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IDENTIFICATION OF GENES RESPONSIBLE FOR MAINTENANCE OF DIFFERENTIATION CAPABILITY IN DENTAL PULP STEM CELLS

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
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in

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through
The Department of Comparative Biomedical Sciences

by
Michael Brian Flanagan
B.S., Spring Hill College, 2003
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ABSTRACT

Stem cells exist in various tissues, including dental follicles and dental pulps. Adult stem cells (ASC) can be isolated from patients for autologous transplantation, which eliminates the risk of immune rejection with low or no tumorigenesis. However, one of the challenges is that ASC progressively lose their differentiation ability when cultured *in vitro*. This prevents expansion of large quantities of high-potential stem cells for therapeutics, especially for stem cells with limited tissue source, such as dental pulp stem cells (DPSC). The goal of this study is to define possible molecular regulation causing loss of differentiation. To achieve this goal, we determined that DPSC at passages 3 and 5 (early passage) possessed strong differentiation capability, and such differentiation capability is completely lost at passage 11 (late passage).

Using whole-genome microarray to compare the transcriptomes, we found that the expression of 34 genes were decreased for more than 10-fold in p11 DPSC when compared to p3. After confirming gene expression with RT-PCR, heat shock protein B8 (*HspB8*) and the GIPC PDZ domain-containing family (*Gipc2*) were selected for siRNA knockdown study. Knockdown of *HspB8* in early-passage DPSC resulted in the cells losing differentiation, but knockdown of *Gipc2* had no effect, suggesting that *HspB8* plays an important role in maintaining DPSC differentiation. To further study *HspB8*, we constructed 2 vectors, one containing the coding sequence (CDS) and 3' untranslated region (3'UTR) and another containing only the CDS. Transfection of the vectors into early passage DPSC dramatically increased both *HspB8* mRNA and protein. However, transfection of the vectors into the late passage DPSC resulted in overexpression of *HspB8* mRNA, but increase of HspB8 protein was seen only in CDS transfection. Given that 3'UTR of mRNA is the major target region for microRNAs (miRNAs), the results indicate that miRNAs are responsible for down-regulation of *HspB8* in long-term

culture of DPSCs. We conclude that high-level *HspB8* expression is essential for differentiation of DPSC, and down-regulation of *HspB8* in cultured DPSC is likely due to increased expression of miRNAs. These are novel findings regarding *HspB8* and miRNAs on the regulation of stem cell fate.

CHAPTER 1: STEM CELLS AND DENTAL PULP STEM CELLS FOR RESEARCH AND THERAPY

1.1 INTRODUCTION

Stem cells are undifferentiated cells which possess the abilities of self-renewal and differentiation into specialized cells. These two abilities make them attractive for therapeutic applications, such as regeneration of diseased and injured organs and tissues. The earliest form of stem cell therapy was the use of transplanted bone marrow to restore hematopoiesis in irradiated bone tissue [1-3]. Currently, bone marrow transplant is still an effective method to restore hematopoiesis for treatment of leukemia [4, 5]. Recent studies have shown that stem cells can move towards tumors (i.e. tumor-tropism), and the usefulness of stem cells as vectors for targeted delivery of therapeutic agents to cancers is currently under investigation [6-9]. Besides therapeutic applications, stem cells also provide a useful model to study tissue development [10].

Stem cells existing in nature can be classified into two major categories, according to their tissue origins: embryonic stem cells (ESC) and adult stem cells (ASC). As their names suggest, ESC are derived from tissues found during embryonic development, specifically in the blastocyst at the early stages of development. ESC possess strong capabilities of self-renewal and differentiation capabilities. They are pluripotent – meaning they can differentiate into cell types of any germ layer [11, 12]. Another important characteristic of ESC is that they can be maintained and proliferated *in vitro* for an indefinite time without losing their stem cell properties of self-renewal and differentiation capability. However, there are a number of technical and ethical issues in using ESC. One of the major drawbacks in the use of ESC is immune rejection because autologous transplantation of ESC is not possible. Use of ESC presents an elevated risk of tumorigenesis after transplantation into patients [13]. In addition,

isolation of ESC requires destruction of embryos, which raises issues on both ethical and legal fronts [14, 15].

In contrast to ESC, ASC are derived from adult tissues, and studies suggest that stem cells exist in most, if not all, adult tissues. ASC possess several advantages. They can be harvested from the patient's tissues and used for that same patient for autologous transplantation. Another major advantage of autologous stem cell transplantation is that it does not cause immune rejection. ASC are multipotent – they can differentiate into limited cell types. This limited potency allows ASC to differentiate into a unique cell type easily after transplantation. Another important feature of ASC is their low tumorigenicity. Transplantation of ASC does not result in teratoma formation, which is a risk associated with transplantation of ESC [13]. However, one major drawback of ASC is that their differentiation property cannot be maintained for long period of time *in vitro*; i.e. ASC reduce or lose their differentiation capability during *in vitro* expansion [16, 17]. Primary isolated ASC can be used for only a few passages before their differentiation potential is lost. A large quantity of high potential ASC cannot be obtained through continuous expansion of the primary isolated cells. Thus, loss of differentiation hampers their therapeutic applications.

Besides naturally-occurring stem cells, a third category of stem cells are artificial stem cells, which include the induced-pluripotent stem cells (iPS) [18, 19] and stem cells from somatic nuclear transfer (SCNT) [20]. The iPS cells are produced by introducing key transcription factors (TFs) into somatic cells, which reprogram those cells to behave similarly to ESC. This technique was first demonstrated in fibroblasts of mouse [18] and human origin [19]. The transcription factors used for such transformation are *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*. Initially, introduction of these genes required the use of viral transfection methods [18]. However, this technique

presented its own set of challenges, as these TFs are oncogenic, and may produce more harm than good if left unchecked [21, 22]. More recent studies have successfully generated iPS cells without the use of viral vectors, allowing the removal of these genes after reprogramming [23-25].

Stem cells from SCNT represent another attractive area as patient-specific cells can be generated, and harvesting such cells raises less ethical issues. These cells are generated through the insertion of somatic cell nuclei into the cytoplasm of an enucleated oocyte [26], a technique that had previously been performed using the nuclei of ES cells [27]. These newly formed cells are capable of blastocyst formation, from which stem cells can be harvested. When the blastocysts are transplanted into a surrogate mother, they can potentially form viable offspring [26]. However, developing stem cells using SCNT is unreliable, with successful stem cell formation occurring 1-2% of the time in mice [28]. Moreover, since SCNT requires the use of oocytes, developing human stem cell lines with this technique requires the addressing of ethical and legal problems [29], which are not unlike the problems faced with isolation of human ESC [14, 15].

1.2 DENTAL STEM CELLS

Obtaining adult tissues for stem cell isolation usually requires some invasive procedures. However, teeth are often extracted and discarded as medical waste in dental treatment, and human deciduous teeth are exfoliated. Use of these teeth for isolation of dental tissue stem cells does not require additional surgical procedures. Thus, they provide an attractive resource of tissues for isolating stem cells [30].

Stem cells have been isolated from various dental tissues. The dental follicle, a connective tissue sac surrounding the unerupted tooth, has been used to isolate dental follicle

stem cells (DFSC) [31-34]. DFSC have been shown to differentiate into calcium-depositing cementoblasts/osteoblasts, adipocytes, and multipolar neurons *in vitro* [32]. Transplantation of DFSC *in vivo* into mice yields cementum-producing tissue [35] and results in cementum formation [36]. Moreover, DFSC could differentiate into periodontal ligament [37], which is responsible for anchoring the mature tooth to the surrounding alveolar bone.

Stem cells are also isolated from the dental pulp, which arises from the neural crest during tooth development [38]. When isolated from deciduous teeth in humans, they are called stem cells from human exfoliated deciduous teeth (SHED) [39, 40]. SHEDs are actually derived from the pulp of human deciduous teeth [39], and are capable of differentiating into osteogenic, odontogenic, and adipogenic cells, as well as neural tissue [40]. Due to the nature of deciduous teeth, they provide a highly accessible and non-invasive tissue source for isolation of a special type of dental pulp stem cells (DPSC).

Besides DFSC and DPSC, stem cells have been obtained from other dental tissues, such as the periodontal ligament (PDLSC) [41], and the apical papilla of immature permanent teeth [42]. PDLSC bear the STRO-1^+ marker [41], a stem cell marker found in bone marrow-derived stromal cells (MSC) and DPSC [40, 43-45]. They are capable of differentiating *in vitro* into cementum-secreting cells, as well as adipocytes and collagen-secreting cells. In addition, *in vivo* transplants of PDLSC were capable of forming periodontal ligament-like tissue [41]. Stem cells from the apical papilla (SCAP) were first isolated by Sonoyama et al. in 2008, and were determined to bear resemblance to DPSC [42]. At the same time, SCAP are $CD24^+$, which makes them distinct from $CD24^-$ DPSC [30]. Further studies show that SCAP are capable of differentiating *in vitro* into osteoblast/odontoblasts, as well as adipocytes [46].

1.3 DIFFERENTIATION AND THERAPEUTIC POTENTIAL OF DENTAL PULP STEM CELLS

DPSC can differentiate into fibroblasts, osteoblasts, odontoblasts, and adipocytes [44, 47], as well as neurogenic and myogenic tissues *in vitro* [47]. A number of studies have suggested the potential uses of DPSC in tissue regeneration. These cells display the ability to grow dental-pulp-like tissue in a matrix of dentin [40, 44]. When grown on a perforated collagen scaffold, human DPSC differentiate into odontoblast-like cells in the presence of DMP-1 [48], or into dentin-like tissue in the presence of BMP2 [49]. Nam et al. showed that growth of human DPSC on porous granules of calcium phosphate, even in the absence of induction, promoted odontoblastic differentiation and dentin deposition [50]. When used in combination with a collagen scaffold and DMP-1, DPSC have been shown to regenerate dentin in perforated canine molars *in vivo* [51]. Additionally, DPSC can potentially be used for the repair or treatment of non-dental tissues. DPSC in canines, as well as stem cells from deciduous canine teeth (DTSC) were shown to develop into new bone when injected into mandible bone defects *in vivo* [52]. Nesti et al. have shown that human DPSC, when co-cultured with mesencephalic neurons, attenuated neuronal destruction from 1-methyl-4-phenylpyridinium (MPP+) and rotenone [53]. Because both MPP+ and rotenone promote the disruption and death of dopaminergic neurons [54] and are used for *in vitro* models to mimic the destruction of dopaminergic neurons that occurs in Parkinson's disease [55, 56], this result suggests that injection of DPSC may delay the progress of neurodegenerative disorders, such as Parkinson's disease. Other studies have shown that SHEDs can enhance wound healing [57] and differentiate into neural progenitor cells [58] and endothelial cells [59]. These studies provide strong evidence to demonstrate that dental pulp stem cells are an attractive source of stem cells for regeneration of dental and non-dental tissues.

1.4 DIFFICULTIES AND PROBLEMS IN APPLICATIONS OF ASC

The use of stem cells for tissue engineering or regeneration requires a large quantity of stem cells. However, it is difficult or often impossible to obtain sufficient quantities of stem cells from primary adult tissues due to the following reasons: First, the frequency of ASC in adult tissues is very low – generally less than 0.1% of the cell population in any given adult tissue [60]. Second, invasive procedures are usually required to obtain the tissues, and the use of small amount of tissue for ASC isolation is desired. Finally, in many cases only a limited amount of tissue is available. This is especially true for the dental tissues. For example, only a small amount of dental pulp could be obtained from each tooth. As such, *in vitro* expansion of the primarily isolated ASC is required to acquire sufficient cells for clinical applications. The complications with *in vitro* expansion of ASC are twofold: first, the cells become senescent and lose their self-renewal property after a certain number of cell doublings; and second, the cells gradually reduce and ultimately lose their differentiation capability as cell passage increases. Thus, it is difficult, often impossible, to obtain a sufficient quantity of high quality/potential ASC via *in vitro* expansion, and this has greatly hampered the clinical application of ASC.

Loss of stem cell qualities during *in vitro* expansion or culture of ASC is a common phenomenon. Various types of adult stromal cells demonstrate a loss of proliferation, termed proliferative senescence [61]. Like any non-immortal somatic cell, ASC will become senescent (non-dividing) after approximately 40-60 doublings [62].

In addition to a proliferative senescence, ASC also experience a decline in their capacity to differentiate, which is termed functional senescence by Bonab et al. [63]. They observed a 20% decrease of differentiation into osteoblasts after 8 passages in cultured MSC. This reduction progressed to a 25% decrease by p10. In addition, they reported that the decline in adipocyte

differentiation occurred sooner [63]. Similar to other ASC, dental stem cells are capable of differentiation within certain passages of *in vitro* culture [16, 17]. Takeda et al. reported that human DPSC lose the ability to differentiate at various passages, depending on the desired ultimate cell type. They reported a strong decrease from p3 to p10 in the activity of alkaline phosphatase (ALP) [16], indicating the reduction of differentiation into osteoblasts and odontoblast [40, 64]. In *in vivo* studies, DPSC could form dentin-like tissues when transplanted at p4, however when p10 DPSC were transplanted, formation of dentin-like tissue was not observed [16].

Stem cells lose their therapeutic value upon loss of their differentiation capability. ESC can maintain their stem cell properties (self-renewal and differentiation capabilities) indefinitely *in vitro* under proper conditions. This evokes a big question: is it possible to develop methods to maintain the proliferation and differentiation capabilities of ASC? Studies have attempted to delay the senescence and to prolong the differentiation capability of ASC by improving culture medium. Gharibi and Hughes report that a great increase in proliferation and delayed senescence of *in vitro* cultured mesenchymal stem cells (MSC) can be achieved by supplementing the culture medium with basic fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF)-bb, ascorbic acid (AA), and epidermal growth factor (EGF). However, this supplementation did not delay the loss of differentiation. They also noticed that the loss of differentiation was not reflected by any changes in stem cell surface marker expression [65].

1.5 MOLECULAR REGULATION OF STEM CELL PROPERTIES

Self-renewal and differentiation capabilities are the two fundamental properties of stem cells. Studies have been attempted to elucidate the molecular regulations of such capabilities. Of particular note are the genes of transcription factors such as *Oct4*, *Sox2*, and *Nanog*. These genes

have been shown to maintain a pluripotent state in stem cells, and they are used to reprogram somatic cells into stem cells. These genes are heavily regulated by a large number of other genes [66-69]. Many other genes were also found to maintain stem cell properties. For example *Esrrg* preserves the undifferentiated state [68], while *Wnt* genes promote ESC differentiation [70]. A more thorough list of stem cell-related genes can be found in Table 1.1.

Micro-RNAs (miRNAs) are short, non-coding RNAs generated from genomic DNA that bind to target mRNAs to exert post-transcriptional regulation (such as translational repression, mRNA destabilization, and/or cleavage) via the RNA-induced silencing complex (RISC) (Figure 1.1). miRNAs have emerged as critical molecular regulators for maintaining the functions of stem cells by fine tuning the protein levels of various factors [91]. A majority of the current knowledge on miRNA regulation of stem cells was obtained from ESC, although the role of miRNAs in regulating ASC was also explored. In hematopoietic stem cells (HSC), miRNA-128 (miR-128) and miR-181 are expressed to prevent differentiation, whereas miR-16, miR-103, and miR-107 are expressed to prevent proliferation [92]. miRNAs have been reported to regulate various forms of differentiation including monocytogenesis, myogenesis, cardiogenesis, neurogenesis, and osteogenesis [91]. One study indicated that miR-125 inhibits osteoblast differentiation by down-regulation of cell proliferation by an unknown mechanism [93]. Another study revealed that osteogenic differentiation of adipose-derived stem cells was inhibited because miR-26a down-regulated the translation of Smad mRNA, a transcription factor required for osteogenesis [94]. These studies support the idea that miRNAs can function in maintaining ASC fate in distinct ways.

Table 1.1 Genes related to stem cell function

Gene	Stem Cell-Related Functions
BMP4	Promotes expansion of human adipose-derived stem cells [71], induces trophoblast formation in human ESC [72]
c-Myc	Key factor in reprogramming adult fibroblasts into iPS cells [18, 19]
Dax1	Nuclear receptor, part of Oct4 signaling network [66], maintains ES cells in undifferentiated state [73]
Dusp7	Knockdown induces differentiation of ES cells [68]
EGR1	Limits proliferation and migration of hematopoietic stem cells (HSC) [74]
Esrrb	Transcription factor in Oct4 signaling network [66], promotes self-renewal and pluripotency by activating *Oct4* expression [67]
FoxO1,3,4	Cell cycle arrest and quiescence [75]
FoxM1	Maintains pluripotency of P19 embryonal carcinoma cells, knockdown reduces *Oct4* and *Nanog* levels [76]
KitL	Promotes the differentiation of ES cells to germ-like precursor cell [77], promotes differentiation of mast cells from hematopoietic stem cells [78]
Klf4	Key factor in reprogramming adult fibroblasts into iPS cells [18, 19], maintains undifferentiated state of ESC [79]
Klf5	Maintains undifferentiated state of ESC [79, 80]
LIN 28	Can be used to reprogram iPS cells from human somatic cells [81]
Mki67ip	Knockdown reduces levels of *Oct4*, *Sox2*, and *Nanog*, knockdown induces differentiation of ES cells [68]
Nanog	Maintains pluripotency of ES cells [82]
Oct4	Key factor in reprogramming adult fibroblasts into iPS cells [18, 19]
Sall1	Part of Oct4 signaling network [66], binds to Nanog and Sox2 in ES cells [83]
Sox2	Key factor in reprogramming adult fibroblasts into iPS cells [18, 19], essential for maintaining ES cells [84]
Tcfcp2L1	Transcription factor in Oct4 signaling network [66]
Timp2	Knockdown reduces levels of *Oct4*, *Sox2*, and *Nanog*, knockdown induces differentiation of ES cells, overexpression reduces ES cell differentiation [68]
Wnt16	Secreted non-canonical protein, interacts with Notch in embryonic HSC specification [85], the isoform Wnt16B is a marker for senescence [86]
Zfp143	Promotes self-renewal of ES cells through binding with Oct4 to promote Nanog transcription [69]
Zfp219	Member of Oct4 binding network [87], regulates differentiation in adult stem cells [88]
Zfp462	Maintains chromatin structure in pluripotent cells [89], maintains pluripotency in P19 embryonic carcinoma cells [90]

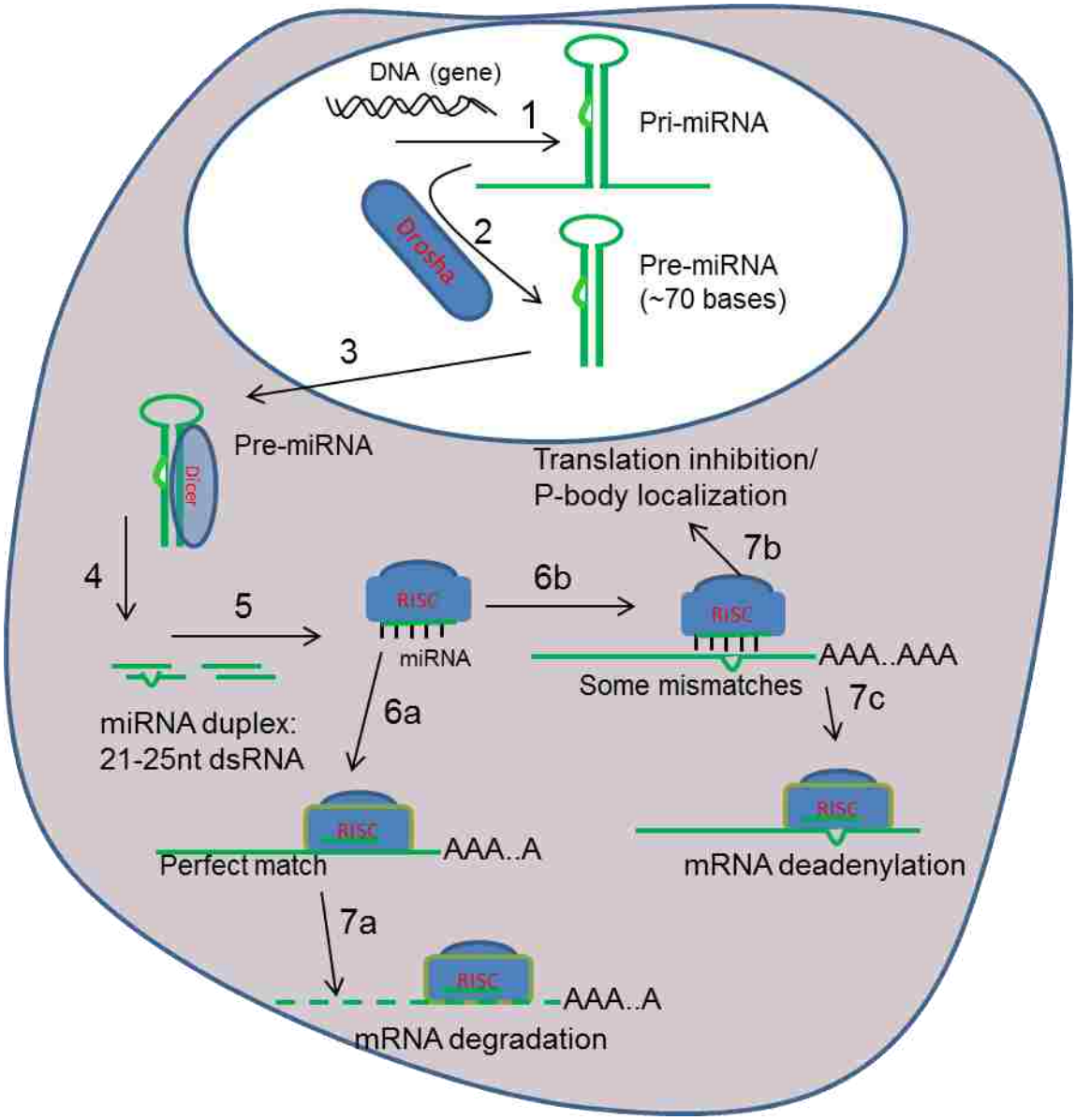

Figure 1.1 Schematic illustration of biogenesis of miRNAs and post-transcriptional regulation of gene expression in animal cells by a miRNA. 1. Transcription of DNA into pri-miRNA; 2. Drosha processes pri-miRNA into pre-miRNA; 3. Nuclear exporting pre-miRNA; 4. Processing of pre-miRNA into miRNA duplex by dicer; 5. Formation of RNA-induced silencing complex (RISC) with guide strand; 6. RISC searches for matching mRNA, 7a. mRNA degradation when miRNA perfectly matching mRNA sequence, 7b. Translation inhibition when miRNA partially matching mRNA; 7c. mRNA deadenylation

