C.

Thus, the results based on cell-counting and Alamar blue reduction assay suggest that the optimal temperature for rapid growth of DFSCs would be in mild heat-stress conditions (38 to 40°C).

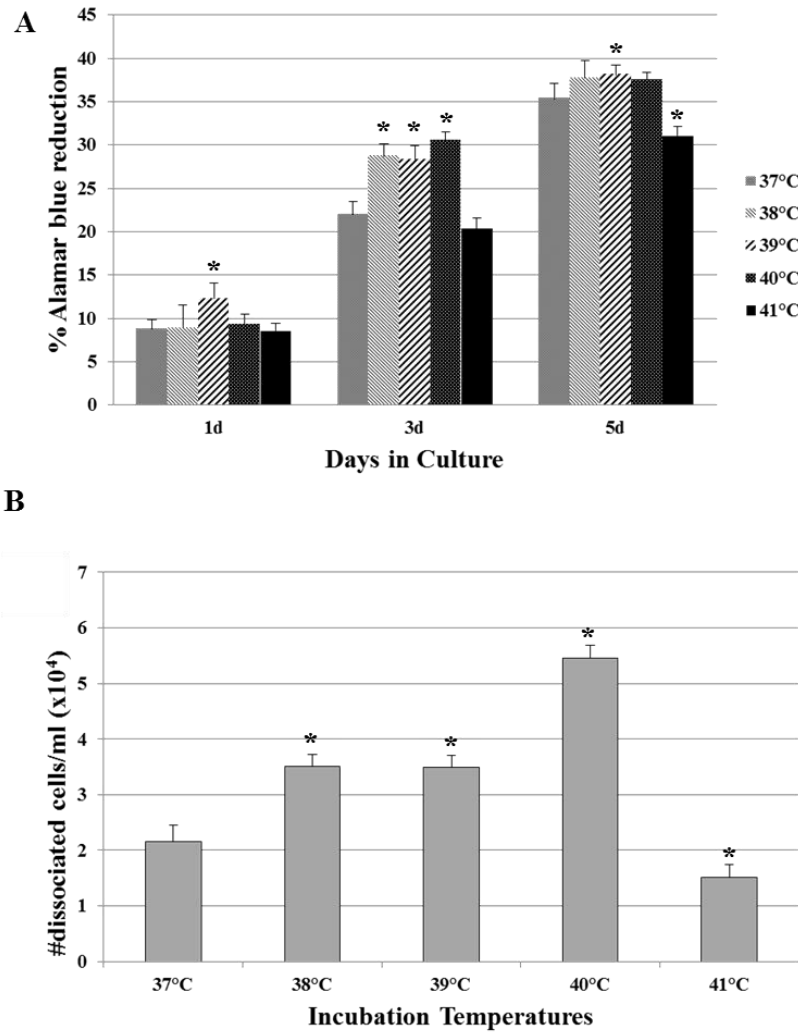


Figure 4.1 Proliferation of DFSCs under different temperatures (37°C-41°C). (A) Proliferation was continuously monitored with Alamar blue reduction assay after 1, 3 and 5 days of incubation. (B) Final cell numbers after 5 days of culture were assessed by cell counting. Note that a significant increase of cell proliferation was observed at the heat-stress temperatures of 38°C-40°C after 3 and 5 days of incubation as compared to the 37°C (control), whereas the cell number after 5 days of incubation was significantly lower at 41°C compared to other temperatures. * indicates significant difference at $P \leq 0.05$ when compared to the control (37°C). DFSCs: dental follicle stem cells.

4.3.2 Differentiation of DFSCs at elevated temperatures

Subjecting the DFSCs to osteogenic differentiation at different temperatures, we observed that osteogenesis, as shown by Alizarin Red staining, could be occasionally detected after as early as 7 days of induction only at 39 and 40°C treatments (Figure 4.2; Section A). After 14 days of induction, osteogenesis occurred at all treatments, and osteogenesis increased coincidentally with the increase of the temperatures from 37 to 40°C with the maximum seen at 39 and 40°C (Figure 4.2; Section B). However, when DFSCs were incubated in 41°C for osteogenic induction, a complete loss of osteogenesis usually was observed, although reduced osteogenesis sometimes was still seen (Figure 4.2; Section C).

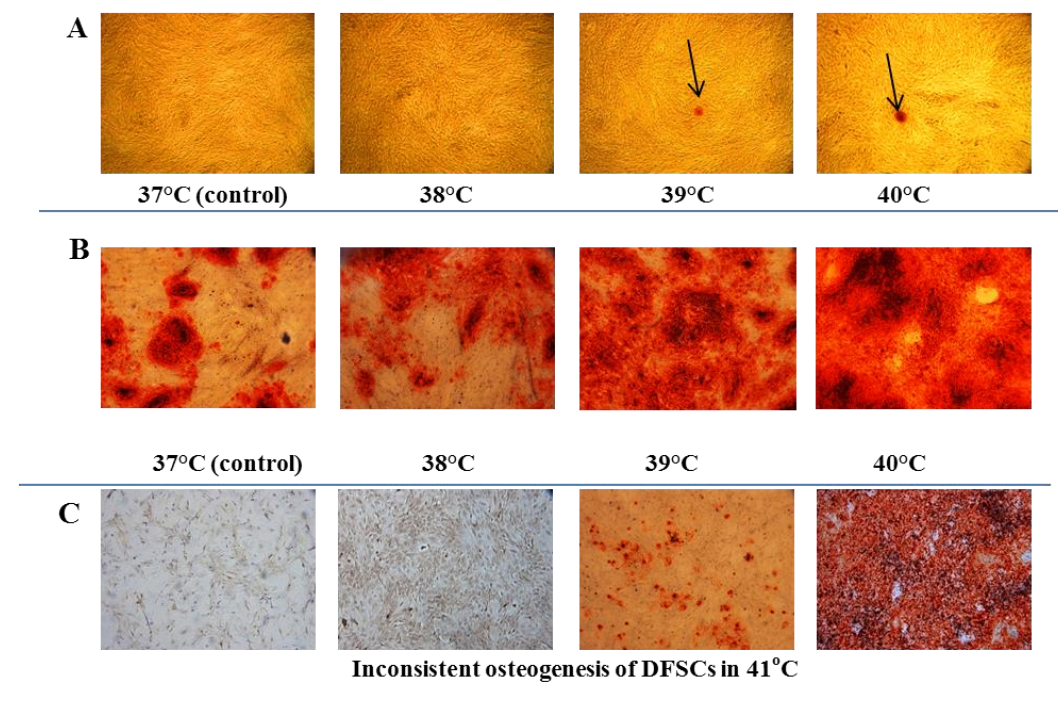


Figure 4.2 Effect of temperatures on induction of osteogenic differentiation of DFSCs as determined by Alizarin Red staining. (A) Mineral deposition was detected after 7 days of induction only from cells incubated at 39 and 40°C (arrows). (B) An increase of staining was seen as temperatures elevating from 37°C-40°C after 14 days of induction. (C) When DFSCs were subjected to osteogenic differentiation at 41°C for 14 days, variability of osteogenesis was observed ranging from loss of osteogenic capability to abnormal osteogenesis. DFSCs: dental follicle stem cells.

4.3.3 Expression of stem cell-related markers

DFSCs were collected after 7 days of incubation in stem cell growth medium at different temperatures for gene expression analysis. RGE of selected stem cell markers was determined by real-time RT-PCR as shown in Figure 4.3; Section A. Increased expression of ALP, BCRP, BMP3, COL9A1, C-KIT and ESRRB was observed when cells were cultured at 38°C-40°C, as compared to the controls grown at 37°C. In general, the increase in gene expression was coincident with the increase of temperatures. Statistical analysis determined that the increase was significant for ALP, BCRP, and COL9A1 at 39 and 40°C as compared to the 37°C control. DFSCs grown in different temperatures were also stained for membrane ALP. The result showed that a great number of cells grown in 38°C-40°C stained high level of membrane ALP (Figure 4.3; Section C), whereas low ALP staining was observed in DFSCs incubated at 41°C.

When DFCs were incubated at elevated temperatures, a slight increase of ALP and BCRP was observed with no statistical significance (Figure 4.3; Section B). The RGE in DFCs was generally lower than in DFSCs, except for BMP3. For example, at 40°C treatment, ALP increased greater than 90% (i.e., RGE >1.9) in DFSCs, whereas in DFCs the increase was only 20%. Decrease of expression of COL9A1 and C-KIT in the DFCs was observed at certain heat-stress conditions, but statistical differences could not be detected. Thus, in general, heat-stress treatments appeared to cause no significant change of expression of these stem cell marker genes in the DFCs. Interestingly, heat-stress at 40°C showed a significant increase of BMP3 expression in the DFCs (Figure 4.3; Section B), but not in DFSCs (Figure 4.3; Section A).

For assessment of the expression of osteogenic markers during osteogenic induction, DFSCs were collected after 7 and 14 days of osteogenic induction at different temperatures (Figure 4.4). Real-time RT-PCR analysis of selected osteogenic markers showed that after 7

