EFFECT OF ASPIRIN ON OSTEOGENIC DIFFERENTIATION AND GENE EXPRESSION PROFILES IN PERIODONTAL LIGAMENT STEM CELLS

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ABSTRACT

Periodontal ligament (PDL) contains a unique population of mesenchymal stem cells (MSCs), also known as PDL stem cells (PDLSCs). The regenerative properties of PDLSCs hold great potential for its use in stem cells based therapy, particularly for periodontal or bone regeneration. Aspirin (ASA) is a widely used non-steroidal anti-inflammatory drug (NSAID) that has been reported to modulate a variety of diseases such as cardiovascular, diabetes and cancer. There have not been many studies examining the effect of ASA on stem cells, notably PDLSCs. The present study investigated the effects of ASA on the proliferation rate, osteogenic differentiation rate, expression of growth factor-associated genes and global gene expression profile in PDLSCs. MSCs from PDL were isolated from normal permanent human teeth (n=3). The MSCs identity was validated by immunophenotyping and tri-lineage differentiation capacity assays. The cell proliferation rate was measured through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PCR array was used to profile the expression of 84 growth-factor associated genes in PDLSCs upon ASA treatment. The effect of ASA on the osteogenic potential of PDLSCs was evaluated through mineralization assay, using Alizarin Red S (ARS) staining. Microarray analysis was used to study the effects of ASA (200, 500, and 1,000 μM) on the gene expression profiles in PDLSCs during osteogenic differentiation. The results indicated that ASA treatment affect PDLSCs proliferation rate. When grown in basal media, ASA reduced PDLSCs proliferation rate, in time (24, 48, and 72 hrs) and dose (10 to 10,000 μM) dependent manners. However, ASA (at 500 and 1000 μM) significantly increased PDLSCs proliferation and osteogenic differentiation rates when they were grown in osteogenic media. The PCR array analyses indicated that ASA was able to modulate the expression of growth factor-associated genes in PDLSCs. Using a fold-change (FC) of 2.0 as a threshold value, the analyses indicated that 19 growth

factor-associated genes were differentially expressed (DE), of which 12 were upregulated and 7 were downregulated. Microarray study revealed that ASA was able to modulate PDLSCs gene expression profile. At 200 µM, 315 genes were DE, involving 151 upregulated and 164 downregulated genes. At 500 µM, 794 genes were DE, involving of 364 upregulated and 430 downregulated genes. At 1000 µM, the number of DE genes increased to 2035, of which 735 were upregulated and 1300 were downregulated. Bioinformatics analyses of the gene expression data revealed that the majority of DE genes (for 500 and 1,000 µM ASA treatment) are involved in osteogenic differentiation. The gene network analysis was carried out using Ingenuity Pathway Analysis (IPA) software, and this revealed that the number of gene groups involved in cell adhesion and extracellular matrix components were increased. Functional enrichment analysis using DAVID and PANTHER revealed a similar finding as revealed by IPA. The present study showed that ASA was able to modulate the expression of growth-factor associated genes and enhance osteogenic potential in PDLSCs. This study indicated that ASA could enhance PDLSCs functions and provide evidence for the potential use of ASA with PDLSCs for regenerative dentistry applications, particularly in the areas of periodontal health and regeneration.

ABSTRAK

Periodontal ligamen (PDL) mengandungi populasi sel stem mesenkima (MSC) yang unik, juga dikenali sebagai sel stem PDL (PDLSCs). Sifat-sifat regeneratif PDLSCs mempunyai potensi yang besar untuk digunakan dalam sel-sel stem terapi, terutamanya bagi regenerative periodontal atau tulang. Aspirin (ASA) digunakan secara meluas sebagai Ubat antiradang bukan steroid (NSAIDs) untuk memodulasi pelbagai penyakit seperti jantung, diabetes dan kanser. Tidak banyak kajian yang telah dijalankan untuk mengkaji kesan ASA terhadap sel-sel stem, terutamanya PDLSCs. Kajian ini telah dijalankan untuk mengkaji kesan aspirin (ASA) terhadap kapasiti proliferatif ligamen periodontal sel stem (PDLSCs), pengekspresan pertumbuhan gen faktor berkaitan di dalam PDLSCs dan pembezaan pengekpresan profil gen terhadap kesan ASA dalam pembezaan osteogenik daripada PDLSCs. MSCs daripada periodontal ligamen tisu diekstrak daripada gigi kekal manusia yang normal (n=3). Identiti MSCs telah dikenalpasti melalui kaedah immunophenotyping dan tri-lineage differentiation asai. Sel proliferasi aktiviti dinilai melalui asai 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). PCR array telah digunakan untuk menilai ekspresi profil 84 gen pengekspresan pertumbuhan gen faktor berkaitan. Potensi osteogenik dinilai melalui nodul mineral dengan menggunakan Alizarin Red S (ARS) pewarnaan dan data mikroatur analisis untuk mengkaji perbezaan gene ekspresi di dalam osteogenesis pembezaan di dalam PDLSCs. Hasil kajian menunjukkan kesan ASA menjejaskan kadar proliferasi PDLSCs. Di dalam basal media, ASA mengurangkan kadar proliferasi PDLSCs di setiap perbezaan masa (24, 48, dan 72 jam) dan dos (10 hingga 10, 000 μM). Walau bagaimanapun, ASA (500 dan 1000 μM) meningkat dengan ketara dalam kadar proliferasi PDLSCs dan kadar pembezaan osteogenik di dalam media osteogenik. PCR array analisis menunujukkan bahawa ASA berkebolehan memodulasi pengekpresan pertumbuhan gen faktor berkaitan dalam PDLSCs. Dengan

menggunakan perubahan gandaan (FC) 2 sebagai nilai ambang, pembezaan pengekspresan gen analisis menunjukkan 19 gen adalah yang berbeza secara signifikan (DE) di mana 12 gen beregulasi menaik dan 7 gen beregulasi menurun. Kajian mikroatur analisis mendedahkan bahawa ASA dapat memodulasi PDLSCs profil ekspresi gen. Pada rawatan dos 200 µM, 315 gen adalah DE dikenalpasti termasuk 151 gen beregulasi menaik dan 164 gen beregulasi menurun. Pada rawatan dos 500 µM, 794 DE dikenalpasti, termasuk 364 gen beregulasi menaik dan 430 gen beregulasi menurun. Manakala pada rawatan dos 1,000 µM, bilangan nombor gen DE menaik kepada 2035. Di mana 735 adalah beregulasi menaik dan 1300 adalah beregulasi menurun.

Bioinformatik analisis gen pengekpresan mendedahkan bahawa majoriti gen DE (untuk 500 dan 1,000 µM untuk rawatan ASA) terlibat dalam pembezaan osteogenik. Analisis rangkaian gen telah dijalankan dengan menggunakan perisian Ingenuity Analisis Pathway (IPA), dan ini menunjukkan bahawa bilangan kumpulan gen yang terlibat dalam sel lekatan dan komponen matriks extracellular telah meningkat. Functional enrichment analisis dengan menggunakan DAVID dan PANTHER menunjukkan keputusan yang sama seperti yang dinyatakan oleh IPA. Kajian ini mendapati bahawa ASA berkebolehan memodulasi pengekpresan pertumbuhan gen faktor berkaitan dan meningkatkan potensi osteogenik dalam ungkapan pertumbuhan faktor-gen yang dikaitkan dan meningkatkan potensi. Hasil kajian ini menunjukkan bahawa ASA meningkatkan potensi penggunaaan PDLSCs di dalam aplikasi regeneratif pergigian, terutamanya dalam bidang kesihatan periodontal dan pertumbuhan semula.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABMSCs	:	Alveolar bone-derived MSCs
ACAN	:	Aggrecan
ADH1A	:	Alcohol dehydrogenase 1A (class I), alpha polypeptide
ADRA2C	:	Adrenoceptor alpha 2C
a-FGF	:	Acidic-FGF
ALP	:	Alkaline phosphatase
AMH	:	Anti-Mullerian hormone
ANGPTL1	:	Angiopoietin-like 1
ARS	:	Alizarin Red S
ARSA	:	Alizarin Red S positive area
ASA	:	Aspirin
BCL2L11	:	BCL2-like 11 (apoptosis facilitator)
BDNF	:	Brain-derived neurotrotrophic factor
b-FGF	:	Basic-FGF
BM	:	Bone marrow
BMMSCs	:	Bone marrow mesenchymal stem cells
BMP10	:	Bone morphogenetic protein 10
BMP2	:	Bone morphogenetic protein 2
BMPER	:	BMP binding endothelial regulator
BMPR-I	:	BMP-type I
BMPs	:	Bone morphogenic proteins
BMRPR-II	:	BMP-type II receptor
BMS1P5	:	BMS1 pseudogene 5

BP	:	Biological processes
BSA	:	Bovine serum albumin
C3	:	Complement component 3
cbfa1	:	Core binding factor alpha 1 and
CC	:	Cellular components
CD	:	Cluster of differentiation
CDH2	:	Cadherin 2, type 1, N-cadherin (neuronal)
CDKN1C	:	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
cDNA	:	Complementary deoxyribonucleic acid
CFU	:	Colony forming unit
CHRDL1	:	Chordin-like 1
CLC	:	Chloride ion channel
CLIC6	:	Chloride intracellular channel 6
CLSTN3	:	Calsyntenin 3
COX-1	:	Cyclooxygenase-1
COX-2	:	Cyclooxygenase-2
CSF2	:	Colony stimulating factor (CSF2)
CSF3	:	Colony stimulating factor (CSF3)
CXCL12	:	Chemokine (C-X-C motif) ligand 12
DAPI	:	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DBSCs	:	Dental bud stem cells
DEG	:	Differentially expressed genes
DFPCs	:	Dental follicles progenitor cells
DIO2	:	Deiodinase, iodothyronine, type II
DKK1	:	Dickkopf-related protein 1

DLK2	:	Delta-like 2 homolog (Drosophila)
DLX5	:	Distal–less homebox
DMSO	:	Dimethyl sulfoxide
DOK3	:	Docking protein 3
DPP9-AS1	:	DPP9 antisense RNA 1
DPRXP4	:	Divergent-paired related homeobox pseudogene 4
DPSCs	:	Dental pulp stem cells
E2F7	:	E2F transcription factor 7
ECM	:	Extracellular matrix
EFTUD1	:	Elongation factor Tu GTP binding domain containing 1
ELOVL3	:	ELOVL fatty acid elongase 3
ENDOV	:	Endonuclease V
EPGN	:	Epithelial mitogen
EREG	:	Epiregulin
ESCs	:	Embryonic stem cells
FABP5	:	Fatty acid binding protein 5 (psoriasis-associated)
FAM107A	:	Family with sequence similarity 107, member A
FAM169A	:	Family with sequence similarity 169, member A
FBS	:	Fetal bovine serum
FC	:	Fold change
FGF5	:	Fibroblast growth factor 5
FGF9	:	Fibroblast growth factor 9
FGFs	:	Fibroblast growth factor
FITC	:	Fluorescein Isothiocyanate
FMO1	:	Flavin containing monooxygenase 1

FMO3	:	Flavin containing monooxygenase 3
FN1	:	Fibronectin 1
GAPDH	:	Glyceraldehyde-3-Phosphate
GCHFR	:	GTP cyclohydrolase I feedback regulator
GDF10	:	Growth Differentiation Factor 10
GDF11	:	Growth Differentiation Factor 11
GF	:	Growth Factor
GGT5	:	Gamma-glutamyltransferase 5 (GGT5)
GMSCs	:	Gingiva-derived MSCs
GO	:	Gene ontology
GOLGA8O	:	Golgin A8 family, member O
GPRC5B	:	G protein-coupled receptor, class C, group 5, member B
GRB14	:	Growth factor receptor-bound protein 14
GTPBP6	:	GTP binding protein 6
HA/TCP	:	Hydroxyapatite/tricalcium phosphate
HDGF	:	Hepatoma-derived growth factor
HLA-DR	:	Human Leukocyte Antigen - antigen D Related
HLH	:	Helix-loop-helix factors
HR	:	Hair growth associated
hUCMs	:	Human umbilical cord matrix-derived mesenchymal stem cells
IBMX	:	3-isobutyl-1-methyxanthine
IBSP	:	Bone sialoprotein
ICAM1	:	Intercellular adhesion molecule 1
IFIH1	:	Interferon induced with helicase C domain 1

IFIT1	:	Interferon-induced protein with tetratricopeptide repeats1
IFN- γ	:	Interferon gamma
IGF2	:	Insulin-Like Growth Factor 2
IL	:	Interleukin
IL2	:	Interleukin 2
IL6	:	Interleukin 6
INA	:	Internexin neuronal intermediate filament protein, alpha
INHBA	:	Inhibin beta A
INHBB	:	Inhibin beta B
IPA	:	Ingenuity Pathways Analysis
IRF5	:	Interferon regulatory factor 5
ISLR	:	Immunoglobulin superfamily containing leucine-rich repeat
ITGA5	:	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
JAG1	:	Jagged 1
JAG2	:	Jagged-2
KCNJ8	:	Potassium inwardly-rectifying channel, subfamily J, member 8
KCNMA1	:	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1
KIRREL3	:	Kin of IRRE like 3 (Drosophila)
KRTAP1-5	:	Keratin associated protein 1-5
KRTAP19-8	:	Keratin associated protein 19-8
LEFTY1	:	Left-right determination factors1
LEFTY2	:	Left-right determination factors1
LFNG	:	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase
LIF	:	Leukemia inhibitory factor

LILRA1	:	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 1
LSP1	:	Lymphocyte-specific protein 1
LTBP4	:	Latent Transforming Growth Factor Beta Binding Protein 4
M-CSF	:	Macrophage colony-stimulating factor
MDK	:	Midkine (Neurite Growth-Promoting Factor 2)
METTL15	:	Methyltransferase like 15
MF	:	Molecular functions
MGP	:	Matrix Gla protein
MI	:	Myocardial infarction
MSC	:	Mesenchymal stem cells
Msx-2	:	Msh homebox homologue-2
MT1E	:	Metallothionein 1E
MTT	:	3-(4,5-dimethylthiazohl-2-yl)-2,5-diphenyltetrazolium bromide
MYBPHL	:	Myosin binding protein H-like
MyoD	:	Myosin D
ND4	:	Mitochondrially encoded NADH dehydrogenase 4
ND5	:	Mitochondrially encoded NADH dehydrogenase 5
NDP	:	Norrie Disease (Pseudoglioma)
NETO2	:	Neuropilin (NRP) and tolloid (TLL)-like 2
NPAS1	:	Neuronal PAS domain protein 1
NRG1	:	Neuregulin 1
NRG2	:	Neuregulin 2
NRG3	:	Neuregulin 3
NRTN	:	Neurturin

NSAIDs	:	Non-steroidal anti-inflammatory drugs
OCN	:	Osteocalcin
OD	:	Optical density (OD)
OPN	:	Osteopontin
OR5L2	:	Olfactory receptor, family 5, subfamily L, member 2
OSGIN1	:	Oxidative stress induced growth inhibitor 1
OTUD6A	:	OTU deubiquitinase 6A
P2RY2	:	Purinergic receptor P2Y, G-protein coupled, 2
PANTHER	:	Protein Analysis Through Evolutionary Relationships
PBS	:	Phosphate-buffered saline
PCA	:	Principle component analysis
PDE1A	:	Phosphodiesterase 1A, calmodulin-dependent
PDGF-BB	:	Platelet derived growth factor-BB
PDGFRL	:	Platelet-derived growth factor receptor-like
PDL	:	Periodontal ligament
PDLSCs	:	Periodontal ligament stem cells
PGE2	:	Prostaglandin E2
PHLPP2	:	PH domain and leucine rich repeat protein phosphatase 2
PITPNM3	:	PITPNM family member 3
PITPNM3	:	PITPNM family member 3
PLCB2	:	Phospholipase C, beta 2
PPARγ2	:	Peroxisome proliferator-activated receptor γ2
PSPN	:	Persephin
PTH	:	Parathyroid hormone
PTN	:	Pleiotrophin

qRT-PCR	:	Quantitative real-time PCR
RANKL	:	Nuclear factor kappa B-ligand
RGMA	:	Repulsive guidance molecule family member a
RGS4	:	Regulator of G-protein signaling 4
RUNX2	:	Runt-related transcription factor 2
SAA1	:	Serum amyloid A1
SAA2	:	Serum amyloid A2
SCAP	:	Stem cells from apical papilla
SCARA5	:	Scavenger receptor class A, member 5 (putative)
SCs	:	Stem cells
SEMA7A	:	Semaphorin 7A, GPI membrane anchor
SEPP1	:	Selenoprotein P, plasma, 1
SERPINA6	:	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6
SERTAD4	:	SERTA domain containing 4
sGAG	:	Sulphated glycosaminoglycans
SHED	:	Human exfoliated deciduous teeth
SLC35F5	:	Solute carrier family 35, member F5
SLC43A2	:	Solute carrier family 43 (amino acid system L transporter), member 2
SLCO1A2	:	Solute Carrier Organic Anion Transporter Family, Member 1A2
SPP1	:	Secreted phosphoprotein 1
TDGF1	:	Teratocarcinoma-derived growth factor 1
TERT	:	Telomerase reverse transcriptase
TGF-β	:	Transforming growth factor-β
TGPCs	:	Tooth germ progenitor cells
THPO	:	Thrombopoietin

TMEM151A	:	Transmembrane protein 151A
TNF-α	:	Tumor necrosis factor alpha
TR	:	Telomerase RNA
TRIL	:	TLR4 interactor with leucine-rich repeats
TRITC	:	Tetramethylrhodamine
TYMP	:	Thymidine phosphorylase
VEGFA	:	Vascular endothelial growth factors-A
VEGFC	:	Vascular endothelial growth factors-C
VEGFs	:	Vascular endothelial growth factors
VIT	:	Vitrin
WISP2	:	WNT1 inducible signaling pathway protein 2
ZDHHC11	:	Zinc finger, DHHC-type containing 11
ZNF157	:	Zinc finger protein 157
ZNF316	:	Zinc finger protein 316

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Mesenchymal stem cells (MSC) are adult stem cells that have two remarkable features, namely the capacity of self-renewal and ability to differentiate into a number of different tissues (multipotency), including differentiation into cells of the mesenchymal lineage such as osteoblasts, chondrocytes, and adipocytes. MSCs can also differentiate into ectodermal (neural cells) and endoderm (hepatic cells) cell lineages (Gronthos *et al.*, 2000; Miura *et al.*, 2003; Seo *et al.*, 2004). The minimal criteria in defining MSCs include: 1) the ability to adhere to plastic surfaces under culture conditions; 2) the cells are positive for the following cell surface markers CD73, CD90, and CD105, and negative for CD14, CD19, CD34, CD45, or CD11b, CD79α and HLA-DR; and 3) be capable of differentiating into osteoblasts, adipocytes, and chondrocytes *in vitro* (Dominici *et al.*, 2006).

MSCs can be isolated from many tissues including bone marrow, umbilical cords (Wharton's jelly), adipose and dental tissues. MSCs were first discovered by Friedenstein and his associates in the rodent bone marrow, which they called bone marrow mesenchymal stem cells (BMMSCs) (Friedenstein, 1970). Since then, knowledge from the study of BMMSCs has been used as the "gold standard" in the approach to the study of stem cells (Ullah *et al.*, 2015). The therapeutic potential of BMMSCs has been well established in *in vitro* and *in vivo* studies (Colnot, 2011; Ma *et al.*, 2014). However, the procedure for obtaining BMMSCs is invasive and the harvesting yield is low (Huang *et al.*, 2009). Thus, the isolation of MSCs from dental tissues as an alternative to BMMSCs provides an appealing alternative. The dental stem cells can be obtained with ease as they are readily accessible in the oral environment and can even be obtained from extracted teeth that are usually discarded.