1.6 OBJECTIVES AND SIGNIFICANCE OF THIS DISSERTATION RESEARCH

Use of patient-specific stem cells for autologous transplantation in regenerative medicine eliminates the risk of immune rejection after transplantation. DPSC are a valuable source of stem

cells for tissue engineering and regeneration. Studies have determined that DPSC can be induced into specialized cells with strong calcium deposition capability, suggesting that DPSC would be an attractive source of stem cells for regeneration related to bone and hard dental tissue. Yet, for any given patient, teeth that can be used for stem cell isolation are limited, and isolation of large quantities of stem cells from teeth is difficult. *In vitro* expansion of primarily isolated DPSC is needed for therapeutic uses of such cells. Studies have also shown that DPSC reduce their differentiation capabilities during *in vitro* culture [16, 17]. Thus, it is difficult to obtain large amounts of high quality DPSC through *in vitro* expansion. The overall goal of this dissertation research is to identify potential molecular regulation of the loss of differentiation capability in DPSC. Our overall hypothesis is that reduced expression of certain genes causes the loss of differentiation capability of DPSC during *in vitro* culture. The results of this study would aid in understanding the mechanisms of loss of differentiation seen in dental stem cells, as well as in other ASC. Elucidation of such a mechanism would facilitate the development of methods for maintaining the stem cell properties of ASC in long-term culture. In turn, this would permit growth of larger quantities of such cells for clinical applications. This is especially important for DPSC, due to the small size of the dental pulp and limited source available for stem cell isolation. Sufficient DPSC cannot be obtained from primary isolation. *In vitro* expansion of primarily isolated DPSC is needed for therapeutic uses of such cells. It is ideal that primary isolated high-differentiation potential DPSC can be expanded *in vitro* into large quantities without the loss of this differentiation potential and saved for future autologous therapeutic applications.

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CHAPTER 2: LOSS OF DIFFERENTIATION POTENTIAL IN DENTAL PULP STEM CELLS DURING *IN VITRO* CULTURE

2.1 INTRODUCTION

Dental pulp stem cells (DPSC) are adult stem cells that were first isolated in human teeth by Gronthos et al. in 2000 [1]. They share a number of similarities with bone marrow-derived stromal cells (BMSC), such as the development of colony-forming unit-fibroblasts (CFU-F) *in vitro* [2], or expression of stem cell markers, including VCAM-1, fibronectin, alkaline phosphatase (ALP) [1], as well as the stromal cell marker STRO-1 [3, 4]. When transplanted *in vivo*, DPSC have been shown to form dentin [1], as well as respond to *in vitro* induction into neurogenic and myogenic tissues [5]. In mice, DPSC have been shown to possess neural crest migration signals [6]. During embryonic development, these signals direct the migration of cranial neural crest (CNC) cells towards the dental mesenchyme [7]. These cells have the capability to differentiate into neuronal cells and glial cells, as well as the skeletal and connective tissues of the head and neck [8]. Consistent with this finding are reports that DPSC possess multi-differentiation in neural crest lineage for odontoblasts, chondrocytes, adipocytes [9, 10], neural tissue [10, 11], and smooth muscles [10]. This multifaceted differentiation potential makes DPSC valuable for regeneration of dental and non-dental tissues.

Like other ASC, the differentiation capability of DPSC is reduced when cultured *in vitro*. Takeda et al. have shown that, when subjected to osteogenic induction *in vitro*, human DPSC are capable of developing into calcium-depositing cells at early passages [12]. Transplantation of early-passage DPSC *in vivo* yielded formation of dentin and pulp-like connective tissue that was surrounded by a thin layer of odontoblast-like connective tissue. However, such differentiation potential was reduced at passage 6 (p6) and total loss of differentiation was observed around p10 [12]. This research aims to determine the potential molecular regulation of the loss of

differentiation using rat DPSCs. To achieve this goal, we first need to know the differentiation potential of rat DPSC under our current culture conditions, such as when they start to lose the differentiation potential, and when they completely lose the capability

Loss of differentiation under *in vitro* conditions mitigates the value of ASC. Various studies have been attempted to preserve the differentiation potential of stem cells, such as the addition of growth factors to culture medium [13]; however, the effect has been limited. Elucidating the mechanism of the loss of differentiation would facilitate development of techniques to preserve the differentiation potential of cultured stem cells. In searching the current literature, we found little information on the topic. Takeda et al. found that, from early to late passage, 719 genes had their expression levels reduced by half or more. In addition, they report that an increase in Wnt16 is suspected to play a role in the loss of differentiation potential in human DPSC [12].

Transcription factors (TFs) are DNA binding proteins that function to regulate expression of various genes. The vital role of TFs has been demonstrated in reprogramming somatic non-stem cells into pluripotent stem cells through the introduction of key TFs [14, 15]. Given the important roles of TFs, we speculate that TFs are involved in maintaining the differentiation capability of DPSC. Thus, another objective of this chapter was to compare the expression of selected stem cell-related TFs in the early and late DPSC to determine if these TFs change their expression as the passages of DPSC advance.

It is generally accepted that ASC reduce overall differentiation potential as progression of cell passages. However, one study showed that passage 9 (p9) DPSC presented greater osteogenic differentiation than did p1 DPSC [16]. They concluded that this was because DPSC

spontanenously underwent differentiation into an osteoblasts lineage in growth medium [16]. The last objective of this study was to clarify this controversy.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Dental Pulp Collection

Sprague Dawley rats were handled in accordance with IACUC protocol. Dental pulps were extracted from the first molars of 5-7 days old postnatal rats. Briefly, the mandibles were surgically removed and surrounding soft tissues were cleaned off. First molars were removed from the tooth crypt. The pulps were extracted from the molars and sliced into 1-2mm sections, then transferred to a sterile tube filled with 5mL stem cell growth medium, i.e., MEM-α supplemented with +20% fetal bovine serum (FBS) and antibiotics [17].

2.2.2 Establishment of Cell Cultures from Rat Dental Pulp

The tubes containing dental pulp tissues and medium were centrifuged at 3000rpm for 1 minute and media was aspirated. Next, 10mL of 1% trypsin-EDTA was added to suspend the tissue pellet and incubated at 37°C for 10 minutes to obtain the cell suspension. After 5 minutes of centrifugation, the trypsin was removed and the cell pellet was re-suspended in the appropriate medium. For DPSC cultures, the cells were grown in stem cell growth medium consisting of α-MEM, 20% fetal bovine serum (FBS), and antibiotics. Cells were incubated at 37°C and 5% CO_2 in humidified incubators. At 90% confluence, cells were detached using trypsin-EDTA and passaged to a fresh T-75 flask with an initial density of 200,000 cells per T-75 flask. For dental pulp cell (DPC) culture, cells were grown in MEM with 10% newborn calf serum (NCS), 1mM sodium pyruvate, and antibiotics as described by Wise et. al [18].

Established dental pulp-derived cells (DPSC and DPC) at p3 were tested for differentiation (see section 2.2.3), and then processed for cryopreservation in liquid nitrogen for

future experiments. Briefly, cells were detached from T-75 flasks and centrifuged at 3000rpm for 5 minutes, and medium was aspirated. Next, the cell pellet was re-suspended in freezing medium, consisting of 50% stem cell growth medium, 40% FBS, and 10% dimethyl sulfoxide (DMSO) to an approximate concentration of 5.0 $x10^5$ cells/mL. The cell suspension was transferred to cryopreservation tubes and frozen at -80°C overnight before moving to liquid nitrogen for long-term storage.

2.2.3 Assessment of Differentiation Capability of Dental Pulp Stem Cells

To assess if the established cells possess differentiation potential, both DPSC and DPC were seeded to 6-well culture plates ($10cm^2$) at a rate of 5 x 10^4 cells/well, with differentiation medium consisting of low glucose DMEM (Gibco) supplemented with 10% FBS and differentiation induction reagents (50μg/mL ascorbic acid, 100nM dexamethasone, and 10mM β-glycerolphosphate) [17]. This medium has been reported to induce osteogenic differentiation of various adult stem cells in several publications [17, 19, 20]**,** including induction of osteogenesis of dental follicle stem cells in our lab [21]. Cells incubated in the basal low-glucose DMEM +10% FBS without the supplemented induction reagents were included as a control. In this study, DPC and DPSC were incubated in the induction medium for 7 to 14 days, with medium changes every 4 days. After the designated time of induction, calcium deposition was assessed by Alizarin red staining [22]. For Alizarin red staining, cells were washed once with PBS and then fixed in 10% neutral buffered formalin for 5 minutes. Cells were then washed in sterile milli-Q H_2O twice and stained in a 1% Alizarin red S solution for 20 minutes. Immediately afterwards, cells were washed 4 times with sterile H_2O. The degree of Alizarin Red staining was graded with scales outlined in Table 2.1.

For gene expression analysis, established DPC and DPSC were seeded in T-25 flasks ($25cm^2$) at a rate of 1.5×10^5 cells/flask in α-MEM+20% FBS and grown for designated times. Next, the cells were collected into RLT buffer (Qiagen) for RNA isolation to compare gene expression of selected stem cell-related TFs using real-time RT-PCR.

Table 2.1 Alizarin red staining scale

Grade	Description
0	Complete absence of staining
1	Staining barely visible, or localized to only 1-3 small locations
2	Staining clearly observed, but covers less than 50% of the stained area
3	Staining observed in 50% or more of area, with only small areas of non-staining
4	Positive staining observed throughout the entire area

2.2.4 Experiments to Determine Differentiation Potential of Different Passages of DPSC

DPSC were recovered from liquid nitrogen, and cultured in T-25 flasks as described earlier. Cells at 90% confluency were passaged into new flasks at a 1:3 ratio (i.e. 1 flask was passaged to 3 flasks of the same size). Cells at passages 3, 5, 7, 9, and 11 were collected and subjected to differentiation induction. Briefly, cells were seeded to sterile 6-well and T-25 flasks. Differentiation induction was initiated when cells reached 80% confluence, as described in 2.2.3. After 2 weeks of induction, cells were stained with Alizarin red or collected into RLT buffer for gene expression analysis, as described in 2.2.3.

2.2.5 RNA Isolation

Cells were collected and lysed in RLT buffer, then centrifuged at 13000 rpm for 5 minutes. The supernatant was then collected and transferred to a fresh tube. Isolation was performed using the RNeasy DNAse Digest kit (Qiagen). Briefly, the lysate was mixed with 70% ethanol at a 1:1 ratio and transferred to a RNA-binding mini-spin column. The column was then washed with RW1 buffer, followed by in-column DNAse I digestion (Qiagen) for 15 minutes.

The column was washed again before elution with 30 μL nuclease-free water. Concentration of RNA was determined using a Nanodrop 8000 (Thermo Scientific).

2.2.6. Reverse-Transcription and Quantitative Real-time PCR

M-MLV reverse transcriptase (Invitrogen) was used to generate a template strand of cDNA from 1000-2000ng isolated total RNA. Each RNA sample was mixed with 250 ng random primer in a 0.5 mL microtube and placed in a 65°C water bath for 5 minutes. The tube was immediately transferred to ice for 2 minutes, followed by addition of reverse-transcription buffer containing M-MLV reverse transcriptase. Reverse transcription was performed at 37°C for 50 minutes and followed by incubation at 70°C for 15 minutes to deactivate the reaction. The resulting cDNA templates were then used for SYBR green real-time PCR to detect cycle threshold (C_T) in an Applied Biosystems 7300 sequence detector. Each reaction contained 1-2 μL template cDNA, 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems), and 200 – 600 nM gene-specific primers in a total reaction volume of 25μL. A full list of primers used for PCR is listed in Table 2.2. The C_T of each gene was normalized to *β-actin* to obtain the

Table 2.2 Primers used in real-time RT-PCR

Gene	RefSeq	Primer Sequence
Actin	NM_031144.3	Forward: 5'-CTAAGGCCAACCGTGAAAAGAT-3' Reverse: 5'-AGAGGCATACAGGGACAACACA-3'
Ambn	NM_012900.1	Forward: 5'-GCTCCTGTTCCTGTCCCTAGT-3' Reverse: 5'-TTCCCAACTGTCTCATTGTCTC-3'
BCRP	NM_181381.2	Forward: 5'-GAAAGACCCACGGGGATTAT-3' Reverse: 5'-CCCATCACAACGTCATCTTG-3'
BMP4	NM_012827.2	Forward: 5'-TGATACCTGAGACCGGGAAG-3' Reverse: 5'-AGAAGTGTCGCCTCGAAGTC-3'
BMP6	NM_133530.1	Forward: 5'-CTTACAGGAGCATCAGCACAGA-3' Reverse: 5'-GTCACCACCCACAGATTGCTA-3'
BSP	NM_012587.2	Forward: 5'-ACGCTGGAAAGTTGGAGTTAGCTG-3' Reverse: 5'-TTCCTCTTCCTCGTCGCTTTCCTT-3'
c-MYC	NM_012603.2	Forward: 5'-AAAGGCCCCCAAGGTAGTTA-3' Reverse: 5'-CTCGCCGTTTCCTCAGTAAG-3'

(Table 2.2 continued)

Gene	RefSeq	Primer Sequence
Dax1	NM_053317.1	Forward: 5'-GAGCAGATCAAACACCAGCA-3' Reverse: 5'-CCACCTGTGGATCCTTGAGT-3'
DMP1	NM_203493.3	Forward: 5'-CCAGGACAGTAGCCGATCC-3' Reverse: 5'-TTCAATGTTTTTGGGGTGGT-3'
DSPP	NM_012790.2	Forward: 5'-GGGAAGCTCAGTGGAAGTAAAG-3' Reverse: 5'-CTGCTGTGTCCCATGTTGTTAT-3'
Dusp7	NM_001100547.1	Forward: 5'-CAGCCAATCTGGCAATTTTT-3' Reverse: 5'-AACCAAGGAGAGCCTGAACA-3'
EGR1	NM_012551.2	Forward: 5'-AACACTTTGTGGCCTGAACC-3' Reverse: 5'-AGGCAGAGGAAGACGATGAA-3'
Esrrb	NM_001008516.2	Forward: 5'-GTGCCTGAAGGGGATATCAA-3' Reverse: 5'-AGAAACCTGGGATGTGCTTG-3'
FoxM1	NM_031633.3	Forward: 5'-GGAGCCTACTCAAGCACAGG-3' Reverse: 5'-GGGATAGCCACCACTTGTGT-3'
FoxO1	NM_001191846.2	Forward: 5'-AACCAGTCCAACTCGACCAC-3' Reverse: 5'-TGCTCATAAAGTCGGTGCTG-3'
FoxO3	NM_001106395.1	Forward: 5'-TCTCCCGTCAGCCAGTCTAT-3' Reverse: 5'-GTCACTGGGGAACTTGTCGT-3'
FoxO4	NM_001106943.1	Forward: 5'-CCTGGCCTACCCAGATTGTA-3' Reverse: 5'-AAGGCAGCAGAAACCAGAAA-3'
KitL	NM_021843.4, NM_021843.4*	Forward: 5'-GCTGTGAAACCTGCACTGAA-3' Reverse: 5'-ATGGCGCAAGTAGACTGGAC-3'
Klf4	NM_053713.1	Forward: 5'- GTGCAGCTTGCAGCAGTAAC-3' Reverse: 5'- GTGGGATAGCGAGTTGGAAA-3'
Klf5	NM_053394.2	Forward: 5'-ACCTACTTTCCCCCATCACC-3' Reverse: 5'-TAGCAGCATAGGACGGAGGT-3'
Lin28	NM_001109269.1	Forward: 5'- CCCAGTGTCACCCTGTCTTT-3' Reverse: 5'- TTCCCCACAAAAGCTTTCAC-3'
Mki67	NM_139186.2	Forward: 5'-GGAAGAGCAAGCAGTGATCC-3' Reverse: 5'-CCTGTCCATGTTTGCATGTC-3'
Mmp13	NM_133530.1	Forward: 5'-TTTATTGTTGCTGCCCATGA-3' Reverse: 5'-GAGAGACTGGATTCCTTGAACG-3'
Msh2	NM_031058.1	Forward: 5'-TGGATTCCACCCAGAGAAAG-3' Reverse: 5'-TCTCTCCGCCTGGTAAAATG-3'
Mst1	NM_024352.1	Forward: 5'-TACCATGGCTCAGGTGAACA-3' Reverse: 5'-TGGGTGTGAATTGTGGCTTA-3'
Musashi	NM_148890.1	Forward: 5'-TGCTGGGTATTGGCATGTTA-3' Reverse: 5'-TAGGTGTAACCAGGGGCAAG-3'

(Table 2.2 continued)

Gene	RefSeq	Primer Sequence
Nanog	NM_001100781.1	Forward: 5'-TACCTCAGCCTCCAGCAGAT-3' Reverse: 5'-CATTGGTTTTTCTGCCACCT-3'
Notch1	NM_001105721.1	Forward: 5'-CTCAACACACTGGGCTCTTTC-3' Reverse: 5'-ACACCCTCATAACCTGGCATAC-3'
Oct4	NM_001009178.2	Forward: 5'-AGAACCGTGTGAGGTGGAAC-3' Reverse: 5'-CACTCGAACCACATCCCTCT-3'
Ocn	NM_013414.1	Forward: 5'-ACTGCATTCTGCCTCTCTGAC-3' Reverse: 5'-TATTCACCACCTTACTGCCCTCCT-3'
RunX2	NM_001278483.1, NM_001278484.1**	Forward: 5'-GCCGGGAATGATGAGAAC-3' Reverse: 5'- GAGGATTTGTGAAGACCG -3'
Sall1	NM_001107415.2	Forward: 5'-GTGGCAAGGGTGAAGACAGT-3' Reverse: 5'-ACAGAGGGTTGGTGAAGGTG-3'
Sox2	NM_001109181.1	Forward: 5'-ATTACCCGCAGCAAAATGAC-3' Reverse: 5'-CTAGTCGGCATCACGGTTTT-3'
Sp7	NM_001037632.1	Forward: 5'-CCTACTTACCCGTCTGACTTTG-3' Reverse: 5'-CAACTGCCTTGGGCTTATAGA-3'
Spp1	NM_012881.2	Forward: 5'-GCTTGGCTTACGGACTGAGG-3' Reverse: 5'-GCAACTGGGATGACCTTGATA-3'
Tcfcp2L1	NM_001107170.1	Forward: 5'-GGGACAGGACCAAAAGTCAA-3' Reverse: 5'-TGGGGTTCAAACACAGACAA-3'
Timp2	NM_021989.2	Forward: 5'-GCATCACCCAGAAGAAGAGC-3' Reverse: 5'-GTCCATCCAGAGGCACTCAT-3'
Wnt16	NM_001109223.1	Forward: 5'-GAGCTGTGCAAGAGGAAACC-3' Reverse: 5'-AGTGGCGACCATACAGTTCC-3'
Zfp143	NM_001012169.1	Forward: 5'-AGCAGCCATCTCTGGAAGAA-3' Reverse: 5'-GAAAGGCTCTTCCTCCTGCT-3'
Zfp219	NM_001007681.1	Forward: 5'-AGGAGAGTGGGCAAGCAATA-3' Reverse: 5'-CAGCAGCACATCCTCTACCA-3'
Zfp462	XM_342840.5	Forward: 5'-CAAGGCTCTGAGTGGGAAAG-3' Reverse: 5'-CAGCTGCATACTGTCCTCCA-3'

* *KitL* mRNA has two separate isoforms, with isoform 2 having a slightly shorter CDS region, in addition to the translated protein having slightly different C-terminus and being shorter than isoform 1. The primers listed flank a conserved sequence.