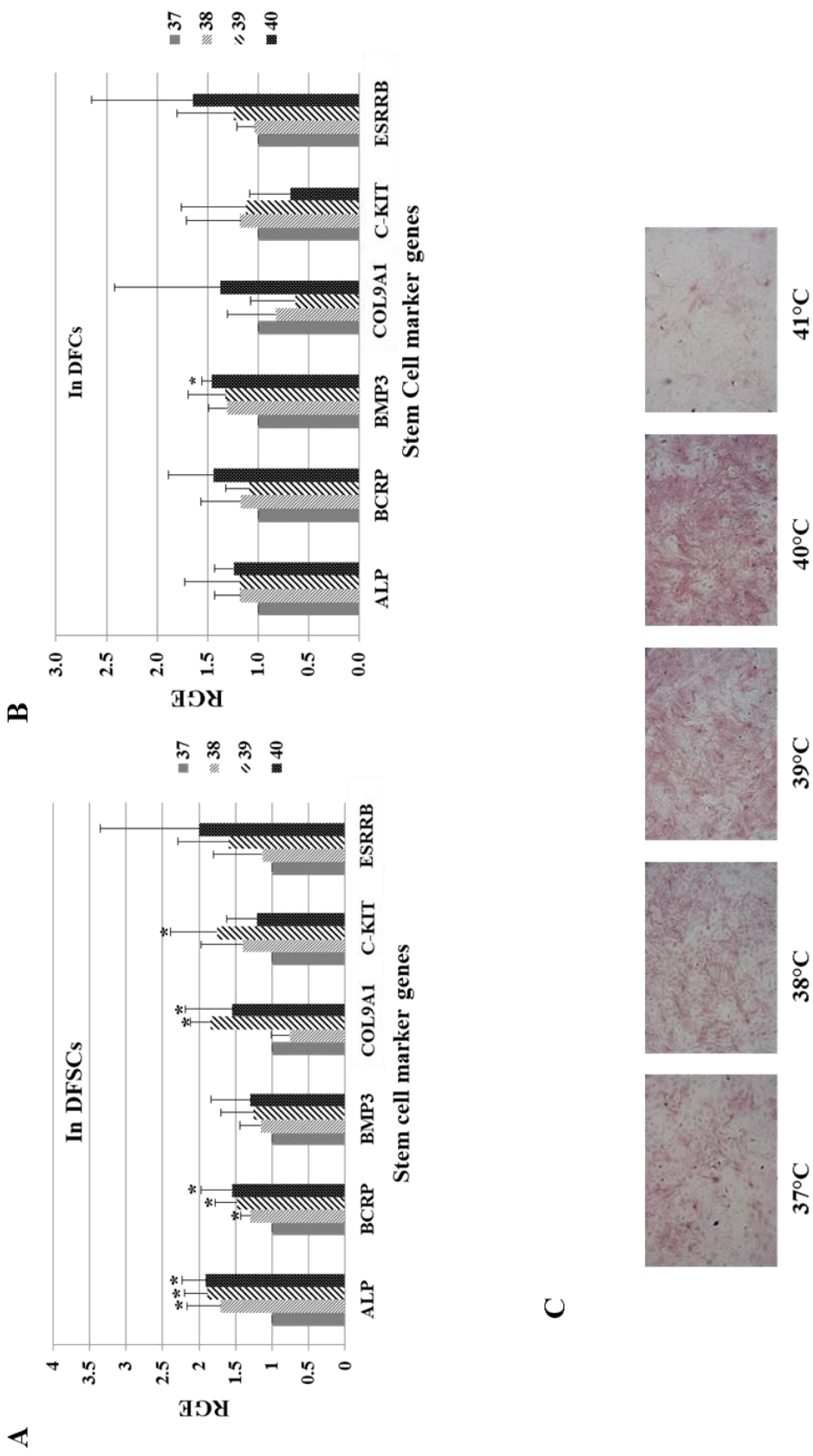


Figure 4.3 Expression of selected stem cell marker genes in the DFSCs and DFCs after 1 week incubation in different temperatures as determined by real-time RT-PCR. RGE was calculated from 4 replicates in the DFSCs (A) and in the DFCs (B). The bar (RGE) labeled with * indicates significant difference at $P\leq0.05$ when compared to the control. (C) Alkaline phosphatase staining of DFSCs demonstrated that the number of cells staining positive for ALP increased at 39°C and 40°C as compared to the 37°C control, whereas a decrease of ALP positive cells was observed at 41°C. ALP: alkaline phosphatase; BCRP: breast cancer resistance protein; BMP3: bone morphogenetic protein 3; COL9A1: collagen Type IX A1; C-KIT: receptor tyrosine kinases; DFCs: dental follicle cells; DFSCs: dental follicle stem cells; ESRRB: estrogen-related receptor beta; RGE: relative gene expression.

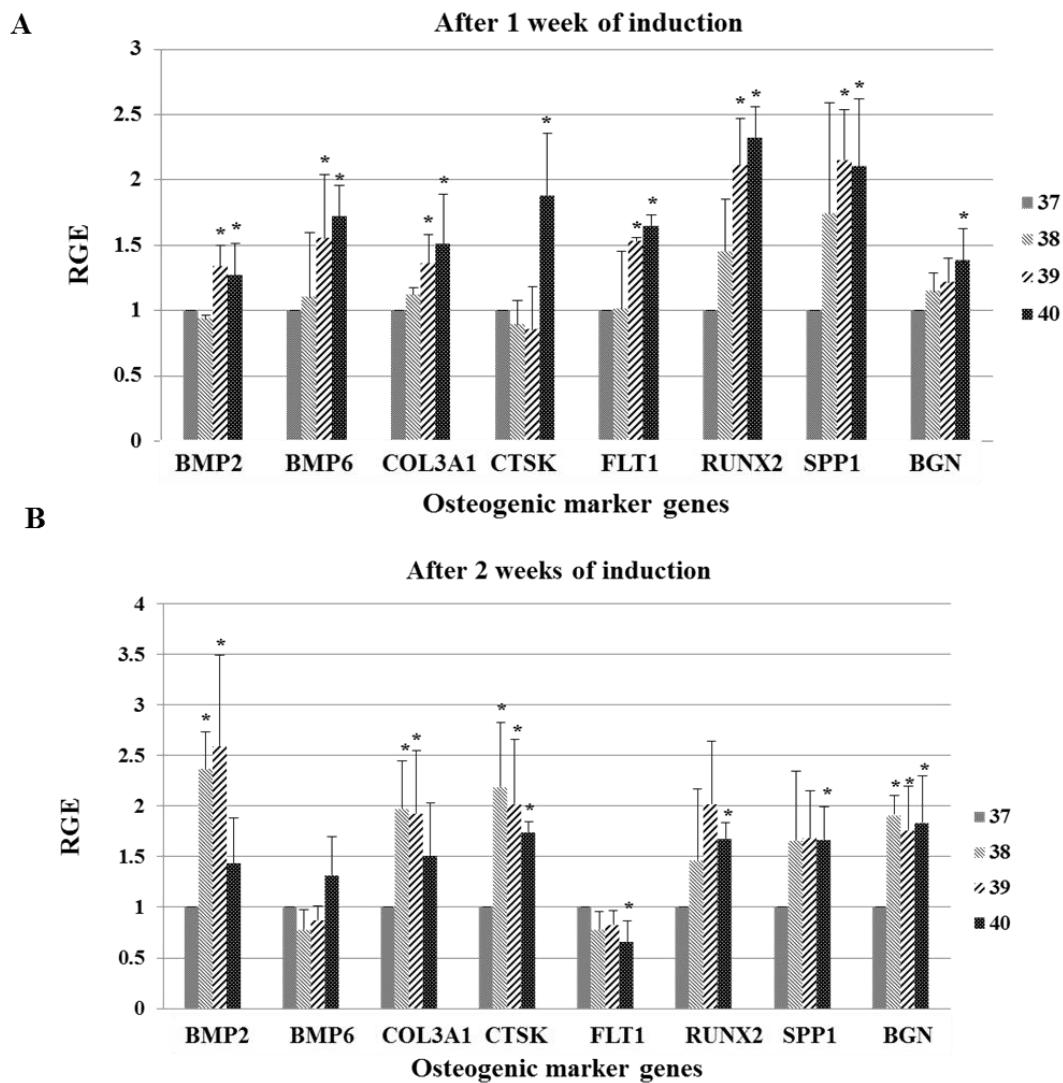


Figure 4.4 Expression of osteoblast marker genes in the DFSCs subjected to osteogenic induction. RGE was determined by real-time RT-PCR after 7 days of induction (A) and after 14 days of induction (B). RGE was calculated from 3 experiments. The bar (RGE) labeled with * indicates significant difference at $P \leq 0.05$ as compared to the control (37 °C). Note that the significantly increased expression of BMP6 and FLT1 was observed in the heat-stress treatments compared to the control after 7 days of osteogenic induction. However, the expression of BMP6 and FLT1 was reduced for heat-stress treatments after 14 days of induction. BMP2 : bone morphogenetic protein 2; BMP6: bone morphogenetic protein 6; BGN: biglycan; COL3A1: Collagen Type III A1; CTSK: cathepsin K; DFSCs: dental follicle stem cells; FLT1: fms-related tyrosine kinase 1; RUNX2: runt-related transcription factor 2; RGE: relative gene expression; SPP1: Secreted phosphoprotein 1.

days of induction, expression of BMP2, COL3A1 and BGN was slightly enhanced at 39°C and 40°C with a maximum of 50% increase as compared to the control. In contrast, more than a 50% increase in expression of BMP6, FLT1, RUNX2 and SPP1 was detected at elevated temperatures versus 37°C. Of them, more than a 2-fold increase of RUNX2 and SPP1 was seen in some treatments (Figure 4.4; Section A). Generally, as the temperature increased from 37 to 40°C, expression of these osteogenic markers also increased with the exception of CTSK. Statistical analysis indicated that increased gene expression was significant at 39 and 40°C when compared to the control (Figure 4.4; Section A).

Expression of the osteogenic markers was also determined after 14 days of induction at different temperatures (Figure 4.4; Section B). Expression of BMP2, COL3A1, CTSK and BGN was still significantly higher in elevated temperature treatments than in the control. However, no significant increase was seen in expression of BMP6 and FLT1 between elevated temperature treatments and the 37°C control. A significant decrease of FLT1 expression was observed after 14 days of induction at 40°C.

4.4 DISCUSSION

DFSCs have been isolated from different species including humans and rats [3, 4], and their potential uses in tissue regeneration have been investigated. Due to the small size and limited source of the tissue, isolation of large quantity of the DFSCs is difficult. Rapid expansion of primary isolation to obtain sufficient quantity of such stem cells with high differentiation potential is usually necessary and desired for application of such stem cells. Evidence from our previous studies has shown that DFSCs are likely more tolerant to heat-stress than their non-stem cell counterparts. We reported that DFSCs grew more rapidly under certain heat-stress conditions than at 37°C [12]. In this study, we determined that the optimal

temperature for *in vitro* proliferation of DFSCs was 39 to 40°C. Studies have suggested that most AdSCs exist in the tissues at quiescent stage [5, 6], and cell injury signals could activate quiescent stem cells to start rapid proliferation and differentiation for tissue repair [8]. For example, in the case of hepatic diseases, various stem cells are activated and recruited to the injury site for liver regeneration [10]. In this study, DFSCs grown in a mild heat-stress condition equivalent to common fever resulted in rapid cell proliferation.

Recent findings suggest that heat-stress could promote neural differentiation of mouse embryonal carcinoma stem cells, and that HSPs appear to regulate this differentiation [15]. To determine if heat-stress treatments can enhance differentiation capability of DFSCs, DFSCs were subjected to osteogenic induction at temperatures ranging from 37°C-41°C. DFSCs from 38°C - 40°C treatments resulted in greater osteogenesis than did the 37°C control, indicating that proper heat-stress treatments could promote differentiation. Our previous study revealed that DFSCs express higher levels of several HSPs than do their non-stem cell counterparts [12]; however, further studies are needed to determine the roles of HSPs in promoting osteogenesis of DFSCs. Taken together, the results suggest that the elevated temperature could likely serve as a signal to activate the tissue stem cells to undergo proliferation and differentiation.