To date, eight unique populations of dental-derived MSCs have been isolated and characterized (Liu *et al*., 2015a). Dental pulp stem cells (DPSCs) are the first type of MSCs isolated from human dental tissue (Gronthos *et al.*, 2000). Since then, the isolation of various dental MSCs have been reported, such as human exfoliated deciduous teeth (SHED) (Miura *et al.*, 2003), periodontal ligament stem cells (PDLSCs) (Seo *et al.*, 2004), dental follicle progenitor cells (DFPCs) (Morsczeck *et al.*, 2005), alveolar bone-derived MSCs (ABMSCs) (Matsubara *et al.*, 2005), gingiva-derived MSCs (GMSCs) (Zhang *et al*., 2009), stem cells from apical papilla (SCAP) and tooth germ progenitor cells (TGPCs) (Ikeda *et al.*, 2008). The PDLSCs are able to generate cementum-forming cells (cementoblast) or bone-forming cells, thus highlighting their potential use in cell based therapy for periodontal/bone regeneration (Huang *et al*., 2009).

Periodontal regeneration is a method of regenerative therapy to return the periodontal tissues (including gingiva, root cementum, alveolar bone, and the periodontal ligament) to their original healthy condition through restoration of form and function of lost structures (Bosshardt & Sculean, 2009). Non-steroidal anti-inflammatory drugs (NSAID) are widely used as an analgesic agent in healthcare. It may be used in managing orthopaedic patients pre/post-surgery, to address acute or chronic inflammation. However, such use of NSAIDs may have undesirable impact on stem cells function, particularly in periodontal or bone regeneration (Chang *et al.*, 2007; Chang *et al.*, 2005; De Luna-Bertos *et al.*, 2012; Ho *et al.*, 1999; Ho *et al.*, 2001).

Aspirin (ASA) is renowned NSAID which has been used for decades. ASA has been reported to modulate a variety of conditions related to human disease, such as cardiovascular disease, periodontal health, cancer and diabetes (Heasman & Hughes,

2014; Sutcliffe *et al.*, 2013). The impact of ASA on stem cells properties has been reported in a number of studies (Liu *et al.*, 2011a; Cao *et al*., 2015; Liu *et al*., 2015b). However, not many studies have examined the effects of ASA on MSCs or osteoblast precursor cells. The beneficial or adverse effects of ASA on the survival and function of MSCs, particularly in osteogenic differentiation needs to be further investigated.

1.1 Objectives

The objectives of this study were to investigate the effects of ASA on the osteogenic potential and expression profile of genes in PDLSCs. The study was conducted by dividing into three phases as following:

Phase I: Isolation and characterization of mesenchymal derived stem cells (MSCs) from periodontal ligament (PDL) tissue.

Phase II: Analysis of the effects of ASA on the cell proliferation rate and expression profile of growth factors-associated genes in PDLSCs using PCR-array.

Phase III: Studying the effects of ASA on osteogenic differentiation of PDLSCs:

a. Analysis of the effect of ASA on PDLSCs cell proliferation in osteogenic differentiation media.
b. Analysis on the effect of ASA on the osteogenic potential of PDLSCs.
c. Analysis of the effect of ASA on PDLSCs gene expression profiles during osteogenic differentiation through microarray analysis.
d. Bioinformatics analysis of gene expression data to gain insights on the mechanism / impact of ASA on PDLSCs osteogenic differentiation.

CHAPTER 2: LITERATURE REVIEW

2.1 Definition and types of stem cells

Stem cells (SCs) are unspecialized or non-differentiated cells which are capable of indefinite cellular division throughout the lifetime of an organism. SCs have two uniquely important properties: self-renewal and multipotency. Self-renewal is the ability of SCs to divide indefinitely to make more copies of itself, perpetuating the stem cell pool throughout the life of an organism, at the same time, maintaining the undifferentiated state (He *et al.*, 2009). Stem cell multipotency refers to its unique ability to develop or differentiate into various types of specialized cells. SCs are able to differentiate into specialized cells under specific cues and conditions (Morrison & Judith Kimble, 2006). Multipotent cells can develop into more than one cell type. However, pluripotent cells can give rise to all of the cell types of an organism.

Stem cells are generally divided into embryonic stem cells (ESCs) and adult stem cells, based on their primary origins and differentiation capacity. ESCs are derived from the inner cell mass of blastocysts and they are considered as pluripotent, capable of differentiating into cells of the endodermal, ectodermal and mesodermal origins (Thomson *et al.*, 1998). ESCs is capable of retaining its undifferentiated states *in vitro* indefinitely, enabling the production of continuous cultures almost indefinitely (Shah *et al.*, 2014). However, the study or manipulation of ESCs brought about ethical and political controversies that can pose challenging issues that is not easy to be reconciled.

2.2 Mesenchymal stem cells (MCSs) and their characteristics

MSCs were first isolated by Friedenstein and his associates from rodent bone marrow. They were identified as colony forming unit (CFU) fibroblasts, capable of differentiating into bone, chondrocytes or adipocytes, and can reconstitute the hematopoietic microenvironment (Friedenstein, 1970). MSCs are adult stem cells originating from the mesodermic layer. They possess two remarkable features similar to SCs, i.e., self-renewal capacity and multipotency, i.e. differentiation into various specialized cells of the mesenchymal lineage such as osteoblasts, chondrocytes, and adipocytes (Seo *et al.*, 2004). In addition, MSCs also have the capacity to differentiate into a variety of cells of mesodermal and endodermal origin such as hepatocytes (Chagraoui *et al.*, 2003), neural cells (Woodbury *et al.*, 2000) and epithelial cells (Spees *et al.*, 2003). Due to such properties, MSCs have been shown to be effective in tissue regeneration (Wei *et al.*, 2013).

MSCs have been extensively characterised through morphology and immunophenotyping analyses, displaying unique cell surface markers. MSCs exhibit fibroblast-like appearance, with an elongated shape and express the following cell surface markers: CD49a/CD29, CD44, STRO-1, CD90, CD105, CD106, CD146, CD140b, CD166 and CD271 (Lin *et al.*, 2009). Phenotypically, MSCs also show cell surface markers expression that is similar to perivascular, bone and neural cells (Lin et al., 2009), suggesting a common link between MSCs and different cell types (Gronthos & Simmons, 1995; Pittenger *et al.*, 1999; Prockop *et al.*, 2001; Gronthos *et al.*, 2003). Although MSCs of different origins may share a common phenotype, differences in terms of proliferative and differentiation/developmental capacities have been reported (Gronthos *et al.*, 2003; Kuznetsov *et al.*, 1997; Muraglia *et al.*, 2000) .

2.3 Tissue sources of MSCs

MSCs can be found in a variety of tissues such as human umbilical cords (Kim *et al.*, 2013a), adipose tissues (Zuk *et al.*, 2002), skeletal muscle, tendons, and trabecular bones (Bi *et al.*, 2007). There have also been attempts to harvest MSCs from various types of dental tissues. To date, eight unique populations of dental-derived MSCs have been isolated (Figure 2.1) and characterized (Liu *et al.*, 2015a). The first types of human dental MSCs were identified from pulp tissues, and are known as dental pulp stem cells (DPSCs) (Gronthos *et al.*, 2000).

Thereafter, isolation of additional dental MSCs such as human exfoliated deciduous teeth (SHED) (Miura *et al.*, 2003), periodontal ligament stem cells (PDLSCs) (Seo *et al.*, 2004), dental follicles progenitor cells (DFPCs) (Morsczeck *et al.*, 2005), alveolar bone-derived MSCs (ABMSCs) (Matsubara *et al.*, 2005), gingiva-derived MSCs (GMSCs) (Zhang *et al.*, 2009b), stem cells from apical papilla (SCAP) (Sonoyama *et al.*, 2008) and tooth germ progenitor cells (TGPCs) (Ikeda *et al.*, 2008) have been reported.

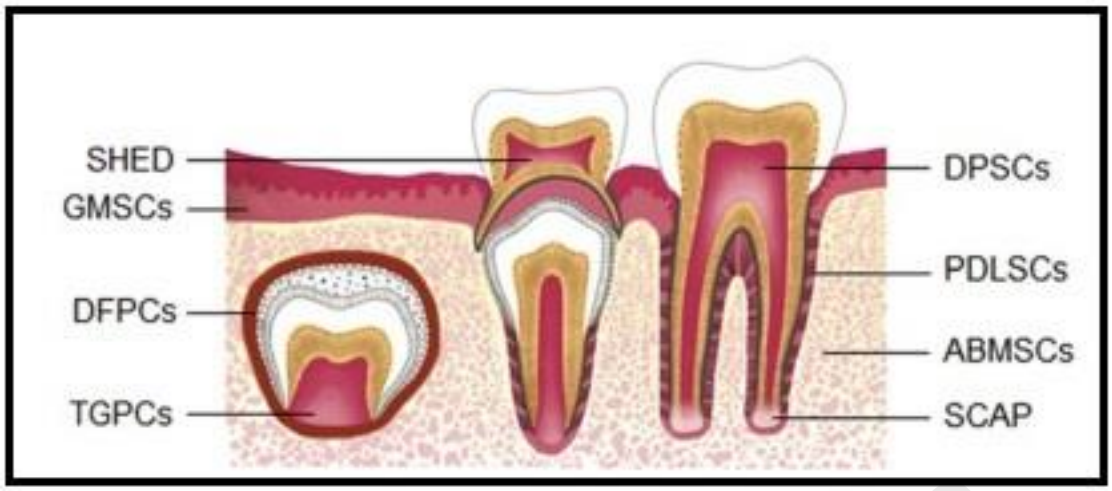

Figure 2.1: Location of dental stem cells [adaptation from (Liu et al., 2015a)].

2.3.1 Dental Pulp Stem Cells (DPSCs)

DPSCs, primarily known as postnatal dental pulp stem cells, were first isolated from permanent dental pulp tissue (Gronthos *et al.*, 2000). They express several MSCs surface markers including CD73, CD90, and CD105 but lack expression of hematopoietic markers (CD14, CD34 and CD45) (Gronthos *et al.*, 2000). DPSCs possess immunosuppressive properties and are also prone to forming ectopic dental-pulp like complexes (Gronthos *et al.*, 2000). DPSCs have the tendency to differentiate into osteoblasts, chondrocytes, cardiomycytes, and hepatocytes *in vitro*.

2.3.2 Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

SHED were first isolated from the remnant pulps of exfoliated deciduous teeth (Miura *et al.*, 2003) and have higher proliferation rates compared to DPSCs, increased cell population doubling rates, and are able to form sphere-like cell clusters. SHED display MSCs cell surface markers (Dominici *et al.*, 2006; Huang *et al.*, 2009) and also

express ESC markers (Oct-4 and Nanog), neuronal markers (nestin) and the stage-specific embryonic antigens, SSEA-3 and SSEA-4 (Miura *et al.*, 2003). SHED displayed trilineage differentiation capacity, similar to other MSCs.

2.3.3 Dental Follicle Stem Cells (DFSCs)

Dental follicle stem cells are derived from ectomesenchyme that surround the developing tooth germ cells prior to eruption. Similar to other dental stem cells, they possess an extensive proliferative ability and expressed MSCs markers but lack expression of hematopoietic markers. The dental follicle contains stem cells and lineage progenitor cells that can differentiate into cementoblast, osteoblast, and PDL-like cells (Morsczeck *et al.*, 2005). DFSCs have been reported to express putative stem cell markers including Notch-1 and Nestin, as well as demonstrated the expression of higher insulin-like growth factor 2 (IGF-2) (Morsczeck *et al.*, 2005).

2.3.4 Stem cells from the Apical Papilla (SCAP)

Sonoyama *et al.* reported the isolation of another unique type of MSCs from dental tissue, known as SCAP (Sonoyama *et al.*, 2008). The MSCs were isolated from the apical papilla of human immature permanent tooth (Sonoyama *et al.*, 2006; Sonoyama *et al.*, 2008). Root formation begins with the apical papilla proliferation of epithelial cells from the cervical loops in developing tooth. The dental papilla contributes to tooth formation and eventually converts to pulp tissue within the pulp chamber.

The apical zone lies in between the apical papilla and pulp. SCAP expresses various MSC markers and represent a novel population of multipotent stem cells that posses a greater proliferative capacity for dentine regeneration than DPSCs. SCAP also have a tendency to develop into odontoblast-like cells and adipocytes in *in vitro* studies (Sonoyama *et al.*, 2006; Sonoyama *et al.*, 2008). Morever, they have the potential for forming odontoblast-like cells produce dentine *in vivo* and are likely to be the source of primary odontoblasts for root dentin formation. In addition, they are capable to express high levels of important molecules in mediating proliferation, survival, and telomerase enzyme. SCAP also exhibit higher proliferation rates and mineralization potential compared to DPSCs.

2.3.5 Gingiva-derived MSCs (GMSCs)

GMSCs are MSCs isolated from the human gingiva. They exhibit stem cell-like properties (clonogenicity, self-renewal, and multi-lineage differentiation), immunomodulatory functions, anti-inflammatory as well as being a promising cell source for cell-based treatments in experimental inflammatory diseases (Zhang *et al*., 2009). GMSCs show positive expression of CD29, CD105, CD90 and STRO-1 (Zhang *et al.*, 2009; Wang *et al.*, 2011).

2.4 Identification and characterization of periodontal ligament stem cells (PDLSCs)

Periodontal ligament (PDL) is a soft dynamic connective tissue located between the cementum of the root and the alveolar bone (Coura *et al.*, 2008; Fortino *et al.*, 2014) (Figure 2.2). PDL tissues consist of heterogeneous populations of cells including fibroblast, epithelial, endothelial, osteoblast, and cementoblast cells (Ibi *et al.*, 2007; Tomokiyo *et al.*, 2008). It plays pivotal roles in maintaining homeostasis and regeneration of the periodontal tissues (Huang *et al.*, 2009; Zhang *et al.*, 2015). The periodontal ligament cells are formed by the cells residing within dental follicle cells during embryogenesis (Lin *et al*., 2009).

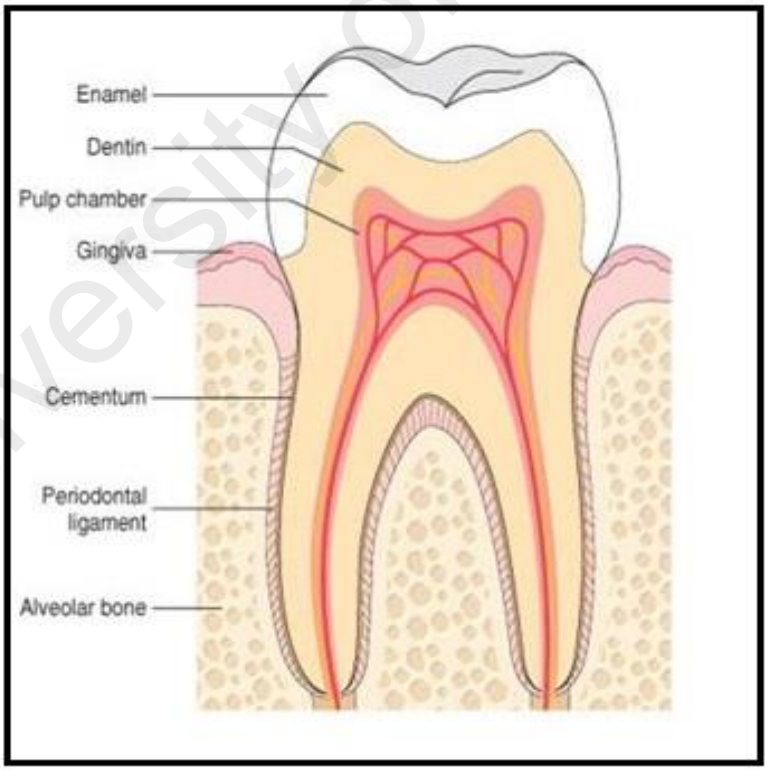

Figure 2.2: Location of periodontal ligament tissue [adapted from (Archana *et al.*, 2014)].

The human PDLSC was first isolated from human impacted third molars (Seo *et al.*, 2004). Numerous studies have then indicated that MSCs isolated from PDL have the properties that are similar to BMMSCs (Seo *et al.*, 2004; Huang *et al.*, 2009; Xu *et al.*, 2009; Silvério et al., 2010). Similar to BMMSCs, the PDLSCs demonstrated the ability to self-renewal and to generate clonogenic adherent colonies, with spindle and elongated-shaped cells. Morever, PDLSCs are capable of forming various types of mesodermal origin cells such as osteoblasts, chondrocytes, cementoblast, adipocytes, and neural-like cells (Seo *et al.*, 2004; Coura *et al.*, 2008; Huang *et al.*, 2009; Xu *et al.*, 2009).

In addition, PDLSCs differentiate into ectodermal (neuronal-like cells) and endodermal (pancreas, hepatocyte and cardiac like cells) lineages. Several *in vitro* studies have shown that PDLSCs possess many osteoblasts-like cell properties similar to BMMSCs (Gay *et al.*, 2007; Huang *et al.*, 2009). Dental stem cells including PDLSCs have been shown to express perivascular markers such as CD146/MUC18, and alpha-smooth muscle actin and pericyte antigen associated (3G5) markers, similar to BMMSCs (Seo *et al.*, 2004). The primary explants of PDLSCs are capable of producing mineralized calcium deposits *in vitro* (Arceo *et al.*, 1991; Cho *et al.*, 1992) and, further, they demonstrated the formation of mineralization nodules which tested positive with Alizarin Red S (ARS) staining (Seo *et al.*, 2004).

PDLSCs show similar characteristics to MSCs as they are positive for the following cell surface markers: CD 166, CD146, CD105, CD 73, CD90, CD29, and STRO-1 (Feng *et al.*, 2010; Yang *et al.*, 2013). However, PDLSCs showed negative expressions of the following hematopoietic markers: CD34 and CD45 (Feng *et al.*,

2010). PDLSCs stored in liquid nitrogen retained high proliferative capacity, even though they may show reduction in cell numbers upon storage (Seo *et al.*, 2004).

PDLSCs are hypoimmunogenic and were able to inhibit the proliferation of allogeneic T-cells through the upregulation of cyclooxygenase2 (COX2) and prostaglandin E2 (PGE2) (Ding *et al.*, 2010). PDLSCs could suppress the proliferation, differentiation, and migration of B-cells through cell-to-cell contact, which were mediated by programmed cell death protein-1 (Ousheng *et al.*, 2013). The low immunogenicity and immunosuppressive effects towards T- and B-cells allow the use of allogeneic PDLSCs in periodontal regeneration.

2.4.1 Growth potential of PDLSCs

PDLSCs could maintain their higher growth potential rate beyond 100 population doublings before entering senescence, compared to BMMSCs, which can only achieve 50 populations doubling before senescence were noted. Similar to other MSCs, PDLs undergo senescence-associated growth arrest as culture passage number increases, and this appears to make it different from ESCs, which are considered as immortal stem cells (Bocker *et al.*, 2008).

2.4.2 Periodontal regeneration using PDLSCs

The presence of various MSCs residing in dental or craniofacial tissue has invited clinical investigations into the potential use of such SCs for the regeneration of tissues in the orofacial region. PDL is predicted to provide the best-suited source of

progenitor cells for periodontal regeneration therapy. Dental stem cells, including PDLSCs, are derived from neural crest cells (ectomesenchyme), and this makes them an attractive source of MSCs for craniofacial regeneration owing to their higher potential or plasticity of differentiation into craniofacial tissues (Coura et al., 2008).

Although MSCs express a common set of genetic markers, it has been observed that different sources of MSCs have their own distinct phenotypes and biological properties. Thus, MSCs of different sources came from their own specific microenvironment, which could influence their plasticity or differentiation capacity (Musina *et al.*, 2005; Musina *et al.*, 2006)). This notion is supported by the observation of lower odontogenic potential showed by BMMSC compared to dental stem cells (Yu *et al.*, 2007; Huang *et al.*, 2009).

The study of PDLSCs use for orofacial regeneration purposes may bring further understanding of the factors that regulate the formation of mineralized matrices and other associated connective tissues during regenerative processes. PDLSCs are able to form mineralized nodules under appropriate culture conditions. They are observed to express several bone related gene marker such as alkaline phosphatase and bone sialoprotein (IBSP), with expressions comparable to BMMSCs (Lekic *et al.*, 2001; Pitaru *et al*.2002).