***RunX2* mRNA has two separate isoforms, with isoform 2 having a shorter 5' untranslated region and CDS region, as well as the translated protein having slightly different N-terminus and being shorter than isoform 1. The primers listed flank a sequence conserved between the two isoforms.

ΔC_T with the equation: $\Delta C_T = C_{T\ gene} - C_{T\ actin}$. We calculuated the difference in ΔC_T between experimental and control samples ($\Delta\Delta C_T$) using the equation: $\Delta\Delta C_T = \Delta C_{T\ experiment} - \Delta C_{T\ control}$.Relative gene expression (RGE) was calculated using the following equation: $RGE = 2^{-(\Delta\Delta Ct)}$ [23]. The control was set to 1 with the standard error of the mean (SEM) of 0.

2.3 RESULTS

2.3.1 Growth Pattern of Dental Pulp Derived Cells

Two culture systems were used to culture cells derived from dental pulps. For DPSC grown in stem cell medium, cultures reached 80% confluence 4-6 days after seeding. Early-passage DPSC had a fibroblast-like appearance. Within 7-8 days, the cells reached 100% confluence (Figure 2.1). As passaging progressed, DPSC morphology gradually transitioned to a spindle-like shape, and cells were capable of forming colonies. In contrast, DPC grown in MEM + 10% NCS did not display the same fibroblast-like appearance. Cells reached 100% confluency within 6 days, which was similar to DPSC, but they were not able to form colonies.

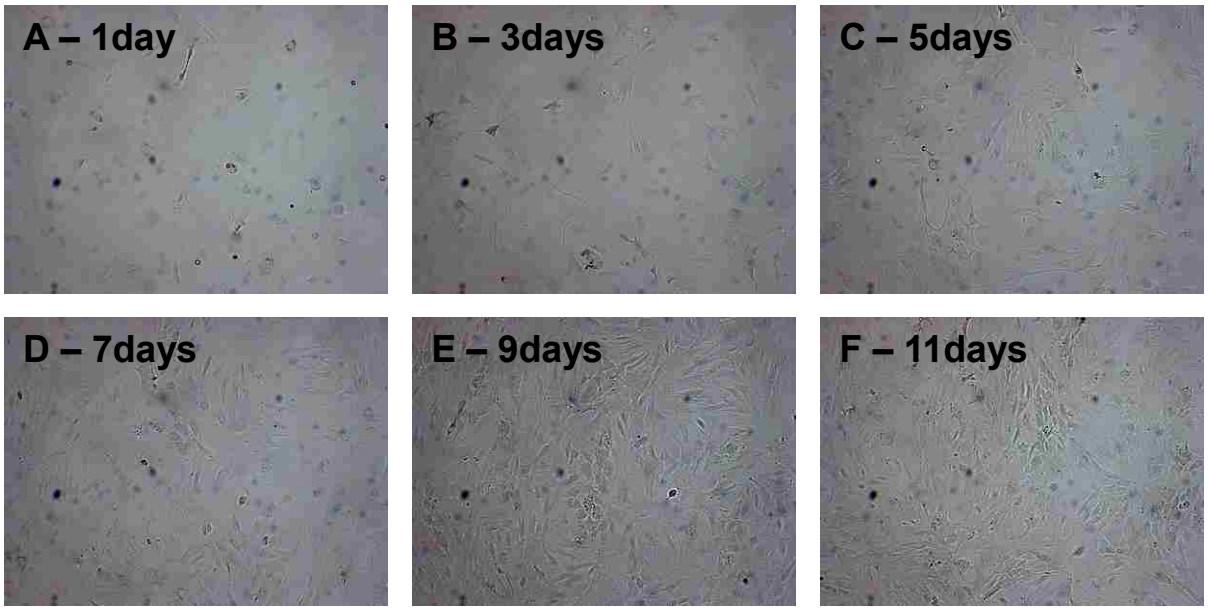

Figure 2.1 Culture and growth of early-passage DPSC. Note that DPSC reached full confluency around 9 days of *in vitro* culture. Each frame represents to the same field of view over the entire 11 days of culture. 100x magnification

2.3.2 Differentiation Capability of Dental Pulp Derived Cells

When both DPC and DPSC were incubated in differentiation-induction medium, Alizarin red staining indicated that calcium deposits could be detected in DPSC as early as after 7 days of induction. These calcium depositions were shown as scattered spots in the plate at day 7 (Figure 2.2; Section A). After 14 days of induction, the calcium deposits were spread to the entire plate (Figure 2.2; Section B). In contrast to DPSC, DPC did not show any positive staining with

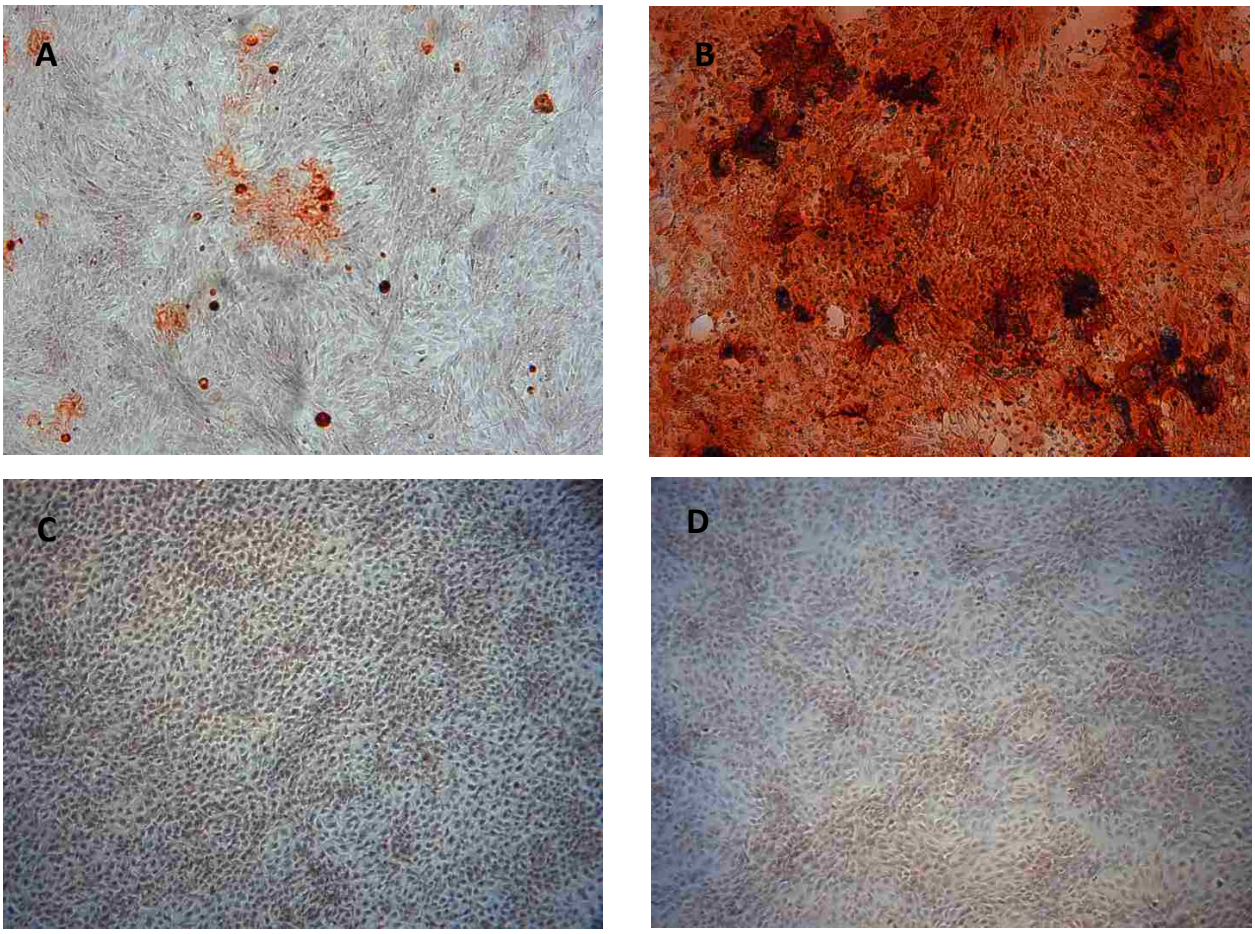

Figure 2.2 Comparing the differentiation capabilities of early-passage DPSC and DPC. Cells were subjected to osteogenic induction for 1 or 2 weeks and stained with Alizarin red. Positive staining was seen in DPSC as soon as 1 week (A), and increased staining was seen after 2 weeks of induction (B). In contrast, no staining was observed in DPC after 1 or 2 weeks of induction (C and D). All images 50x magnification.

Alizarin red after both 7 and 14 days of induction (Figure 2.2; Sections C & D), suggesting that no stem cells were present in the DPC population.

Because calcium can be deposited by osteoblasts, odontoblasts, and/or ameloblasts, we further assessed the expression of marker genes specific for osteoblasts (*Bsp*, *Ocn*, *Runx2*, *Sp7*, *Spp1*), ondontoblasts (*DSPP*), and ameloblasts (*Ambn*) with real-time RT-PCR to assess the differentiation lineage of DPSC. The results are shown in Figure 2.3. We observed an increase in expression for marker genes of osteoblasts, odontoblasts, and ameloblasts after differentiation induction. The greatest increase in expression occurred in Ocn, with a 10-fold increase after 7 days of induction, and an increase greater than 200-fold after 14 days. After 7 days of culture in induction medium, Bsp expression appeared unchanged; however, its expression had increased to 10-fold expression after 14 days of induction. This upregulation in expression after differentiation was also observed for the other osteoblast markers *Runx2* and *Sp7*. Expression of the odontoblast marker *DSPP* increased sharply from 3-fold after 7 days to 112-fold after 14 days. Expression of the ameloblast marker *Ambn* had rapidly increased to 50-fold and 80-fold after 7 and 14 days of induction, respectively (Figure 2.3). These results of marker gene expression indicate that DPSC are capable of multipotent differentiation toward osteoblasts, odontoblasts, and ameloblasts under our induction conditions.

2.3.3 Differentiation Potential of DPSC at Different Passages

To test the differentiation capability of DPSC during *in vitro* expansion/culture, the DPSC were passaged at about 90% confluency, and different passages of cells were tested to evaluate their differentiation capability, as detailed in Section 2.2.4. Gradual reduction of calcium deposition was observed with progression of serial passaging, as shown by Alizarin red staining. The representative images for each passage tested are reported in Figure 2.4. The

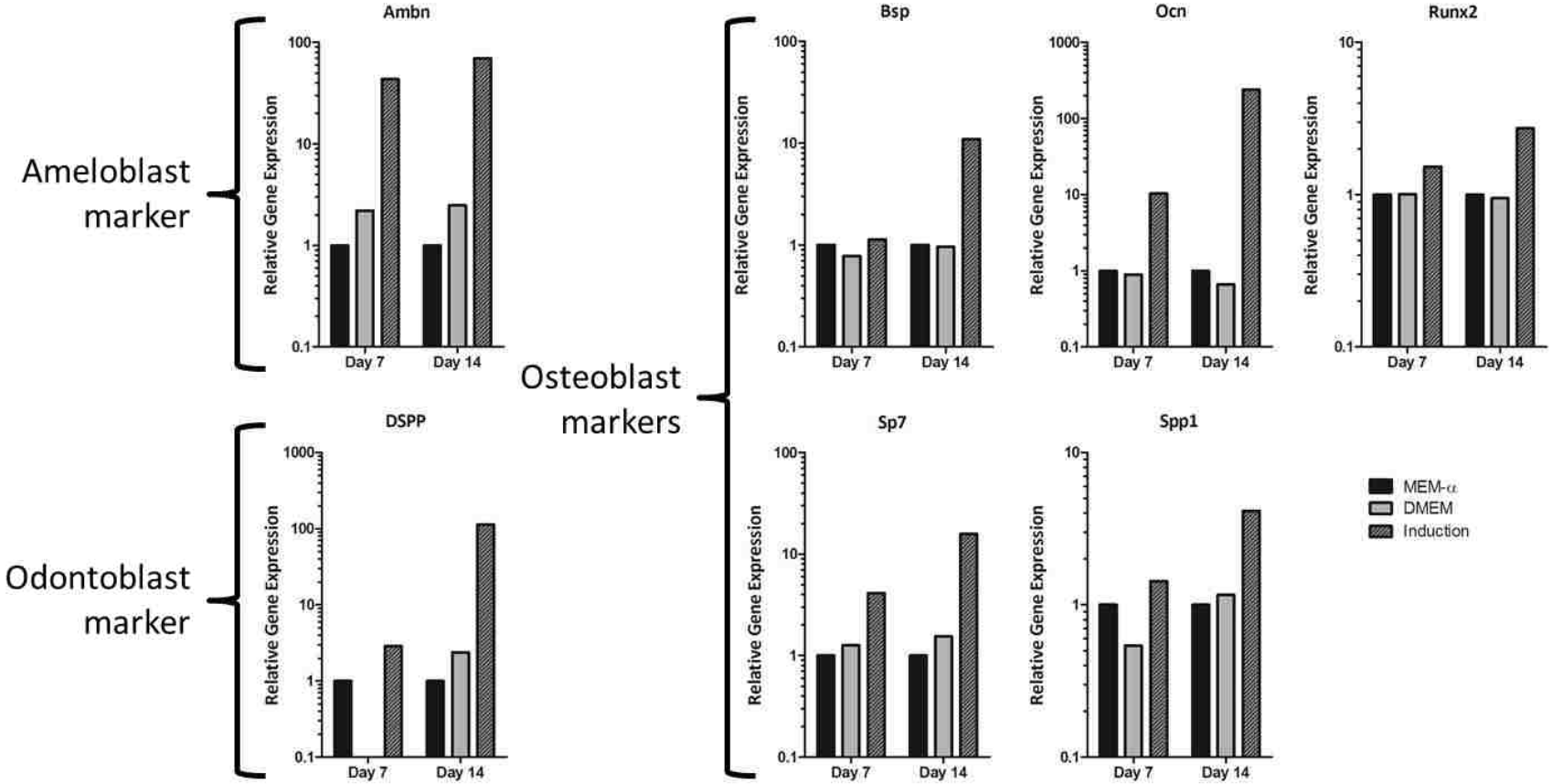

Figure 2.3 Culture of DPSC in induction medium increases the expression of differentiation marker genes as determined by real-time RT-PCR

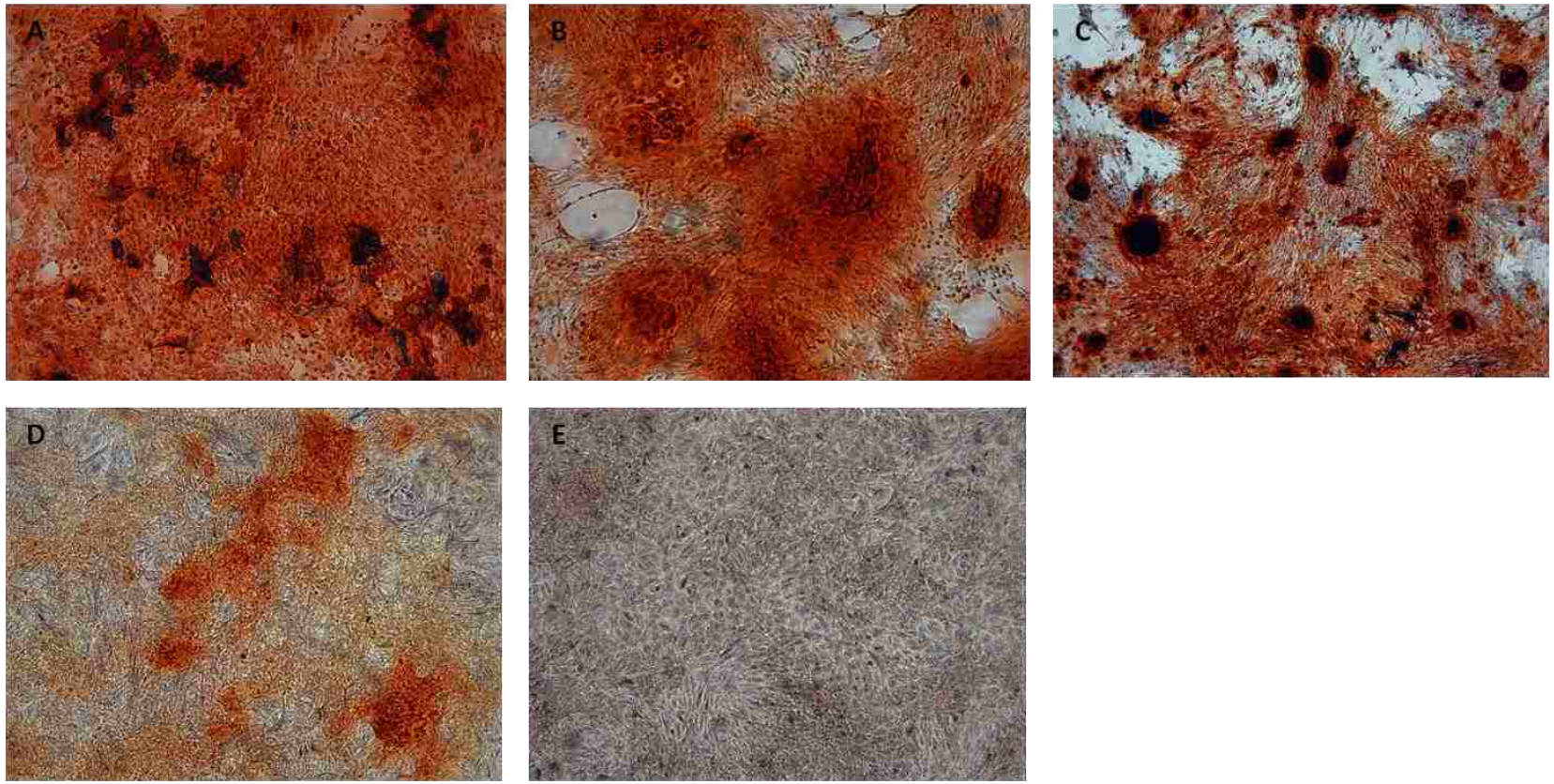

Figure 2.4 Reduced differentiation after induction of DPSC across serial passages, as determined by calcium deposition with Alizarin red staining. Note that the gradual reduction of calcium-deposition in passages 3, 5 and 7 (A, B and C), and sharp decrease of staining in passages 9 (D) and no staining at passage 11 (E). 50x magnification

degree of staining was graded on a 0-4 scale as described in Table 2.1. Non-parametric statistical analysis was conducted using the SAS program for analysis of variance (ANOVA) and least significant difference (LSD). The results are presented in Figure 2.5. The results showed that p3 cells had a strong differentiation potential. Differentiation potential was somewhat reduced in p5 with no statistical difference, but significantly reduced at p7 (Figures 2.4, 2.5). However, by p9 and p11 their differentiation potential was dramatically reduced. Generally, no Alizarin red staining could be seen in p11 DPSC after 14 days of induction, suggesting that the DPSC had totally lost their differentiation capability by this passage. Based on the results of differentiation potential, we classified p3 to p5 DPSC as early-passage DPSC, which possess strong differentiation capability; and p9 to p11 as late-passage DPSC with significantly and greatly reduced differentiation. capability. DPSC at p7 appeared as a transition passage before complete loss of differentiation capability.