Based on the marker gene expression, the DFSCs had a significantly increased expression of the selected markers including ALP, BCRP, COL9A1 and C-KIT after incubation at elevated temperatures. This could be due to an increase of stem cell numbers in the cell population and/or enhanced expression of the markers by heat-stress treatments. Regarding the former, it should be kept in mind that the DFSCs population used in this study was heterogeneous in that it contained stem cells and non-stem cells. Because stem cells possess greater heat-tolerance and grow more rapidly than non-stem cells in heat-stress conditions, the

number of stem cells in this heterogeneous population could be increased after proliferation in elevated temperatures [12]. Another possibility is that the heat-stress induced the expression of the marker genes. We grew the non-stem cell dental follicle cells (DFCs) in the elevated temperatures and found that heat-stress did not result in a significant change in expression of these genes in the DFCs. Thus, increased expression of the genes should largely come from the stem cells in the population. We speculate that the increased expression of the genes in the DFSCs under elevated temperatures could likely enhance the cell's heat resistant capability, and thus allow the DFSCs to endure the heat-stress condition as discussed in the following.

ALP has been reported as a universal marker for all types of pluripotent stem cells [16, 17]. Undifferentiated stem cells have elevated levels of ALP on their cell membranes and thus membrane ALP staining is used to detect these cells [18]. Enhancement of ALP activity by oxidative and heat-stresses has been observed in intestinal epithelial cells and dental pulp cells, respectively [19, 20]. When rat pulp cells were subjected to 42°C heat-stress for 30 minutes, ALP activity was increased in the cells up to 14 days [20]. Our study indicated that ALP expression in DFSCs also was enhanced by heat-stress. The role of ALP in protecting stem cells from heat-stress is currently unknown.

BCRP is abundantly expressed in various AdSCs [21] to serve as a detoxification mechanism of stem cells [22]. We have shown that DFSCs can express BCRP [3]. It has been suggested that BCRP may play a physiological role in survival of stem cells under a hypoxic environment [23]. The significant increase of BCRP expression seen in heat-stressed DFSCs suggests that BCRP may also play a role in the survival of stem cells under heat-stress conditions.

C-KIT has been found on the surface of hematopoietic stem cells, as well as in mesenchymal stem cells [24, 25]. Binding of stem cell factor (SCF) to C-KIT activates various signal transductions which play roles in proliferation and differentiation. In addition, the SCF/C-kit pathway functions to protect cells from stress-induced cell damage. For example, it has been suggested that SCF/ C-KIT can activate an anti-apoptotic pathway to promote cell survival [24-27]. Because heat-stress can induce apoptosis [28, 29], the increased expression of C-KIT in heat-stressed DFSCs may prevent activation of the apoptotic pathway under elevated temperatures and thus increase cell survival.

When DFSCs were subjected to osteogenic induction at different temperatures, osteogenesis could occur as early as 7 days in cells incubated at 39 or 40°C, as detected by Alizarin Red staining. No osteogenesis could be seen in the 37°C control, suggesting that the osteogenic process of DFSCs was accelerated at the elevated temperature. This was supported by the increased expression of osteogenic genes (BMP2, BMP6, COL3A1, FLT1, RUNX2 and SPP1) seen in the heat-stressed cells as compared to the control cells after 7 days of induction.

After 14 days of induction, real-time RT-PCR showed that expression of the osteogenic markers (except BMP6 and FLT1) in the elevated temperature treatments remained at a higher level than in the control, whereas RGE of BMP6 and FLT1 was greatly reduced in elevated temperature treatments. Given that BMP6 is the earliest of the BMPs to be expressed during osteogenic differentiation [30], its high level of expression at day 7 followed by leveling off at day 14 seen in this study suggests that BMP6 is likely important for osteogenic differentiation of DFSCs at the initiation/early stage.

FLT1 (also known as vascular endothelial growth factor receptor1--VEGFR1) and FLK1 (VEGFR2) are the two receptors that bind to VEGF for various biological effects, such as in

angiogenesis and hematopoiesis. In developing mice, metaphyseal blood vessels and trabecular bone formation were impaired when some VEGF activity was blocked [31]. When exogenous VEGF was applied to mouse femur fractures, blood vessel formation, ossification and new bone (callus) maturation were enhanced. Moreover, inhibition of VEGF dramatically slowed down the healing of a tibial cortical bone defect [32]. Thus, VEGF signaling plays important roles not only for angiogenesis, but also for osteogenesis. In that vein, a recent study showed that inhibiting both FLT1 and FLK1 activity resulted in decrease of ALP activity during osteoblastic differentiation of cultured human periosteal-derived cells. However, inhibiting FLK1 activity did not alter the ALP activity [33], suggesting that Flt1 would be the receptor for VEGF signaling in osteogenesis. In the present study, we observed that FLT1 expression and osteogenesis increased at a concurrent manner in the differentiating DFSCs at the initiation stage, and its expression was decreased in the DFSCs once differentiation was complete. This result provides additional evidence that VEGF/FLT1 could be the signal pathway for osteogenic differentiation.

In conclusion, this study has demonstrated that elevated temperature could serve as a signal to activate stem cells (e.g. DFSCs) from quiescence to undergo proliferation and differentiation. The results are significant in application of stem cells. From a clinical perspective, the results could help to design treatment guidelines in stem cell therapy. For example, prior to transplantation, the stem cells could be incubated at certain elevated temperatures to boost their osteogenic capability. A heat pad may be applied to the injury site after a stem cell transplant to promote proliferation and differentiation of the transplanted stem cells, and in turn to accelerate the healing process. However, proper heat (e.g., 39°C) and time must be carefully considered to avoid thermal damage to tissues when a heat pad is used.

The results of this study indicated that culturing DFSCs under mild heat-stress could effectively promote proliferation and osteogenic differentiation. The optimal temperatures to grow and differentiate DFSCs appeared to be 39 to 40°C. This finding might be used to develop a cell culture condition for rapid *in vitro* expansion of DFSCs for therapeutic applications.

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CHAPTER 5: EVALUATION OF OSTEOGENIC POTENTIAL OF DENTAL FOLLICLE STEM CELLS (DFSCS) FOR TREATMENT OF CRANIOFACIAL DEFECTS

5.1 INTRODUCTION

Various factors such as trauma, infection, tumor, and congenital deformities can cause craniofacial defects [1]. Conventional approaches for treatment of craniofacial bone defects usually require the use of autograft or alloplastic materials. Both, however, have disadvantages that limit their applications in clinics. For autograft, obtaining autogenous bone requires extra surgical procedures with associated co-morbidities [2, 3]. Although alloplastic materials can be used for the treatment of such defects with no need of extra surgery, immune rejection and infection can occur, resulting in treatment failure. The concept of harvesting adult stem cells (AdSCs), followed by expansion and transplantation for tissue regeneration, has been proposed. In that vein, utilization of stem cells for treatment of craniofacial defects has been attempted [4-8].

Stem cells have been isolated from various tissues. Invasive surgical procedures are usually needed to obtain tissues for stem cell isolation. Since impacted teeth are often extracted in dental clinics and discarded, the use of extracted teeth for stem cell isolation does not require extra surgery. Dental follicle (DF), a loose ectomesenchymal tissue surrounding unerupted teeth, has been shown to contain progenitor/stem cells. Thus, dental follicle stem cells (DFSCs) have been isolated and tested for stem cell properties [9-11]. DFSCs possess strong osteogenic capability to differentiate toward the osteoblast lineage [12, Chapter 2], which makes them suitable for repair of skeletal defects. In addition, since the DF is derived from the neural crest, the same origin of craniofacial tissues, it may be preferential to utilize DFSCs for treatment of defects in this region.

Previous studies have attempted to evaluate the hard-tissue forming potential of DFSCs *in vivo*. Subcutaneous transplantation of bovine [13] and human DFSCs [14] mixed with hydroxyapatite (HA) ceramics showed formation of mineralized structure on the border of the HA. Two other studies have investigated DFSCs for bone regeneration in critical-size defects created in the calvarium of immune-deficient rats. The first study demonstrated the differentiation potential of porcine DFSCs into osteogenic lineage cells [15]. Similar results were observed from an independent study showing the *in vivo* bone formation potential of human DFSCs [16]. Both studies were done by transplanting DFSC pellets without loading cells into scaffolds. However, scaffolds are important components for tissue engineering because they mimic the extracellular matrix and provide a three-dimensional structure for cell attachment and vascularization [17]. In particular scaffolds are required for regeneration of large-size defects. In attempts to utilize AdSCs for regeneration of skeletal defects, both undifferentiated [4, 5] and osteo-induced stem cells [18] have been used. Hence, it would be beneficial to compare bone regeneration capability between undifferentiated and osteoinduced stem cells.

In the current study, we evaluated bone regeneration potential of DFSCs in rat calvarial critical-size defects using immunocompetent rats. DFSCs isolated from littermates were seeded into polycaprolactone (PCL) or polycaprolactone plus hydroxyapatite (PCL-HA). Prior to transplantation, we examined DFSC viability and attachment in interaction with selected scaffolds *in vitro*. Next, bone regeneration potential of both undifferentiated and osteo-induced rat DFSCs was examined in a rat calvarial model.

5.2 MATERIALS AND METHODS

5.2.1 Animals

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University (LSU). Immunocompetent Sprague Dawley (SD) rats were bred in the vivarium of the Department of Laboratory Medicine, School of Veterinary Medicine, LSU to produce postnatal pups. For each litter, female rat pups at postnatal day 6 were used for the isolation of DFs, and male pups were kept with their mothers until they were weaned at 5 weeks old. Then each of the 3 males was caged separately until 5 months old for surgical and stem cell transplantation procedures.

5.2.2 Establishment of DFSC cultures

DFSCs were established as described in Chapter 2 (Section 2.2.1). Four different DFSC cultures were established from four different litters. They were propagated until passage 3 (P3). Their osteogenic capability was evaluated by induction for differentiation, and calcium deposition was determined using Alizarin Red staining as described in Section 2.2.2. Then cells were transferred to liquid nitrogen for long-term storage. Cells were recovered from cryopreservation when the littermates of the donors reached the designated age for transplantation.

5.2.3 Scaffold preparation and cell seeding

PCL (100% polycaprolactone) and PCL-HA (75% polycaprolactone and 25% hydroxyapatite) were prepared by dissolving in dioxane (Sigma-Aldrich, St. Louis, MO, USA). Briefly, two 10 ml solutions were prepared by adding 0.8 g of PCL (100% PCL) or 0.6 g PCL and 0.2 g HA (75% PCL: 25% HA) in a glass vial. The solutions were heated to 120°C and mixed until the polymer dissolved. Next the solutions were poured into polydimethylsiloxane

(PDMS) templates and immediately incubated at -20°C for 24 hours, followed by freeze-drying at -80°C for 48 hours. The scaffolds had a pore size of 200-300 µm. Two different sizes of scaffolds were used in this study. The scaffolds with a diameter of 10 mm and thickness of 5 mm were used for *in vitro* experiments, and the scaffolds with a diameter of 5 mm and thickness of 0.5 mm were used for *in vivo* transplantation. Prior to cell seeding, the scaffolds were sterilized with ethylene oxide.