ALP activity was also observed by day 14 in PDLSCs and day 7 in BMMSCs under osteogenic cultures conditions (Gay *et al.*, 2007). IBSP expression was observed by day 7 for both cells but more intense staining is seen in PDLSCS cultures. In addition, PDLSCs are also capable of generating cementoblast-like cells and periodontal

ligament-like tissue *in vitro*, highlighting potential therapeutics application of PDLSCs for PDL regeneration (Huang *et al*., 2009).

A number of studies have reported PDLSCs potential in periodontal regeneration (Gronthos *et al.*, 2000; Gronthos *et al.*, 2000;Miura *et al.*, 2003; Seo *et al.*, 2004; Moshaverinia *et al.*, 2014; Tang *et al.*, 2014; An *et al.*, 2015). Those studies indicated that PDLSCs were able to show osteogenic differentiation, forming mineralized matrices in the presence of β-Glycerophosphate, ascorbic acid and dexamethasone (An *et al.*, 2015). L-ascorbic acid, dexamethasone and β-glycerophosphate are *in vitro* osteogenic inducers that are supplemented in osteogenic media (Jaiswal *et al.*, 1997; Kim *et al.*, 2003) but these inducers are not useful for *in vivo* studies.

PDLSCs that were expanded *in vitro* and transplanted into immunocompromised rodents have been shown to generate cementum/PDL-like cells and connective tissues, and this highlighted the potential use of PDLSCs in addressing periodontal defects *in vivo* (Seo *et al*., 2004). PDLSCs have also been reported to induce formation of periodontal ligament-like structures *in vivo*, in the presence of scaffolds such as hydroxyapatite/tricalcium phosphate (HA/TCP) acting as a carrier (Sonoyama *et al.*, 2006). The PDLSCs were observed to form cementum on the surface of the HA/TCP carrier and Sharpey's fibers after a few weeks of transplantation (Sonoyama *et al.*, 2006). These findings suggested that PDLSCs could be useful for dental root and periodontal regeneration.

2.5 Bone

The human bone is a type of mineralized connective tissue that is also known as osseous tissue, which forms the rigid supporting structure of the human body-the skeleton. The bone tissue undergoes a continuous process of remodeling *in vivo*. The formation of new bone tissue is known as ossification (or osteogenesis). The osteogenic potential of adult stem cells is often investigated if the cells are newly isolated from the human tissue, to confirm/ascertain their osteogenic differentiation capability, particularly BMMSCs or stem cells from the oro-facial regions. Bone consists of four types of cells: (i) osteoblast, (ii) osteoclast, (iii) osteocyte, and (iv) bone lining cells (Downey & Siegel, 2006), as shown in Figure 2.3. Bone cells work in harmony to maintain a balance between bone formation and resorption, to eventually control bone structure and function.

2.5.1 Osteoblast

Osteoblasts are bone forming cells which originated from MSCs. Osteoblasts make about 4 to 6% of total bone cells (Capulli *et al.*, 2014) and they are found along the bone surface with a cuboidal shape (Figure 2.3). They synthesize the bone matrix and subsequent matrix mineralization. At the end of the bone-forming phase, osteoblasts can have one of four different fates; some of the osteoblast cells are trapped as they are buried in the bone matrix as osteocyte; some of the osteoblasts form the bone–lining cells and the majority of the osteoblasts undergo apoptosis and also transdifferentiate into cells that deposit chondroid or chondroid bone (Noble *et al.*, 1997; Jilka *et al.*, 1998; Manolagas, 2000; Li *et al.*, 2004; Franz-Odendaal *et al.*, 2006).

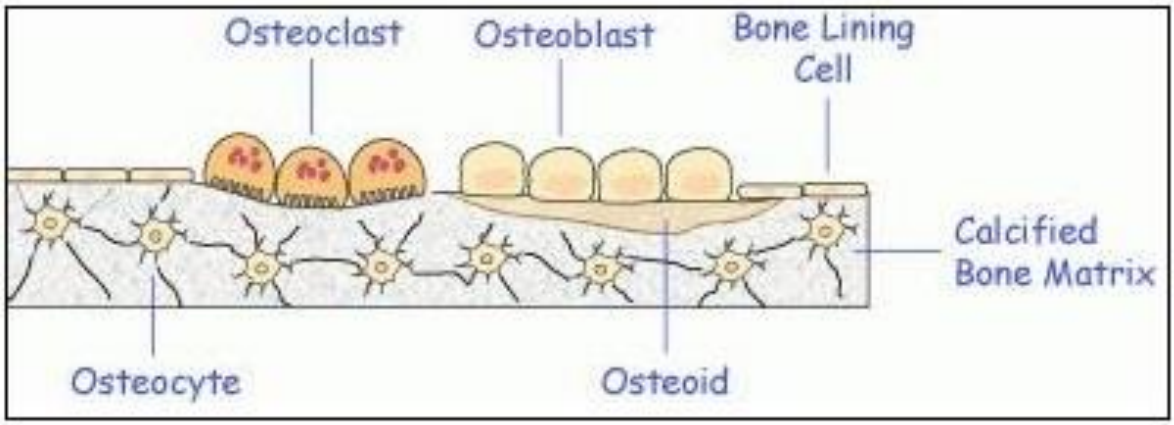

Figure 2.3: Types of bone cells.

2.5.2 Bone Lining Cells

Bone lining cells are quiescent flat-shaped osteoblasts that exhibit a thin and flat nuclear profile. They have been shown to prevent the direct interaction between the osteoclast and bone matrix. The cells in the bone lining are expressed receptor activators of the nuclear factor kappa B-ligand (RANKL) which plays a critical role during osteoclast differentiation (Erik Lubberts, 2002).

2.5.3 Osteocytes

Osteocytes represent the most abundant cells in the bone, consisting of about 90 to 95% of total bone cells. They are terminally differentiated osteoblast cells and function to support bone structure by maintaining a connection with each other and the bone surface via multiple filipodial cellular processes. As such, they act as mechanosensors where the osteocytes will transduce signals from the bending or stretching of the bone into biologic activity. The major function of osteocytes is that they affect remodeling by translating mechanical strainings into biochemical signals between each other and to cells on the bone surface (Lanyon, 1993).

2.5.4 Osteoclasts

Osteoclast cells are derived from hematopoietic stem cells that which developed and adhere to the bone matrix. Several steps are involved in osteoclast differentiation of hematopoietic stem cells, to give rise to colony-forming granulocytes/macrophages units. During the initiation phase, the hematopoietic stem cells are recruited to the specific area of the bone, to differentiate into cells of the monocyte/macrophage lineage in the bone marrow. The monocyte/macrophage lineage in the bone marrow acts as an osteoclast precursor that will attach to the bone matrix to differentiate into mature osteoclasts in response to osteoclastogenesis factors, macrophage colony-stimulating factor (M-CSF), and nuclear factor kappa B-ligand (RANKL) that initiate bone resorption (Boyle *et al.*, 2003). M-CSF will bind to its receptor found in osteoclast precursor cells and this stimulated their proliferation (osteoclastogenesis) and inhibited their apoptosis.

2.5.5 Bone extracellular matrix

The bone extracellular matrix consists of both a non-mineralized organic matrix and an inorganic matrix (Downey & Siegel, 2006). The organic component is constituted of mainly collagen proteins, pre-dominantly type I, and several non-collagenous proteins including bone sialoproteins (IBSP), osteopontin (OPN), osteocalcin (OCN), proteoglycan, glycosylated proteins, and gla-proteins as well as growth factors and cytokine components (Downey & Siegel, 2006). The inorganic matrix component plays an important role that is related to the storage of ions. It is estimated that mineral salts consist of 99% calcium and 85% phosphorus as well as little amounts of magnesium and sodium.

2.6 Osteoblastogenesis

Osteoblastogenesis is the process of osteoblast development, consisting of three major phases: (i) lineage commitment, (ii) proliferation phase known as pre-osteoblast, (iii) matrix maturation and (iv) matrix mineralization of osteoblasts which are characterized by the expression of distinctive osteoblast markers. The first transition involves the commitment of MSCs to osteogenic lineage. The lineage commitment of multipotent mesenchymal cells is driven by the selective expression of the so-called "master transcriptional regulators".

This step is regulated by transcription factors and the expressions of lineage inducing factors are committed either to osteoblast or chondroblast, depending upon the threshold levels of Sox, myosin D (MyoD) and runt-related transcription factor 2 (Matsubara *et al*., 2008). Meanwhile MyoD directs these cells into the myogenic pathway, where Sox9 enhances chondrogenesis development, while Peroxisome proliferator-activated receptor γ2 (PPARγ2) promotes adipogenesis.

After lineage commitment, a second transition is associated with mitotic duplication, giving rise to another stem cell type and a committed osteoprogenitor. A wide range of osteogenic cell types are involved during this stage and range from MSCs through to the more committed pre-osteoblast and chondro-osteoprogenitors, which are characterized by the production of proteins such as collagen, fibronectin, and growth factors (i.e. BMP2/TGF-β). The osteoblast differentiation (Figure 2.4) is regulated by several specific transcription factors such as runx2 (Qi *et al.*, 2003) and osterix (SP7) (Nakashima *et al.*, 2002), along with other transcription mediators such as msh

homebox homologue-2 (Msx-2) and distal–less homebox (DLX5) (Harada & Rodan, 2003).

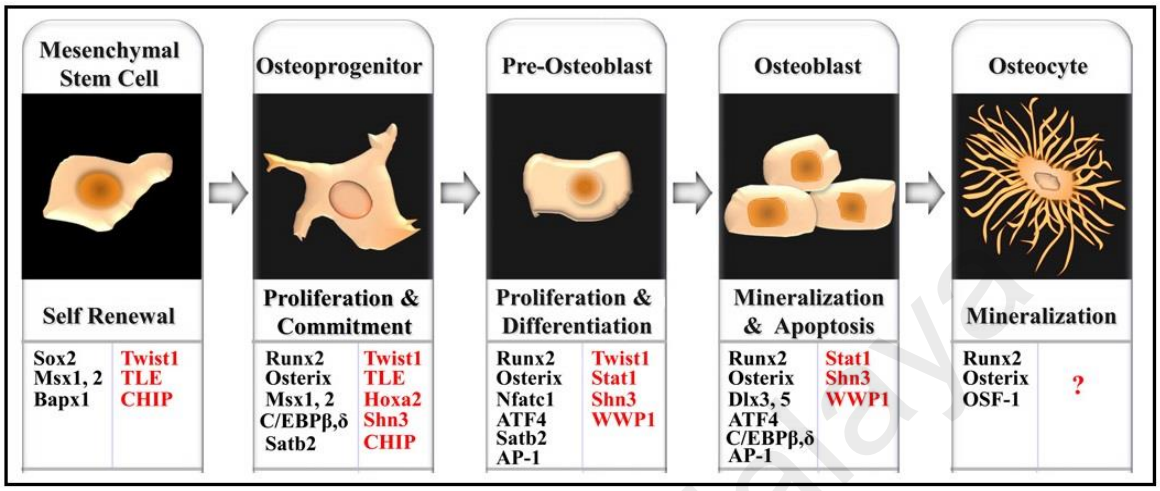

Figure 2.4: The bone formation process (adapted from (Javed *et al.*, 2010).

In addition, the transcription factors support the proliferation of MSCs and also suppress genes that induce post-proliferative stage of osteoblast differentiation, including TWIST and helix-loop-helix factors (HLH). Osteoprogenitor cells then give rise to a transit-amplifying compartment (Watt *et al.*, 1995) that is associated with proliferative activity. The pre-osteoblast is an intermediate stage where the extracellular matrix (ECM) protein starts to be synthesized, with high amounts of IBSP expression and commitment to the osteoblastic lineage then follow suit. This highly regulated program of gene expression and cellular differentiation is governed by the expression and activity of transcription factors. These factors also play an important role in maintaining the rate of proliferation of progenitor cells in an undifferentiated state (Jensen *et al.*, 2010).

Subsequently, after the proliferation stage the cells exit mitosis, and induce the expression of genes involved in the mineralization of the extracellular matrix such as

osteocalcin, OPN, and collagenase. At this level, there is an expression of ALP, which is an early marker of the post-proliferative osteoblast phenotype, and production of collagen matrix. The MSCs exit from the cell cycle and the cessation of cell proliferation is indicated by the accumulation of type 1 collagen and a marked reduction in histone expression. In order to support the expression osteoblast-related genes, collagen ECM promotes a signalling cascade by both cell matrix and cell-cell interactions (Franceschi & Xiao, 2003).

For example, runx2 mRNA or protein levels and its transcriptional activity suggest that post-translational modification and/or protein-protein interactions may regulate this factor. Runx2 can be phosphorylated and activated by the mitogen-activated protein kinase (MAPK) pathway. This pathway can be stimulated by a variety of signals including those initiated by extracellular matrix (ECM), osteogenic growth factors like bone morphogenic proteins (BMPs) and fibroblast growth factor-2 (FGF2), mechanical loading and hormones such as parathyroid hormone (PTH) (Abelev *et al.*, 2009).

The final stage of osteoblastogenesis is characterized by the deposition of minerals in ECM together with the expression of osteoblast marker genes such as IBSP, OCN, and OPN. At this stage, the downregulation of matrix maturation takes place and strong expression of gene associated with the mineralization that leads to the formation and accumulation of hydroxyapatite crystals occurs. Osteoblasts cells produce ECM that are mineralized and become inactive bone lining cells or osteocytes, entrapped within the mineralized matrix (Javed *et al.*, 2010). The majority of osteoblast cells do not achieve the end stage of an osteocyte and undergo apoptosis. It can be

observed when apoptotic osteoblasts show increased expressions of Bad and Bax, and the suppression of Bcl2, a pro-cell survival gene (Xing & Boyce, 2005).

2.7 Growth Factors (GFs): regulation of proliferation and differentiation of MSCs

Growth factors (GFs) are known biological mediators with crucial roles in tissue proliferation and repair. Fibroblast growth factor 2 (FGF2), platelet derived growth factor-BB (PDGF-BB), bone morphogenetic proteins (BMP2/6/12), and brain-derived neurotrotrophic factor (BDNF) are among GF candidates for periodontal regeneration (Lin *et al.*, 2015). The following section discusses the importance of the above growth factors in stem cell proliferation and differentiation, particularly in relation to osteoblast differentiation.

2.7.1 BMPs family members and their roles in osteogenic differentiation

BMPs are multifunctional cytokines and identified as members of the transforming growth factor-β (TGF-β) superfamily (Celeste *et al.*, 1990). There are more than 20 BMPs family members, of which BMP2, BMP4, BMP6, BMP7 and BMP9 have been shown to play important roles in ossification. The effects of BMP6 and BMP9 are the strongest inducer, imparting the most prominent factors for robust and mature bone ossification to take place (Kang *et al.*, 2004), as shown in Figure 2.5. BMP3 has been shown to act as a negative regulator of bone ossification induced by BMP2, BMP6, and BMP7. In contrast, BMP9 plays a vital role during the entire process of osteoblast formation. It has also been reported that BMP2 did not affect cell

proliferation, but it is able to enhance immortalized human marrow stromal cell line hMS(2–6) osteoblast differentiation. This is achieved by an increase in mineralized nodule formation and enhanced expression and secretion of ALP and type I procollagen (Gori *et al.*, 1999).

BMP2 plays an important role in MSCs osteoblast differentiation through the activation of the SMAD signaling pathway. It regulates essential factors in bone formation and osteogenesis-related genes such as ALP type I collagen, osteocalcin, and IBSP (Yang *et al*., 2014). BMP2 also regulates the expression of Osterix/sp7 through runx2-dependent and -independent mechanisms. Osterix is a transcriptional factor that plays an important role in osteoblast formation and osteoblastogenesis, and in addition, the homebox gene, MSX-2, is also upregulated by BMP2 (Matsubara *et al.*, 2008). It is noted that the expression of Osterix by BMP2 can be mediated through either of two different transcriptional factors, runx2 and MSX-2 (Matsubara *et al.*, 2008).

Another BMPs family member that plays an important role in bone formation is BMP4. BMP4 was found in less differentiated osteoprogenitor cells and not by differentiated osteoblasts. It plays an important role in the early stages of bone fracture healing (Nakase *et al.*, 1994). BMP4 indirectly stimulates bone formation by inhibiting osteoclastogenesis (Yang *et al.*, 2014). This is achieved by BMP4 stimulation of osteoprotegerin (OPG) expression, which is an osteoclastogenesis inhibitor protein (Tazoe *et al.*, 2003).

It has also been reported that BMP4 induced an increase in the expression level of OPG in mouse bone marrow derived from stromal cell lines via the MAPK signaling pathway (Tazoe *et al.*, 2003). Another study reported that BMP4 and VEGF acted

synergistically to enhance cartilage formation in the early stages of endochondral bone formation from muscle-derived stem cells (Peng *et al.*, 2002). These early stage actions coupled with accelerated cartilage resorption eventually led to a significant enhancement of osteoblast differentiation.

BMP6 is similar to BMP5, osteogenic protein (OP)-1/BMP-7, and OP-2/BMP-8 (Ebisawa *et al.*, 1999). It has been demonstrated that BMP6 could increase ALP activity in osteoblast lineage cells including C2C12 cells, MC3T3-E1 cells, and ROB-C26 cells and induce bone formation through transduced signals mainly through Smad5 and possibly through Smad1 (Ebisawa *et al.*, 1999).

These findings suggest that, similar to BMP-2 and OP-1/BMP-7 (Katagiri *et al.*, 1994; Takeda *et al.*, 1998), BMP-6 converts the differentiation pathway of myoblastic cell lines into that of osteoblast lineage, and induces osteoblast differentiation and maturation in osteoprogenitor and osteoblastic cell lines. The profile of binding of BMP-6 to type I and type II receptors was similar to that of OP-1/BMP-7 in C2C12 cells and MC3T3-E1 cells; BMP-6 strongly bound to activin receptor-like kinase (ALK)-2 (also termed ActR-I), together with type II receptors, i.e. BMP type II receptor (BMPR-II) and activin type II receptor (ActR-II). In addition, BMP-6 weakly bound to BMPR-IA (ALK-3), to which BMP-2 also bound.

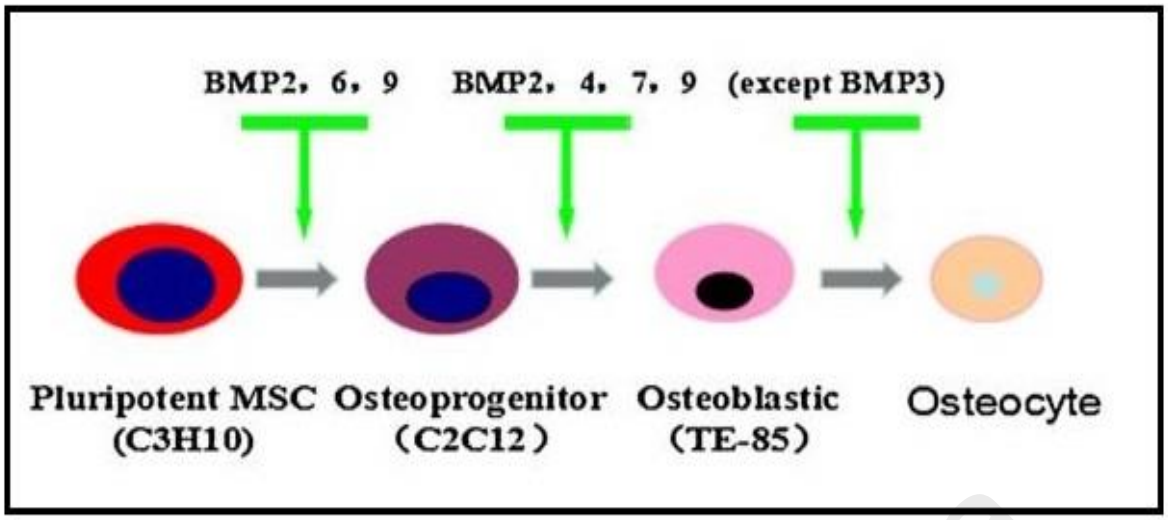

Figure 2.5: BMPs play a role in osteoblast formation [adapted from (Yang *et al.*, 2014)].