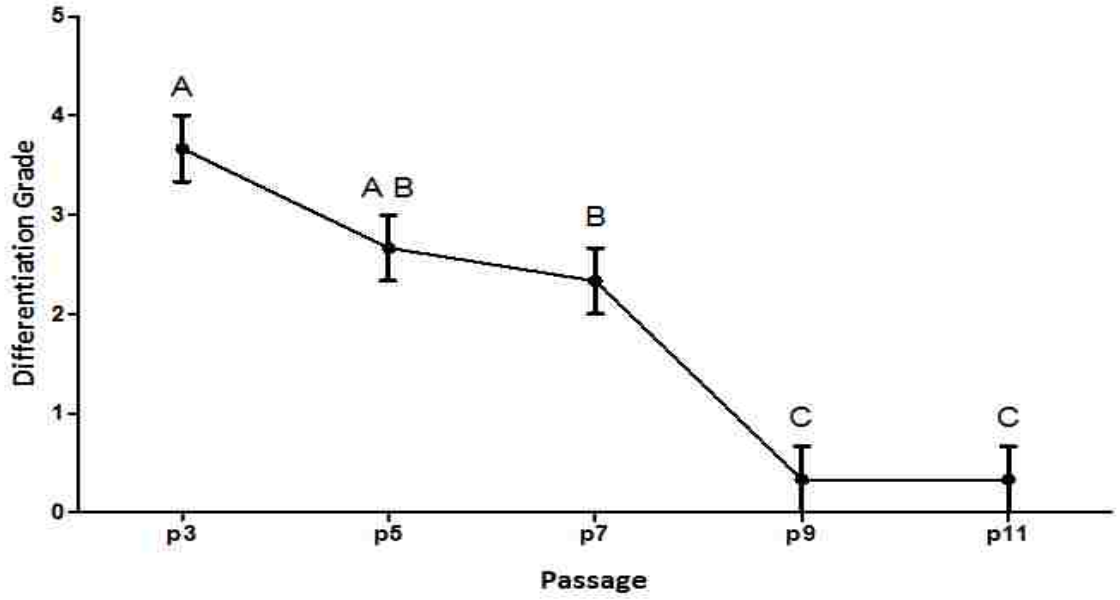

Figure 2.5 Reduction of differentiation capability in DPSC as cell passage increases. Note that cells significantly reduced differentiation at p7 as compared to p3, and almost completely lost differentiation after p9. Groups that do not share the same letter are statistically significant at $P<0.05$ (mean ± SEM, n=3).

2.3.4 Loss of Differentiation in DPSC Was Not Due to Overgrowth of DPC

The above experiments showed that DPSC gradually reduced their differentiation capability during *in vitro* culture, and by p11 they no longer responded to differentiation induction, which is similar to non-stem cell DPC. Because established DPSC were heterogeneous, likely to contain non-stem cells in the population, there was a concern if the loss of differentiation seen in the late passage DPSC was due to overgrowth of non-stem cell DPC. To address this concern, we observed that the morphology of the two cell populations remained distinct, as depicted in Figure 2.6. We further compared the expression of some stem cell marker genes in early and late passages DPSC vs. DPC under the same culture condition. These results are shown in Figure 2.7. We found that many of these marker genes, including *Esrrb, DMP1, Musashi,* and *Kit ligand* (*KitL*), retained a high level of expression in late-passage DPSC, which were similar to early passage. More importantly, expression of those genes was generally more than 10 to 100-fold higher in late-passage DPSC as compared to DPC (Figure 2.7), suggesting that the late-passage DPSC and non-stem cell DPC were distinct populations.

Comparing the early and late passages of DPSC, we found no significant changes in expression of most of the stem cell marker genes tested such as *Esrrb, DMP1, Musashi,* and *KitL*. However, significant changes of expression of *ALP, Notch1,* and *BCRP* were observed when comparing the early passage to late passage. Of these, decreased expression of *ALP* and *BCRP* in the late passage was observed.

2.3.5 Expression of Transcription Factors in Different Passages of DPSC

To study the possible transcription factors (TFs) that may be responsible for the loss of differentiation in DPSC, a total of 30 TFs associated with stem cell function (see Table 2.2) were screened for expression of the transcription factors and selected stem cell related genes in the

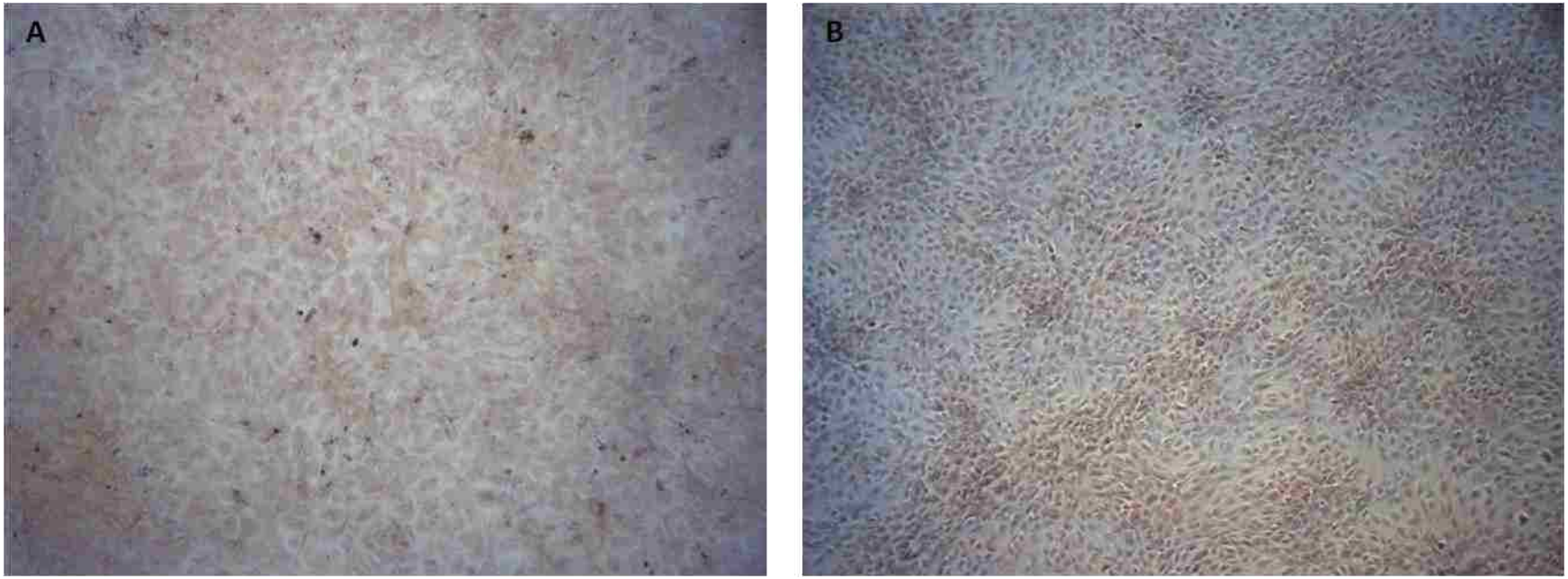

Figure 2.6 Comparing the morphological differences between late-passage DPSC (A) and non-stem DPC (B). 50x magnification

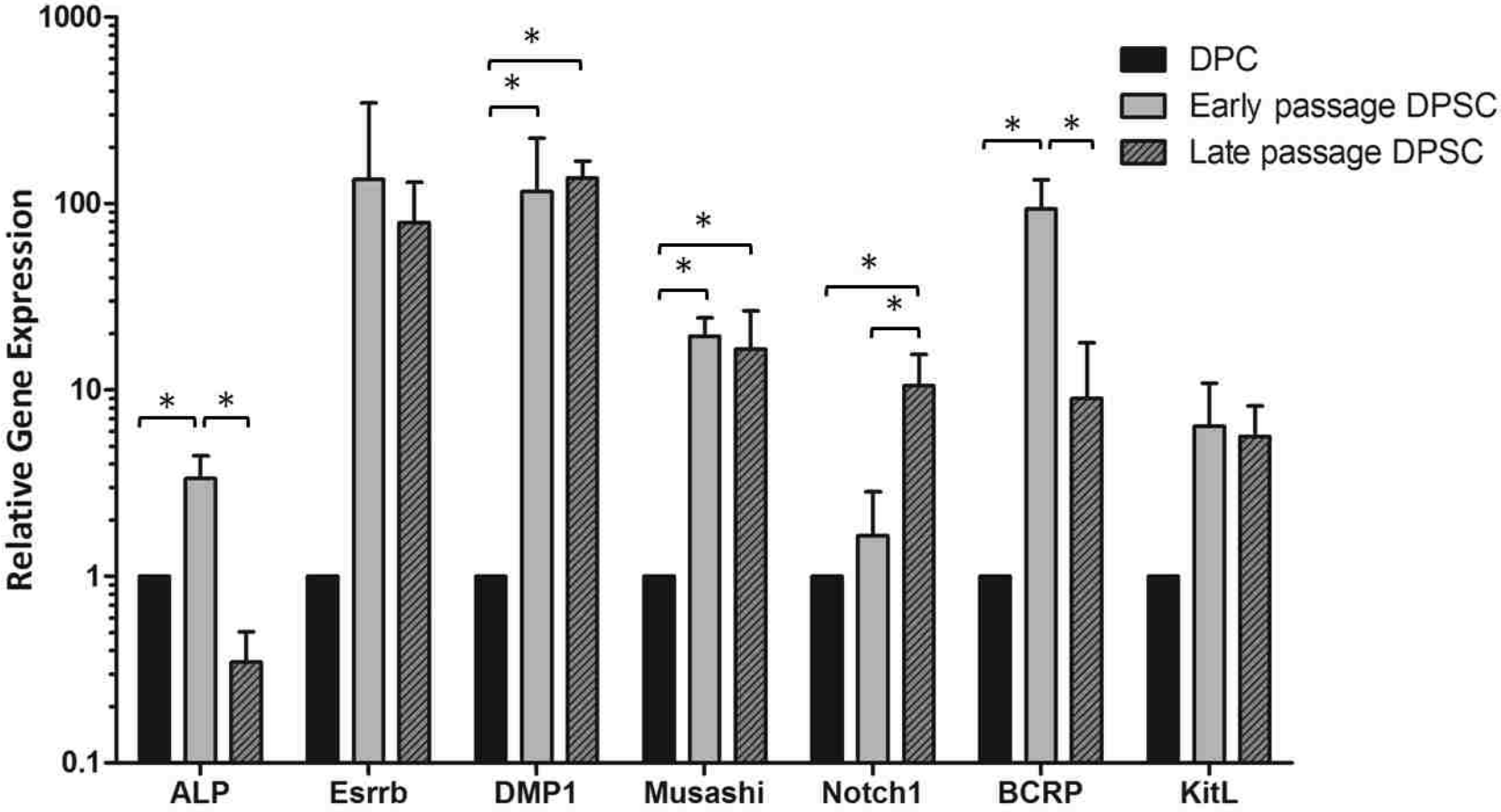

Figure 2.7 Expression of stem cell marker genes in early, late-passage DPSC and DPC. RGE was normalized to gene expression in DPC (Y-axis is $\log_{10}$ scale, mean ± SEM, n=3, * indicates statistical significance of $p < 0.05$).

early and late passage DPSC using real-time RT-PCR. Our objective was to determine a change in expression of these genes in late passage DPSC when compared to the earlier passages. The majority of the genes screened did not show any noticeable change between early and late passage DPSC (data not shown). The most notable decrease-in-expression genes in late-passage DPSC are displayed in Figure 2.8. Although the genes *Klf5*, *Tcfcp2L1*, and *Zfp462* showed differences, no statistical significance was detected in those genes by ANOVA and LSD. We observed huge variations in expression of some of the genes among different batch of DPSC as reflected by large error bars (Figure 2.8).

2.4 DISCUSSION

Alizarin red has been used to stain calcium deposition in many labs. When subjecting early-passage DPSC to differentiation induction medium, strong calcium deposition was detected after 2 weeks of culture as revealed by Alizarin red staining, suggesting that the cells had differentiated into calcium-depositing cells. Because osteoblasts, odontoblasts, and ameloblasts are all capable of depositing calcium, Alizarin red staining alone could not determine if the DPSC were induced to differentiate into a particular type of calcium-depositing cells. Thus, we examined the expression of marker genes in these DPSC-derived calcium depositing cells, and we found that expression of the markers for osteoblasts, odontoblasts, and ameloblasts was dramatically increased after differentiation induction. Increased expression of these differentiation markers indicates that DPSC were capable of differentiating into osteoblasts, odontoblasts, and ameloblasts. Studies by others have shown that colonies derived from human DPSC show a diversity in cell surface markers, suggesting that DPSC cultures are heterogeneous, containing different populations of cells [1]. Thus, it is also possible that

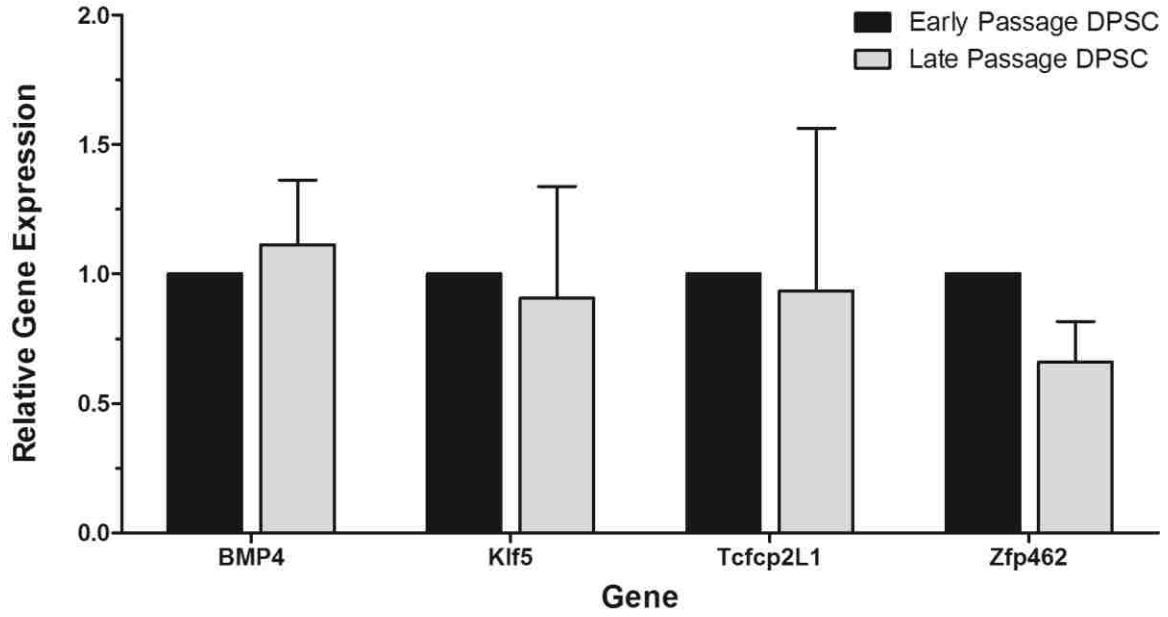

Figure 2.8 Comparison of the expression of TFs and stem cell-related genes in early and late-passage DPSC. No statistically significant change in expression of the transcription factors was observed (mean ± SEM, n=6)

osteoblastogenesis, odontoblastogenesis, and ameloblastogenesis are the result of differentiation of different subpopulations of stem cells existing in DPSC.

Loss of differentiation has been reported in long-term culture of stem cells derived from different types of tissues. This cellular aging has been observed in hematopoietic stem cells in mice [24]. In humans, bone marrow stromal/stem cells (BMSC) demonstrate a change in morphology, from a fibroblast-like spindle shape to a flat, broadened shape, after 34 – 42 doublings *in vitro*, resulting in decreased differentiation of these cells [25]. Many studies reported that BMSC largely lose their *in vitro* differentiation capability around p6 [26, 27], but some studies have shown that differentiation reduction could occur as early as the first [28] or second passage [29]. Wall et al. found that stem cells derived from human adipose tissue lose the ability to differentiate into adipocytes as cells reach p10 [30]. It should be noted that not all ASC experience this loss of function at the same rate. For example, MSC isolated from human

umbilical cord can be grown *in vitro* for a longer period than those isolated from bone marrow [31]. This study revealed that, similar to other ASC, DPSC start to reduce differentiation capability at p7, and largely or completely lose differentiation around p11 (Figures 2.4, 2.5).

By p11, DPSC were not capable of differentiating into calcium depositing cells (Figures 2.4, 2.5) when subjected to induction. However, the expression of stem cell markers remained constant in early and later passages, and expression was much higher than in non-stem cell DPC. The results of our experiments indicate that the cells did not undergo spontaneous differentiation in the medium without ascorbic acid, dexamethasone, and β-glycerolphosphate. This result was inconsistent with the observations of Yu et al, who reported that DPSC underwent spontaneous differentiation into osteoblasts [16]. After reviewing the experiments of Yu et al, we found that although they used similar medium for establishing and maintaining DPSC, their medium contained ascorbic acid and bovine pituitary extract [16]. Ascorbic acid is one of the reagents used for inducing osteogenic differentiation [18]. Thus, it is likely that supplementation of ascorbic acid in the medium induced differentiation, while the effect of bovine pituitary extract could not be excluded.

Individual colonies derived from single cells in the DPSC tested positive for different surface proteins, suggesting that DPSC are a heterogeneous cell population, containing different cell types [1]. Because the majority of the cells in the dental pulp are non-stem cells, it is not surprising that the primary isolated DPSC contained non-stem cells. One may ask if the reduction or loss of differentiation seen in the late passages was due to overgrowth of non-stem cells, such that they might become predominant in the late-passage DPSC population after long-term culture. To address this concern, we compared the expression of common stem cell markers and found that most markers did not significantly change their expression in later passages, as

compared to early passage. More importantly, comparing the late passage DPSC to their counterpart non-stem cell DPC, we found that expression of these stem cell marker genes was much higher in late-passage DPSC than in DPC (Figure 2.7). In addition, they also displayed distinct cell morphology (Figure 2.6). Thus, the results of this study provide evidence that the loss of differentiation potential in the late passage DPSC was not due to overgrowth of non-stem cells, and loss of differentiation capability was likely due to intrinsic changes.

To further explore the intrinsic changes in DPSC that may cause the loss of differentiation, we studied the expression of 30 stem cell-related TFs in the early and late passages of DPSC, and found that none of them showed significant changes between these two passages, suggesting that TFs may not be the key factors causing the loss of differentiation during long-term culture of DPSC.

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CHAPTER 3: WHOLE TRANSCRIPTOME ANALYSIS OF EARLY AND LATE PASSAGES OF DENTAL PULP STEM CELLS TO IDENTIFY POTENTIAL GENES CAUSING LOSS OF DIFFERENTIATION IN LONG-TERM CULTURE

3.1 INTRODUCTION

In Chapter 2, we determined that the differentiation ability of DPSC is reduced as cell passage increases. Established DPSC at early passages, such as passage 3 and 5 (p3 and p5) possess strong differentiation capability, but as these cells progress they completely lose that differentiation ability at passages 9 to 11 (p9 to p11). We conducted experiments to prove that the loss of differentiation capability was caused by intrinsic changes in DPSC during passaging. Thus, we analyzed the expression of 30 stem cell-related transcription factors (TFs), but found that late passage DPSC did not significantly change expression of these TFs when compared to those in early passages. In order to identify genes causing the loss of differentiation, we decided to compare the transcriptomes of early and late-passage DPSC. The objectives of this chapter were (a) to determine what genes were significantly down-regulated in late-passage DPSC when compared to the early-passage, and (b) to identify candidate genes that might be involved in regulating differentiation of DPSC. These objectives were accomplished by using whole genome microarray, real-time RT-PCR, and gene knockdown techniques.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture

DPSC were established using a published protocol for isolation of dental stem cells [1]. Briefly, dental pulps were isolated from day six rat pups and trypsinized to obtain cell suspension. For DPSC culture, the cells were grown in α-MEM with 20% fetal bovine serum (FBS). Cells were then incubated at 37°C and 5% CO_2 in humidified incubators. At 90% confluence, cells were detached using trypsin-EDTA and passaged to a fresh T-75 flask with an initial density of 200,000 cells per T-75 flask.

3.2.2 Whole-Genome Microarray Analysis

Whole-genome microarrays provide a powerful tool to study the transcription of the entire genome, known as the transcriptome, in a given cell population. To compare the transcriptomes of the early and late passages of DPSC, whole-genome microarray analysis was employed to obtain the gene transcription profile of p3 and p11 DPSC. For microarray study, cells were cultured and passaged as described above in Section 3.2.1 until designated passages. P3 and p11 cells were seeded in T-25 flasks and grown to confluence. Next, cells were collected and processed for RNA isolation, as described in Section 3.2.5. Concurrently, the cells were seeded to 6-well plates and subjected to differentiation analysis, as described in Section 3.2.4, to ensure strong differentiation capability of p3 cells and loss of differentiation of p11 cells.

Microarray analysis was conducted by Phalanx Biotech (Belmont, CA) using a Rat OneArray containing long oligonucleotides (~60-mer in length) to detect 24,358 transcripts. The microarray also includes 980 control oligonucleotides for validation and normalization of the data. Microarray analysis was run in triplicate for each RNA sample. Raw signal intensity of each transcript was obtained and analyzed. Data was obtained from two sets of DPSC samples.