A total of 10^5 cells were seeded into each scaffold using the following procedures: Cells were suspended in 100 µl stem cell growth medium. Half of the cells were pipetted into the top surface of the scaffold. After 20 minutes incubation, the scaffolds were flipped, and the rest of the cells were pipetted into the other surface of the scaffold followed by incubation for another 20 minutes. This incubation period allowed the cells to penetrate into the porous scaffolds. Cell-scaffold constructs were then incubated in either stem cell growth medium or osteogenic induction medium for the designated time according to different experimental purposes.

5.2.4 Evaluation of cell attachment loaded into scaffolds

Cell-scaffold constructs were placed in 48-well plates and incubated in 2 ml stem cell growth medium consisting of α -MEM (Invitrogen, Grand Island, NY, USA) + 20% FBS (Atlanta Biologicals, Flowery Branch, GA, USA) at 37°C and 5% CO₂. After 3 days of incubation, the constructs were sectioned and fixed with 2% paraformaldehyde and 1.25% glutaraldehyde in a 0.1 M sodium carbonate (CAC) buffer, pH 7.4 for 1 hour. Next they were post-fixed in 1% osmium tetroxide in 0.1 M CAC buffer for another 1 hour, followed by dehydrating through an ethanol series (30%-100%) and drying in a CO₂ critical point dryer. The samples were then coated with gold and subjected to scanning electron microscopy (SEM, FEI Quanta 200 ESEM,

Hillsboro, Oregon, USA) for examination of cell attachment. Scaffolds without cells but cultured and processed under the same conditions were also examined by SEM as controls.

5.2.5 Assessment of cell viability loaded into scaffolds

Cell–scaffold constructs were placed in 48-well plates and incubated in 2 ml stem cell growth medium. PCL and PCL-HA scaffolds without DFSCs were incubated in stem cell growth medium and served as negative controls. Cell viability was evaluated after 1 and 3 days of incubation using Alamar blue reduction assay [19]. Briefly, the culture medium was removed and replaced with 1 ml of assay medium consisting of α -MEM, 10% FBS and 10% Alamar blue (Invitrogen, Grand Island, NY, USA). After 2 hours of incubation, 100 μ l of assay medium was loaded into 96-well plates and the optical density was read at 570 nm and 595 nm. The Alamar blue reduction was calculated using manufacturer's protocol.

5.2.6 *In vivo* transplantation of DFSCs

SD male rats at an age of 5 months with an average body weight of 415 g were used for *in vivo* DFSC transplantation study. Surgical procedures are illustrated in Figure 5.1. Prior to surgery, the animals were anaesthetized using isoflurane inhalation (Vet one, Boise, ID, USA). Animals were placed on a hot water blanket during the operation to prevent hypothermia. Skin around the incision area on parietal bone was shaved after disinfection with Povidone-iodine 5% (Purdue Pharma, Stamford, CT, USA). An incision was made with a surgical blade from the nasofrontal area to the anterior area of the occipital protuberance. Then two 5mm-diameter full thickness defects were created around the sagittal suture with a trephine bur (XEMAX, Napa, CA, USA) and a low speed dental drill under constant irrigation with sterile saline. The operation was done carefully to avoid injuring the dura mater or underlying blood vessels and sinus. The cell–scaffold constructs were rinsed 3 times with sterile PBS buffer to remove the

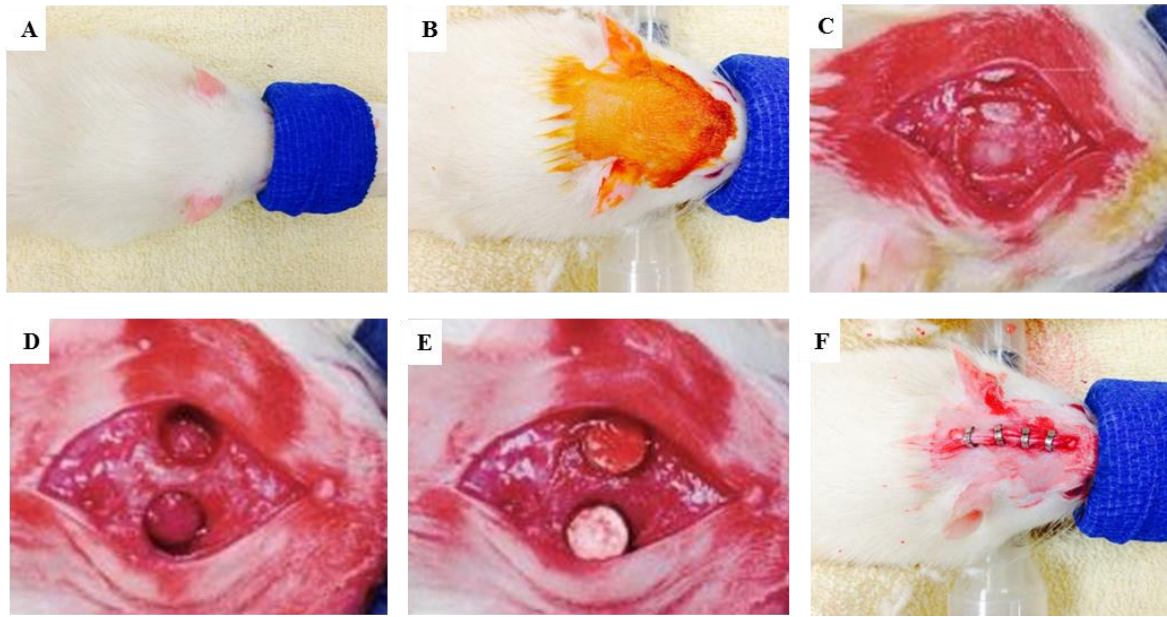


Figure 5.1 Surgical procedures for transplantation of DFSCs to treat critical-size defects on rat calvarial bone. (A) Experimental rat was anaesthetized using isoflurane inhalation. (B) Skin in the incision area was shaved and disinfected with iodopovidone solution. (C) A midline incision was created from the nasofrontal area to the anterior area of the occipital protuberance. (D) Two 5mm full thickness defects were created using trephine bur. (E) Implants were placed into the defects. (F) The scalp was closed using Michael clips.

medium and then inserted into the defects. In each litter (cohort) experimental animals were randomly assigned to the treatment groups (Table 5.1). Two types of DFSCs have been used for transplantation (Table 5.1). DFSCs refer to undifferentiated DFSCs, and iDFSCs refer to the DFSCs subjected to osteo-induction for 7 days prior to transplantation. After transplantation, the scalp was closed using Michael clips. Following the surgery, the animals were placed in a warm and soft-bedded plastic cage for recovery. An injection of buprenorphine (Reckitt Benckiser Healthcare Ltd, Hull, UK) 0.05 mg/kg was administrated every 12 hours for 3 days post-operation as an analgesia. Endroflaxacin (Bayer, Shawnee Mission, Kansas, USA) was added to their drinking water to prevent infection for 7 days. In each cohort, half of the animals (4 rats) were sacrificed after 4 weeks post-operatively, and the other half were sacrificed after 8 weeks. To collect the samples, the animals were euthanized using CO₂ asphyxiation followed by

cervical dislocation or decapitation. Skulls were harvested surgically, and skins were removed completely from the skulls. The skulls were fixed in 10% Neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for further analysis or examination to determine the treatment effects.

Table 5.1 Treatments for *in vivo* transplantation

Treatment Groups*	Description
Empty	For spontaneous healing as the negative control
PCL only PCL-HA only	Scaffolds without cells were incubated for 3 days in stem cell growth medium.
PCL + DFSCs PCL-HA + DFSCs	The cell–scaffold constructs were incubated in stem cell growth medium for 3 days prior to transplantation.
PCL + iDFSCs PCL-HA + iDFSCs	The cell–scaffold constructs were incubated in stem cell growth medium for 3 days followed by 7 days induction in osteogenic medium prior to transplantation.

DFSCs: dental follicle stem cells; iDFSCs: osteo-induced dental follicle stem cells; PCL: polycaprolactone; PCL-HA: polycaprolactone plus hydroxyapatite.

*In each cohort (N=4), two animals were used for each treatment group. One animal was sacrificed at 4 weeks post-transplantation, and the other was sacrificed after 8 weeks.

5.2.7 Evaluation of bone regeneration

The assessment of bone regeneration was done with micro-CT and histological analysis. First, harvested skulls were assessed for new bone formation using a micro-CT imaging system (Skyscan 1074, Micro photonics, Inc., Allentown, PA, USA) at the Department of Physics and Astronomy, LSU. Samples were mounted on a rotating stage. The following parameters were used for examination of all samples: X-ray voltage of 40 kVp, current of 1000 μ A, and exposure time of 550 milliseconds for each of the 360 rotational steps. From the micro-CT data, two-dimensional images were reconstructed using NRecon, version 1.6.9.4 (Bruker microCT,

Kontich, Belgium). Next, three-dimensional images were made using CTvox, version 2.6.0 (Bruker microCT, Kontich, Belgium). Following image intensity modification, quantitative analysis of new bone formation was performed using Matlab 2013a software (MathWorks, Natick, MA, USA). The percentage of newly regenerated bone was calculated from the fraction of the number of voxels of new bone over the number of voxels of the entire defect. Procedures for micro-CT data analysis are illustrated in Figure 5.2.

Following micro-CT scanning, skulls were trimmed using bone cutting forceps and transferred to fixation solution (10% Neutral-buffered formalin) for 7 days. Samples were then decalcified with Cal-Ex™ Decalcifier (Fisher Scientific, Pittsburgh, PA, USA) for 14 days. Each defect sample was embedded with paraffin. Five μm serial sections were prepared parallel to the sagittal plane. Sections were mounted on Sperfrost plus® slides (Fisher Scientific, Pittsburgh, PA, USA). They were then deparaffinized in xylene and rehydrated in 95 and 75% ethanol. Next, the slides with odd numbers were stained with Hematoxylin and Eosin (H & E) while even-number slides were stained with Masson Trichrome. For H & E staining, slides were stained in Hematoxylin solution (0.5% in distilled water) for 20 minutes followed by 1 minute of staining in Eosin (0.25% in 80% ethanol). For Masson Trichrome staining, sections were first stained in Weigert's iron Hematoxylin working solution for 10 minutes, and then stained in Biebrich Scarlet-Acid Fuchsin for 2 minutes and Aniline Blue Solution for another 2 minutes. After staining, sections were covered with mounting medium and cover slipped. Digital images from stained sections were taken with an optical microscope (Olympus BX48, Center Valley, PA, USA).