2.7.2 Vascular endothelial growth factors (VEGFs)

VEGF family members are well-known as potent angiogenesis inducers *in vivo* and *in vitro* (Giacca & Zacchigna, 2012). It was noted that co-injection of MSCs with VEGF to myocardial infarction (MI) hearts increased MSC proliferation and increased cell engraftment, and resulted improvement of cardiac functions.

2.7.3 Platelet derived Growth Factors (PDGFs)

Platelet derived Growth Fcators (PDGFs) are growth factors that promote cell proliferation, migration as well as cell differentiation (Li *et al*., 2014a). PDGF are dimers of disulfide-linked polypeptide chains that encode four different PDGF chains (PDGF-A, PDGF-B, PDGF-C and PDGF-D) (Andrae *et al.*, 2008; Hoch & Soriano, 2003). PDGF will execute their functions when PDGFs molecules will bind to the cell surface receptors, PDGFRs, which are member of tyrosine kinase (Hoch & Soriano, 2003; Andrae *et al.*, 2008).

MSCs express high levels of PDGF-A and PDGF-C and low levels of PDGF-b and PDGF-D (Rodrigues *et al.*, 2010). PDGF-BB has been found to induce both proliferation and migration in MSCs (Tamama et al., 2006; Fierro *et al.*, 2007). However, one study reported that PDGF-β inhibits osteogenic differentiation while the effect of PDGF-α was subtle (Tokunaga A, 2008).

2.7.4 Fibroblast growth factors (FGFs)

FGFs have been isolated from many sources and although have a pivotal role in cell proliferation, they also display multitude functions in the epithelial and mesenchymal cells (Hurley *et al.*, 2002). Structurally, FGFs contain 22 members and their molecular weight ranges between 17 to 34 kDda (Yun *et al.*, 2010). FGFs are divided into two classes: acidic (a-FGF) or basic (b-FGF), and these were originally isolated from the brain and pituitary glands as growth factors for fibroblasts (Yun *et al.*, 2010). bFGFs showed various biological functions including development, differentiation, angiogenesis, and wound healing.

The expressions of FGFs play major roles in bone development, and are found in mesenchymal progenitor and osteoblast cells. In addition, a study has shown that b-FGF, especially FGF2, maintains the proliferation of as well trilineage differentiation capacity in MSCs through the early mitogenic cycles although, eventually, all the MSCs differentiate into the chondrogenic line (Rodrigues *et al.*, 2010). FGF2 was reported to markedly enhance the growth rates and the life spans of MSCs from rabbit, canine, and human bone marrows in monolayer cultures (Tsutsumi *et al.*, 2001).

bFGF could enhance the levels of cAMP, ALP, OCN, mineralization and differentiation of osteogenic precursors cells of rat stromal bone marrow cells (rSBMC) isolated from young adult rat. This suggests bFGF is able to stimulate rSBMC growth and biochemical functions (Pitaru *et al.*, 1993). The exposure of bFGF to rat BMMSCs enhances *in vitro* osteogenic development in the presence of dexamethasone as the inducer (Hanada *et al.*, 1997). The treatment of rat BMMSC in combination with bFGF and BMP2 was also shown to synergistically enhance osteogenic potential of the stem cells, compared to BMP2 treatment alone.

FGF-18 was reported to be expressed in mesenchymal tissues during differentiation of osteoblasts in calvarial bone development and in the perichondrium of developing bones (Ohbayashi *et al.*, 2002). It appears to positively affect osteogensis, by regulating cell proliferation and differentiation, while at the same time, able to negatively regulate chondrogenesis (Ohbayashi *et al.*, 2002). The biological activities of FGFs are dependent on the presence of BMP proteins. FGF4 and FGFR signals play important roles during BMP2-induced bone formation as observed in rats (Kubota *et al.*, 2002).

2.8 Mechanism of MSCs osteogenic differentiation

2.8.1 FGF signaling controls osteoblast gene expression

FGFs are heparin-binding proteins and function by binding to the following: tyrosine kinase in the intracellular region of FGR receptors (FGFRs), non-transducing heparan sulfate-containing proteoglycans (HSPGs), the cysteine-rich receptor (CFR), and binding proteins (BPs) (Hurley *et al.*, 2002). FGFRs contain an extracellular ligand-binding domain, a transmembrane region, and an intracellular divided tyrosine

kinase domain. The FGF-FGFRs binding results in the autophosphorlyation of tyrosine residues in the intracellular region of the FGFR leading to the activation of intracellular downstream signaling pathways especially in the three important pathways of mitogen-activated protein kinase (Ras/MAPK), protein kinase B, protein kinase C and phospholipase C, and also p21 pathways (Dailey *et al.*, 2005).

There are four distinct types of FGFR tyrosine kinase receptors (FGFR1, FGFR2, FGFR3, and FGFR4) with different FGF- binding properties. FGFRs are expressed on many different cell types and regulate proliferation, differentiation, and survival. FGFR1 and FGFR2 are expressed in MSCs prior to deposition on extracellular bone matrix during bone development as shown in Figure 2.6. FGFR1 was recently shown to be an important transducer of FGF2 signals in proliferating osteoprogenitor cells and subsequent differentiation during short term cultivation (Ling *et al.*, 2006).

When FGFR1 is blockied, the inhibition of the FGF signaling pathway is resulted and leading to the abrogation or impairment in the osteogenic differentiation of human BMMSCs (Jacob *et al.*, 2006). FGF signaling is associated with main downstream pathways such as: 1) RAS/MAP kinase signaling pathway, 2) P13 Kinase/AKT signaling pathway, and 3) PLCγ signaling pathway. The key event in the FGF signaling pathway is the phosphorylation of tyrosine residues in the docking protein and fibroblast growth factor receptor substrates 2-alpha (FRS2-alpha) which provide new binding sites for direct or indirect recruitment of proteins that are responsible for both activation and attenuation signaling (Lax *et al.*, 2002).

FRS2-alpha recruits a complex consisting of an adaptor protein, the guanine nucleotide exchange factor 2 (GRB2), the son of sevenless, the tyrosine phosphatase (SHP2), and the docking GRB2-associated binding protein (GAB1). The formation of the FRS2 signaling complex results in the activation of the RAS/MAP kinase signaling pathway (Dailey *et al.*, 2005) and the PI3 kinase/AKT pathways (Lamothe *et al.*, 2004).

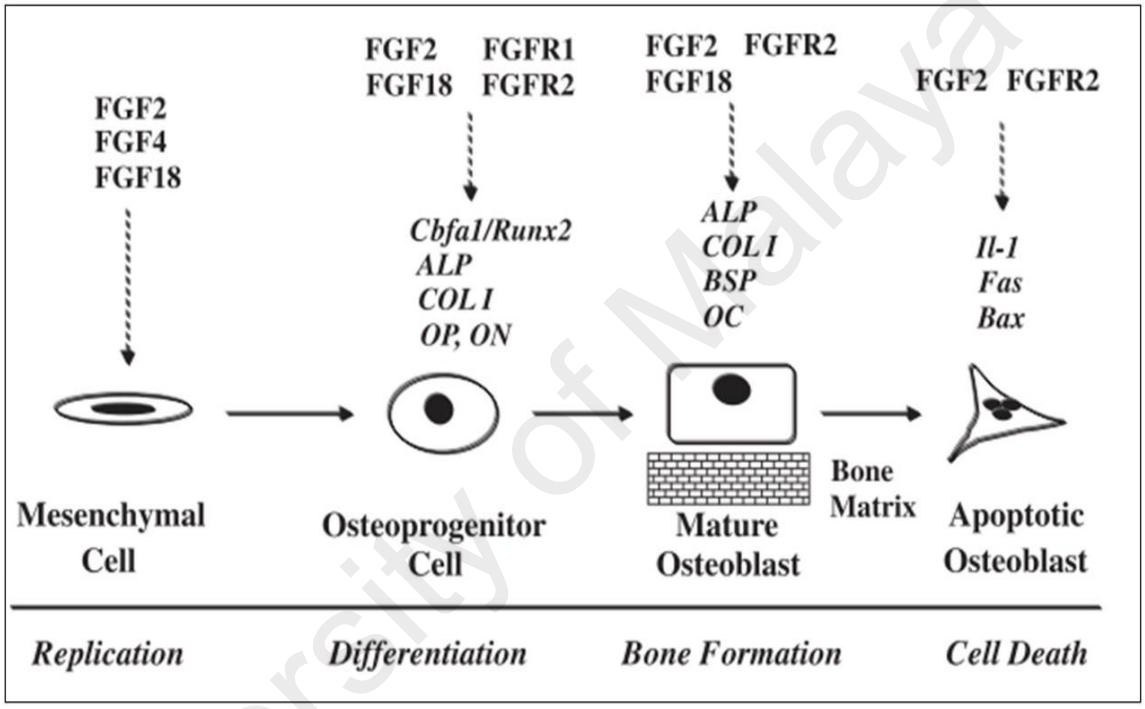

Figure 2.6: Roles of FGF and FGR in osteoblast differentiation and fate. FGF acts through FGFR (dashed arrows) to control genes involved in osteoblast proliferation, differentiation and apoptosis [adapted from (Marie, 2003)].

FGF signaling plays important roles in MSCs osteogenic differentiation. It can stimulate the proliferation of osteoblast at an early stage, allowing osteoblastic lineage commitment to take place. FGF signaling regulates the expression of various genes that are involved in osteoblast differentiation. Its effect on osteoblast marker genes varied, depending on the cell types either directly or indirectly. The direct effect of FGF signaling is mediated by transcription factors (i.e. runX2/Cbfa1) while the indirect effect is mediated by soluble factors or cell matrix interactions (Marie, 2003).

The stimulation of runx2/Cbfa1 expression by FGF2 provides an important molecular mechanism by which FGF/FGFR signaling directly activates the expression of osteoblast genes that are dependent on runx2/Cbfa1. FGFs signaling could also have indirect effects on growth factor, matrix degradation, and cell proteins, as shown in Table 2.1. In such cases, FGFs signaling could affect osteoblast differentiation. Another example of the indirect effect of FGF signaling is to regulate osteogenic differentiation through the interaction of the growth factor signaling pathway.

In addition, FGF can also influence other growth factors that influence osteoblast formation, and this could enhance bone formation. For example, FGFs increase VEGF and the hepatocyte growth factor (HGF) levels, both of which are mitogenic factors for osteoprogenitor cells (Tokuda *et al.*, 2000). FGFs also regulate the genes involved in matrix degradation. FGF signaling regulates the expression of IL6 which is important mediator of the effects of bFGF on bone cells (Kawaguchi *et al.*, 1995; Hurley *et al.*, 1996). FGF2 modulates the bone matrix by regulating the expression of collagenase activity. In fetal rat osteoblast-enriched (Ob) cells, it has been observed that bFGF can stimulate collagenase 3 gene promoter activity through AP-1 promoter binding site (Varghese *et al.*, 2000) and stromelysin-3 transcription.

Table 2.1: Gene regulation by FGF/ FGF signaling in bone [adapted from (Marie, 2003)]

Transcription factors	Matrix and cell proteins	Growth factors	Matrix degradation	Apoptopic proteins
AP-1 Cbfa1/Runx2 Twist	Col I ON, OP BSP OC N-Cadherin Connexin-43 Noggin	TGFβ IGF1 IGFBP5 VEGF HGF	MMP1 Collagenase-3 TIMPs Stromelysin MMP1	Bax IL-1 Fas

2.8.2 Signal transduction pathways for BMP-induced osteoinductive differentiation

The BMP signalling pathway encompasses two categories: 1) a classical SMAD-dependent pathway where the BMP triggers a cellular response mainly through the SMAD pathway (Massague, 1998), and 2) a non-classical SMAD-independent mitogen activated protein kinase (MAPK) pathway. In the former, BMPs initiate their signaling cascade by binding to a dimeric complex of two transmembrane serine–threonine kinase receptors. The BMP receptors can be divided into type I and type II (Yang *et al.*, 2014).

The activation of intracellular SMAD proteins occurs when BMPs bind to two different types of BMPs receptors i.e., BMP-type I (BMPR-I) and BMP-type II receptor (BMRPR-II). Upon binding to the BMPR-II, BMPRI is recruited to form an activated quaternary complex and phosphorylation occurs to activate SMAD proteins (Lin & Hankenson, 2011). Receptor SMADs bind to a co-SMAD and translocate to the nucleus to serve as transcription factors.

SMADs were divided into two categories: 1) R-SMADs (SMAD-1, SMAD-5 and SMAD-8); 2) Co-mediator SMADs. At first, the BMPs will bind to the type-I and type-II receptor complexes as shown in Figure 2.7. Then, the type-II receptor complex was activated and phosphorylates the GS-domain type-I receptor. The type-I receptor subsequently recognized the R-SMADs in the cytoplasm and phosphorylation occurred. The phosphorylated R-SMADs together with SMAD-4 turn to the complex form and activate an essential factor for the bone formation transcriptional factor, runx2/Cbfa1 in the nucleus (Nishimura *et al.*, 2003).

Meanwhile in the non-classical BMPs-MAPK pathway, BMPs will bind to either type-I or type-II receptors to activate the MAPKs signaling pathway and further transduce the signaling into the nucleus via the ERK pathway, p-38 pathway, and JNK-pathway. This signal transduction plays an important role in bone formation (Nohe *et al.*, 2004; Matsubara *et al.*, 2008).).

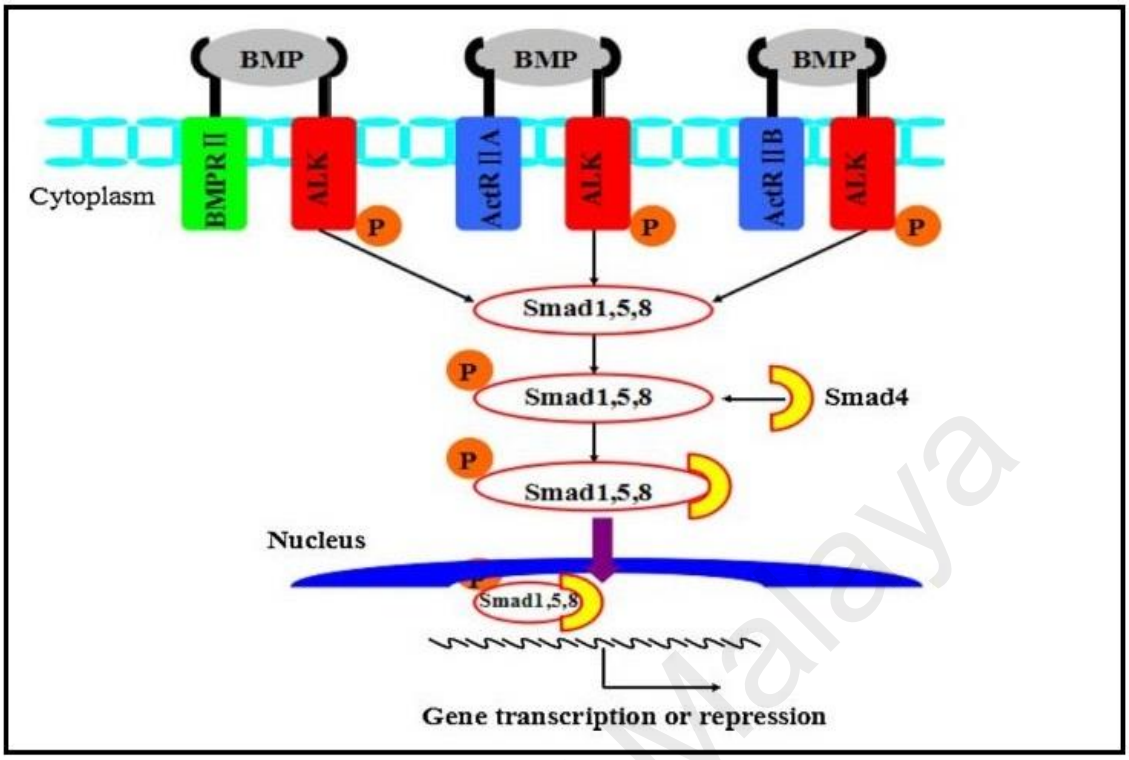

Figure 2.7: Classical BMPs signal transduction pathway [adaptation from (Yang *et al*., 2014)].

2.9 Integrin–Extracellular Matrix (ECM) interactions to direct MSCs differentiation via cell adhesion/transduction

Extracellular matrix (ECM) proteins were observed to have varying abilities in supporting osteogenesis of hsMCs (Salasznyk *et al.*, 2004; Klees *et al.*, 2005; Salasznyk *et al.*, 2007; Doran *et al.*, 2010; Mauney & Volloch, 2010). Integrin is a transmembrane protein consisting of associated α and β subunit plasmic tails and provide the primary link between mesenchymal stem cells (MSCs) and their surrounding extracellular matrix (ECM). It represents a major family of cell-surface receptors that facilitate adhesion between cells and the surrounding ECM. Upon binding of integrin to ECM proteins, intracellular signaling cascades are resulted, and this modulates cells phenotype and genotype, thereby affecting adhesion, proliferation and differentiation.

MSCs expressed a broad range of integrins including α1β1, α2β1, α5β1, α6β1, αVβ3, and αVβ5 (Gronthos *et al.*, 2001). The variety of integrins results in a different combination of α and β subunits that can bind to a specific repertoire of ECM protein molecules or peptides. The specific repertoires include fibronectin, laminin, collagens, vitronectin, OPN, bone sialoprotein, and dentin matrix protein-1 (Docheva *et al.*, 2007). In MSCs, the binding of integrin and fibronectin can facilitate osteogenic potential. Different domains of fibronectin have different affinity for multiple integrins including α5β1 and αVβ3 and thus have varying abilities to promote osteogenesis.

It is widely acknowledged that the type of ECM and integrin present can influence MSC differentiation. This is supported in a previous study on BMMSCs where cultures in osteogenic conditions in the presence of fibronectin and OPN, and the mineralization matrix and commitment to the osteoblast lineage were increased (Schwab *et al.*, 2013). It has also been reported that osteogenic induction upregulates integrin receptors α5β1 and αVβ3, and that the differentiation of dental bud stem cells (DBSCs) toward the osteoblastic lineage increased when grown with FN, OPN, and vitronectin (Di Benedetto *et al.*, 2015).

2.10 Prostaglandin (PG), Cyclooxygenase and their roles in bone metabolism

Prostaglandin (PG) is known as prostanoids are formed when arachidonic acid is released from the plasma membrane by phospholipases (PLAs) and metabolized by the activity of prostaglandin G/H synthase or cyclooxygenase (Casimiro *et al.*, 2010). COX is a bifunctional enzymes contains cyclooxygenase and peroxidase activity (Smith *et al.*, 2000). PG synthesis is triggered by COX activity. The COX enzyme exists in two

different forms, namely COX-1 and COX-2. The former is a housekeeping gene, constitutively expressed and plays important roles in physiological function such as platelet aggregation, gastric lining protection and homeostasis (Crofford, 1997). COX-2 is an inducible enzyme that is implicated in pathophysiological processes, which can lead to inflammation, pain and fever (Turini & DuBois, 2002).

The COX-1 is expressed in both normal and fractured bones while COX-2 is up regulated especially during the initial stages of the bone repair process. Both osteoblast and osteoclasts produce prostaglandins, where PGE2 is the most abundant PG synthesized. COX and PG play important roles in bone homeostasis (Raisz, 1995; Min *et al.*, 1998; Zhang *et al.*, 2002) specifically COX-2 which has a key role in prostaglandin-E2 (PGE-2) production and important in osteoblast formation (Zhang *et al.*, 2002). The effect of PGs on bone metabolism is mediated through the prostaglandin E receptor types 2 and 4 (EP2 and EP4). In osteoblast formation, PG could increase the proliferation and differentiation of osteoblasts while in bone resorption, PG could increase the activity of the osteoclast.