3.2.3 Analysis of Microarray Data

A generous amount of gene expression data were generated from whole genome microarray analysis. Proper analysis and data mining was critical. There are two basic types of data mining methods for whole genome microarray studies, as described by Schulze et al. [2] and schematically shown in Figure 3.1. Because Method A (Figure 3.1; Section A) was proposed to answer specific biological questions, and our biological question was to find the gene whose expression was down-regulated in late-passage DPSC, this method was used for analyzing and mining the microarray data in this study.

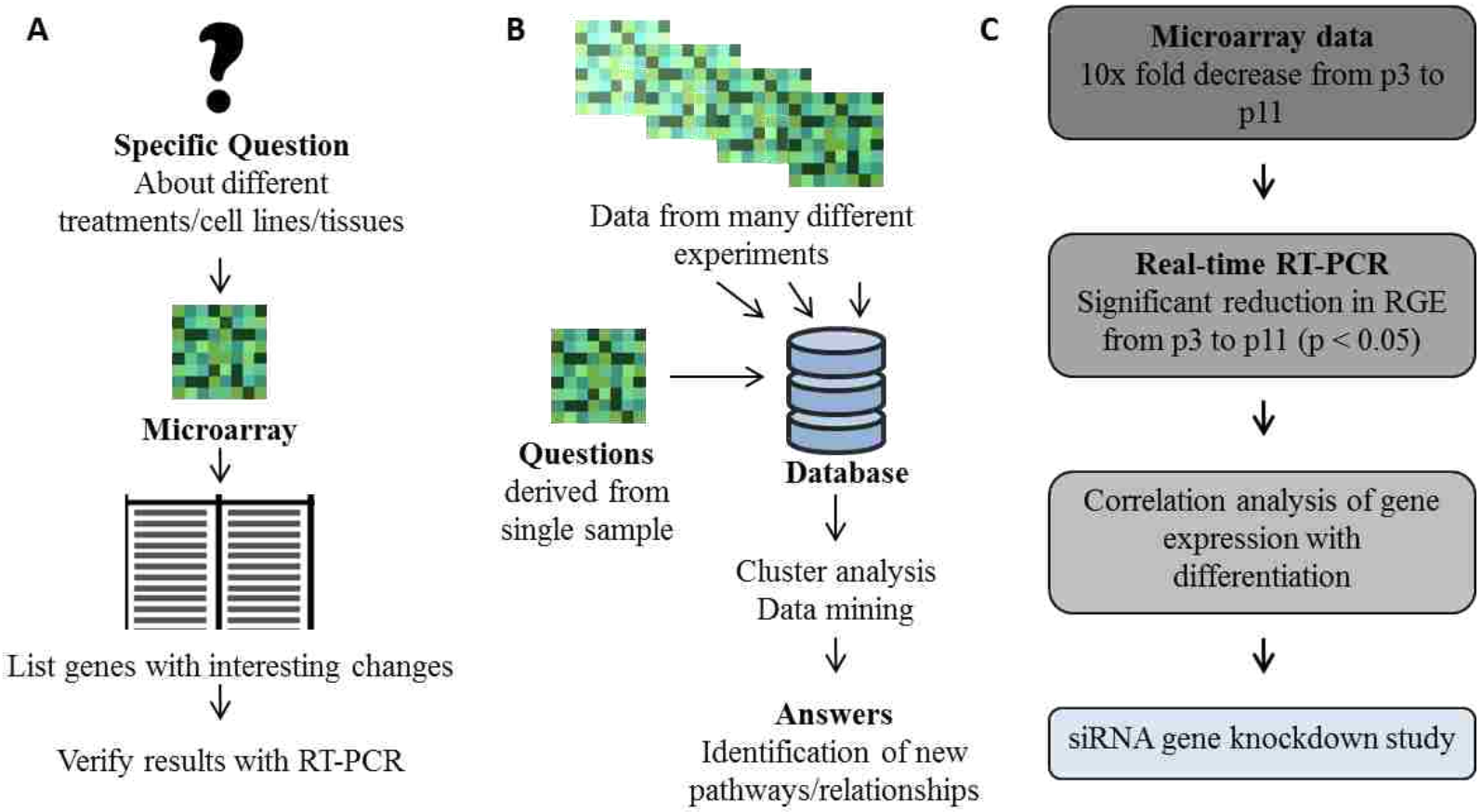


Figure 3.1 Data mining in whole genome microarray analyses and schematic flowchart to determine the candidate genes in this study. The method to analyze data from whole-genome microarrays can be classified into two archetypes. (A) Microarray analysis which compares expression patterns between two experimental samples to address specific questions. (B) Microarray data from experimental samples are compared to a vast database from collection of other microarrays to discover new gene pathways and relationships. (C) The flowchart used in this chapter to identify candidate genes involved in loss of differentiation in DPSC.

In order to process the large quantities of information obtained from the microarray, we sought to reduce the overall population of genes to a smaller population by way of establishing more rigorous criteria for selection of the genes. Our post-microarray analysis followed a similar pattern described by Schulze et al. [2], and our criteria has been outlined in Figure 3.1. Briefly, we first compared signal intensity levels between the two separate sets of DPSC samples. We sought to eliminate those genes that did not display a large decrease in microarray intensity from early to late passage. After doing so, we used real-time RT-PCR to verify the changes of expression observed in the microarray data. Next, correlation analysis was conducted to determine the correlation between differentiation and gene expression. Finally, siRNA was used to knockdown the expression of selected genes to determine if they possessed the effect to regulate differentiation of DPSC.

3.2.4 Differentiation of DPSC

Cells were seeded either to sterile 6-well plates at a rate of 5×10^4 cells/well, or to sterile T-25 flasks at a rate of 1.5×10^5 cells/plate prior to differentiation treatment. Induction medium was prepared using low glucose DMEM supplemented with 10% FBS, 0.05 mM ascorbic acid, 100 nM dexamethasone, and 10m M β-glycerolphosphate [3]. Cells were incubated in this induction medium for 14 days prior to staining with Alizarin red S to detect calcium deposition [4]. Cells were also collected for RNA isolation to analyze gene expression.

3.2.5 RNA Isolation

Cells were collected by trypsinization and pelleted by centrifugation. The pellet was lysed using 350μL Buffer RLT (Qiagen). Next, RNA was isolated from the lysate using an automated Qiagen RNeasy DNAse Digest protocol on a Qiacube instrument (Qiagen). Briefly, 70% ethanol was added to the sample at a 1:1 ratio and the mixture was transferred to an RNA-

binding spin column. The bound RNA was then washed before incubating in DNAse I reagent for 15 minutes (Qiagen). The column was washed again, and RNA was eluted with 30 μL nuclease-free water. After isolation, RNA concentration was measured using a Nanodrop 8000 (Thermo). For microarray analysis, RNA Integrity was measured using an RNA 6000 Nano LabChip Kit (Agilent Technologies), per the manufacturer's instructions, using an Agilent 2100 Bioanalyzer. RNA samples that met the following requirements were used for microarray analysis:

1. Minimum RNA integrity number (RIN) of 8.0
2. OD260/280 > 1.8 and OD260/230 > 1.8
3. Minimum RNA quantity of 6 μg in a concentration greater than 200 ng/μL

3.2.6 Real-Time RT-PCR

Template cDNA was generated from isolated RNA using M-MLV reverse transcriptase (Invitrogen) with the same methods as described in Chapter 2 (2.2.6). Briefly, 1000-2000 ng sample RNA was mixed with 250 ng random primer and heated to 65°C for five minutes. Reverse transcriptase was added, and the reaction was incubated for 50 minutes at 37°C before deactivation by incubating at 70°C for 15 minutes. The cDNA were stored at -20°C until ready for analysis. The primer sequences used for PCR are listed in Table 3.1. All reactions were conducted in 25 μL per reaction containing 1-2 μL cDNA with an ABI 7300 sequence detector (Applied Biosystems). We used the SYBR real-time PCR to obtain cycle threshold (C_T) values for calculation of relative gene expression (RGE). *β-actin* was used as control to obtain ΔC_T and $\Delta\Delta C_T$ was calculated as described in Chapter 2. RGE was determined using the following formula: $RGE = 2^{-(\Delta\Delta Ct)}$[5].

Table 3.1 Real-time PCR primers used in this chapter

Gene	RefSeq	Primers
Acad9	NM_181768.2	F: 5'-CTTGGGGAGATCATCAGCAT-3' R: 5'-GCAGATACTTGGCCTTCTGC-3'
Alx1	NM_012921.1	F: 5'-GATGCCAGAGAAGAGCGAAC-3' R: 5'-TAGCTGCAAACTGGTGAACG-3'
Apbb1ip	NM_001100577.1	F: 5'-GAACCATCCAGGCTCAGAAA-3' R: 5'-CAGCAGCATTTGCTCCATAA-3'
Arhgap20	NM_213629.1	F: 5'-TCGGACTCTGCTGATTGATG-3' R: 5'-ATTTTGGCCGAGACAAACAG-3'
Clgn	NM_001109472.1	F: 5'-AGATCGGATGTGGAGAATGG-3' R: 5'-AGGACTCCAGATTCCCTGGT-3'
Colec11	XM_002729610.1	F: 5'-TTTATCCCCCTTGTGAGACG-3' R: 5'-CCACTGTTTGCTAGCTGCTG-3'
Egflam	NM_001108938.2	F: 5'-TAAAGCCCTGTGGACCAAAC-3' R: 5'-TCCAGAGCGTGCACTGATAC-3'
Eya2	NM_130427.1	F: 5'-TTGGTCACCACCACACAACT-3' R: 5'-CAGCTCTCCTTGCCTGTCTT-3'
Fam159b	XM_574844.3	F: 5'-GGCTTCGCAGACCTCAAGTA-3' R: 5'-CAGCAATTCCCAATCCAATC-3'
Gipc2	NM_001037210.1	F: 5'-CACTTGGACTCACCATCACG-3' R: 5'-ATTCGATATGATCCCCCACA-3'
HspB8	NM_053612.2	F: 5'-TCTCCAGAGGGTCTGCTCAT-3' R: 5'-GCAGGTGACTTCCTGGTTGT-3'
PCOLCE2	NM_001127640.1	F: 5'-AGGACGCCTTGAAAAACCTT-3' R: 5'-GCTGGTTCTTCGGAGCTATG-3'
Sial	NM_012587.2	F: 5'-TACGGGGTAGAGACCACAGC-3' R: 5'-TCGTCGCTTTCCTTCATTTT-3'
Slc6a15	NM_172321.1	F: 5'-TGGATTCGCAAGCTGTGTAG-3' R: 5'-CCAAGGGAGAGGTTGTTGAA-3'
Tcfap2c	NM_201420.2	F: 5'-CTGAGAACCTAGGGCTGCAC-3' R: 5'-GGTCCTTTGCGAATGACAGT-3'
Thbd	NM_031771.2	F: 5'-TATGACAAGCGAGGGTAGGG-3' R: 5'-GGGACACTCTGGGATCTTCA-3'
Wif1	NM_053738.1	F: 5'-GGAGGGACCTGTTTTTACCC-3' R: 5'-ATGCATTTACCTCCGTTTCG-3'
Zfp423	NM_053583.2	F: 5'-CGACTCCGGGATCATAACAT-3' R: 5'-TCCGAGAAGAAAGTCCGAGA-3'

3.2.7 Statistical Analyses

Beginning in p3, serial passages of DPSC were grown through p11, and cells were collected for RNA isolation in early and late passages (i.e. p3, p5, p9, and p11). Potential candidate genes were selected from the microarray results obtained from the microarray analysis

described in Section 3.2.3. Relative gene expression (RGE) of those genes was measured using real-time RT-PCR to compare p3 to p11. Correlation of gene expression and differentiation was calculated by performing Spearman correlation analysis between the RGE value and differentiation observed (see Figure 2.5, page 32). SAS program version 9.2 was used for the correlation analysis.

All other statistical analyses were performed using SAS 9.2 using the GLM ANOVA procedure. Statistical significance was determined by comparing for least significant difference (LSD), with $p < 0.05$ being statistically significant.

3.2.8 Gene Knockdown Experiment

To determine if expression of candidate genes had any effect on differentiation, we used siRNA-mediated knockdown to reduce the expression of the candidate genes in early-passage DPSC. We designed and ordered dicer-substrate siRNA (DsiRNA) oligos using the online RNAi Design Tool, available on Integrated DNA Technologies's website [6]. A list of the siRNA sequences used in this experiment can be found in Table 3.2. These siRNA were reconstituted to a concentration of 100 μM and diluted to a working stock concentration of 10 μM for cell transfection, as detailed below.

Approximately 30 minutes prior to transfection, all culture medium was aspirated from the cultures and replaced with 2 mL fresh stem cell media in each flask or well. The Lipofectamine RNAiMax Transfection reagent (Invitrogen) was used to transfect DPSC with DsiRNA. Briefly, 9 μL of 10 μM DsiRNA stock was added to 150 μL OptiMEM. Concurrently, 9 μL of RNAiMax reagent was added to a separate 150 μL volume of OptiMEM. The two solutions were then mixed at a 1:1 ratio and incubated at room temperature for five minutes to form the lipofection complex. Immediately following incubation, 250 μL of the appropriate

Table 3.2 Sequences used for DsiRNA transfection

Gene	RefSeq	Sequence*
Gipc2	NM_001037210.1	Antisense: 5'-rGrUrCrCrUrArUrUrUrCrArArArUrGrCrCrUrUrCrUrUrGrGrGrUrU-3' Sense: 5'-rCrCrCrArArGrArArGrGrCrArUrUrUrGrArArArUrArGrGAC-3'
HspB8	NM_053612.2	Antisense: 5'-rGrGrArGrArCrArArUrCrCrCrArCrCrUrUrCrUrUrGrCrUrGrCrUrU-3' Sense: 5'-rGrCrArGrCrArArGrArArGrGrUrGrGrGrArUrUrGrUrCrUCC-3'

*The sequences listed are RNA sequences, with "r" indicating the ribose in between nucleotides.

complex was added to each flask or well, and the cells were incubated at 37°C for 24 hours. After incubation, the medium containing the lipofection complex was removed and fresh stem cell culture medium was added. Cells were cultured normally after this.

To determine knockdown efficiency, cells were collected every four days until day 13 post-transfection for total mRNA isolation. We then used real-time PCR to determine the relative expression levels of these genes (RGE) when compared to a transfection control concurrently with each treatment groups. The primers for real-time PCR are listed in Table 3.1.

To determine if knockdown of the candidate genes decreases differentiation ability of DPSC, differentiation induction medium was added to the cells 48 hours after initiating transfection. The induction medium was prepared using low glucose DMEM supplemented with 10% FBS, 0.05 mM ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerolphosphate [3]. Cells were incubated in induction medium for 14 days prior to staining with alizarin red S [4].

3.3 RESULTS

3.3.1 Comparison of the Transcriptomes of Early and Late Passages of DPSC

There were 76 genes showing at least a two-fold differential expression in p3 and p11 DPSC. Of the 76 genes, 34 genes had decreased their expression more than 10-fold in p11, as

compared to p3. The detailed fold-differential expression of these genes from microarray analysis can be found in Table 3.3. We selected 18 genes from these 34 genes, based on their displaying increased expression in p3 consistent in both microarray data sets, for further analysis using real-time RT-PCR with primers listed in Table 3.1. A list of these 18 genes and their fold changes in the microarray analysis can be found in Table 3.4.

Table 3.3 Microarray genes with greater than 10-fold change in intensity

Change in Intensity	Gene
> 100 fold	*Egflam*
100 fold – 50 fold	*Acad9, Thbd*
50 fold – 30 fold	*Colec11, Myh11, Sial, Slc6a15, Tmem117*
30 fold – 15 fold	*Alx1, Ccdc64, Cyp27a1, Eya2, Fam159b, Hspb8, Tc2n, Tcfap2c, Wif1, Zfp423*
15 fold – 10 fold	*Abcg2, Apbb1ip, Arhgap20, Cdh10, Clgn, Cyp2j3, Fgf12, Gipc2, Khdrbs3, LOC100360067, Msx2, Olr377, PCOLCE2, Pls1, Reln, Slpi*

3.3.2 Confirmation of Expression of Selected Genes with RT-PCR

Real-time RT-PCR was conducted to determine the expression of the 18 genes in p3 and p11 DPSC. Relative gene expression (RGE) was calculated from the C_T values by normalizing to p3 as a control, with RGE of 1. RGE values less than 1.0 indicated decreased expression when compared to p3. While Acad9 displayed an upward trend that contradicted the microarray data (Figure 3.2), most of the selected genes reduced their expression as cell passages increased to p11, however, some of them were not statistically significant (Figure 3.2). The following 13 genes displayed significant reduction in expression in p11 when compared to p3: *Apbb1ip*, *Clgn*, *Colec11*, *Eya2*, *Fam159b*, *Gipc2*, *HspB8*, *Pcolce2*, *Slc6a15*, *Tcfap2c*, *Thbd*, *Wif1*, and *Zfp423* (Figure 3.2). These genes were selected for correlation analysis to determine the correlation between differentiation and expression of those genes in different passages.

Table 3.4 Genes identified from microarray and selected for real-time RT-PCR analysis and their fold-change in signal intensity comparing p3 to p11 in two sets of RNA samples

Gene	RefSeq	Known Function	Fold Change (p3/p11 Set 1)	Fold Change (p3/p11 Set 2)
Acad9	NM_181768.2	Mitochondiral protein associated with fatty acid digestion	74.4	34.6
Alx1	NM_012921.1	Alias "CART1," embryonic gene expressed in craniofacial development	21.7	13.4
Apbb1ip	NM_001100577.1	Alias "RIAM," Interacts with Rap1-GTP, promotes integrin-mediated adhesion	13.9	54.0
Arhgap20	NM_213629.1	Inactivates Rho protein, causing neurite outgrowth	12.8	10.4
Clgn	NM_001109472.1	Molecular chaperone protein, primarily associated with spermatogenesis	14.3	23.2
Colec11	XM_002729610.1	Strongly expressed embryonically in craniofacial cartilage, heart, kidney, & vertebral bodies	35.5	313.5
Egflam	NM_001108938.2	Alias "Pikachurin," ECM protein associated with photoreceptor synapse formation	134.9	10.5
Eya2	NM_130427.1	Transcriptional coactivator, regulates physiological hypertrophy	19.1	88.1
Fam159b	XM_574844.3	Sequence with unknown function	18.9	25.4
Gipc2	NM_001037210.1	Associates with TGF-β receptors, Wnt pathway messenger	12.8	12.1
HspB8	NM_053612.2	Alias "Hsp22," Small heat-shock protein family member, expressed in motor neurons & cardiac tissue	17.7	6.2
PCOLCE2	NM_001127640.1	Binds to BMP1 to enhance the lysis of Proapolipoprotein	13.7	10.9

(Table 3.4 continued)

Gene	RefSeq	Known Function	Fold Change (p3/p11 Set 1)	Fold Change (p3/p11 Set 2)
Sial	NM_012587.2	Alias "Ibsp," Encodes BSP, Osteogenic marker, ECM protein which	35.3	8.0
Slc6a15	NM_172321.1	Neuronal transporter protein, used to transport amino acids	47.4	9.4
Tcfap2c	NM_201420.2	Embryonic stem cell gene, expressed primarily in germ cells	26.7	8.1
Thbd	NM_031771.2	Transmembrane protein in endothelial cells, binds to Thrombin to regulate coagulation	83.7	81.5
Wif1	NM_053738.1	Extracellular protein that binds to Wnt and inhibits its effects	18.1	19.8
Zfp423	NM_053583.2	Expressed in preadipocyte stromal tissue prior to adipogenesis	16.1	6.6

3.3.3 Correlation of Gene Expression and Differentiation

We conducted Spearman correlation analysis to determine any correlation between expression of the selected genes and differentiation capability in different passages of DPSC. The results of this correlation analysis are listed in Table 3.5. The individual RGE of each gene, as well as the non-parametric differentiation scores for different passages (p3, p5, p9 and p11), can be found in the Appendix. The correlation analysis showed that *Gipc2* has the maximal correlation with a correlation coefficient of 0.86, followed by *Apbb1ip* and *Fam159b* with correlation coefficients of 0.82. The correlation coefficients of other genes were below 0.80 (Table 3.5).