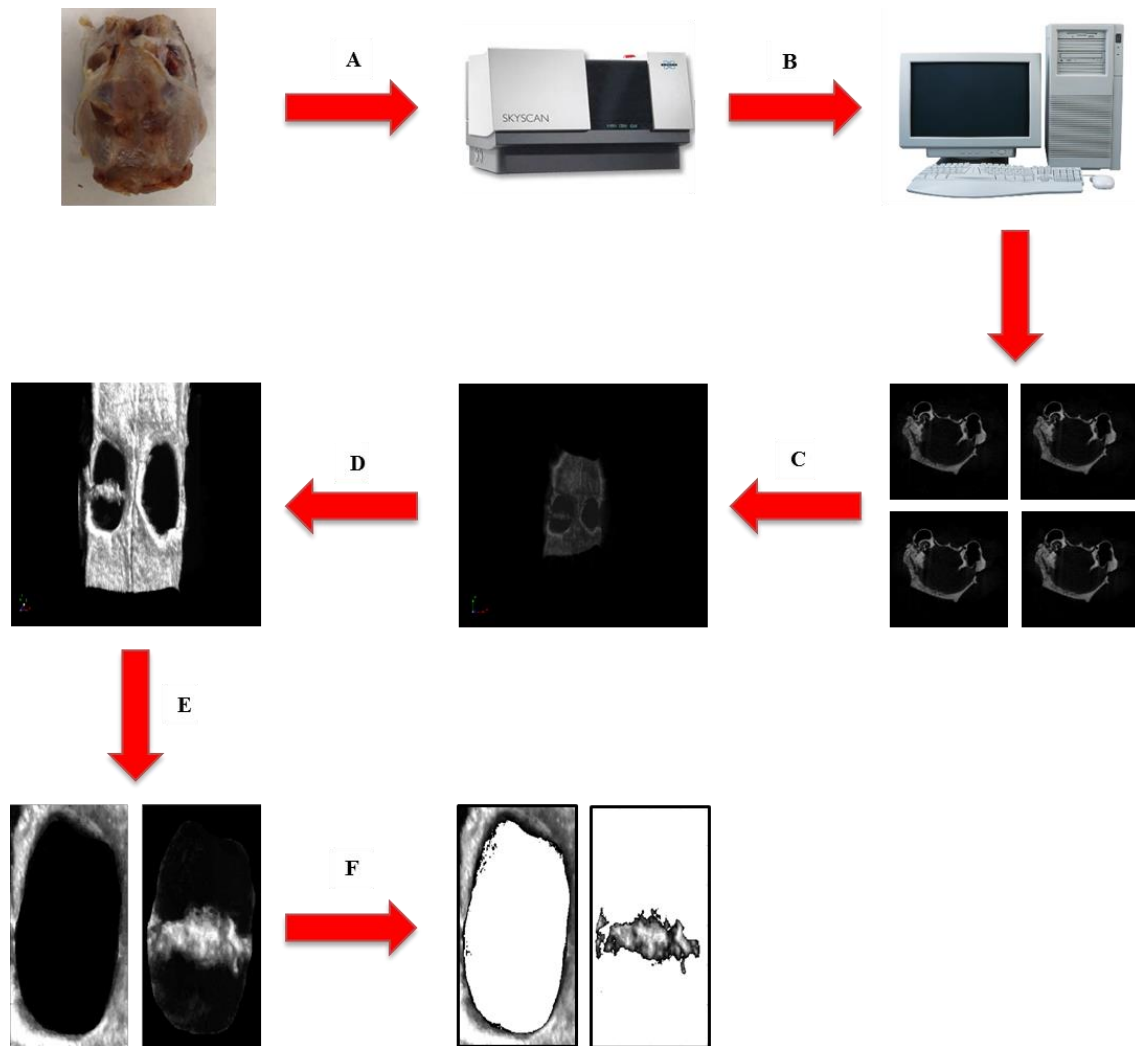


Figure 5.2 Schematic illustrations of the procedures for micro-CT analysis of bone regeneration after transplantation of DFSCs to the rat calvarial defects. (A) Micro-CT scanning of rat skull by Skyscan 1074. (B) Construction of 2D slices by NRecon software. (C) Construction of a 3D image by CTvox software. (D) Image intensity modification. (E) Bone and defect segmentation. (F) Quantitative analysis of new bone formation using Matlab 2013a. 2D: two-dimensional; 3D: three-dimensional; CT: computed tomography.

5.2.8 Statistical analysis

Statistical analyses were performed using SAS program version 9.3 (SAS, Cary, NC, USA). Analysis of variance (ANOVA) and least significant difference (LSD) were used for multiple comparisons of the treatment effects. Means were separated by LSD with $P \leq 0.05$ being statistically significant.

5.3 RESULTS

5.3.1 Evaluation of osteogenic capability of established DFSCs

Four different DFSC cultures established from 4 different litters were used in this study. The osteogenic capability of DFSC cultures was verified at P3 by *in vitro* osteogenic induction for 14 days. Following induction, calcium deposition was visualized using Alizarin Red staining. Robust staining was observed in all cultures, indicating that they possessed strong osteogenic differentiation capability (Figure 5.3) and could be used for *in vivo* transplantation.

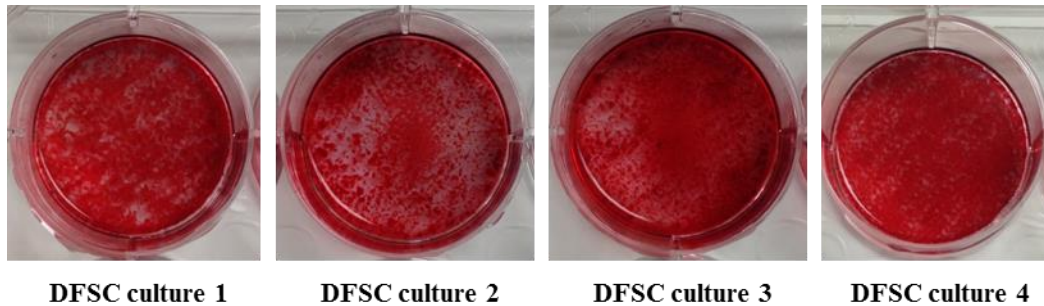


Figure 5.3 *In vitro* evaluation of osteogenesis of DFSC cultures. Note that all four DFSC cultures established from different litters possessed strong osteogenic capability at passage 3 after 2 weeks of induction as shown by Alizarin Red staining. DFSC: dental follicle stem cell.

5.3.2 Evaluation of DFSC attachment loaded into PCL and PCL-HA scaffolds

DFSCs, as well as other mesenchymal stem cells, are adherent cells. Therefore, cell attachment to the culture surface is required before they can grow and differentiate. We examined cell attachment by SEM at day 3 after loading DFSCs into scaffolds. SEM micrographs showing cell attachment at different magnifications (500x-3000x) are presented in Figure 5.4. Attachment and infiltration of DFSCs in the porous structure of both scaffolds (Figure 5.4; Sections D-F and J-L) were observed, as compared to the control scaffolds without cells (Figure 5.4; Sections A-C and J-I).

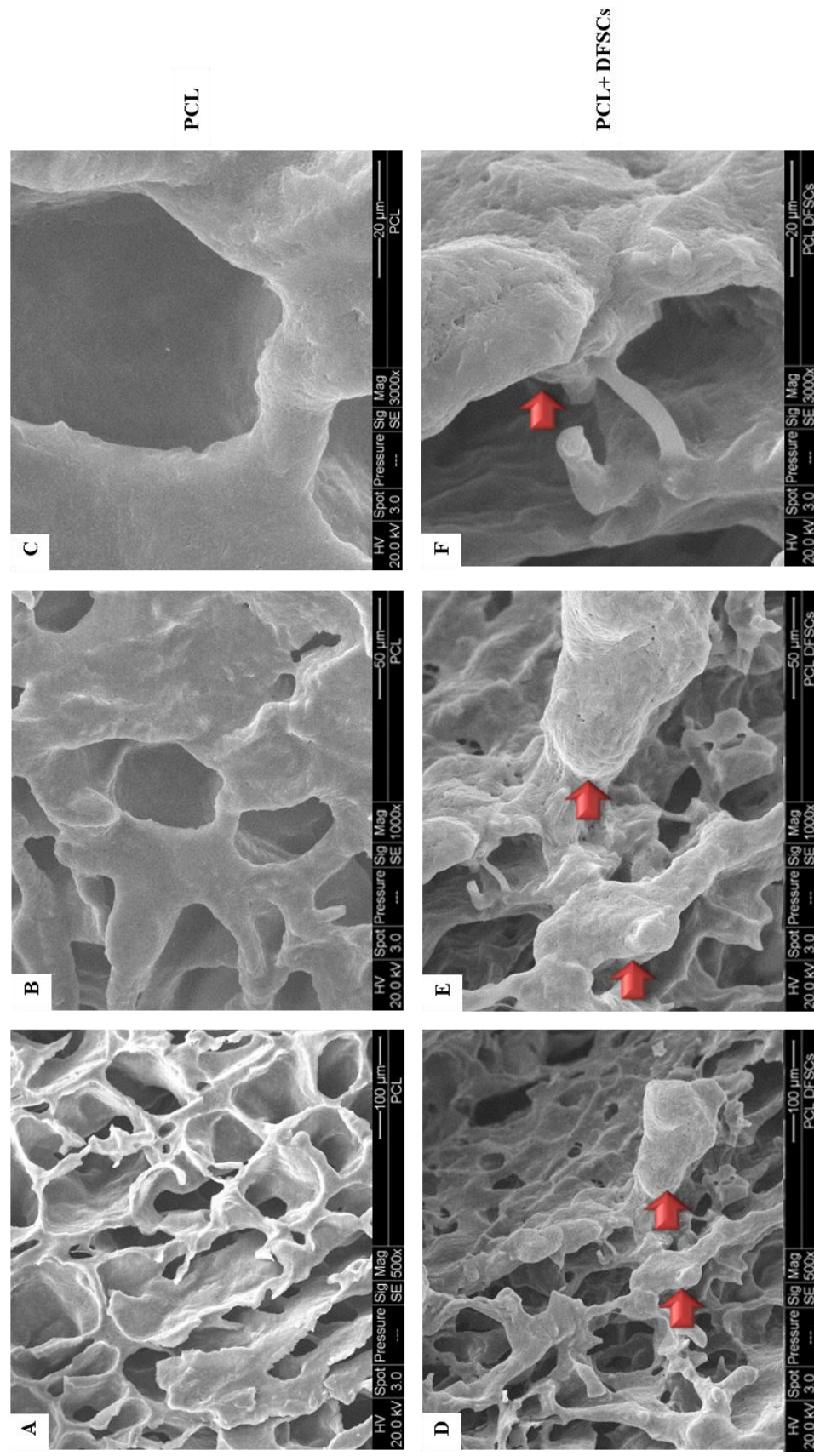
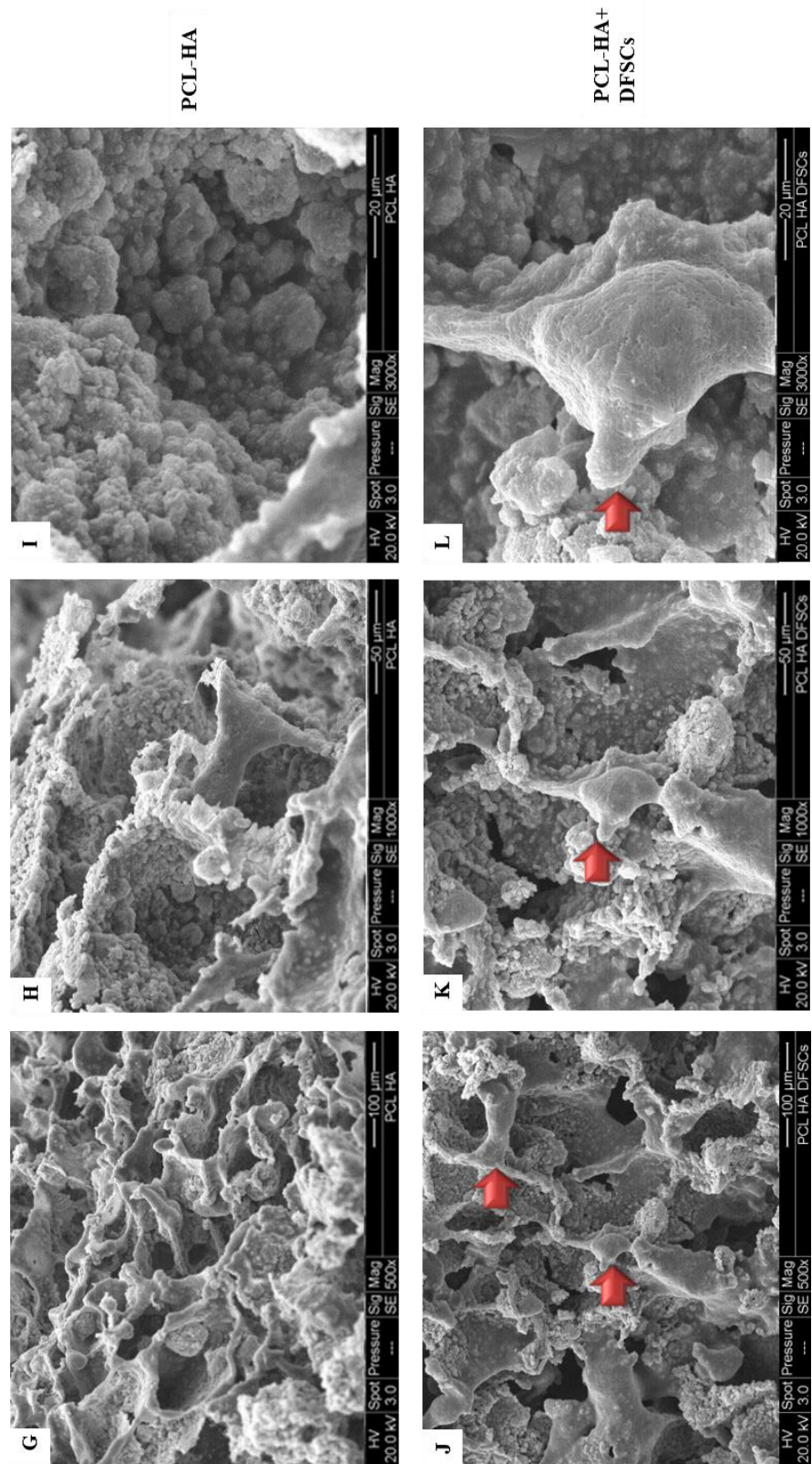