2.11 Aspirin (ASA) and its mechanism

Aspirin (ASA) is also known as acetylsalicylic acid and type of non-steroidal inflammatory drug (NSAID) that is mainly used as analgesic, anti-pyretic and anti-inflammatory. ASA exerts its anti-inflammatory action by suppressing the production of prostanoids (thromboxanes, prostacyclines, and PGs) (Casimiro *et al.*, 2010), that are produced by COX-1 and COX-2 enzymes. The inhibition of cyclooxygenase, particularly COX-2 prevents the elevation of PGs levels, molecules which are related to

induction of inflammation, which can lead to various kind of pain sensation (Gray *et al.*, 2002). The inhibition prevents the pro-inflammatory signaling pathways such as NF-Kβ pathway (Yin *et al.*, 1998). Moreover, ASA action resulted in the inhibition of IKK-β kinase activity which in turn prevents the activation of the NF-kB pathway (Yin *et al.*, 1998).

2.12 Effect of NSAIDs on osteoblast cell culture and osteoblast precursor cells or MSCs

There has been limited number of studies which investigated the effect of NSAIDs on osteoblast cells. Ho *et al.*, (1999) reported that NSAIDs capable to affect osteoblast proliferation and the earlier stage of differentiation. Further analysis indicated that both PGs and NSAIDs inhibited DNA synthesis and mitosis, in a time- and concentration-dependent manner (Ho *et al.*, 1999) . Indomethacin and ketorolac also negatively affect cell proliferation and differentiation/osteoblastic differentiation, although the mechanisms were not through PGE1 and PGE2.

Other studies have also reported that NSAIDs (ketorolac, indomethacin, diclofenac, and piroxicam) exerted cytotoxic effects on osteoblast cultures derived from fetal rat calvaria bone (Arlt *et al.*, 2001; Ho *et al.*, 2001). Ketorolac and indomethacin were observed to arrest the cell cycle at the G_0/G_1 phase. All the NSAIDs tested showed cytotoxic effect in a concentration-dependent manner. Among them, indomethacin showed the greatest toxicity, followed by diclofenac > ketorolac > piroxicam. The cytotoxic effect of NSAIDs on osteoblast was PG-independent. These observations suggest that the cell cycle arrest induced by the NSAIDs is an important suppressive mechanism of bone repair and remodeling *in vivo*.

According to report by De Luna-Bertos et al. (2012), the treatment of the MG63 human cell line with therapeutic doses of aspirin (1–10 μM) was not cytotoxic, did not induce apoptosis/necrosis or resulted to cell cycle changes (De Luna-Bertos *et al.*, 2012). They reported that ASA in therapeutic doses appeared to have no adverse effect on osteoblast growth *in vitro***.** The proliferation of MG-63 cells was not significantly inhibited by 0.1, 1, or 10 μM aspirin at 24-hour treatment. However, at higher ASA concentrations (20, 100, and 1000 μM), cellular proliferation was negatively affected within 24 hours of treatment. It was previously reported that NSAID dosage is a key factor in modulating cellular proliferation, differentiation and migration (Muller *et al.*, 2011).

NSAIDs (ketorolac, indomethacin, diclofenac and piroxicam) have been reported to suppress cell proliferation and cell cycle kinetics, exert cytotoxicity and impair osteogenesis of BMMSCs in mice (D1 cells) and humans (hBMSCs) (Chang *et al.*, 2007). The NSAIDs drugs showed that all the NSAIDs drugs suppressed proliferation and arrested cell cycle of D1 cells, but no significant cytotoxic effects was found. These observations suggest that the suppression of proliferation by NSAIDs on BMMSCs is not through prostaglandin-related mechanism, but may have been achieved by the modulation of cellular proliferation, particularly through proteins that regulate the cell cycle.

In another study, NSAIDs (ketorolac and indomethacin) were reported to suppress osteoblast proliferation, arrest cell cycle, and induce cell death in osteoblast cultures from rat fetal calvaria (Chang *et al*., 2005). The NSAIDs arrested the cell cycle at the G_0/G_1 phase, induced cytotoxicity and apoptosis in osteoblast cells. The study also indicated that piroxicam is less harmful on the osteoblasts function compared to

indomethacin. The effect of COX-2 inhibitors celecoxib and naproxen on the osteogenic differentiation of BMMSCs in inflammatory and non-inflammatory conditions has also been reported (Yoon *et al.*, 2010).

IL-1β was used to induce inflammation in BMMSCs. The results indicated that osteoblast differentiation, alkaline phosphatase and calcium contents of IL-1β-treated BMMSCs were significantly reduced at high doses of COX-2 inhibitors, compared to the low-dose group. Meanwhile, ALP and calcium contents were not reduced by COX-2 inhibitors in non-inflammatory condition. In addition, COX-2 inhibitor reduced the levels of transcription factors such as runx2, Dlx5, and OCN, which are known to be essential for osteogenesis. The levels of these transcription factors remain constant in the non-inflammatory-conditioned MSCs. These data indicate that the osteogenic potential of MSC is inhibited by the treatment of high-dose NSAIDs under inflammatory conditions.

In a study by Pountos and colleagues, the *in vitro* effects of COX-1 and COX-2 inhibitors (diclofenac sodium, piroxicam, parecoxib, lornoxicam, meloxicam, ketoprofen, and ketorolac) on BMMSCs were reported (Pountos *et al.*, 2011). The results indicated that at a broad range of concentrations (10^{-3} to 100 μg/ml), the NSAIDs did not interfere with the MSCs potential to proliferate and differentiate into osteogenic lineage, but affected the chondrogenic potential with a reduction in sulphated glycosaminoglycans (sGAG) content.

2.13 Beneficial impact of ASA on stem cell and the potential application

The impact of ASA on stem cells properties has been reported in a number of studies (Liu *et al.,* 2011a; Liu *et al*., 2015b; Cao *et al*., 2015). Liu *et al.,* (2011a) reported that repairs in BMMSC-based calvarial bone defects in C57BL/6 mice was enhanced by site-specific ASA treatment, and this was due to the suppression of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) concentrations (and action) at the implantation site (Liu *et al*., 2011a). They found that the TNF-α and IFN-γ that are primarily produced by activated T cells, and these are capable of inducing apoptosis and inhibiting the ability of MSCs to mediate bone repair.

Thus, the host immune system poses significant effects on the fate of transplanted MSC during bone remodeling, where TNF-α and IFN-γ play a critical role (Liu *et al*., 2011a). Another report also showed that the local administration of ASA achieved MSC-based regenerative application through the repair of calvarial bone defects in a mini swine model, and this was postulated to have been achieved through the same mechanism as above, i.e., through the suppression of TNF- α and IFN-γ . Observations from previous studies indicated that ASA showed a positive impact in reducing human alveolar bone loss and improving periodontal status, leading to the suggestion that ASA provides a favourable condition for periodontal tissue health.

However, it was also argued that due to the complexity of periodontitis pathogenesis, it was unlikely that the inhibition of inflammation by NSAIDs was the only factor which contributed to the observed clinical benefit (Heasman & Hughes, 2014). Liu *et al.* (2015b) reported that ASA treatment on stem cells from exfoliated deciduous teeth (SHED) at low dosages (~55 and 277 μM) resulted in the activation of

telomerase reverse transcriptase (TERT) activity, which improved SHED osteogenesis and immunomodulatory properties. The authors suggested that a low dose of ASA therapy is a feasible and efficient pharmacologic approach to activate TERT activity in stem cells, to improve stem cell functions, avoid replicative senescence, and achieve the therapeutic effects of stem cells.

A recent study reported that administration of ASA and human umbilical cord matrix-derived mesenchymal stem cells (hUCMs) reduced the effect of post-ischemic brain injury in the rat induced by artificial stroke (Shams ara *et al.*, 2015). Based on the aforementioned observations, it could be hypothesized that ASA may have positively affect growth factors gene expression in dental stem cells, leading to improvement in periodontal status. Thus, investigating the effect of ASA on stem cells properties, such as those sourced from PDLSCs is of relevance. There is not much data or studies that have been done to investigate the effect of ASA on the viability and expression of growth factor genes in PDLSCs. The outcome of this study could shed additional insights on the impact of ASA on PDLSCs properties. This could provide novel data that may be useful in the field of regenerative dentistry, particularly periodontal regeneration.

CHAPTER 3: METHODOLOGY

3.1 Isolation and culturing human PDLSCs from periodontal ligament tissue

This study was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [Medical Ethics Clearance Number: DF CO1107/0066 (L)] and with the informed consent of the patients. The PDLSCs were isolated from normal and vital tooth. The donors were aged between 18 to 35 years old (n=3) and the teeth were indicated for extraction for orthodontics treatment purposes. The PDLSCs were isolated by using standard protocols with some modifications (Seo *et al.*, 2004). The PDL tissues were scraped off the root surface using a sterilized scalpel and minced into small fragments prior to digestion in a solution of 1 mg/mL collagenase type I (Gibco, Grand Island, NY) for 30 minutes at 37°C.

After neutralization with a 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), the cells were centrifuged and seeded in T75 culture flasks (BD Pharmingen, San Diego CA, USA) using a culture medium containing KO-DMEM, 10% FBS, 0.5% and 10000 µg/mL of penicillin/streptomycin (Invitrogen), 1% 1x Glutamax (Invitrogen), and incubated at 37^{o}C in the presence of 5% CO_2. The medium was replaced every three days until the cells reach 70-80% confluency

3.1.1 Basic characterization of periodontal ligament stem cells (PDLSCs)

3.1.1.1 Growth kinetics of PDLSCs

The growth curve of PDLSCs at passage 3 were analysed by plating them at T25 culture flasks with a cell density of 1×10^3 cells/cm^2 (BD Pharmingen, San Diego CA, USA) until they reached confluency. The cell number was determined through trypan blue assay (Sigma Aldrich). The mean cell number was determined using three independent samples in each experimental group and the results were expressed as mean ± standard deviation (SD).

3.1.1.2 Trilineage differentiation capacity

The cultures were initiated at a density of $1x10^3$ cells/cm^2 in 6-well plates (Nunclon, Roskilde, Denmark) and were grown to confluence and subjected to differentiation into adipogenic, chondrogenic, and osteogenic lineages. The adipogenic induction media contained 10% FBS, 200 mmol/L indomethacin, 0.5 mmol/L 3-isobutyl-1-methyxanthine (IBMX), 10 mg/mL insulin, and 1 mmol/L dexamethasone (Sigma Aldrich). The lipid droplets formed by the cells were visualized through oil red O staining (Sigma Aldrich).

For chondrogenic differentiation, the cells were cultured using media supplemented with ITS+1 (Sigma Aldrich), 50 mmol/L L-ascorbic acid-2 phosphates (Sigma Aldrich), 55 mmol/L sodium pyruvate (Invitrogen), 25 mmol/L L-proline (Sigma Aldrich), and 10 ng/mL of transforming growth factor-β (TGF-β) (Sigma Aldrich). The assessment of proteoglycan accumulation was achieved using alcian blue staining (Sigma Aldrich).

Osteogenic differentiation was done by culturing the cells for 3 weeks using osteogenic media supplemented with 10% FBS, 1% glutamax (Gibco), 100 nM dexamethasone (Sigma Aldrich), 10 mmol/L β-glycerol phosphates (Sigma Aldrich), and 0.2 mM of Ascorbic acid (Sigma Aldrich). Calcium accumulation was visualized using 2% Alizarin Red S staining (Sigma Aldrich).

3.1.1.3 Flow cytometry

The PDLSCs were immunophenotyped through flow cytometry analysis. The human MSC phenotyping kit (MACS MiltenyiBiotec, catalog #130-095-198) was used. The following antibodies were used to mark the cell surface: epitopes-CD90-phycoerythrin (PE), CD73-APC, CD105-PE and CD14-PerCP, CD20-PerCP, CD34-PerCP, and CD45-PerCP. The PDLSCs were trypsinised using trypsin and the cell numbers determined up to $1x10^6$ cells. Two aliquots were prepared for the $1x10^6$ cells and the cell suspension was centrifuged at 300xg for 10 minutes.

Each aliquot was resuspended in 100µL of buffer. 10 µL of the MSC Phenotyping Cocktail was added to aliquot 1 and a similar amount of the Isotype Control Cocktail was added to aliquot 2. Both aliquots were mixed well and incubated for 10 minutes in the dark in the refrigerator at 2-8 °C. The cells were washed by adding 1-2 mL of buffer and centrifuged at 300×g for 10 minutes. The supernatant was aspirated completely. The cell pellet was resuspended in a suitable amount of buffer for analysis by flow cytometry and the results analyzed utilizing MACS Quantify™ software. The results were analyzed utilizing MACS Quantify™ software.

3.2 MTT cell proliferation assay

The proliferation of PDLSCs was assessed using a 3-(4,5-dimethylthiazohl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Chemicon, Milipore) assay. ASA (Sigma Aldrich) was dissolved in ethanol to make a stock solution of 1M. The experimental group was treated with ASA at 10, 100, 200, 500, 1,000, 6,000, 8,000, and 10, 000μM. The PDLSCs were seeded at $3x10^4$ cells in a 96-well plate and incubated overnight at 37^oC in 5% CO_2 atmosphere.

The experimental group was treated at the required ASA concentrations for 24, 48, and 72 hours. The MTT solution was then added and the cells were further incubated at $37^{\circ}C$ for 4 hours. The culture media was then removed and dimethyl sulfoxide (DMSO) was added into each well. The absorbance was then measured at 575 nm. The cell viability was determined by calculating the ratio of optical density (OD) values between the ASA-treated and vehicle-control cells.

3.3 Growth factors gene expression profiling utilizing RT^2 PCR array

Gene expression profiling was done using the human growth factors RT^2 PCR array (Qiagen, catalog number PAHS-041Z). The array could profile 84 growth-factor associated genes, associated to the following categories: angiogenic growth factors, regulators of apoptosis, cell differentiation, and developmental regulators. The PDLSCs were cultured for 24 hours in the absence (experimental control) or presence of 1000 μM ASA. Total RNA was then isolated using the RNA easy Mini kit (Qiagen) and then cDNA amplification uses the SYBR green master mix PCR array kit (Qiagen). For PCR array, the reaction mix was prepared by using the components listed below (Table 3.1).

Table 3.1: Reaction mix of PCR array

Component	Volume (µL) per reaction	Final Concentration
	20µL	
Taqman Universal Master Mix II (2X)	10.0	1X
Taqman Gene Expression Assay (20X)	1.0	1X
cDNA template + H20	9.0	1 to 100ng
Total Volume	20.0	-

The tubes were capped and vortexed to mix the solutions. The tubes were centrifuged to spin down the contents. An appropriate volume of each reaction mixture (50 µL) was transferred to each well of an optical plate (MicroAmp® Optical 96-Well Reaction Plate) as specified in the following table (Table 3.2).

Table 3.2 Reaction mixture of an optical plate

Plate Format	Reaction volume
MicroAmp® Optical 96-Well Reaction Plate	1X
Taqman Gene Expression Assay (20X)	1X
cDNA template + H20	1 to 100ng
Total Volume	-

The plate was covered with a MicroAmp Optical Adhesive Film and briefly centrifuged to spin down the contents and eliminate air bubbles from the solution. The PCR reaction plate was run according to the following thermal cycling parameters (Table 3.3).

Table 3.3: Thermal cycling parameters

System	UNG incubation	Polymerase activation	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/extend
Temperature (^{0}C)	50	95	95	60
Time (mm:ss)	2:00	10:00	00:15	1:00
Volume (µL)	50 µL			

The expression values of target genes were normalized using GAPDH gene. For data analysis, the 7500 Real Time PCR software was used to calculate the fold change (FC) of gene expression between the non-treated and the ASA treated PDLSCs based on the $\Delta\Delta C_T$ method. A cut-off cycle threshold (C_T) value of 35 was assigned for estimation of fold change. Upregulation was defined as having a fold change of > 2.0.

3.4 Array Validation

The results from the RT^2 PCR array study was validated by quantitative real-time PCR (qRT-PCR). Total RNA was then isolated using the RNA easy Mini kit (Qiagen) and then then reverse-transcribed into Cdna (Invitrogen). To prepare the PCR reaction mix for each sample the following were pipetted into a nuclease-free 1.5-mL microcentrifuge tube (Table 3.4).

Table 3.4: PCR reaction mix

Component	96-well plate (Volume µL) for 1 reaction	Final Concentration
Taqman® Fast Advanced Master Mix	10.0	1X
Taqman ®Gene Expression Assay (20x)	1.0	1X
cDNA template	2.0	100 ng to 1pg
Nuclease-free water	7.0	
Total volume per reaction	20.0	-

20 µL of PCR reaction mix was transferred into a 96 well-reaction plate which was sealed with an appropriate cover. The plate was centrifuged and loaded into the instrument. The plate was run by using the parameter values shown in Table 3.5. All determinations were normalized using GAPDH as the reference gene.

Table 3.5: Thermal cycling conditions

System	**Run**	**Plate document/experiment parameters**	**Thermal cycling conditions**		
			Stage	**Temperature (^{0}C)**	**Time (mm:ss)**
Applied Biosystems 7500 Fast Real-Time PCR System	Fast	Reaction volume: 20µL Ramp rate: Fast	Hold	50	2:00
			Hold	95	0:20
			Cycles (40 cycles)	95	0:03
				60	0:30

The following genes were selected for the validation assay, with the corresponding TaqMan® Gene Expression Assay (Thermo Fisher Scientific) reagents set. The TaqMan® primers are as in Table 3.6

Table 3.6: List of the Taqman primers used for validation of RT^2 PCR array

No.	Target Gene	TaqMan® Assay ID
1.	FGF2	Hs00266645_m1
2.	FGF9	Hs00181829_m1
3.	BMP2	Hs00154192_m1
4.	FGF7	Hs00940253_m1
5.	VEGFA	Hs00900055_m1
6.	VEGFC	Hs.435215
7.	IL2	Hs00907779_m1
8.	IL4	Hs00174122_m1
9.	IL10	Hs00961622_m
10.	BMP10	Hs00205566_m1
11.	BMP3	Hs00609638_m1
12.	INHBB	Hs00173582_m1
13.	GAPDH	Hs02758991_g1

3.5 ASA treatment and evaluation of PDLSCs proliferation rate during osteogenic differentiation

The PDLSCs (n=3) were seeded at $3x10^4$ cells in a 96-well plate and maintained in a 5% CO_2 incubator at 37°C for 24 hours. They were exposed to ASA [0 (control), 10, 200, 500, 1000 μM] in osteogenic media containing 10% FBS, 1% L-alanyl-L-glutamine, 100 nM dexamethasone, 10 mmol/L β-glycerol phosphate, and 0.2 mM of ascorbic acid for 21 days. The media was changed every 3 days. MTT assay was conducted at 0, 3, 7, 14, 17, and 21 days to evaluate the proliferation rate and the absorbance was then measured at 575 nm. The cell viability was determined by calculating the ratio of optical density (OD) values between the ASA-treated and vehicle-control cells.

3.6 Effect of ASA on PDLSCs osteogenic potential

The PDLSCs (n=3) were seeded in a 6-well plate and maintained in 5% CO_2 incubator at 37◦C until they reached 70 to 80% confluency. They were then exposed to ASA [0 (control), 10, 200, 500, 1000 μM] in osteogenic media containing 10% FBS, 1% L-alanyl-L-glutamine, 100 nM dexamethasone, 10 mmol/L β-glycerol phosphate, and 0.2 mM of ascorbic acid for 21 days.

The media was changed every 3 days. The mineralization assay was done to evaluate the PDLCs osteogenic potential and the cells stained with 2% ARS at the designated days (3, 7, 14, and 21 days). The Alizarin Red S positive area (ARSA) was analyzed using image J software (developed by the National Institutes of Health, USA) and the results presented as a percentage of ARSA over the total area of the image.

3.7 Microarray based gene expression profiling: effect of ASA on PDLSCs during osteogenic differentiation

The PDLSCs were seeded in T75 culture flasks (BD Pharmingen) and maintained in 5% CO_2 incubator at 37°C for 24 hours until they reached 70% to 80% confluency. Then, the PDLSCs (n=4) were exposed to ASA [0 (control), 200, 500, 1000 μM] in osteogenic media containing 10% FBS, 1% L-alanyl-L-glutamine, 100 nM dexamethasone, 10 mmol/L β-glycerol phosphate and 0.2 mM of ascorbic acid for 21 days. The media was changed every 3 days. Total RNA was extracted at day-21, using the RNAeasy Mini kit (Qiagen). For RNA extraction, the cells numbers were

determined. The cells were transferred to an RNAase-free glass tube and centrifuged at 300 x g for 5 minutes. The supernatants were completely aspirated.