3.3.4 Gene Knockdown Study

Two genes (*Gipc2* and *HspB8*) were selected for knockdown experiments. *Gipc2* was selected because it had the maximal correlation between its expression and differentiation in

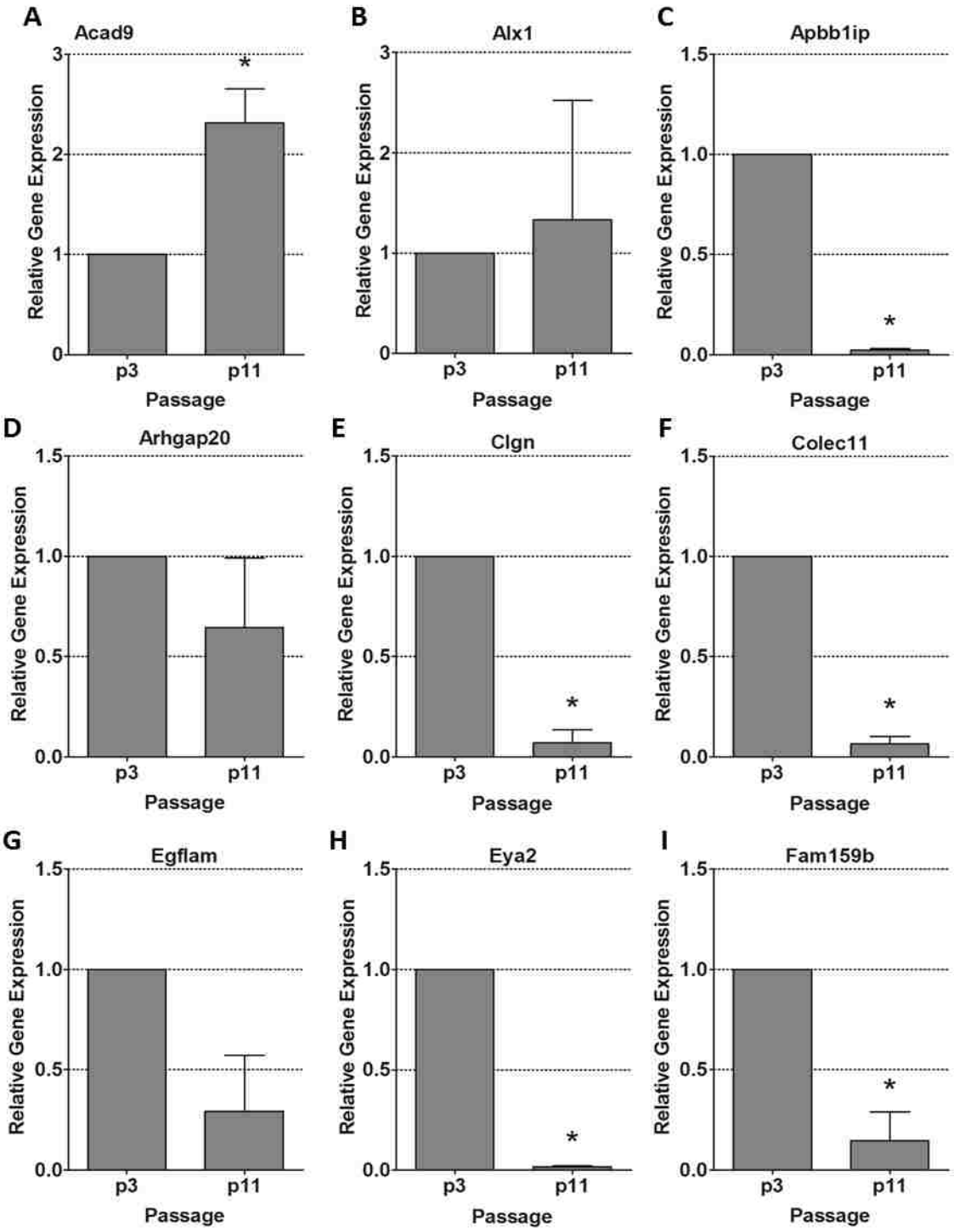
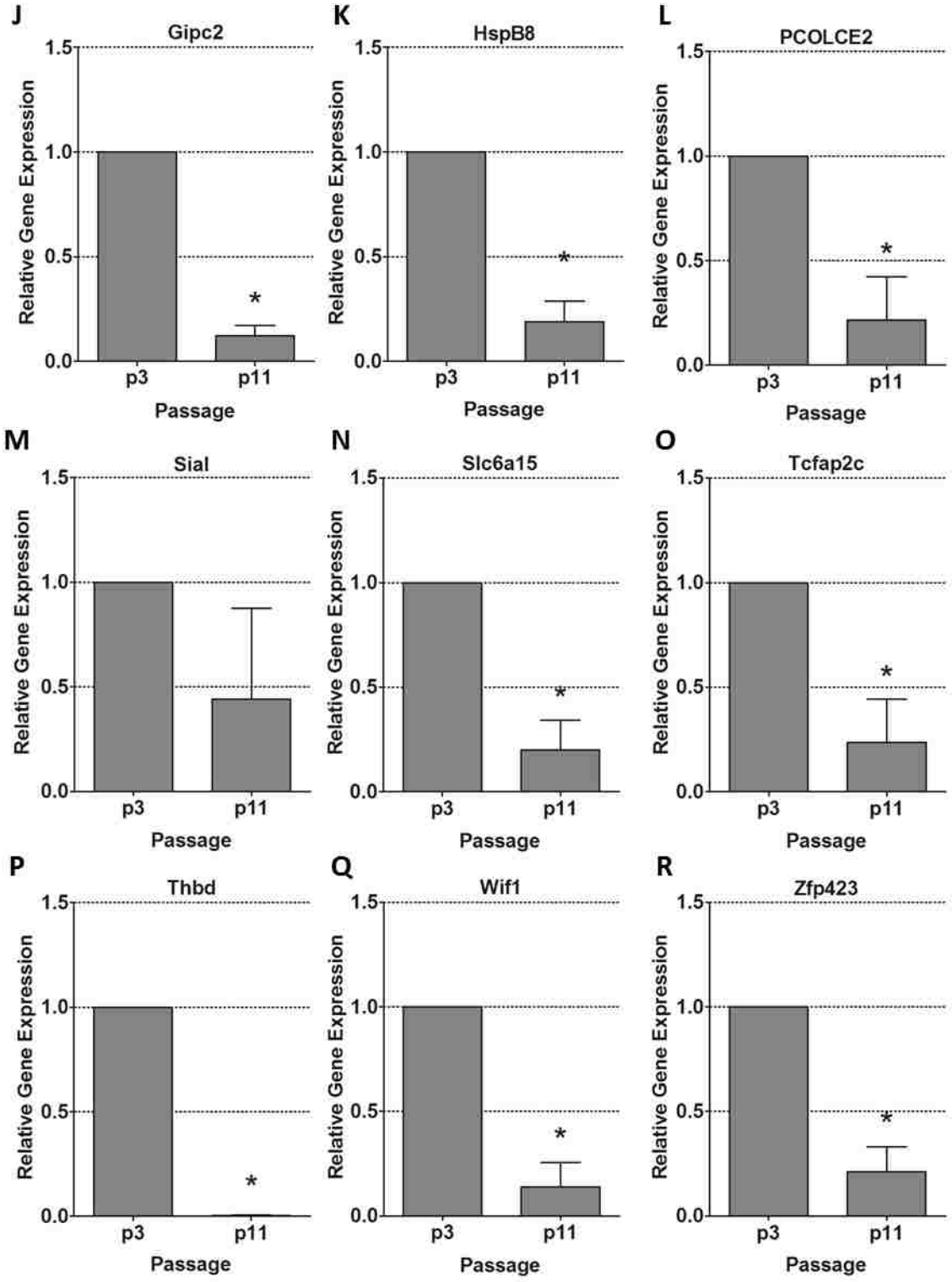


Figure 3.2 Real-time RT-PCR to confirm expression of genes selected from microarray analysis. Comparison of RGE in p3 and p11 DPSC for the genes selected from the initial microarray results. (* indicates statistical significance, $p < 0.05$; n=3). p11 was normalized to p3 which was set as 1.



(Figure 3.2 continued)

Table 3.5 Correlation analysis of differentiation with relative gene expression in different passages of DPSC

Gene	Correlation coefficient
Apbb1ip	0.81989
Clgn	0.76087
Colec11	0.26998
Eya2	0.74080
Fam159b	0.81989
Gipc2	0.85904
HspB8	0.46634
Pcolce2	0.69141
Slc6a15	0.41725
Tcfap2c	0.46634
Thbd	0.72957
Wif1	0.46634
Zfp423	0.26998

different passages. *HspB8* was of interest because dental stem cells appeared to be more thermal tolerant than their non-stem cell counterparts, and we have reported that dental stem cells also expressed higher level of some heat shock proteins than their non-stem cell counterpart [6, 7].

Because 14 days of induction is required for *in vitro* differentiation of DPSC, we conducted a time-course study to check the gene knockdown efficiency. As shown in Figure 3.3, the post-siRNA transfection knockdown effects appear to last through day 13 for both genes tested. The maximum knockdown appeared to be over 90% for up to nine days post-siRNA transfection for both *Gipc2* and *HspB8* (Figure 3.3; Section A). A knockdown efficiency of 65 to 80% was still achieved at day 13, which was statistically significant (Figure 3.3; Section B).

3.3.5 Effect of Gene Knockdown on Differentiation of DPSC

With successful knockdown of gene expression following transfection of early-passage DPSC with siRNA, we tested whether knockdown of the genes would alter differentiation capability. In this experiment, siRNA transfected DPSC were subjected to differentiation induction for 14 days. Alizarin red staining showed that knockdown of *HspB8* resulted in a

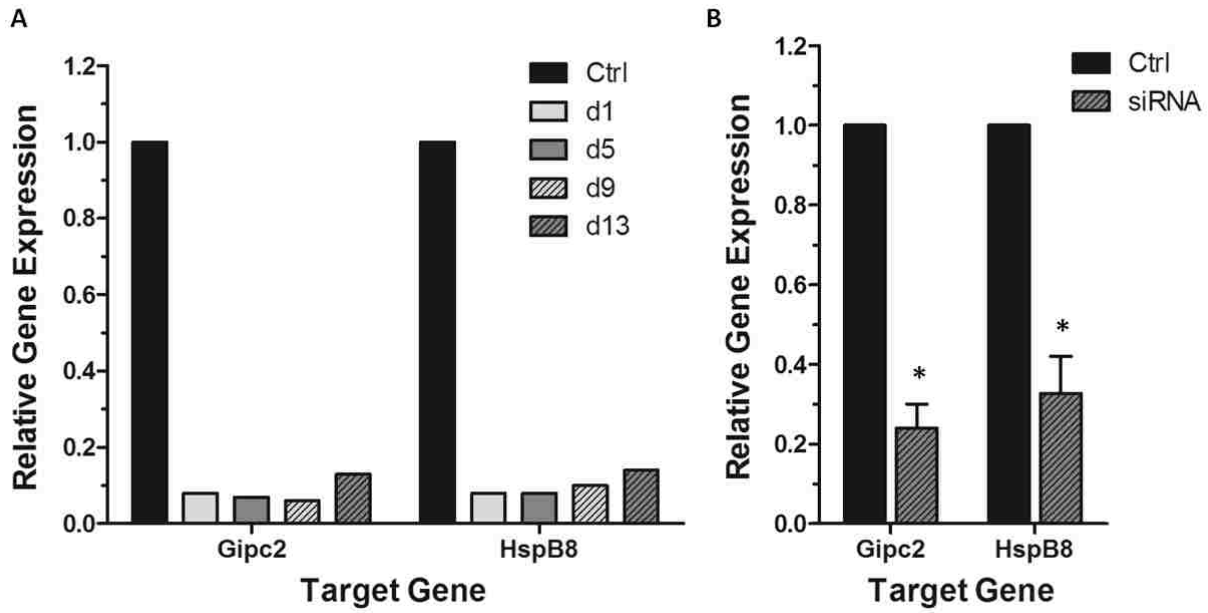


Figure 3.3 Knockdown efficiency of *Gipc2* and *HspB8* expression by siRNA. (A) DPSC were transfected with the appropriate siRNA and cells were collected every four days to determine knockdown efficiency using real-time RT-PCR. siRNA transfected cells displayed a sustained knockdown of Gipc2 or HspB8 through 13 days post-transfection. (B) An average of greater than 65% knockdown was still observed at day 13 after transfection, which was statistically significant.

significant reduction of calcium deposition. In fact, in some replicates, almost no calcium deposition was observed when *HspB8* expression was inhibited, as shown in Figure 3.4. In contrast, knockdown of *Gipc2* appeared to have no effect on calcium deposition, as compared to the transfection control. This experiment suggested that *HspB8* may play a vital role in maintaining differentiation capability of DPSC.

3.4 DISCUSSION

With whole genome DNA microarray, we screened 24,358 transcripts in early-passage and late-passage DPSC. Huge amounts of data were generated from the two sets of DPSC samples. We used the method described by Schulze et al [2] to address our question of mining for candidate genes in causing loss of differentiation during *in vitro* expansion of DPSC.

Figure 3.4 Differentiation of early-passage DPSC after knockdown of the expression of HspB8 and GipC2 as determined by Alizarin red staining. (A) Alizarin red positive calcium deposition shown in the control. (B) Little or no change in calcium deposition was seen in DPSC after knockdown of Gipc2 expression. (C) Knockdown of HspB8 resulted in dramatic reduction of calcium deposition.

Other investigators have found large variability of microarray data [2]. Because of the high variability in microarray analysis, using other techniques such as RT-PCR to verify the microarray results is very important. However, it is unfeasible to verify such large numbers of genes. Thus, selection of genes for RT-PCR verification was necessary. Based on our microarray data, we selected 18 genes whose signal intensity in p3 was 10-fold greater than in p11 for RT-PCR analysis.

With real-time RT-PCR, we obtained the C_T values of these 18 genes in p3 and p11 of DPSC. From the C_T values, we calculated the RGE of the genes to compare gene expression (Figure 3.2). Genes with no significant difference in RGE between p3 vs. p11 were eliminated. These selection procedures allowed us to narrow down to 13 genes for further study.

Finally, we conducted correlation analysis to determine the correlation between gene expression and differentiation potential in different passages of p3, p5, p9 and p 11 DPSC. We selected *Gipc2* and *HspB8* for gene knockdown study. Expression of *Gipc2* showed maximal correlation to reduction of differentiation in different passages, and expression of *HspB8* had a moderate correlation with differentiation across passages. Surprisingly, knockdown of *Gipc2* did

not show any effect on differentiation (Figure 3.4); however, knockdown of *HspB8* did result in dramatic reduction of differentiation.

Gipc2 is a homologue to the first member of its respective gene family, *GIPC1* [8], which was first discovered in 1999 to associate with the cytoplasmic domain of the axon neural protein M-SemF [9]. Little is currently known about *GIPC2*, but its expression has been tied to a number of different forms of gastric cancer [10]. However, when it was discovered, GIPC2 was found to associate with TGF-β receptors or other types of Wnt receptors [8]. Takeda et al. discovered a drastic change in the expression of *Wnt16* through serial passaging of DPSC [11], and the strong correlation of *GIPC2* expression to differentiation hints at a potential relationship between the two genes and differentiation. In this study, we showed that knockdown of Gipc2 had no effect on differentiation of early passage DPSC, suggesting that it may not be involved in regulation of differentiation.

Heat shock proteins (Hsps) have been found to play vital roles in regulating many aspects of stem cells, including self-renewal, differentiation, dormancy, and senescence of stem cells [12]. In addition, our lab had also recently shown that DFSC express higher levels of some Hsps, including small heat shock proteins (HspB1 and HspB2) than their non-stem counterparts [7]. HspB1, also known as Hsp27, has been shown to interact with HspB8 to regulate cell fate [13]. In addition, we had also previously found that DFSC increase their proliferation and differentiation under proper heat stress [14]. HspB8 is also a small heat-shock protein that is required for proper neurite formation in motor neurons [15, 16]. It has also been found to exhibit chaperone-like qualities both in cardiac tissue [17] and during *in vitro* culture [18]. When we consider that DPSC arise from the neural crest [19], as well as possess the capability to differentiate into neural tissue *in vitro* [20], the potential relationship between *HspB8* and DPSC

seems more likely. Our gene knockdown study showed that knockdown of *HspB8* expression indeed resulted in abolishment of differentiation in early-passage DPSC, suggesting that HspB8 may exert a function in differentiation of DPSC, which is a novel finding regarding the role of HspB8. Thus, it was our deep interest to further study the gene expression regulation of this small heat shock protein in DPSC.

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CHAPTER 4: CONSTRUCTION OF HEAT SHOCK PROTEIN B8 (HSPB8) EXPRESSION VECTORS AND EXPLORING THE REGULATORY MECHANISM OF HSPB8 EXPRESSION IN DPSC

4.1 INTRODUCTION

It is well known that heat shock proteins (Hsps) are molecular chaperones that can function to stabilize intracellular proteins and repair damaged proteins under stress conditions. We observed that dental follicle stem cells express higher levels of some Hsps than the non-stem dental follicle cells originating from the same tissue [1]. It is likely that Hsps are required to stabilize factors for maintaining the "stemness" of ASC under *in vitro* condition. Although Hsps are classically considered as cell stress defense proteins, they are also involved in gene expression regulation [2]. Hsps were found to play vital roles in regulating many aspects of stem cells, including self-renewal, differentiation, dormancy, and senescence of stem cells [3].

HspB8, also known as Hsp22, belongs to the Small Heat Shock Protein family and is known for its role in pleiotropic prosurvival effects in cells. There is limited information regarding the role of HspB8 in stem cell behavior or properties. One study indicated that expression of HspB8 was increased during neuron differentiation *in vitro*, and overexpression of HspB8 promoted neuron differentiation [4]. The role of HspB8 in maintaining differentiation potential of stem cells and its regulation in stem cells are largely unknown.

In the previous chapter, we found that expression of HspB8 was significantly and dramatically down-regulated in late-passage DPSC (p11), which have lost differentiation capability. Furthermore, knockdown of *HspB8* expression in early-passage DPSC caused the cells to lose their differentiation ability. Thus, *HspB8* appears to be an important gene (or one of the important genes) to maintain differentiation in DPSC. In this chapter, we attempted to clone the *HspB8* cDNA into a plasmid to construct *HspB8* overexpression vectors. The purpose of constructing the *HspB8* expression vectors is two-fold. First, with the expression vectors, we

could force the late passage DPSC to overexpress *HspB8* by transfecting the vectors into the cells. Such an overexpression study would allow us to assess if HspB8 can function independently to regulate differentiation, or if it depends on other factors to exert the regulatory role. Secondly, because microRNAs (miRNAs) have been shown to play an essential role in stem cell self-renewal and differentiation [5-9], and because miRNAs are believed to regulate a large percentage of genes in mammalian cells [10], the constructed vectors could be used to explore if miRNAs are involved in regulating the expression of *HspB8*. This chapter provided strong evidence to show the possibility of miRNAs in regulating *HspB8* expression.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

DPSC were established using a published protocol for isolation of dental stem cells [11]. Briefly, dental pulps were isolated from rat first mandibular molars and trypsinized to obtain cell suspension. For DPSC culture, the cells were grown in α-MEM with 20% fetal bovine serum (FBS). Cells were then incubated at 37°C and 5% CO_2 in humidified incubators. At 90% confluence, cells were detached using trypsin-EDTA and passaged to a fresh T-75 flask with an initial density of 200,000 cells per T-75 flask.

In preparation for experimentation, cells were either seeded to T-25 flasks or 6-well plates. For experiments that required assaying of total protein, we seeded 1.5×10^5 cells to each T-25 flask. Cells destined for assaying by Alizarin red staining or collection of total mRNA were seeded to 6-well plates at 5.0×10^4 cells per well.

4.2.2 Clone HspB8 cDNA Fragments

To clone *HspB8* cDNA, 2 μg RNA extracted from DPSC was reverse-transcribed into cDNA using Oligo-dT primers to synthesize cDNA of the full mRNA transcript. A pair of PCR

primers (forward 5'-GCACGAATTCCAACAAACACCATGGCTGAC-3' and reverse 5'-GCACAAGCTTGGAAGGTCCCCAGAGAAAAG-3') was designed to amplify the *HspB8* cDNA sequence. This sequence contains the 590bp coding DNA sequence (CDS) and a 383bp 3' untranslated region (3'UTR). PCR was conducted by mixing cDNA and the primers, and run for 35 cycles. The PCR product was electrophoresed and *HspB8* cDNA was purified with a gel extraction kit (Qiagen). This DNA fragment contained restriction enzyme sites of EcoRI (GAATTC) and HindIII (AAGCTT) on the ends. With the same procedures, we also cloned the CDS only region using another primer pair (forward 5'-CTGAATTCATGGCTGACGGGCAAT TGCCT-3' and reverse 5'-CGAAGCTTTTAGGAGCAGGTGACTTCCTGGT-3').