Figure 5.4 Evaluation of DFSC attachment on PCL and PCL-HA scaffolds by SEM. (A-C) Microphotographs of PCL only control. (D-F) Microphotographs showing attachment of DFSCs on PCL scaffold (red arrows). (G-I) microphotographs of PCL-HA only control. (J-L) microphotographs showing attachment of DFSCs on PCL-HA scaffold (red arrows). Note that the porous structures of PCL and PCL-HA provide three-dimensional structure for proliferation and attachment of DFSCs. DFSCs: dental follicle stem cells; PCL: polycaprolactone; PCL-HA: polycaprolactone plus hydroxyapatite; SEM: scanning electron microscope.

(Figure 5.4 continued)



5.3.3 Assessment of DFSC viability/proliferation loaded into PCL and PCL-HA scaffolds

Since induction of differentiation is generally associated with growth arrest, we evaluated the cell growth on the scaffold prior to osteogenic induction. In this regard, the cell viability assay was performed during the culture in stem cell growth medium. Alamar blue reduction assay was conducted at days 1 and 3 after cell seeding into PCL and PCL-HA scaffolds. Scaffolds without stem cells served as negative control. Significant increase of Alamar blue reduction was seen at day 3 as compared to day 1 in both scaffolds, indicating that DFSCs could survive and proliferate in the scaffolds (Figure 5.5). There was no statistical difference between PCL and PCL-HA in Alamar blue reduction assay within given days, suggesting that DFSCs proliferate in a similar manner in both scaffolds.

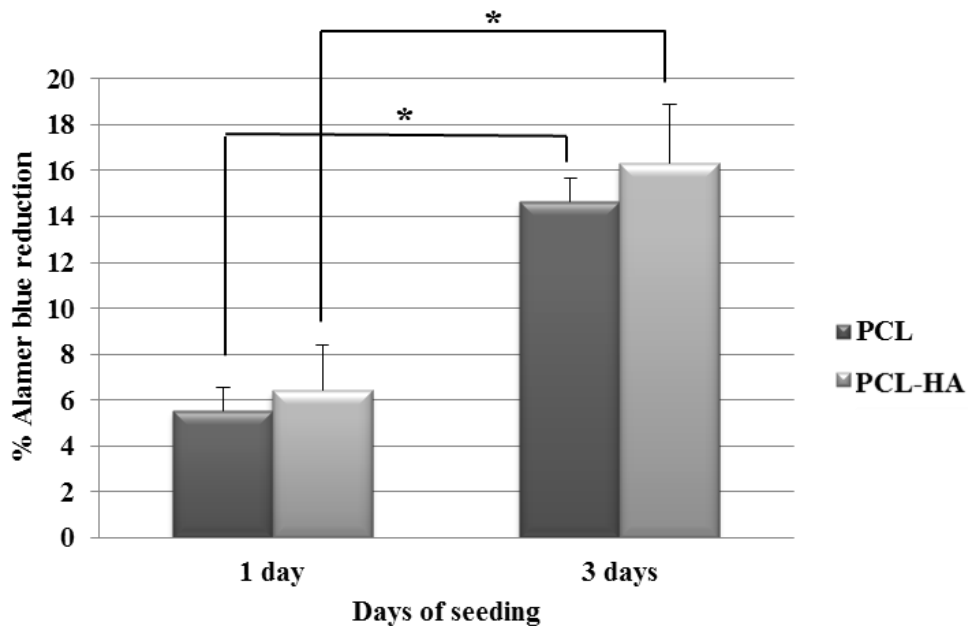


Figure 5.5 Assessment of DFSC viability loaded into PCL and PCL-HA with Alamar blue assay. Note that a significant increase of Alamar blue reduction (Mean \pm SE) was observed at day 3 compared to day 1. For a given day, no difference was seen between PCL and PCL-HA. * indicates significant difference at $P \leq 0.05$ (N=4). DFSCs: dental follicle stem cells; PCL: polycaprolactone; PCL-HA: polycaprolactone plus hydroxyapatite.

5.3.4 Micro-CT analysis of bone regeneration after transplantation of DFSCs

To assess bone regeneration, micro-CT scans were performed at weeks 4 and 8 post-transplantation. A representative case from each treatment group is shown in Figure 5.6 (N=4). At week 4 post-surgery, micro-CT scanning revealed a complete lack of bone regeneration among the defects in all treatment groups (Figure 5.6; Sections A-G). Scans at week 8 showed no bone formation in the negative control (empty defect without cells) (Figure 5.6; Section H). Similarly, defects treated with scaffolds only (PCL or PCL-HA) showed no bone regeneration (Figure 5.6; Sections I and J). In comparison, small areas of bone formation were seen in defects treated with either PCL-HA plus DFSCs (Figure 5.6; Section M), or PCL-HA plus iDFSCs (Figure 5.6; Section N). However, in the defects treated with PCL plus DFSCs (Figure 5.6; Section K) or PCL plus iDFSCs (Figure 5.6; Section L), new bone formation filled almost half of the defects.

Quantitative bone formation was acquired by micro-CT images. Defects treated with PCL plus DFSCs or PCL plus iDFSCs showed a significant increase in the percentage of bone healing with $48.9\% \pm 8.7$ and $51.33\% \pm 2.9$ bone regeneration, respectively (Table 5.2) as compared to the control (0.00%). The osteogenic induced DFSCs (iDFSCs) gave slightly higher bone regeneration than did the DFSCs without induction; however, no significant difference was observed (Table 5.2). Defects treated with PCL-HA plus DFSCs or PCL-HA plus iDFSCs resulted in approximately 5% and 3% bone recovery, respectively, over the 8 weeks, which was significantly lower than the PCL group (Table 5.2).