The cells were disrupted by adding 350 µL buffer RLT to an appropriate volume and vortexed. The lysates were pipeted into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at full speed. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. Up to 700 µL of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15a at ≥8000 x *g* (10,000 rpm). The flow-through was discarded.

700 µL Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. The flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane, and the flow-through discarded. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 2 minutes at≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane, and the flow-through discarded.

The RNeasy spin column was placed in a new 2 ml collection tube and the old collection tube with the flow-through was discarded. The samples were centrifuged at full speed for 1 minute. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA. The quality of RNA (A260/A280, A260/A230) was determined using Nanodrop spectrophotometer ND-2000 (Thermo Scientific Inc.)

The RNA integrity number (RIN) was determined using Agilent Bioanalyzer 2100 (Agilent Technologies). The microarray assay was carried out using Agilent SurePrint G3 Human GE v2 8x60K Microarrays (Agilent Technologies, catalog number G4851B) according to the manufacturer's protocol. 100 ng of total RNA was labeled with Low Input Quick Amp Labeling Kit, One color (Agilent Technologies) following the manufacturer's instruction. In this step, 100 ng of total RNA was converted into double stranded cDNA by priming with an oligo-dT primer containing the recognition site for T7 RNA polymerase. In vitro transcription with T7 RNA polymerase was used to produce cyanine 3-CTP labeled cRNA.

Six hundred ng of labeled cRNA was hybridized onto Agilent SurePrint G3 Human GE 8X60K Microarray for 17 hours at 65°C in hybridization oven (10 rpm). After hybridization, the microarray slide was washed in gene expression wash buffer 1 for 1 minute at room temperature and another minute in gene expression wash buffer 2 at 37°C before scanning the chip on Agilent High Resolution Microarray Scanner (C-model). Raw signal data were extracted from TIFF image with Agilent Feature Extraction Software (V10.7.1.1). Repeated measure test was used for statistical analysis. Genes up or downregulated ($p<0.05$) by two-fold change (FC) were selected for further analysis.

3.7.1 Gene ontology (GO) and functional enrichment analysis

The PANTHER (Protein Analysis Through Evolutionary Relationships) classification system provides intuitive visualizations for GO analysis output and this was used to classify the differentially expressed genes to facilitate data analysis. The functional enrichment analyses were performed using DAVID functional annotation

clustering tool (Huang da *et al.*, 2009) to ascertain the effect of ASA treatment at 200, 500, and 1,000 μM on the gene expression profile. The categories were filtered based on the enrichment score with the lowest p-values. The threshold value of the enrichment score was set at 1.6. DAVID generates an enrichment score for a group of genes indicating the associations of annotation term members in a given experiment.

3.7.2 Functional Annotation and Pathway Analysis

Pathway enrichment analysis was performed using Qiagen's Ingenuity Pathways Analysis (IPA, Ingenuity Systems Inc.). The software determines the significance (p-value) of a particular pathway being represented in the list of differentially expressed genes by Fisher's exact. IPA is also able to show the canonical pathway participated by any of the dysregulated genes, the gene functions, and the potential gene network interactions.

A dataset with gene identifiers and corresponding fold change values were entered into IPA software. These molecules were overlaid onto a global molecular network (contained in the Ingenuity Knowledge Base) during the analyses. Default settings were used to perform the analyses. The functional analysis in IPA allows determination of the biological functions and diseases associated with each network. Since the data composition of Ingenuity Knowledge Base can change over time, the results of the IPA analyses performed at different times may differ in the details uncovered.

3.8 Validation microarray

3.8.1 Real-time PCR (qPCR)

The microarray results were validated by qRT-PCR. A number of genes that were detected to be differentially expressed were selected and they are as listed in Table 3.7. The validation assay was carried out using the corresponding TaqMan® Gene Expression Assay ID (Thermo Fisher Scientific) reagents set (The method same as section 3.4). All determinations were normalized using GAPDH as the reference gene.

Table 3.7: List of TaqMan primers for microarray validation

No.	Taqman primers	Assay ID
1.	Fibroblast growth factor 1 (FGF1)	Hs01092738_m1
2.	Fibroblast growth factor 5 (FGF5)	Hs03676587_s1
3.	Fibroblast growth factor receptor-like 1 (FGFRL1)	Hs00222484_m1
4.	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide) (ITGA5)	Hs01547673_m1
5.	Fibronectin 1 (FN1)	Hs01549976_m1
6.	BMP binding endothelial regulator (BMPER)	Hs00403062_m1
7.	Bone morphogenetic protein 4 (BMP4)	Hs03676628_s1

3.8.2 Double immunofluorescene analyses

The PDLSCs (n=3) were seeded at $3x10^4$ cells in a 4-well glass slide (Lab-Tek®, Nunc) and maintained in a 5% CO_2 incubator at 37°C for 24 hours. They were exposed to ASA [0 (control), 200 μM, 500 μM and 1000 μM] in osteogenic media containing 10% FBS, 1% L-alanyl-L-glutamine, 100 nM dexamethasone, 10 mmol/L β-glycerol phosphate and 0.2 mM of ascorbic acid for 21 days. The experimental group was treated at the designated concentrations of ASA treatment for 21 days. The media was changed every 3 days. After 21 days, the PDLSCs were fixed in 4% paraformaldehyde (Merck) in phosphate-buffered saline (1X PBS) pH 7.4 for 15 minutes at room temperature.

The cells were washed thrice in 1X PBS for 5 minutes each and then permeabilized with 0.25% Triton X-100 and blocked with 10% goat serum for 1 hr at 37°C. The mixtures of two primary antibodies in 1% goat serum at appropriate dilutions were prepared as tabulated in Table 3.8. 100µl of primary antibodies were added into each well and incubated 1-2hr at 37°C and incubated overnight at 37°C and kept in the dark. The mixed solution was removed and washed 3 times in 1x PBS for 5 minutes each. The mixtures of two secondary antibodies in 1% goat serum at appropriate dilutions were prepared as tabulated in Table 3.8. 100µl of secondary antibodies were added into each well and incubated 1-2hr at 37°C and kept in the dark. The mixed solution was removed and washed 3 times in 1x PBS for 5 minutes each. The cells were incubated in DAPI (Sigma) for 20 minutes. One drop of mounting solution was added and the cells were then observed under the microscope.

Table 3.8: List of the antibodies for immunofluorescence study

No.	Primary Antibody	Secondary Antibody
1.	Anti-Fibronectin (Fn) (ab32419) (dilution :1/150)	Goat Anti-Rabbit IgG H&L (TRITC) (ab6718)
2.	Anti-Integrin alpha 5 antibody (ab6131) (dilution: 1/200)	Goat Anti-Mouse IgG H&L (FITC) (ab6785) (dilution:1/1000)
3.	Anti-FGF1 (ab117640) (concentration of 10 µg/ml)	Goat Anti-Mouse IgG H&L (FITC) (ab6785) (dilution:1/1000)
4.	FGFRL1 (ab112917) (dilution :1/100)	Goat Anti-Rabbit IgG H&L (TRITC) (ab6718)

3.8.3 Western blotting

Protein was extracted using RIPA buffer with protease inhibitors (Bandeen-Roche *et al.*, 2009). Fifty micrograms of the samples were heated in 5X loading dye at 95°C for 15 mins and then loaded onto a 10% SDS–PAGE gel. After electrophoresis, the proteins were transferred to an Immobilon® PVDF membrane (Millipore, Bedford, MA) which was blocked with 5% bovine serum albumin (BSA) and incubated overnight with the primary antibody (Table 3.9). The membrane was washed with PBST and incubated with a secondary antibody (Table 3.9) for 1 hour. After the wash cycle, the protein bands were detected with Luminata Crescendo Western HRP substrate detection reagent (Millipore).

Table 3.9: List of antibodies for Western blot

No.	Primary Antibody	Secondary Antibody
1.	Anti-Fibronectin (Fn) (ab32419) (dilution : 1/1000)	Goat Anti-Rabbit Secondary Antibody (Origen)(dilution: 1:3000)
2	Anti-FGF1 (ab117640) (concentration of 10 µg/ml)	Goat Anti-Mouse IgG1 heavy chain (HRP) (ab97240)(dilution:1/2000)
3.	Anti-FGFRL-1 (ab112917) (dilution 1:1000)	Goat Anti-Rabbit Secondary Antibody (Origen)(dilution: 1:3000)
4.	Anti integerin α5 (cell signaling)(dilution: 1/1000)	Goat Anti-Rabbit Secondary Antibody (Origen)(dilution: 1:3000)
5.	Anti-beta Actin (ab8226)(dilution: 1/500)	Goat Anti-Mouse IgG1 heavy chain (HRP) (ab97240)(dilution:1/2000)

3.9 Statistical Analysis

The results are presented as means ± standard deviations (SDs) of at least 3 replicates (n=3). Two-way ANOVA with Bonferroni post-test was performed using software (Graphpad Software, San Diego CA) and p values of <0.05 were considered significant. The PCR array data was analysed at Qiagen's data analysis center (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The p-values were calculated based on a student's t-test of the triplicate 2^ (-Delta Ct) (2 FC) values for each gene in the control and treatment groups with p-values < 0.05 being considered significant.

CHAPTER 4: RESULTS

4.1 Characterization of the stem cells derived from periodontal ligament (PDL)

The PDLSCs were subjected to a number of basic phenotypic characterizations to fulfill the criteria of MSCs (Seo *et al.*, 2004; Zhu & Liang, 2015). The proliferating cells showed adherence to the culture plate and their morphology resembled that of fibroblast in their spindle-like shape (Figure 4.1A [i]). The adipogenic, chondrogenic, and osteogenic differentiation capacities of the isolated MSCs are shown in Figure 4.1A [ii] - [iv].

The chondrogenic differentiation was confirmed by the detection of proteoglycan accumulation, while osteogenic differentiation was confirmed by the detection of the calcium deposition. Adipogenic differentiation was confirmed by the detection of neutral lipid vacuoles accumulation. Immunophenotyping analyses revealed the following expression profile: CD90 (99.98%), CD105 (99.22%), and CD73 (99.98%) with only 5.61% of the cells expressing hematopoietic markers (CD45, CD34, CD20, and CD14).

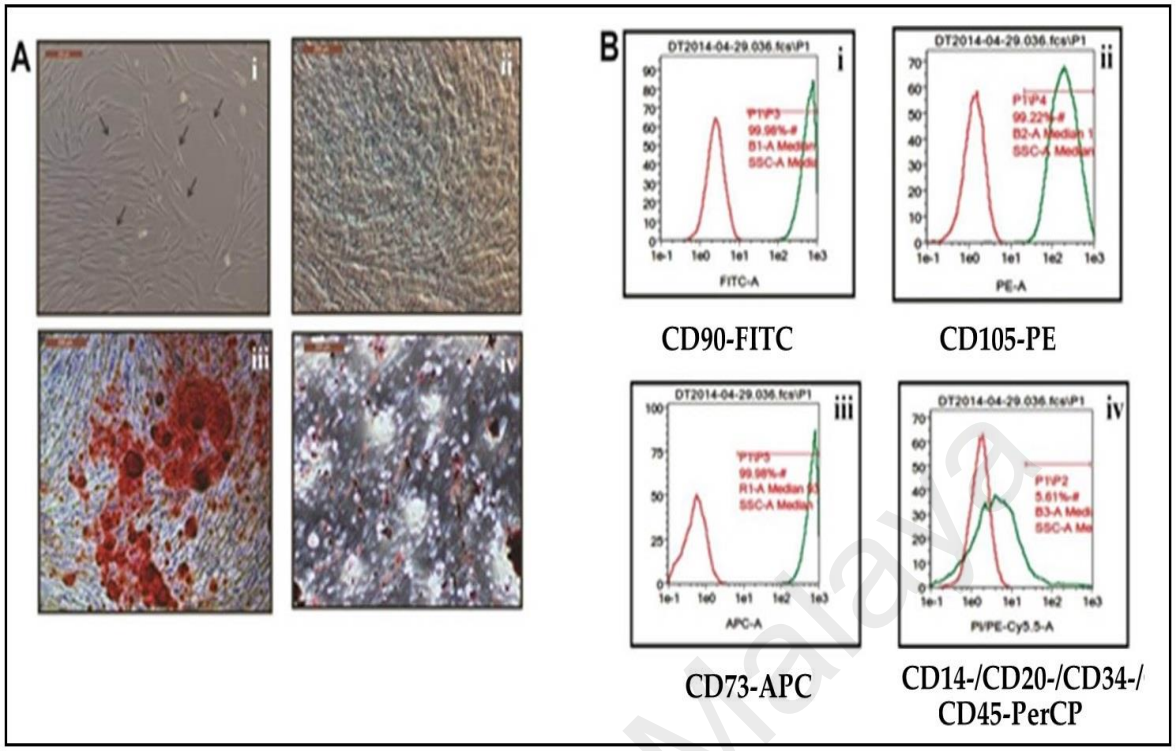

Figure 4.1: Basic characterization of periodontal ligament stem cells (PDLSCs) (n=3). A(i): Morphology of PDLSCs. Arrows indicate spindle-shape PDLSCs. Trilineage differentiation, A(ii): Chondrogenic differentiation A(iii): Osteogenic differentiation A(iv): Adipogenic differentiation. Phase contrast microscope; magnification: 10X; scale bar: 50 µm. 1(B): Analyses PDLSCs cell-surface markers expression. Positive expression was defined as the level of fluorescence greater than 95% of the corresponding isotype-matched control antibodies.

4.2 Growth Curve of PDLSCs

The growth curve (Figure 4.2) shows there were an increasing number of cells during the assay duration. There was however, a 24-hour lag phase as seen in the earlier part of the growth curve. The lag phase is considered to be the adaptation phase to culture conditions.

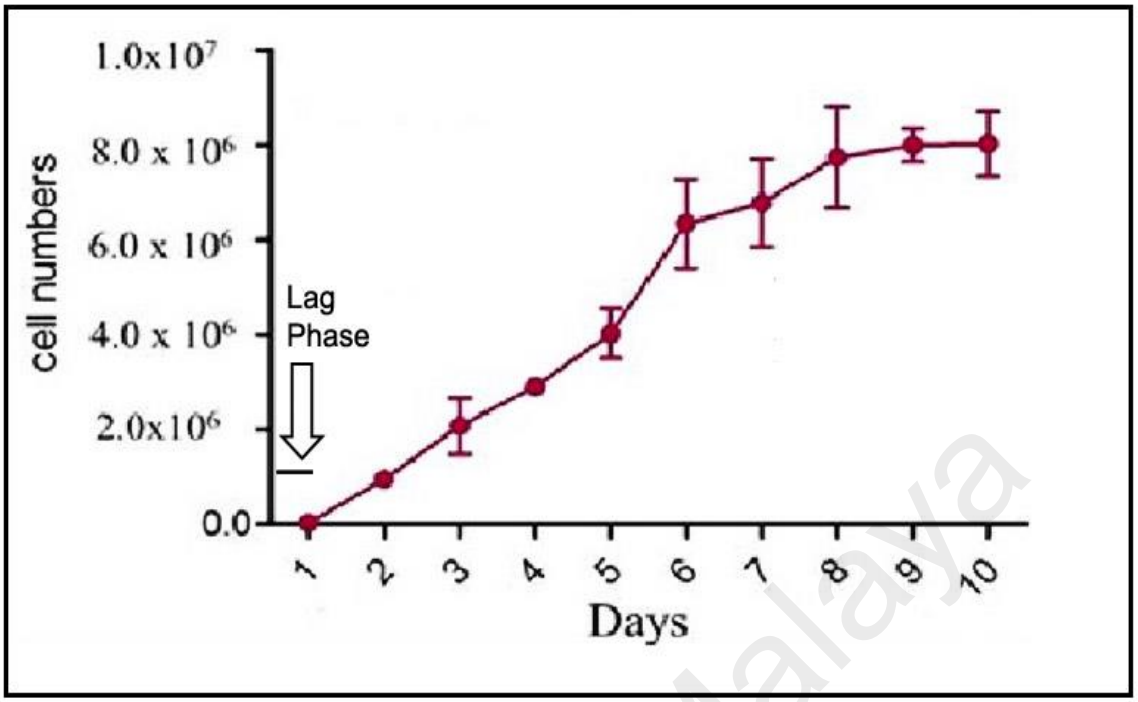

Figure 4.2: Growth kinetics of PDLSCs (n=3) at passage 3. Error bars represent standard deviation.

4.3 Effects of ASA on the PDLSCs morphology and proliferation rate

In the present study, the PDLSCs were treated with ASA at concentrations ranging from 10 to 10,000 µM. The absorbance readings for the treated cells were normalised against the vehicle control, which contained 0.1% v/v ethanol. The effect of ASA on cell proliferation was assessed by MTT assay. In comparison to the vehicle control, ASA treatments (at 24, 48, and 72 hours) reduced the PDLSCs proliferation rate in a time- and dose-dependent manner (Figure 4.3).

At the highest ASA concentration of 1, 000 µM, the treatments at 24, 48, and 72 hours reduced the proliferation rates of the PDLSCs to 87.70%, 85.04%, and 71.80% respectively (Figure 4.3). ASA concentrations ranging from 2,000 µM to 10, 000 µM showed a profound negative impact on the proliferation capacity of the PDLSCs (Figure 4.3).

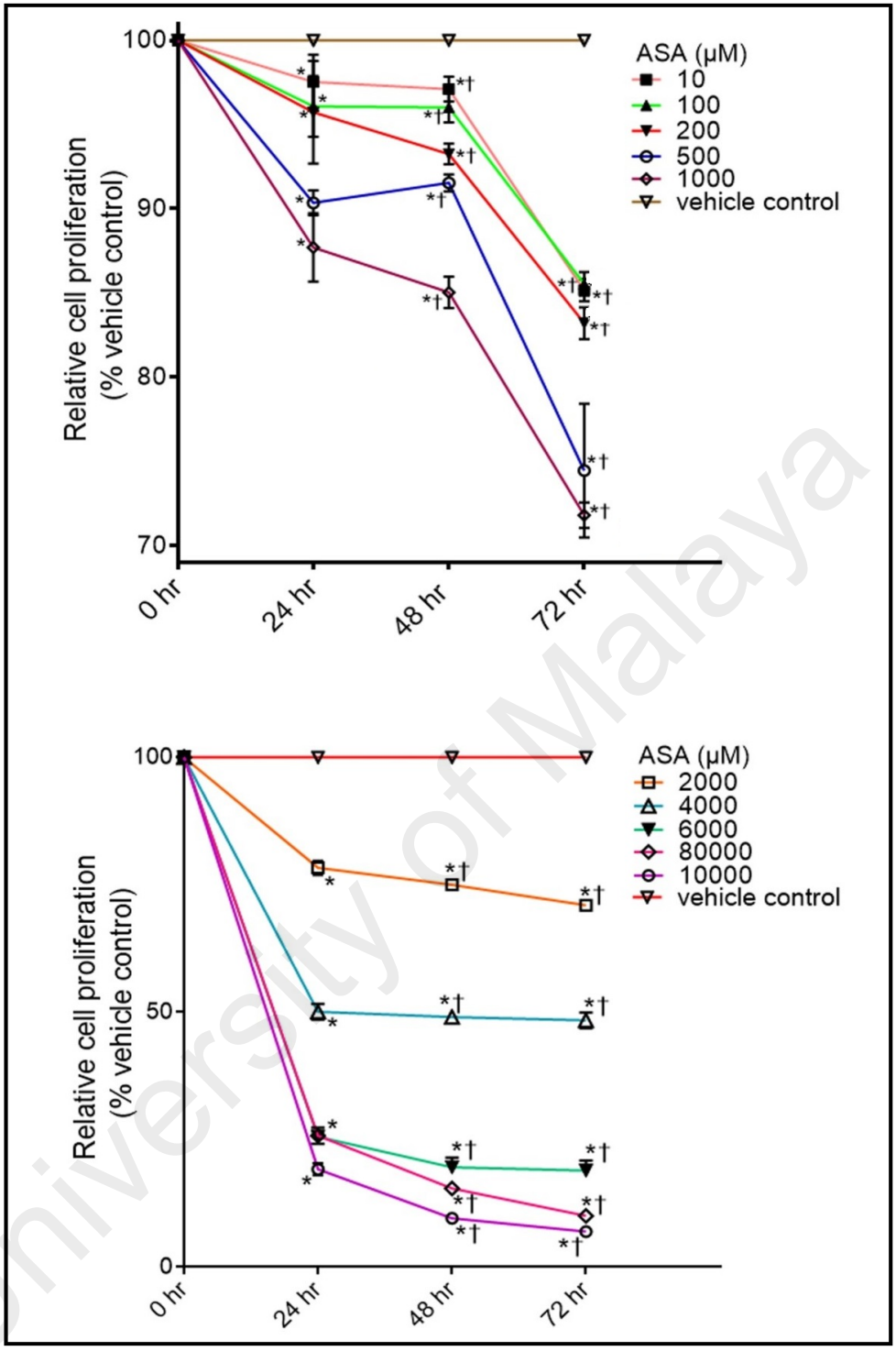

Figure 4.3: MTT cell viability assay. The assay was done following 24, 48 or 72 hour incubation with ASA at the designated concentration. Significant differences between groups ($p<0.05$):*compared to vehicle control; $^{+}$ compared to 24 hr. ‡ Vehicle control. Error bars represent standard deviation.