4.2.3 Construction of the HspB8 Expression Vectors

Each of the cDNA fragments was inserted into the multiple cloning site (MCS) of expression vector pCMV-3Tag-3 (Figure 4.1), such that the transcription of the fragments is driven by the CMV promoter using the procedures detailed below. We first digested the *HspB8* DNA fragment and the plasmid separately with EcoRI and HindIII (New England Biolabs) at 37°C for one hour, followed by enzyme deactivation at 65°C for 20 minutes. To prevent auto-ligation, the digested plasmid and DNA fragment were subjected to agarose gel electrophoresis, followed by gel extraction and purification using the Qiaquick Gel Extraction Kit (Qiagen) to collect the linear plasmid and *HspB8* DNA fragment with sticky ends. Next, the linear plasmid and DNA fragment were mixed in a ligation reaction at a ratio of 0.09 pmol:0.03 pmol with the presence of T4 DNA ligase (New England Biolabs). Ligation was performed by incubating the mixture at 16°C overnight.

Following ligation, the mixture was used to transform competent E. coli cells. Briefly, competent E. coli cells (New England Biolabs) were thawed on ice for 30 minutes. After adding

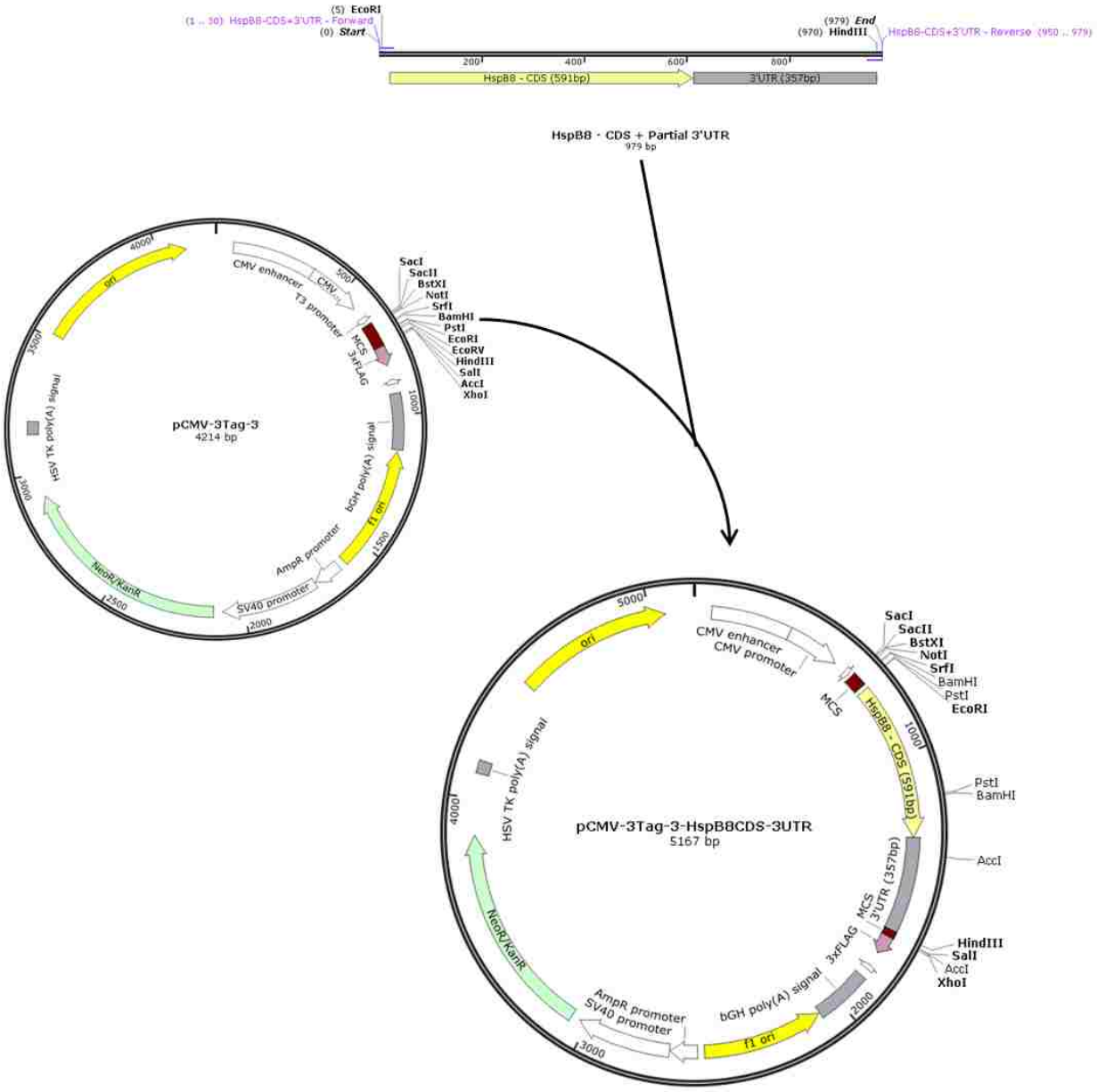

Figure 4.1 Illustration of the Cloning of *HspB8* cDNA CDS+3'UTR into expression vector pCMV-3Tag-3. Rat *HspB8* cDNA was amplified using primers that contained hanging restriction sites for EcoRI and HindIII. The PCR product and the plasmid vector were digested with EcoRI and HindIII. The cDNA fragment was inserted into the vector.

the mixture to the E. coli, the bacteria were incubated for 30 minutes on ice, followed by heat-shock at 42°C for 30 seconds. The competent E. coli were then incubated on ice for five minutes before suspension in SOC medium (New England Biolabs) and incubated on a shaker at 37°C for one hour. Finally, the transformed E. coli were spread to a LB medium plate containing kanamycin as a selection drug and incubated overnight at 37° C for formation of bacterial clones. The plasmids containing putative *HspB8* insertion were isolated from the clones using the QIAPrep Spin Miniprep Kit (Qiagen).

To confirm that the plasmids contained the correct *HspB8* insertion, the newly constructed plasmid, along with the original pCMV-3Tag-3 plasmid, were digested with EcoRI and/or HindIII for fiv0065 hours. Immediately after digestion, aliquots were separated via agarose gel electrophoresis to identify the correct DNA fragment bands.

The new plasmids that displayed the correct plasmid+insertion pattern were sent to LSU GeneLab for sequencing with T3-19 primer (5'-CAATTAACCCTCACTAAAG-3') and T7-19 primer (5'-GTAATACGACTCACTATAG-3'). The sequence received from GeneLab was analyzed using UGENE [12]. The sequence was aligned in BLAST with the RefSeq of rat *HspB8* (NM_053612.2) published in the Gene Bank.

4.2.4 Transfection of DPSC with Gene Expression Vectors

DPSC were grown to 80% confluence. One hour prior to transfection, culture medium was removed, and fresh medium was added to the cultures. DPSC were transfected using the PolyJet™ DNA In Vitro Transfection Reagent (Signagen), a biodegradable polymer DNA transfection reagent, according to the manufacturer's protocol. Briefly, plasmids were diluted in serum-free high glucose DMEM medium (Invitrogen). Concurrently, PolyJet™ reagent was added to a separate volume of DMEM. Then, the plasmid solution and PolyJet™ solution were

mixed in a 1:1 volume ratio and incubated at room temperature for 15 minutes. The combined mixture was then added drop-wise to the cells in culture at 0.1 mL mixture to 1 mL medium. The cells were incubated for 24 hours. Next, the medium containing transfection reagent was removed and replaced with fresh culture medium. This general transfection protocol was applied to the following two transfection experiments.

We first optimized the protocol for transfection of DPSC using the pCMV-βLac-Zco plasmid (Marker Gene Technologies), which contains the *LacZ* reporter gene. To do this, we seeded 2.5 x 10^4 DPSC to each well of a 12-well plate. When cells reached 80% confluence, they were transfected with pCMV-βLac-Zco at the final concentrations of 0.1, 0.2, 0.5, 1.0, or 1.5 μg/mL using the above mentioned PolyJet™ protocol. After 24 hours, the transfection medium was removed and fresh culture medium was added to the cells. After an additional two days of incubation, cells were fixed and stained using X-Gal staining to determine the transfection efficiency. Briefly, cells were rinsed with PBS and fixed in 10% formalin for five minutes, and washed again with PBS. The fixed cells were incubated with X-Gal staining solution at 37°C overnight. Cells were then rinsed once with PBS, followed by rinsing in 100% ethanol for 10-30 minutes. After aspirating the ethanol, the fixed cells were counterstained with eosin Y for 30 seconds, followed by a rinse with 95% ethanol for one minute and three rinses in 100% ethanol for one minute each. The stained plates were then photographed under a microscope. The number of blue transfected cells and total cells in a given area were counted. Transfection efficiency was calculated as the percentage of the transfected cells to total cells.

Using the optimized protocol, we transfected DPSC with the *HspB8* expression vectors. Following transfection at designated times, cells were harvested in Buffer RLT for total RNA extraction using RNeasy mini kit (Qiagen) or harvested into Cytobuster buffer for extraction of

total protein. The RNA samples were treated twice with Turbo DNase I (Ambion) to ensure complete removal of the possible plasmid contamination. Next, 1-2μg RNA was reverse-transcribed into cDNA, followed by PCR to determine the overexpression of *HspB8* and other genes, as listed in Table 4.1. In addition, Western blotting was conducted to analyze the protein samples to determine the translation of HspB8, as detailed below.

4.2.5 Western Blotting

DPSC were harvested and lysed in Cytobuster Protein Extraction Reagent (Millipore) to obtain total protein. Protein was quantified by Pierce BCA assay. Then, equal amounts of the total protein were subjected to Western blotting analysis. Briefly, 15-25 μg of protein from each sample was diluted to 20 μL and mixed with 4 μL of 6X-stock loading buffer consisting of 60% glycerol, 12% sodium dodecyl sulfate (SDS), 0.3M dithiothreitol (DTT), 0.375M Tris (pH 6.8), and 0.06% bromophenol blue. The sample mix was denatured in a 95-100°C water bath for five minutes before being loaded into a 5% polyacrylaminde stacking gel. polyacrylamide gel electrophoresis (PAGE) was conducted at 80V for 15 minutes, followed by separation at 130V for 60-80 minutes. Then, the gel was immediately removed and proteins were transferred to a 0.2 μm Immuno-Blot PVDF membrane (Bio-Rad) by electrophoresis at 100 – 110V for 60 – 100 minutes.

The membranes bound with protein were blocked for one hour at room temperature in 0.05% Tween-20 in PBS (PBST) with 5% blotto dry milk (Santa Cruz Biotechnology). Following blocking, samples were incubated with 0.5-2.0 μg/mL primary antibodies in 2% bovine serum albumin (BSA, Amresco) PBST at 4°C overnight. Following primary antibody incubation, membranes were washed five times in PBST at room temperature for five minutes before incubating with secondary antibody. All secondary antibodies were diluted in PBST

Table 4.1 Primers used for real-time RT-PCR

Gene	RefSeq	Primer Sequence
Actin	NM_031144.3	Forward: 5'-CTAAGGCCAACCGTGAAAAGAT-3' Reverse: 5'-AGAGGCATACAGGGACAACACA-3'
HspB8	NM_053612.2	Forward: 5'-TCTCCAGAGGGTCTGCTCAT-3' Reverse: 5'-GCAGGTGACTTCCTGGTTGT-3'

**RunX2* mRNA has two separate isoforms, with isoform 2 having a shorter 5' untranslated region and CDS region, as well as the translated protein having slightly different N-terminus and being shorter than isoform 1. The primers listed flank a sequence conserved between the two isoforms.

containing 2% BSA, and membranes were incubated with secondary antibodies for 1 – 2 hours at room temperature. Following secondary antibody incubation, the membranes were once again washed five times in PBST. The membranes were then stained using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) for 2-5 minutes to develop protein signals, and the signals were captured using a Chemidoc XRS. Image processing was performed using Quantity One 1-D Analysis Software (Bio-Rad).

4.3 RESULTS

4.3.1 Construction of *HspB8* Expression Plasmid Vector

E. coli clones containing putative *HspB8* expression plasmids were picked for plasmid isolation. Enzymatic digestion and gel electrophoresis indicated the insertion of the correct DNA fragment of *HspB8* CDS or HspB8 CDS + 3'UTR into the EcoRI and HindIII sites of pCMV-3-Tag-3, as shown in Figure 4.2. The plasmids were sent to LSU Genelab for sequencing. The resultant sequences were aligned with the NCBI Genebank *HspB8* mRNA sequence, and the result showed a perfect match in the CDS, with only one mismatch occurring in the 3' untranslated region (3'UTR) in the plasmid containing CDS+3'UTR, which is believed to be a

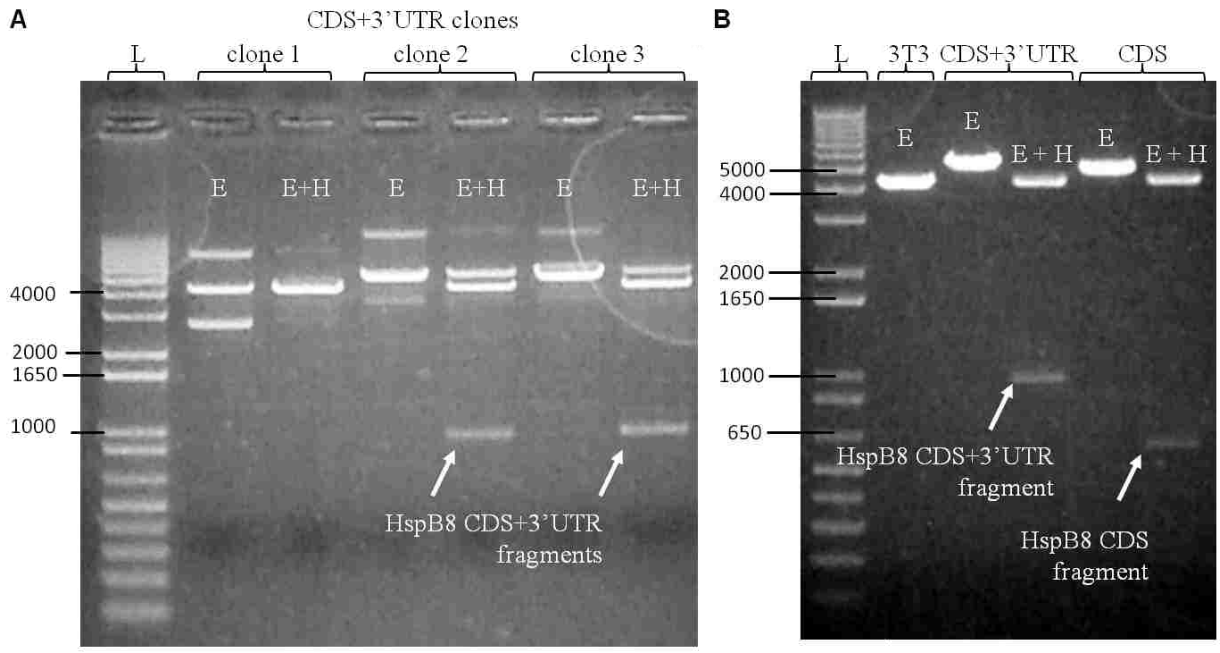

Figure 4.2 Enzymatic digestion of putative plasmid constructs to determine the correct insertion of *HspB8* cDNA into pCMV-3Tag-3 plasmid. (A) Example for screening *E. coli* clones containing putative HspB8 CDS+3'UTR insertion by enzymatic digestion. Digestion of putative plasmid containing *HspB8* CDS and 3'UTR (pCMV-3Tag3-HspB8CDS-3'UTR) yielded correct pattern with the correct DNA fragment insertion indicated by arrows in clones 2 and 3. Note that expected fragment was absent in Clone 1, indicating improper ligation of the plasmid, and this clone was discarded. (B) Showing that enzymatic digestion of pCMV-3Tag3-HspB8CDS-3'UTR and pCMV-3Tag3-HspB8CDS resulted in correct pattern and insertion of HspB8 CDS +3'UTR and HspB8 CDS (arrows) into the plasmids. Key: L – DNA ladder marker; E – sample digested with EcoRI; E+H – sample digested with EcoRI and HindIII

single- nucleotide polymorphism (SNP). These plasmids were respectively named pCMV-3Tag3-HspB8CDS and pCMV-3Tag3-HspB8CDS-3'UTR, , and the *E. coli* clones harboring these plasmids were saved for propagation of the *HspB8* expression vectors.

4.3.2 Optimization of DPSC Transfection

We optimized the PolyJet™ protocol for transfection of DPSC using the Lac-Z reporter plasmid. We found that PolyJet™ could be used to effectively transfect DPSC (Figure 4.3). The optimal transfection efficiency was seen at plasmid concentrations ranging between 0.5 µg/mL

and 1 μg/mL. Averagely, 50% to 60% of cells were transfected at this condition. Greater than 80% cells were transfected in some areas (Figure 4.3).

4.3.3 Transfection of HspB8 Expression Vectors into DPSC

When pCMV-3Tag3-HspB8CDS-3UTR and pCMV-3Tag-CDS were transfected into early and late-passages of DPSC, RT-PCR showed that both passages of DPSC exhibited a strong increase in *HspB8* mRNA expression within 24 hours after transfection with both plasmids, as compared to the control transfection with pCMV-3tag3 (Figure 4.4; Sections A and B).

Protein was collected from the transfected cells for Western blotting analysis to determine the translation of mRNA. Interestingly, increased HspB8 protein expression was seen in the early-passage DPSC transfected with both plasmids; however, in late passage DPSC, a dramatic increase of protein expression was only seen in the cells transfected with the plasmids containing *HspB8* CDS (Figure 4.4; Section C), but not in the cells transfected with the vector containing CDS+3'UTR (Figure 4.4; Section D). Because the 3'UTR is the major target site for miRNAs, this result indicated that miRNAs play an important role in regulation of *HspB8* expression in DPSC. Specifically, reduced expression of HspB8 expression seen in late-passage DPSC was due to high level expression of certain miRNAs.

4.4 DISCUSSION

DPSC lose their differentiation capability alongside decreasing expression of certain genes in late passages. We have previously determined that expression of *Gipc2* and *HspB8* was greatly and significantly reduced in late passage DPSC, where DPSC lose their differentiation ability. The expression of these genes in later passages could be reduced on average by 70% or more when compared to the early passages. We had also previously confirmed that knockdown

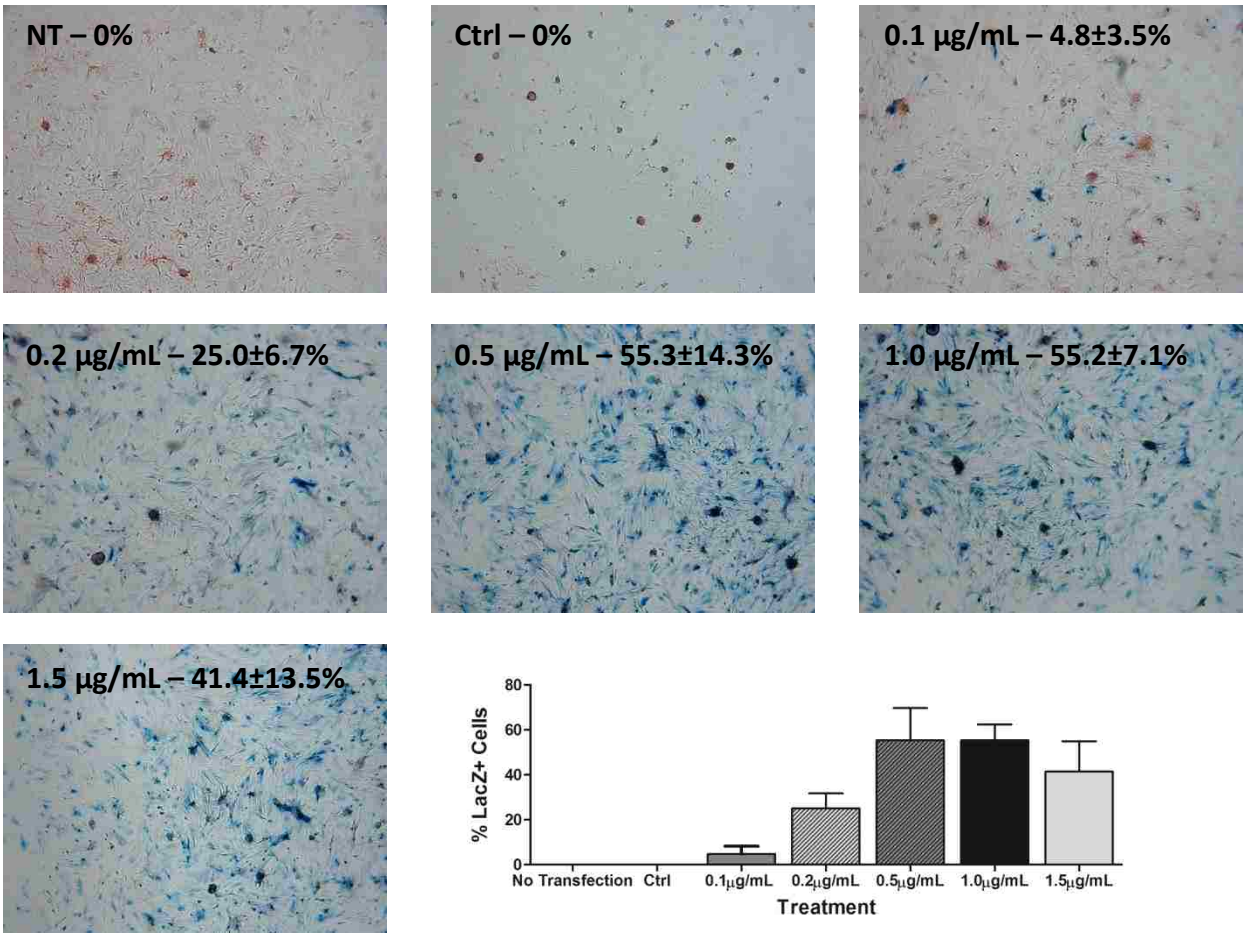

Figure 4.3 Optimization of DPSC Transfection using PolyJet™ DNA *In Vitro* Transfection Reagent. Different concentrations of pCMV-βLac-Zco plasmid were transfected into DPSC with PolyJet™ protocol. X-Gal staining showed that optimal plasmid concentration was between 0.5 to 1.0µg/mL with 55% cells transfected. (mean+SD, n=6) Key: (NT) non-transfected cells, (Ctrl) cells transfected without *LacZ*. All images are 50x magnification.

of *HspB8* caused loss of differentiation in early-passage DPSC, but knockdown of *Gipc2* did not have an effect. We therefore speculate that *HspB8* is involved in maintaining differentiation capability of DPSC.