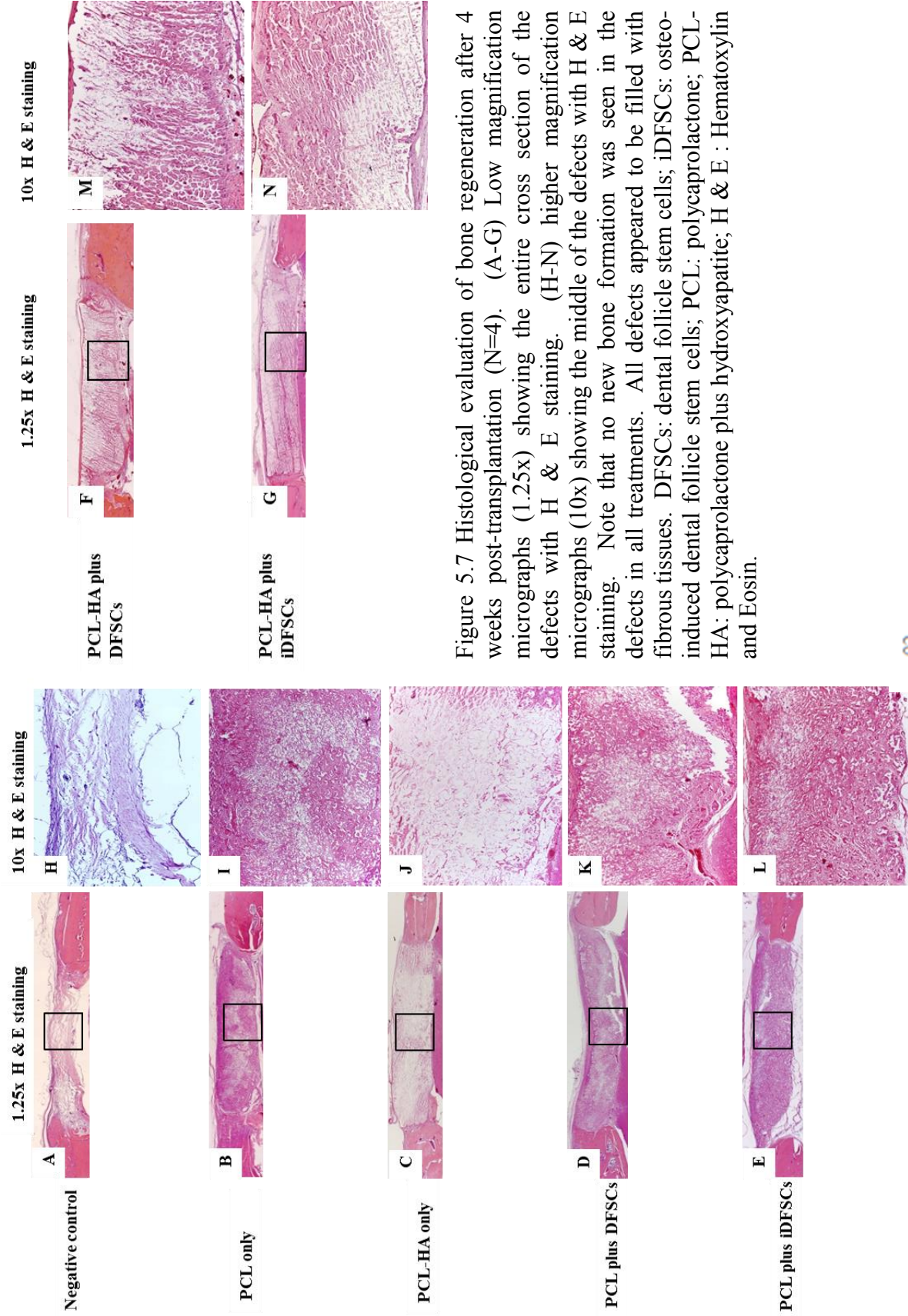
Table 5.2 Percentages of new bone formation in different treatment groups at week 8 post-transplantation as determined by micro-CT (N=4)

Treatment Groups	New Bone Formation (%)
Empty	0.00 \pm 0.00
PCL only	0.00 \pm 0.00
PCL-HA only	0.00 \pm 0.00
PCL + DFSCs	48.9 \pm 8.7 *
PCL + iDFSCs	51.33 \pm 2.9 *
PCL-HA + DFSCs	5.01 \pm 0.7
PCL-HA + iDFSCs	2.61 \pm 1.1

DFSCs: dental follicle stem cells; iDFSCs: osteo-induced dental follicle stems cells; PCL: polycaprolactone; PCL-HA: polycaprolactone plus hydroxyapatite. * indicates significant difference at $P \leq 0.05$ when compared to the controls (empty and scaffolds only).

5.3.5 Histological examination of bone regeneration after transplantation of DFSCs

Since radiological evaluation shows only mineralization, it is necessary to evaluate bone healing by histological examination of bone structure. Representative slides are shown in Figures 5.7 and 5.8 (N=4). No bone regeneration was observed in all treatment groups at week 4 after transplantation (Figure 5.7; Sections A-G). Defects at this time were filled with fibrous tissues as seen in Figure 5.7; Sections H-N. Histological examination of week 8 specimens showed absence of bone regeneration in the empty control defect (Figure 5.8; Sections A and H), as well as in the defects treated with scaffolds only (Figure 5.8; Sections B, C, I and J). A small layer of osteoid was observed in the defects treated with PCL-HA plus DFSCs (Figure 5.8; Sections F, M and Q), or PCL-HA plus iDFSCs (Figure 5.8; Sections G, N and R). In comparison, substantial bone formation was observed in the defects treated with PCL plus DFSCs (Figure 5.8; Sections D, K and O) or PCL plus iDFSCs (Figure 5.8; Sections E, L and P). Formation of woven bone could be seen in those groups at higher magnification (Figure 5.8; Sections O and P). The appearance of new bone formation was similar to what was expected in intramembranous ossification. The bone formation was confirmed by Masson Trichrome staining in those groups (Figure 5.8; Sections S-Z).



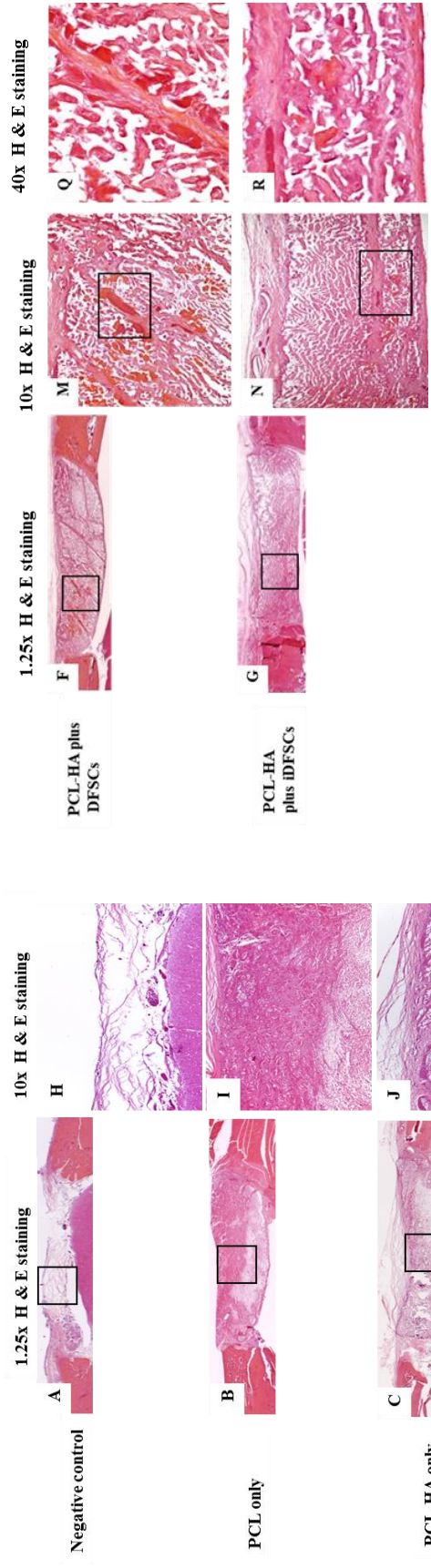
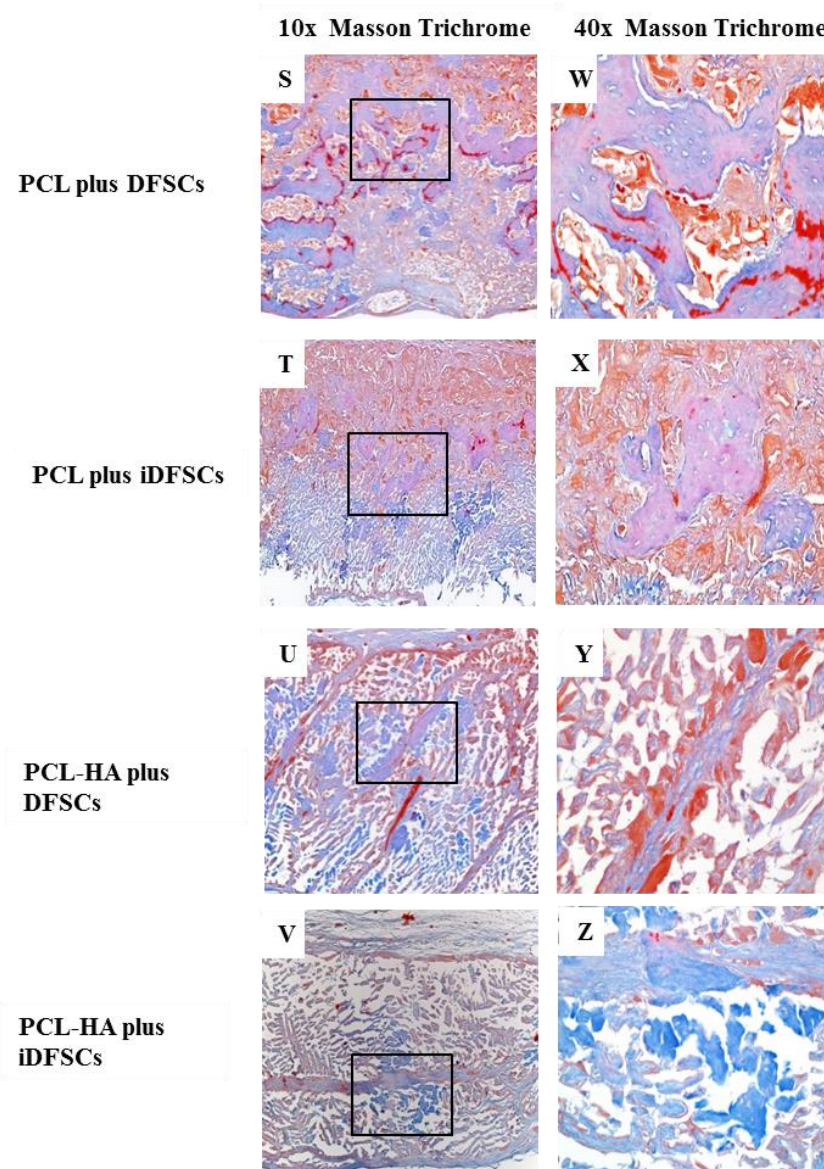


Figure 5.8 Histological evaluation of bone regeneration after 8 weeks post-transplantation (N=4). (A-G) Low magnification (1.25x) H & E stained micrographs showing the entire defects. Note that no bone regeneration was seen in the negative control (A) and in the scaffolds without DFSCs (B, C), and small amount of bone formation was observed in PCL-HA plus DFSCs or iDFSCs (F, G). Substantial bone formation was seen in the defects treated with PCL plus DFSCs (D) or PCL plus iDFSCs (E). Formation of woven bone can be seen in these treatments at higher magnifications (10x and 40x) (K, L, O, P). Slides were also stained with Masson Trichrome to confirm new bone formation (S-Z). DFSCs: dental follicle stem cells; iDFSCs: osteo-induced dental follicle stem cells; PCL: polycaprolactone; PCL-HA: polycaprolactone plus hydroxyapatite; H & E: Hematoxylin and Eosin

(Figure 5.8 continued)



5.4 DISCUSSION

Utilization of dental stem cells, such as DFSCs, has raised interest in the field of regenerative medicine. The advantages of these cells are that they can be isolated from extracted impacted teeth that are normally discarded as medical wastes during the course of dental treatment [20]. Experimental data have demonstrated multipotential differentiation of DFSCs [9-11]. Particularly, their strong osteogenic capability makes them an attractive type of AdSCs for

repairing bone defects, and *in vivo* transplantation of DFSCs has been attempted. In early studies, DFSCs were mixed with ceramic phosphate and then transplanted subcutaneously. Bone formation was seen after transplanting the DFSCs, indicating the *in vivo* bone-forming capacity of these cells [13, 14]. Recent studies reported the success of transplanting DFSC pellets to heal calvarial critical-size defects in immunocompromised rats [15, 16]. This study sought to extend our knowledge regarding the potential application of DFSCs for treatment of skeletal defects. Successful bone regeneration was achieved for treatment of calvarial critical-size defects of immunocompetent rats after transplanting DFSCs loaded into scaffolds.