4.4 The effect of ASA on the expression of growth factors-associated genes in PDLSC

The result for the expression analysis of the 84 growth-factor associated genes using the human growth factors RT^2 PCR array is shown in Table 4.1. Using a 2.0-fold-change as a threshold value, 19 genes were found to be differentially expressed, of which 12 were upregulated and 7 downregulated (Table 4.1). The following 35 genes showed very high Ct values (Ct > 35) in the treated and untreated cells, suggesting that they were expressed only at very low/background levels: BMP3, BMP8B, CLC, CSF2, CSF3, EREG, FGF11, FGF13, FGF14, FGF17, FGF22, FGF6, GDF10, GDF11, IGF2, IL11, IL1A, IL1B, INHBA, JAG2, LEFTY1, LEFTY2, LTBP4, MDK, NDP, NRG2, NRG3, NRTN, OSGIN1, PSPN, SLCO1A2, SPP1, TDGF1, THPO and TYMP.

Table 4.1: List of genes that were significantly up (+) or downregulated (-) in PDLSCs upon ASA treatment. Student's t-test, *p<0.05. Processes: [†]cell differentiation; [‡]angiogenic growth factor; [§]apoptosis regulator; [‖]nervous system development; [¶]embryonic development; [#]muscle development, **developmental regulatory gene; [††]ovarian follicle development; [‡‡]cartilage development

No.	Process(es)	Gene symbol	Fold change (FC)*
1	CD[†]	FGF9	+15.88±0.56
2	AGF[‡], AR[§], NSD[‖]	VEGFA	+13.19±0.86
3	AR[§], CD[†]	IL2	+11.19±0.21
4	ED[¶]	BMP10	+ 6.90±0.34
5	CD[†], AGF[‡]	VEGFC	+ 6.31±0.20
6	AGF[‡], CD[†], NSD[‖], MD[#]	FGF2	+ 6.30±0.14
7	DRG**	FGF7	+ 4.99±0.01
8	CD[†]	IL4	+ 4.59±0.28
9	AR[§], CD[†]	IL10	+ 4.20±0.07
10	OFD[††], CD[†]	INHBB	+ 3.25±0.32
11	CRD[‡‡], CD[†]	BMP2	+ 3.03±0.54
12	DRG**	CSF3	+ 2.24±0.20
13	DRG**	AMH	- 6.59±0.35
14	AGF[‡], CD[†], NSD[‖]	JAG1	- 14.15±2.35
15	ED[¶]	NRG1	- 16.31±0.03
16	DRG**	DKK1	- 31.99±5.24
17	DRG**	BDNF	- 32.10±0.62
18	NSD[‖]	FGF5	- 32.16±0.06
19	NSD[‖]	PTN	- 32.20±0.19

4.5 Validation of RT^2 PCR array result by qRT-PCR

The qRT-PCR results were generally in agreement with the RT^2 PCR array data. The upregulation of the following genes were confirmed: FGF2, FGF7, FGF9, VEGFA, VEGFC, BMP2, BMP10, IL2, IL4, IL10, INHBB and CSF3 (Table 4.2)

Table 4.2: Validation of PCR array data by qRT-PCR. Upregulation was defined as having a fold change of >2.0. Values are expressed as mean ± standard deviation (SD)

No.	Genes name / symbol	Fold Change (FC)	
		PCR array	**Real-time PCR**
1.	Fibroblast growth factor 2 (FGF2)	+ 6.30±0.14	9.99±0.21
2.	Fibroblast growth factor 7 (FGF7)	+ 4.99±0.01	8.10±0.33
3.	Fibroblast growth factor 9 (FGF9)	+15.88±0.56	19.66±0.46
4.	Bone morphogenic protein 2 (BMP2)	+ 3.03±0.54	5.57±0.14
5.	Bone morphogenic protein 10 (BMP10)	+ 6.90±0.34	5.20±0.25
6.	Vascular endothelial growth factor C (VEGFC)	+ 6.31±0.20	4.89±0.36
7.	Vascular endothelial growth factor A (VEGFA)	+13.19±0.86	13.22±0.19
8.	Interleukin 2 (IL2)	+11.19±0.21	10.33±0.12
9.	Interleukin 4 (IL4)	+ 4.59±0.28	6.37±0.12
10.	Interleukin 10 (IL10)	+ 4.20±0.07	4.58±0.25
11.	Colony stimulating factor-3 (CSF3)	+ 2.24±0.20	3.14±0.46
12.	Inhibin, Beta B (INHBB)	+ 3.25±0.32	5.70±0.25

4.6 Ingenuity Pathway Analyses (IPA)

The Ingenuity Pathway Analysis was used to identify the plausible association between the upregulated genes with biological functions and canonical pathways to gain insights on the biological effects of ASA on the PDLSCs function. Figures 4.4 (A) and 4.4 (B) show the top 15 significantly regulated biological functions and canonical pathways respectively upon exposure of PDLSCs to 1,000μM of ASA.

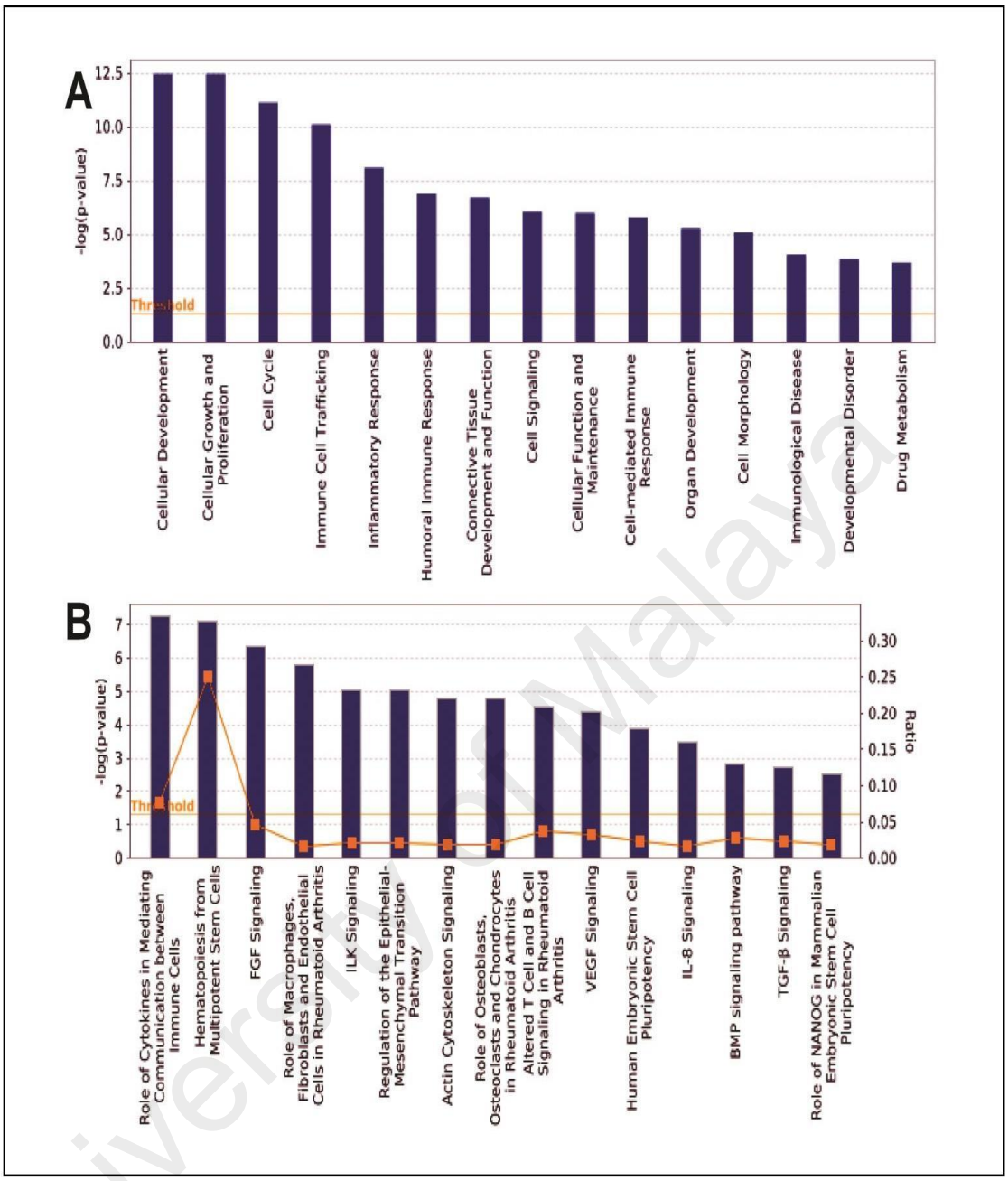

Figure 4.4: Ingenuity Pathway Analyses: the impact of 1,000 µM ASA treatment on biological functions and canonical pathways in PDLSCs. (A) Top 15 biological functions with its -log p-value (B) Top 15 canonical pathway with its –log p-value and ratio.

4.7 Effect of ASA on cell proliferation rate during osteogenic differentiation of PDLSCs

The results showed that ASA is able to modulate the proliferative capacity of PDLSCs. The MTT assay indicated that 500 µM and 1000 µM ASA treatments induced a higher proliferative capacity compared to the untreated cells from days 3 to 21 (Figure 4.5).

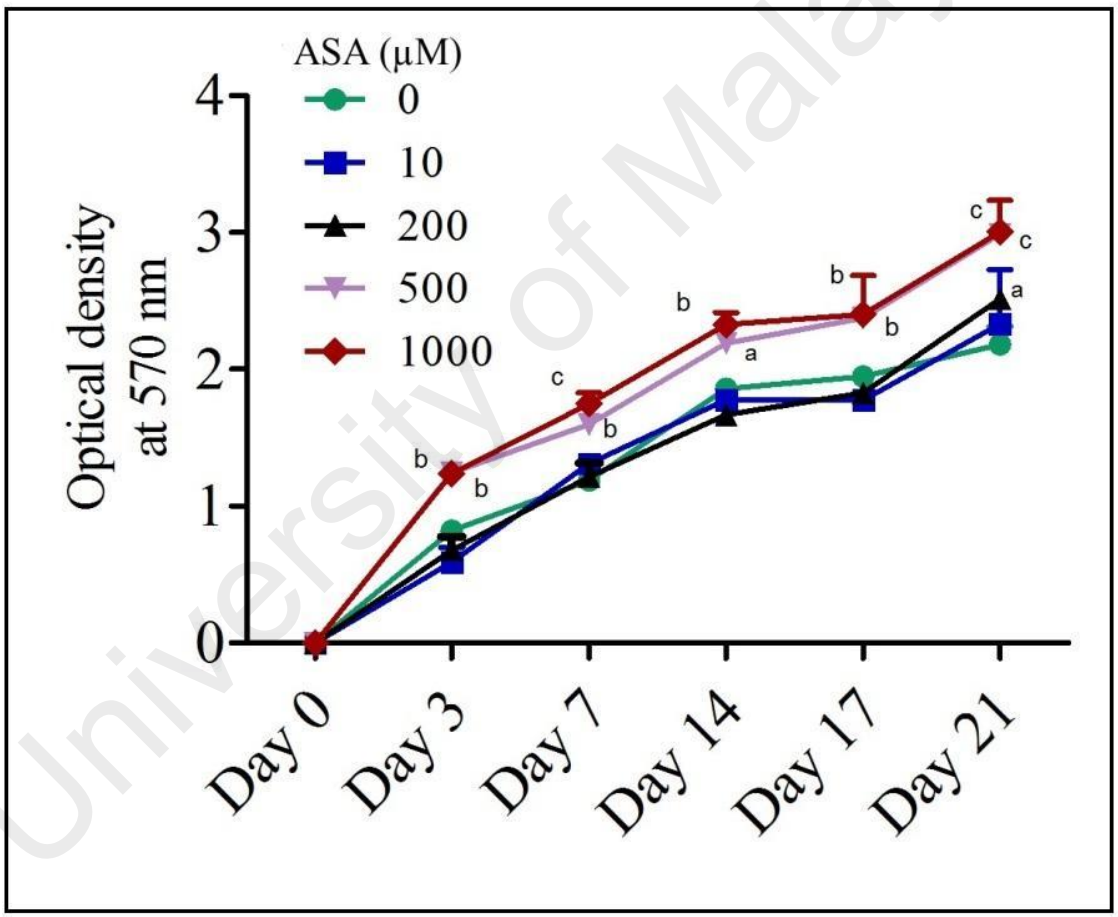

Figure 4.5: The proliferation rate of PDLSCs (n=3) during osteogenic differentiation assay was assessed through MTT assay. Error bars represent standard deviation. Statistically significant difference, $^{a}p<0.05$; $^{b}p<0.01$ and $^{c}p<0.001$.

4.8 ASA treatment and evaluation of osteogenic potential of PDLSCs

The osteogenic differentiation capacity of PDLSCs treated with various ASA concentrations was deduced by estimating calcium deposition using Alizarin red S staining (Figure 4.6). The result indicated that ASA at 500 and 1,000 μM demonstrated higher percentages of calcium mineral nodule formation compared to the lower dose groups and the untreated cells. Overall, significant differences ($p<0.01$) of Alizarin Red S stained area (ARSA) were observed between treated and untreated cells at days 3, 7, 14, and 21 (Figure 4.6).

During the assay, at day 3, there was no any obvious indication of mineralization nodule formation among the samples (treated or untreated). At 1000 μM, the PDLSCs osteogenic potential was consistently enhanced by ASA for days 7, 14, and 21. In contrast, the 10 and 200 μM treatments reduced the PDLSCs osteogenic potential consistently, for days 7 and 21. ASA treatments at 0 (control), 10, 200, 500, and 1000 μM showed the following percentages of ARSA: day 7 - 27.4%, 14.9%, 11.4%, 23.0%, and 31.0%, respectively; day 14 - 47.3%, 30.9%, 38.4%, 40.3% and 49.4%, respectively; day 21- 73.4%, 49.0%, 53.2%, 76.6% and 87.9%, respectively (Figure 4.7).

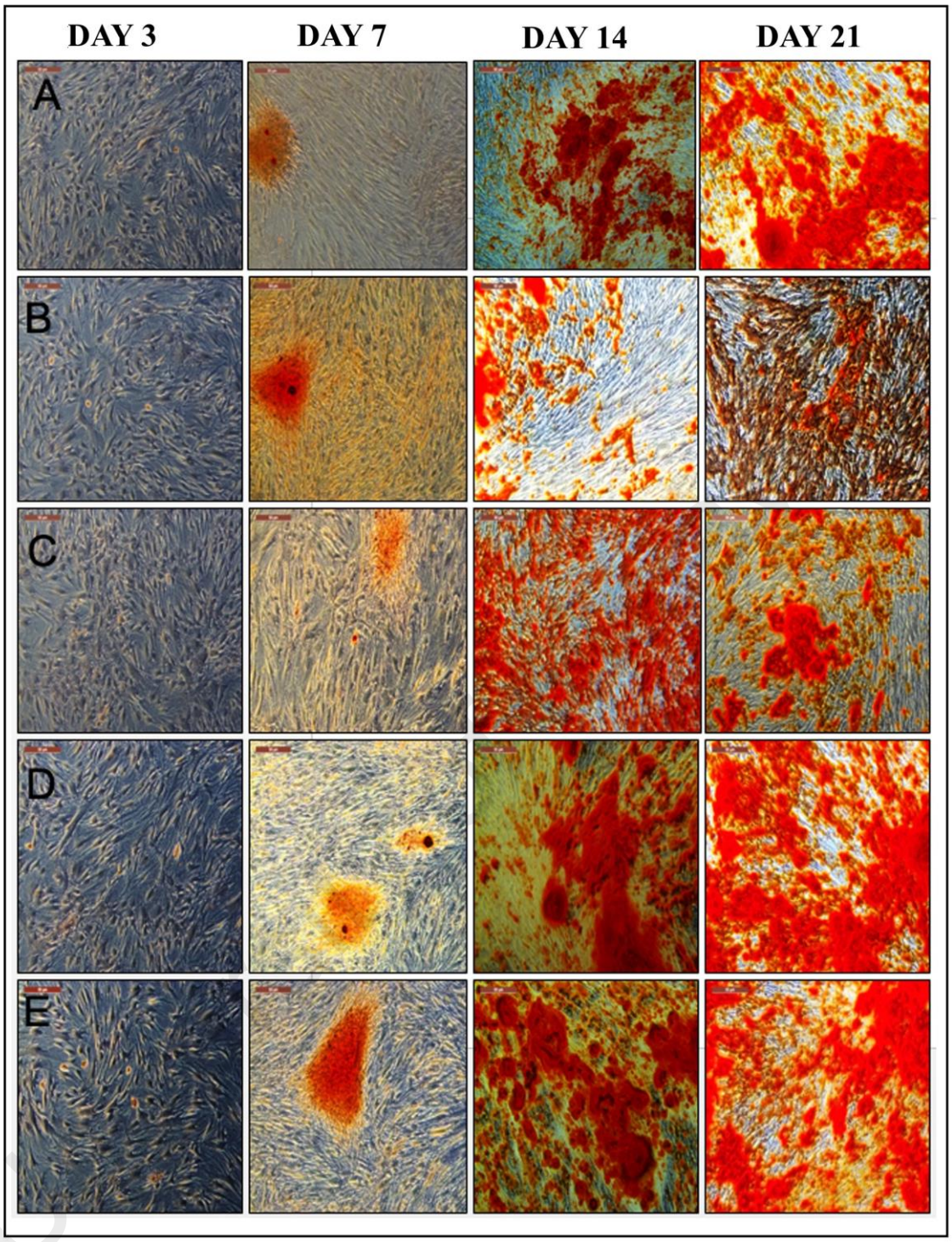

Figure 4.6: PDLSCs osteogenic potential assay. The PDLSCs (n=3) were treated with ASA in osteogenic media at the following concentrations: (A)0 µM [control],(B)10 µM, (C)200 µM, (D)500 µM and (E)1000 µM, respectively. The osteogenic capacity was measured through Alizarin Red S staining (ARS) at the designated days. Phase contrast microscope; magnification: 10X; scale bar: 50 µm.