To further study HspB8, we cloned the different fragments of *HspB8* cDNA into an expression vector. In late-passage DPSC, transfection of the vector expressing *HspB8* CDS+ 3'UTR resulted in an increase in transcription of the genes into mRNA, but translation of the

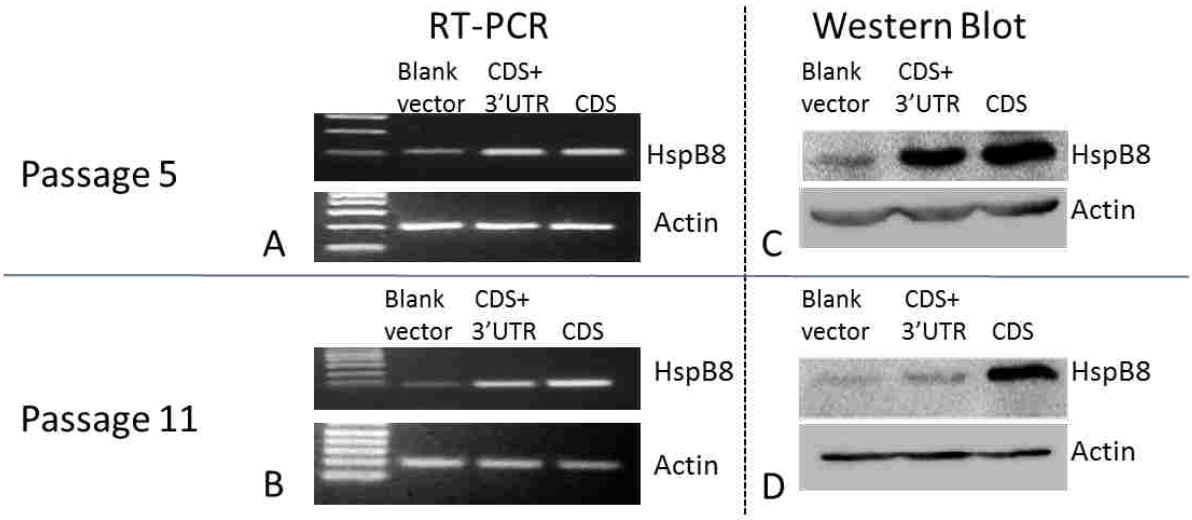

Figure 4.4 Transfection of plasmid vector to overexpress *HspB8* CDS+3UTR or CDS constructs in p5 and p11 DPSC. Note that the increase in *HspB8* mRNA were seen in both CDS+3'UTR and CDS transfections in both p5 (A) and p11 DPSC (B) shown by RT-PCR. Dramatic increase of protein expression was seen in p5 DPSC transfected with both plasmids (C). In contrast, for p11 DPSC, a dramatic increase of protein expression was seen in CDS transfection, but not in CDS+3'UTR transfection (D) as determined by Western blot. Instead, only slight increase of HspB8 protein was seen in CDS+3'UTR transfection (D).

mRNA was inhibited. However, transfection of CDS to the same cells resulted in overexpression of both mRNA and protein (Figure 4.4; Sections B and D). Thus, the presence of the 3'UTR apparently blocked the translation of mRNA in a posttranscriptional manner.

Recent studies revealed that miRNAs are important posttranscriptional regulators [6]. The main function of miRNAs is to downregulate gene expression by several mechanisms, including translational repression, mRNA cleavage, and mRNA deadenylation/destabilization [13]. After examining thousands of protein genes in response to miRNAs, Baek et. al. concluded that the target sites for most miRNA are located in the 3'UTR of mRNA transcripts in animal cells [14]. Given the facts about miRNAs, the above overexpression experiment provides strong evidence that miRNAs play an important role in regulating HspB8 expression.

Moreover, transfection of early passage of DPSC with the aforementioned *HspB8* expression vectors increased expression of mRNA and protein regardless of the presence or absence of the 3'UTR (Figure 4.4; Sections A and C). We speculate that this was because the early passage DPSC had nonexistent or low levels of *HspB8*-targeting miRNAs. As cell passages progressed, DPSC increased expression of *HspB8*-targeting miRNAs, and as a result a decrease in the expression of HspB8 was seen in the p11 DPSC.

In an experiment attempting to restore differentiation capability of late passage DPSC by overexpression of *HspB8*, we did not observe a positive effect (data not shown). We reasoned that it is likely that *HspB8* cannot independently reverse the process, and that other down-regulated genes or factors are also involved in maintaining the differentiation potential of DPSC. Further studies are needed to elucidate those genes.

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CHAPTER 5: CONCLUDING REMARKS

5.1 OVERALL SUMMARY OF FINDINGS

As discussed in Chapter 1, use of ASC for tissue regeneration prevents some problems associated with ESC [1]. On the other hand, the use of ASC requires the constant re-isolation of primary cultures, since *in vitro* culture of ASC reduces their differentiation capabilities over time [2, 3]. For stem cells, such as dental pulp derived stem cells (DPSC), this challenge is further magnified due to the low volume and availability of the tissue source. However, DPSC possess the ability to differentiate into a variety of different tissues, such as adipogenic, chondrogenic, and myogenic tissues [4, 5]. Therefore, any discovery that preserves their differentiation capability during *in vitro* culture would further enhance their utility.

In Chapter 2, we examined the capability of DPSC to differentiate into one of its many potential cell types: the calcium-depositing cells. This ability has been shown to persist *in vitro* for DPSC cultures as late as p9 [3]. We showed that calcium depositing cells derived from DPSC might contain osteoblasts, odontoblasts, and ameloblasts. While serial passaging of DPSC resulted in a change in cell morphology, the cells also eventually lost differentiation potential around p11. However, these cells remained distinct from non-stem dental pulp cells (DPC). In particular, a number of stem cell marker genes, such as *KitL* [6] and *Esrrb* [7], continued to be strongly expressed in the late passage DPSC when compared to DPC. We screened a number of different stem cell-related transcription factors, but none significantly changed/reduced its expression in the late passage DPSC.

The scope of our study expanded in Chapter 3, and we conducted a whole-genome microarray analysis to search for as many potential differences between early and late-passage DPSC as possible. From there, we found 18 genes with a marked decrease in expression in late-

passage DPSC. After confirming the decrease in expression in most of these genes by real-time RT-PCR, we selected two genes, *Gipc2* and *HspB8,* for further study. Using shRNAi, we knocked down the expression of these candidate genes in early-passage DPSC and attempted to induce differentiation. When knockdown of *HspB8* almost completely prevented differentiation in early-passage DPSC, we concluded that high level expression of *HspB8* is important for DPSC to maintain differentiation potential.

In Chapter 4, with evidence that *HspB8* expression is important in differentiation, we sought to clone the cDNA of *HspB8* into plasmid vectors that would constitutively overexpress *HspB8* when transfected into DPSC. We cloned two DNA fragments from *HspB8* cDNA, and inserted them into pCMV-3Tag-3 (Agilent) to construct Hspb8 expression vectors. Fragment 1 contained *HspB8* coding sequence (CDS) and 3'untranslated region (3'UTR), and fragment 2 contained only *HspB8* CDS. These vectors were transfected into p5 and p11 DPSC using the optimized PolyJet™ protocol. Transfection of the vector containing both the *HspB8* CDS and 3'UTR increased *HspB8* mRNA in both early and late passages of DPSC, but the increase of HspB8 protein was only seen in early-passage DPSCs. In contrast, transfection of the vector containing only the *HspB8* CDS (lacking the 3'UTR) resulted in a dramatic increase of *HspB8* mRNA and protein for both early and late passages of DPSCs. Given that the 3'UTR is the major target site for miRNAs, the results strongly suggest that miRNAs are responsible in post-transcriptional regulation to downregulate HspB8 in late-passage DPSCs. This was one of the most important findings of this research.

5.2 SIGNIFICANCE OF RESEARCH

With their relative ease of acquisition, and the ability to differentiate into a variety of tissues, DPSC can potentially serve multiple roles in both the regeneration and study of these

tissues. However, large quantities of functional DPSC must be available before serious research and therapy are to be considered. With dental pulp presenting a small volume of primary tissue, the only other way to obtain large quantities of DPSC is through *in vitro* culture. The mechanism for this loss of differentiation in DPSC remains largely unknown. While an occasional study may link the change of a particular gene to increased passaging [2], direct evidence of a relationship between a gene and changes in DPSC functionality would be of greater benefit to understanding and preventing this loss of differentiation capability. We believe this study helps to further understanding of the molecular regulation of differentiation in other tissue derived stem cells. The finding of miRNAs in regulating differentiation is especially significant, since each miRNA can regulate multiple genes. This may allow us to establish the molecular regulatory network for maintaining differentiation potential in ASC.

5.3 FUTURE STUDIES

HspB8 has already been shown to function in concert with other small heat shock proteins [8, 9]. The possibility that HspB8 works with other proteins to maintain differentiation should be addressed. In addition, the discovery that miRNAs acting on *HspB8* mRNA to inhibit translation is a novel one, and the interaction of *HspB8* transcript with miRNAs is one that deserves further study. Over 1000 miRNAs have been found in animal cells. One big question is which miRNAs actually target *HspB8* mRNA to down regulate its expression in long-term cultures of DPSC. In this regard, using the TargetScan miRNA prediction tool, we found 42 potential miRNAs that may target against HspB8 (Table 5.1) [10]. Immediate future studies would focus on determining which of these miRNAs are elevated in the late passage DPSC, and which miRNAs can act to down regulate HspB8. In addition, it is also our interest to define the exact target sites of miRNAs on the 3'UTR of *HspB8* mRNA. Since each miRNA can target

Table 5.1 miRNAs predicted to target *HspB8* mRNA

miRNA	Seed match on 3'UTR	miRNA Sequence*
rno-miR-3561	138-145	AGACUCACAGAUGGGACCUAU
rno-miR-3562	36-42	AGGGUAGGUCGGUGACGGGGUU
rno-miR-295	54-60	UCUUCACACGGGGUGUAAACUCA
rno-miR-543	92-98	GCUUUUUGUGCGCCCGUUGAA
rno-miR-219-1-3p	93-100	GCCCUGCAGGUCUGCGUUGAGA
rno-miR-3541	114-121	ACGUCACUCCCCCUCCCU
rno-miR-343	116-123	AGACCCGUGUGCCUCCCUCU
rno-miR-3594-5p	137-143	AAGUGUGACGAGACGGGACCC
rno-miR-323	144-150	UCUCCAGCUGGCACAUUACAC
rno-miR-539	158-164	UGUGUGGUUCCUAUUAAAGAGG
rno-miR-3558-3p	166-172	GUGUUUAAGCCUAGAUGUCCCAU
rno-miR-10a-5p	166-172	GUGUUUAAGCCUAGAUGUCCCAU
rno-miR-336	168-174	UCUGAUCUAUACCU---UCCCACU
rno-miR-592	173-179	UGUAGUAGCGUAUAACUGUGUUA
rno-miR-124	199-205	CCGUAAGUGGCGCACGGAAU
rno-miR-215	204-211	ACAGACAGUUUAGUAUCCAGUA
rno-miR-192	204-211	CCGACAGUUAAGUAUCCAGUC
rno-miR-150	216-222	GUGACCAUGUUCCCAACCCUCU
rno-miR-3551-5p	243-250	UACAGUUUUUGUGGUACCACUUCU
rno-miR-3580-5p	275-281	CAGUGGUCUUAUUCAUGUUCGU
rno-miR-194	302-308	AGGUGUACCUCAACGACAAUGU
rno-miR-22	319-325	UGUCAAGAAGUUGACCGUCGAA
rno-miR-431	328-334	ACGUACUGCCGGACGUUCUGU
rno-miR-493	350-356	GACCGUGUGUCAUCUGGAAGU
rno-let-7i	423-429	UUGUCGUGUUUGAUGAUGGAGU
rno-let-7f	423-429	UUGGUAUGUUGGAUGAUGGAGU
rno-let-7c	423-429	UUGGUAUGUUGGAUGAUGGAGU
rno-let-7d	423-429	UUGAUACGUUGGAUGAUGGAGA
rno-let-7a	423-429	UUGAUAUGUUGGAUGAUGGAGU
rno-let-7e	423-429	UUGAUAUGUUGGAGGAUGGAGU
rno-let-7b	423-429	UUGGUGUGUUGGAUGAUGGAGU
rno-miR-98	423-429	UUGUUAUGUUGAAUGAUGGAGU
rno-miR-674-5p	426-432	AUGUGGUGAGGGUAGAGUCACG
rno-miR-3587	427-433	CGUGGUAAACUUU---AGUCACAA
rno-miR-3571	429-435	AUACCUUACAUUUCUUCACACAU
rno-miR-3560	489-495	UUACGUGGGCCCGUUCCUAAAC
rno-miR-3563-3p	497-503	CAAAUGGCAGGGUGUAU

(Table 5.1 continued)

miRNA	Seed match on 3'UTR	miRNA Sequence*
rno-miR-615	531-537	CUAGGCUCGUGGCCCCUGGGGG
rno-miR-3573-5p	573-580	AGGAAAGAUAGUGACGGGGAGU
rno-miR-3583-3p	626-632	GACGUACUUCUCUCUUCAGUCC
rno-miR-674-3p	644-650	AACAAGACUCUACCCUCGACAC
rno-miR-337	806-812	UUUCCGUAGUAUAUC---CUCGACUU

*The miRNA Sequences listed are arranged in a 3' to 5' order

more than one gene, another research topic will be to find out if *HspB8*-targeting miRNAs can also regulate expression of other genes for maintaining differentiation, and what those genes are. For example, Zfp423 also reduced expression in the late passage DPSC, and in a pilot study we noticed that knockdown of its expression also decreased differentiation of early passage DPSC.

In conclusion, this study revealed that DPSC lose differentiation potential around passages 9 or 11. High level expression of *HspB8* is essential for differentiation of DPSC, and down-regulation of HspB8 in cultured DPSC is likely due to increased expression of miRNAs. These are novel findings regarding HspB8 and miRNAs on the regulation of stem cell fate. The potential miRNAs that may regulate HspB8 expression are predicted using a bioinformatics tool.

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APPENDIX A DIFFERENTIATION AND RELATIVE GENE EXPRESSION OF SIGNIFICANT GENES IN MULTIPLE SERIAL PASSAGES OF DPSC

	DPSC L10-7					DPSC L11-27			
	p3	p5	p9	p11		p3	p5	p9	p11
Differentiation	2	2	1	0		4	3	1	1
Apbb1ip	1.00	0.08	0.01	0.01		1.00	0.30	0.08	0.02
Clgn	1.00	0.08	0.03	0.00		1.00	0.03	0.02	0.01
Colec11	1.00	0.19	0.10	0.12		1.00	0.06	0.09	0.07
Eya2	1.00	0.03	0.00	0.02		1.00	0.06	0.01	0.01
Fam159b	1.00	0.02	0.00	0.00		1.00	0.05	0.01	0.01
Gipc2	1.00	0.34	0.04	0.02		1.00	0.42	0.16	0.14
HspB8	1.00	0.29	0.09	0.36		1.00	0.34	0.12	0.17
Thbd	1.00	0.03	0.00	0.00		1.00	0.02	0.00	0.01
Wif1	1.00	0.04	0.00	0.01		1.00	0.17	0.07	0.33
Zfp423	1.00	0.06	0.03	0.08		1.00	0.26	0.38	0.39

APPENDIX B ABBREVIATIONS OF GENE NAMES USED IN THIS DISSERTATION

Abbreviation	Full gene name
Acad9	Acyl-CoA dehydrogenase family, member 9
Alx1	ALX homeobox 1
Ambn	Ameloblastin
Apbb1ip	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein
Arhgap20	Rho GTPase activating protein 20
Bcrp	ATP-binding cassette, sub-family G (WHITE), member 2
BMP4	Bone Morphogenetic Protein 4
BMP6	Bone Morphogenetic Protein 6
BSP	Integrin-binding sialoprotein (alias: Ibsp)
c-Myc	Myelocytomatosis oncogene
Clgn	Calmegin
Colec11	Collectin sub-family member 11
Dax1	Nuclear receptor subfamily 0, group B, member 1 (alias: Nr0b1)
DMP1	Dentin matrix acidic phosphoprotein 1
DSPP	Dentin sialophosphoprotein
Dusp7	Dual specificity phosphatase 7
Egflam	EGF-like, fibronectin type III and laminin G domains
EGR1	Early growth response 1
Esrrb	Estrogen-related receptor beta
Eya2	Eyes absent homolog 2
Fam159b	Family with sequence similarity 159, member B
FoxM1	Forkhead box M1
FoxO1	Forkhead box O1
FoxO3	Forkhead box O3
FoxO4	Forkhead box O4
Gipc1	GIPC PDZ domain containing family, member 1
Gipc2	GIPC PDZ domain containing family, member 2
HspB1	Heat shock protein 1
HspB8	Heat shock protein B8
KitL	KIT ligand
Klf4	Kruppel-like factor 4
Klf5	Kruppel-like factor 5
LacZ	Beta-D-galactosidase
Lin28	Lin-28 homolog A

(Appendix B continued)

Abbreviation	Full gene name
Mki67ip	Nifk nucleolar protein interacting with the FHA domain of MKI67 (Alias: Nifk)
Mmp13	Matrix metallopeptidase 13
Msh2	MutS homolog 2
Mst1	Macrophage stimulating 1 (hepatocyte growth factor-like)
Musashi	Musashi
Nanog	Nanog homeobox
Ocn	Bone gamma-carboxyglutamate (gla) protein (Alias: Bglap)
Oct4	OCT4 Protein
PCOLCE2	Procollagen C-endopeptidase enhancer 2
Runx2	Runt-related transcription factor 2
Sall1	Spalt-like transcription factor 1
Sial	Integrin-binding sialoprotein (alias: Ibsp)
Slc6a15	Solute carrier family 6 (neutral amino acid transporter), member 15
Sox2	SRY (sex determining region Y)-box 2
Sp7	Sp7 transcription factor
Spp1	Secreted phosphoprotein 1
Tcfap2c	Transcription factor AP-2 gamma
Tcfcp2L1	Transcription factor CP2-like 1
Thbd	Thrombomodulin
Timp2	TIMP metallopeptidase inhibitor 2
Wif1	Wnt inhibitory factor 1
Wnt16	Wingless-type MMTV integration site family, member 16
Zfp143	Zinc finger protein 143
Zfp219	Zinc finger protein 219
Zfp423	Zinc finger protein 423
Zfp462	Zinc finger protein 462

VITA

Michael Flanagan was born in Mobile, Alabama. He attended McGill-Toolen Catholic High School in Mobile, from which he graduated in 1999. During his studies at Spring Hill College in Mobile, he developed a strong interest in cellular and molecular biology. In May of 2003, he received his Bachelor of Science degree. He worked privately as an environmental chemist until late 2007, whereupon learning about the research at the Louisiana State University School of Veterinary Medicine rekindled his interest in molecular biology. He entered the graduate school in January 2008 and began his research under the mentorship of Dr. Shulin Li. Under Dr. Li, he published two papers on stem cell transfection, with a focus on adipose-derived stromal cells. In May of 2010, he transferred into the laboratory of Shaomian Yao. Mike is currently completing his Doctoral Degree in Dr. Yao's Lab at the Louisiana State University School of Veterinary Medicine.