Studies have suggested that scaffold is essential for supporting a three-dimensional structure for cell attachment and vascularization [17], particularly in treatment of large defects. We tested two types of scaffolds, PCL and PCL-HA for loading DFSCs. Although DFSCs can attach and proliferate in both scaffolds, PCL appears to be remarkably better than PCL-HA for DFSCs differentiation, resulting in greater bone regeneration. HA has been added to a variety of scaffolds as an osteoinductive material [4, 5, 21]. It has been shown that addition of HA to the scaffolds increases cell attachment and differentiation [22]. HA at percentage of 25%-30% has been used in previous studies [5, 23]. In this study, PCL-HA scaffold contained 25% of HA; however, we did not observe any beneficial effect in bone regeneration when 25% HA was used in the scaffold. It is likely that the ratio of HA in the scaffold construct should be adjusted properly for seeding different types of AdSCs.

This study revealed that no visible bone regeneration was seen at 4 weeks after transplantation of rat DFSCs to critical-size defects, and it took as long as 8 weeks to see the healing effect. Studies using other types of AdSCs, including adipose-derived stem cells (ASCs) and bone marrow stem cells (BMSCs), showed the bone healing at 4 weeks post-transplantation

[4,5]. Cowan *et al.*, reported 10% and 40% bone formation at 4 weeks after transplantation of mouse ASCs and BMSCs, respectively [4]. They observed that the regenerated bone filled almost half of the defects at 8 weeks, which is similar to our observation. However, in a human ASC transplantation study, almost complete healing of the defects could be achieved by 8 weeks [5]. The bone regeneration seen in our study was generally slower than the AdSC transplantation studies reported by others. This could be due to the following reasons: (A) AdSCs from different tissues and different species were transplanted; (B) immunocompromised animals were used in the reported study, and immunocompetent animals were used in our study. Although we did not observe any inflammatory reaction in our transplantation experiments, unnoticeable immune response might exist after transplantation, causing the delay of the healing process.

In the current study, we evaluated bone regeneration of DFSCs without treating cells with any growth factors. Various cytokines and growth factors, such as bone morphogenetic proteins (BMPs) and insulin-like growth factor-1 (IGF-1), are known to enhance osteogenic repair of AdSCs [5, 24, 25]. Our laboratory has shown that bone morphogenetic protein 6 (BMP6) and dentin matrix protein 1(DMP1) can enhance the osteogenic capability of DFSCs *in vitro* [12, Chapter 3]. It would be interesting to determine whether addition of these growth factors to scaffolds can accelerate the bone regeneration from DFSCs. In addition, we revealed that appropriate stressors, such as heat-stress treatments, can activate DFSCs to proliferate and differentiate *in vitro* (Chapter 4). Future study should examine if incubation of DFSCs at certain elevated temperatures, prior to transplantation, may boost their osteogenic capability *in vivo*.

There is a controversy within the literature regarding the necessity of pre-differentiation of AdSCs prior to transplantation [5, 19, 26]. This study determined that pre-induction of DFSCs slightly enhanced bone formation; however, there was no significant difference between

them. This suggests that pre-induction of DFSCs may not be necessary prior to transplantation. This would be beneficial to the patient, since it reduces the overall treatment time.

Several studies have demonstrated that transplanted cells not only contribute to new bone formation, but they can also release the signals to recruit the host's own stem cells to the defect site for tissue regeneration [27]. It is unclear whether the bone regeneration seen in this study resulted completely from the transplanted DFSCs, or the host's own stem cells also contributed to the bone regeneration. To clarify this, DFSCs labeled with a reporter gene, such as GFP transgenic rat, can be transplanted for tracking the regeneration. In this study, because DFSCs isolated from female pups were transplanted into adult male rats, it is possible to determine the contributions of the transplanted cells versus host cells to bone formation through sex chromosome detection [4].

In conclusion, this study demonstrated that DFSCs are a promising cell type for repair of craniofacial defects. Our data suggest that DFSCs, without prior induction, can be directly transplanted into craniofacial defects for bone repair in clinics. In addition, PCL appears to be a suitable scaffold material for seeding DFSCs for bone regeneration in repairing craniofacial defects.

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CHAPTER 6: CONCLUDING REMARKS

6.1 SUMMARY OF FINDINGS AND SIGNIFICANCE OF THE RESEARCH

Utilization of stem cells for treatment of diseased and damaged tissues represents a novel approach in regenerative medicine. Chapter 1 of this dissertation summarizes the basic knowledge of stem cells, characteristics of dental stem cells, and their potential applications in tissue regeneration. Particular emphasis has been given to the dental follicle stem cells (DFSCs). One of the advantages of DFSCs is that they can be isolated from extracted impacted teeth, which are normally discarded as medical waste during the course of dental treatment, while isolation of other tissue stem cells usually requires extra invasive procedures. This dissertation focuses on the evaluation of *in vitro* and *in vivo* osteogenic capability of DFSCs, which would provide important information toward the clinical applications of DFSCs for regeneration of bone defects.

In Chapter 2, we demonstrated the differentiation of DFSCs toward calcium-depositing cells. Further examination of marker gene expression revealed that the DFSCs primarily differentiated toward the osteoblast lineage under our current induction conditions. However, similar to other adult stem cells (AdSCs) [1, 2], such osteogenic capability during *in vitro* expansion was reduced. Maximal osteogenesis appeared at passage 3 (P3) to passage 5 (P5). Thus, for *in vivo* transplantation of DFSCs, P3 to P5 cells should be used. Studies in Chapter 2 also revealed that DFSCs express high levels of stem cell-related genes, including receptor tyrosine kinases (C-KIT), neurogenic locus notch homolog protein 1 (NOTCH1), and dentin matrix protein 1 (DMP1) as compared to the non-stem dental follicle cells (DFCs) of the same origin. These genes may serve as additional molecular markers for identification of DFSCs. Among the stem cell-related genes identified in Chapter 2, DMP1 showed a maximal differential

expression in DFSCs than in non-stem cell DFCs. Given that DMP1 has been reported to function as a nucleator for hydroxyapatite maturation as well as transcriptional factor [3-5], in Chapter 3, we further evaluated the role of DMP1 in regulating the osteogenic differentiation of DFSCs. We found that this high level expression of DMP1 is likely necessary to maintain the osteogenic differentiation capability of DFSCs. Moreover, our results indicate that DMP1 regulates expression of other osteogenic genes in the DFSCs, and DMP1 can be added to the osteogenic induction medium to improve osteogenesis of long-term cultured DFSCs.

AdSCs are known to be maintained in a slow cycling and quiescent state under normal physiological conditions [6, 7]. Certain factors such as cell injury signals can activate the quiescent stem cells to carry out their repairing role when needed [8, 9]. We attempted to use heat-stress to activate DFSCs; the results are reported in Chapter 4. Our experiments showed that under heat-stress conditions (39°C-40°C), DFSCs increased proliferation, osteogenic differentiation, and expression of stem cell-related marker genes. The results are significant in the application of DFSCs: (a) DFSCs may be cultured in an elevated temperature for rapid *in vitro* expansion prior to transplantation, and (b) DFSCs could be incubated at elevated temperatures to boost their osteogenic capability for transplantation.

One of the ultimate goals of stem cell studies is to use them for repairing tissue damage or defects via tissue regeneration. To explore the use of DFSCs for bone regeneration, DFSCs were seeded into scaffolds and transplanted into the calvarial defects of rats. The results are reported in Chapter 5. Our study indicates that pre-induction of DFSCs for osteogenesis is not necessary prior to transplantation. The synthetic polymer PCL appears to be a suitable scaffold for seeding DFSCs. The successful *in vivo* bone regeneration from DFSCs in immunocompetent

rats shown in this study is an important step toward the establishment of protocols for clinical applications of DFSCs.

6.2 FUTURE STUDIES

This dissertation focuses on osteogenic differentiation and bone regeneration capability of DFSCs. Approximately 50% of the bone regeneration of critical-size defects was achieved after transplanting DFSCs-PCL scaffold at 8 weeks post-transplantation (Chapter 5). It would be necessary to determine whether transplantation of DFSCs can result in full recovery of the defects, and the time needed for such recovery. Various cytokines and growth factors, such as bone morphogenetic proteins (BMPs) and insulin-like growth factor-1 (IGF-1) are known to enhance osteogenic regeneration of AdSCs [10, 11]. We have shown that the growth factors, such as bone morphogenetic protein 6 (BMP6) and dentin matrix protein 1(DMP1), can enhance the osteogenic capability of DFSCs [12, Chapter 3]. It would be interesting to determine whether coating scaffolds with these growth factors can accelerate healing of skeletal defects, particularly in large-size defects in stem cell based therapies. As shown in the Chapter 4, stressors, such as heat-stress treatments (38–40 °C), could activate DFSCs proliferation and differentiation *in vitro* (Chapter 4). It would be valuable to determine whether incubation of DFSCs at certain elevated temperatures, prior to transplantation, may boost their osteogenic capability *in vivo*.

Due to the small size of the DFs, limited quantities of the DFSCs can be obtained from a single tooth. This dissertation demonstrates that *in vitro* expansion of DFSCs at high passages results in reduction of their osteogenic capability (Chapter 2). Development of novel culture techniques to maintain differentiation potential during expansion of DFSCs would facilitate in

obtaining sufficient high-potential cells for regeneration of large defects. This would be one important area in future studies.

For human therapies, DFSCs from the same patient for autogenous transplantation may not be always available. Although this research suggests that the DFSCs from the siblings can be used safely for allogeneic transplantation without activation of a notable immune response in rats, the results may not be readily extrapolated to human therapies because genetic background of humans is much more complicated than the rat colonies used in this study. HLA matching is necessary for safe transplantation of sibling DFSCs in humans; however, our results imply that transplantation of the DFSCs from siblings is expected to have a much lower chance of immune response/rejection.

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VITA

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