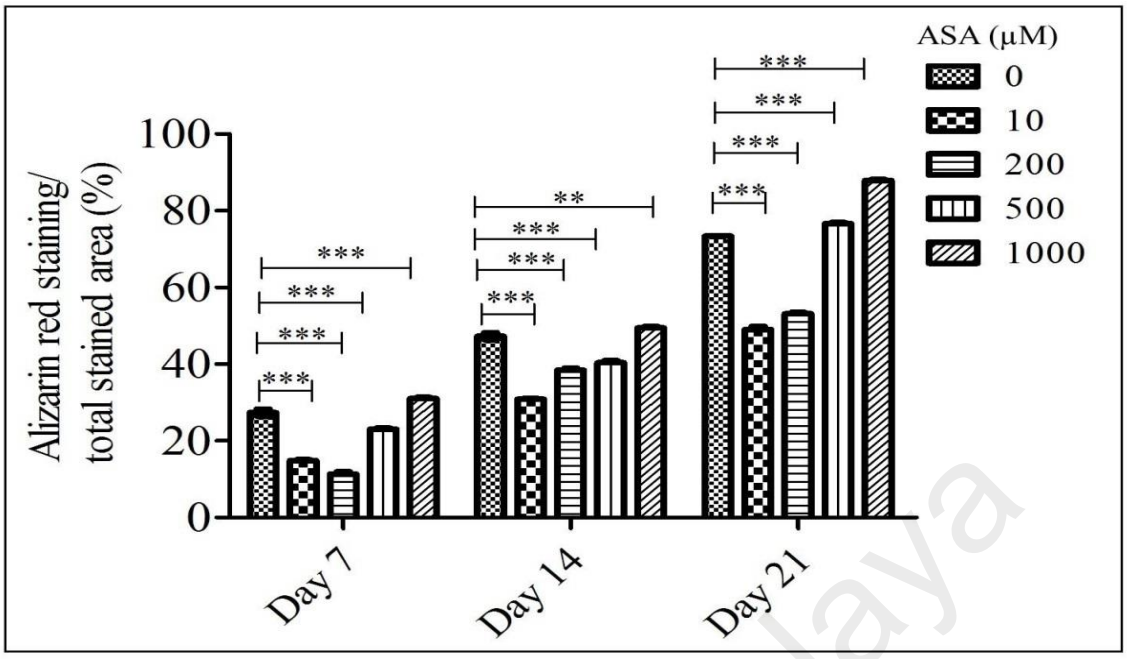

Figure 4.7: Total area stained by Alizarin Red S (ARS) staining was used to assess the PDLSCs osteogenic potential at the designated days. Percentage of mineralized area/total red image intensity of ARS staining performed using image J Red intensity over ½ maximum over area image was quantified (as a percentage of the image). Error bars represent standard deviation. Statistically significant difference, **p<0.05; ***p<0.01.

4.9 Microarray based gene expression study - the effect of ASA on PDLSCs gene expression profile

4.9.1 Quality and quantity of RNA isolated from the PDLSCs

Table 4.3 shows the quantity and quality of RNA for all samples 4 different donors (n=4). The yield of RNA was between 92 ng/μL to 1136.5 ng/μL. The RNA integrity number (RIN) for the samples were between 3.0 to 9.8. The median RIN value was 8.25. However, one of the samples (donor 1, red fonts) was eventually excluded, due to its low RIN value for 1,000 μM ASA treatment.

Table 4.3: Quality and quantity of RNA samples in the study

Sample	Concentration	Ratio (A260/A280)	Ratio (A260/A230)	Yield	RIN
Donor 1	control/non-treated	2.04	2.11	449.8	9.5
Donor 1	200 μM	2.0	2.0	350	9.5
Donor 1	500 μM	2.07	1.45	109.9	8.2
Donor 1	1,000 μM	2.06	1.20	92	3.0
Donor 2	control/non-treated	2.08	2.11	1136.5	9.8
Donor 2	200 μM	2.05	2.11	832.5	9.8
Donor 2	500 μM	2.05	2.04	521.8	9.3
Donor 2	1,000 μM	2.05	1.71	429.5	9.4
Donor 3	control/non-treated	2.10	2.12	1536.8	9.8
Donor 3	200 μM	2.08	2.10	671.3	6.50
Donor 3	500 μM	2.08	2.12	865.7	6.10
Donor 3	1,000 μM	2.07	2.10	267.1	6.60
Donor 4	control/non-treated	2.04	2.17	1013	9.4
Donor 4	200 μM	2.04	1.95	750.5	8.9
Donor 4	500 μM	2.05	2.19	256.2	9.2
Donor 4	1,000 μM	2.05	1.73	325.8	7

4.9.2 Analysis of microarray gene expressions

Figure 4.8 shows the Box-and-Whisker plots (BWP) for the raw data of the gene expression study, displayed in a ratio form for all of the samples included in this study. Each donor was labeled 1a through 4d, as indicated in Table 4.4, with the corresponding ASA concentration used in the study.

Table 4.4: List of donors

Label	Donor	Concentration(s)
1a	1	Non-treated (0)
1b	1	200 μM
1c	1	500 μM
1d	1	1,000 μM
2a	2	Non-treated (0)
2b	2	200 μM
2c	2	500 μM
2d	2	1,000 μM
3a	3	Non-treated (0)
3b	3	200 μM
3c	3	500 μM
3d	3	1,000 μM
4a	4	Non-treated (0)
4b	4	200 μM
4c	4	500 μM
4d	4	1,000 μM

The BWP displays the feature intensity distributions on a per-microarray basis. Boxes represent the interquartile range, with the 75th and 25th percentiles at the top and bottom respectively and the line in the middle of the blue box representing the 50th percentile, or median value of the distribution. Jointly with intensity that exceeds 1.5 times the interquartile range is represented by the red line. A BWP is meant to represent the rest of the distribution, with their terminations representing the lowest and highest feature intensity values.

After normalization, a box plot showed similar data distribution between all samples (equal medians) except for one sample (patient 1(d)). This dissimilarity might be attributed to the sample being of poor quality as seen from the RIN number of 3 for that sample. In view of this, all data from sample 1 was removed due to the paired nature of the experimental setup. Figure 4.8A shows the box plot for all the samples and Figure 4.8B shows the box plot after removal of the sample with low RIN number.

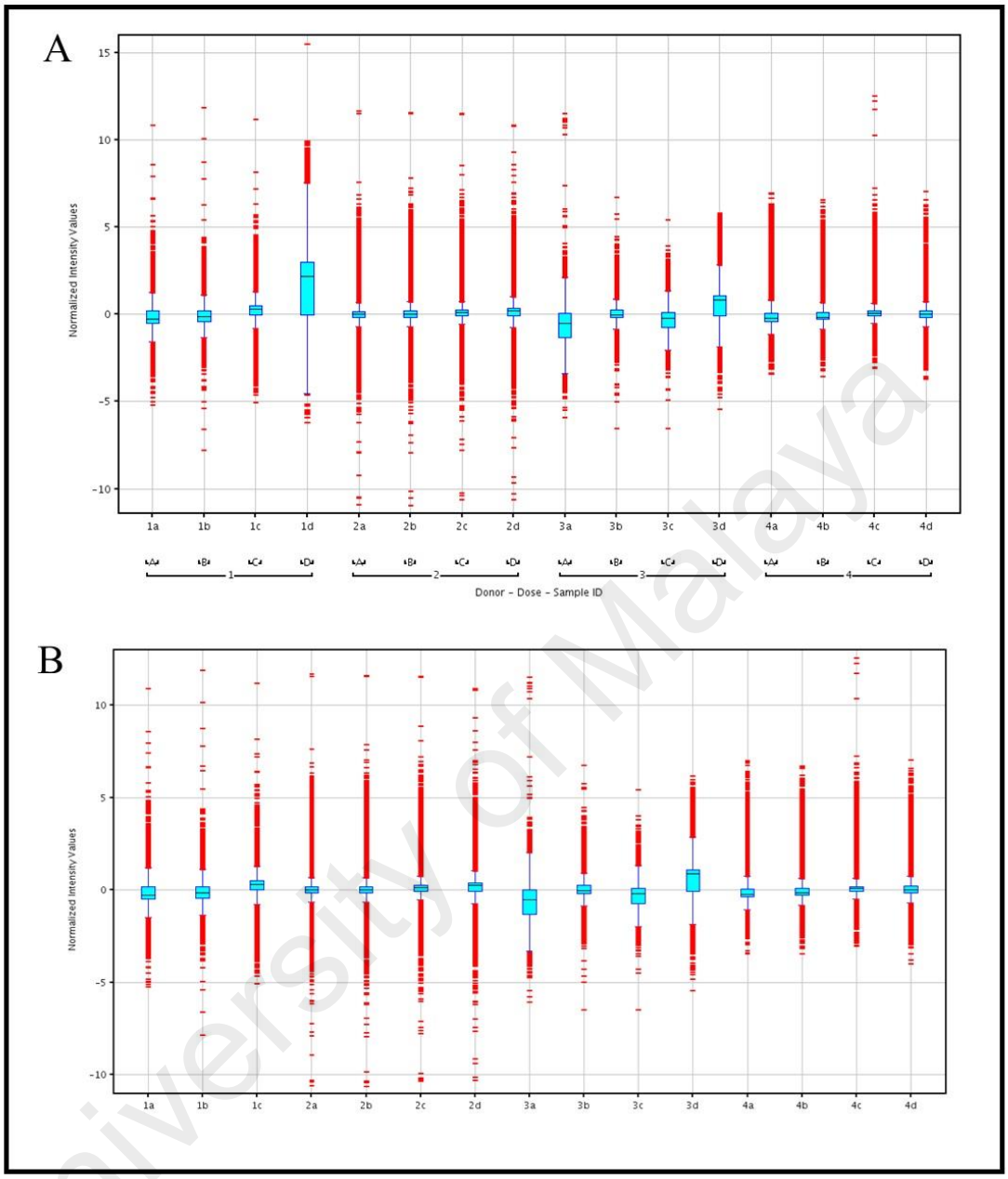

Figure 4.8: Box-and-Whisker plots (BWP) (A): BWP for all samples; (B): BWP after removal sample patient 1(d).

4.9.3 Principle Component Analysis (PCA)

Principle Component Analysis (PCA) was also conducted to examine the quality of a number of 20, 603 biological features after normalization of the raw data (Figure 4.9A). Since sample 1(D) was identified as an outlier and it had to remove for further analysis, a PCA plot with distinct separation of sample group was shown (Figure 4.8B). In Figure 4.9A sample donor 1(D) was similarly identified as an outlier as suggested by the box plot and justifies its removal from further analysis. Figure 4.9B shows the same plot after sample 1 is removed.

Samples were better clustered based on the donor 1D, which is expected due to the relatedness of the sample. Sample 3 seemed to have poorer clustering which might be due to the fact that a few of the samples (3(b), 3(c) and 3(d)) showed lower RIN numbers (6.10 – 6.60), compared to other samples (7- 9.8). However, sample 3 was still kept in the analysis as their RIN number (of about 6) suggested the RNA quality of the samples were good enough and acceptable for the study.

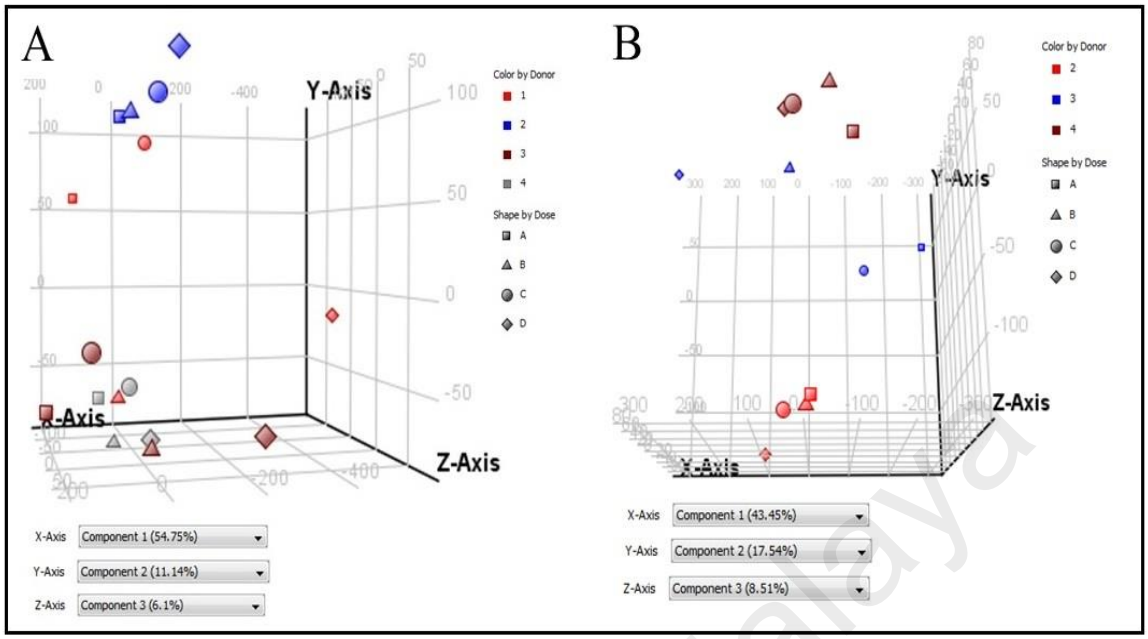

Figure 4.9: Principle Component Analysis (PCA) (A): PCA plot of 20, 603 of biological features on microarray; (B) PCA plot of 20, 603 of biological features on microarray after removal of sample 1.

4.9.4 ASA modulation of gene expression profile during PDLSCs osteogenic differentiation

Data processing, normalization, and error modeling was performed using Genespring GX (Agilent Technologies). In the present study, the Agilent microarray used contained 8 arrays/slide, of which 60,000 features are found in each of the arrays. Each feature/gene identifier could be mapped to the relevant/corresponding gene. The highest expression value was selected in the case of genes with multi-identifiers.

Using a FC of 2.0 as a cut-off threshold value ($p<0.05$), the results detected 968 differentially expressed genes (DEG) (Table 4.5). At 200 μM ASA treatment, 17 DEGs were noted, including 14 upregulated and 3 downregulated ones. At 500 μM, 145 DEGs were identified, including 53 up-regulated and 92 down regulated genes. In

contrast, at 1,000 M, 819 DEGs were identified, which included 335 up-regulated and 484 down-regulated genes.

Meanwhile, using a FC of 1.75 as a cut-off threshold value ($p<0.05$), 1620 were found to be differentially expressed (Table 4.5). At 200 μM ASA treatment, 61 DEGs were noted, including 41 upregulated and 20 downregulated genes. At 500 μM, 305 DEGs were identified, including 122 upregulated and 183 downregulated ones. In contrast, at 1,000 M, 1254 DEGs were noted including 633 that were upregulated and 987 downregulated.

Using an FC of 1.5 as a cut-off threshold value ($p<0.05$), 3,144 were found to be DEGs (Table 7). At 200 μM ASA treatment, 315 DEGs were noted, including 151 upregulated and 164 down-regulated genes. At 500 μM, 794 DEGs were identified, including 364 upregulated and 430 down regulated genes. In contrast, at 1,000 M, 2035 DEGs were noted, which included 1,250 upregulated and 1,894 downregulated genes.

The common shared upregulated gene between 200 μM vs 500 μM and 1, 000 μM is a zinc finger protein 316 (ZNF316) gene. Meanwhile the most common downregulated genes between 200 μM vs 500 μM and 1,000 μM were family with a sequence similarity of 107, member A (FAM107A), potassium inwardly-rectifying channel, subfamily J, member 8 (KCNJ8) and scavenger receptor class A, member 5 (putative) (SCARA5).

Table 4.5: ASA and DE genes during osteogenic differentiation of PDLSCs (p< 0.05)

ASA (µM)	FC >± 1.5 (p<0.05)		FC >± 1.75 (p<0.05)		FC >± 2(p<0.05)	
	Upregulated Genes	Downregulated Genes	Upregulated Genes	Downregulated Genes	Upregulated Genes	Downregulated Genes
200	151	164	41	20	14	3
500	364	430	122	183	53	92
1,000	735	1300	470	784	335	484
Total	1250	1894	633	987	402	579

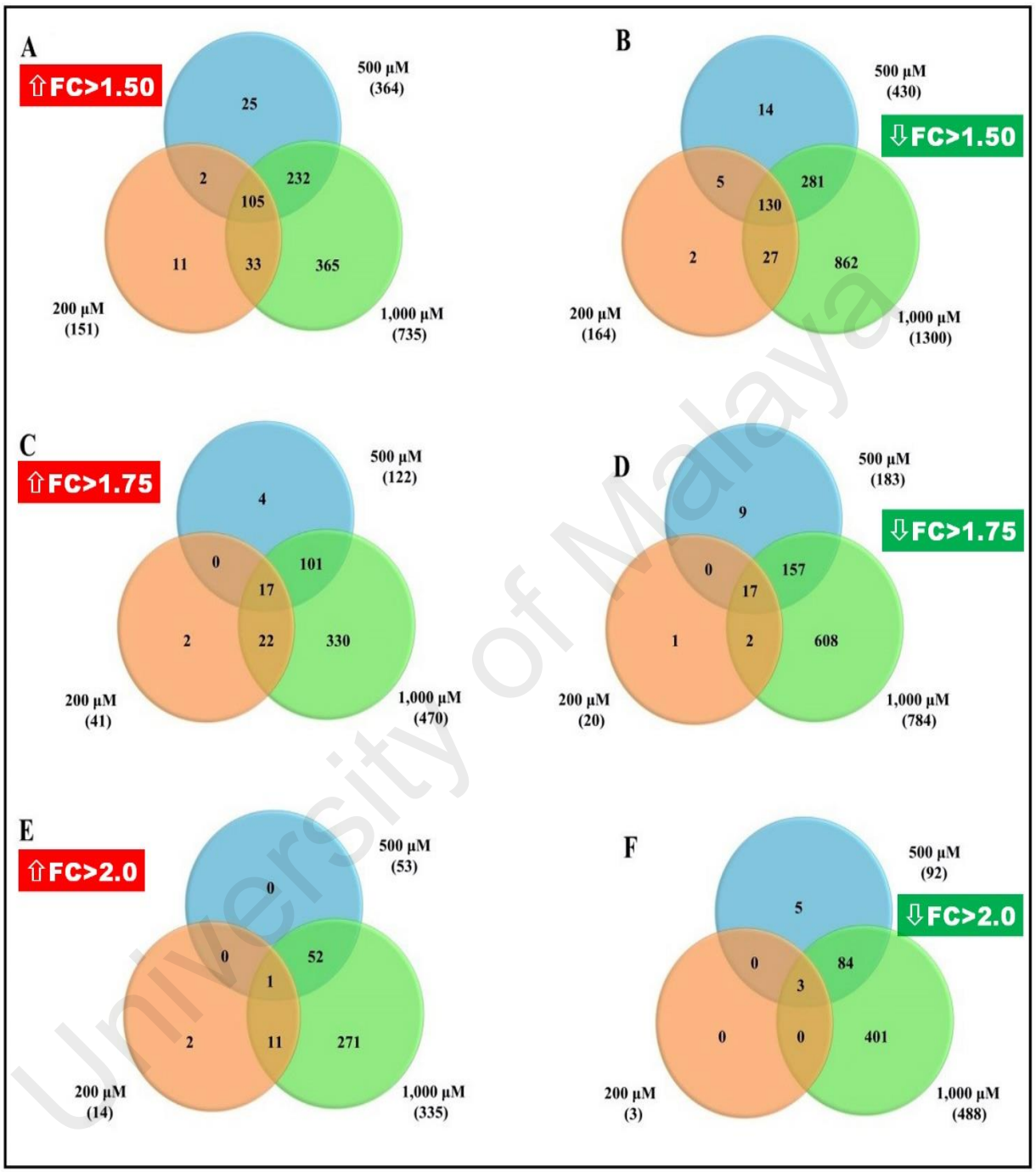

Figure 4.10: Venn diagram representing the number of DEGs that are shared among the 200, 500 and 1,000 µM ASA treated samples. Number in the brackets represent total number of DEGs for the corresponding ASA treatment (A) Up-regulated, FC1.5 (B) Down-regulated, FC 1.5 (C) Up-regulated, FC 1.75 (D) Down-regulated, FC 1.75 (C) Up-regulated, FC 2 (F) Down-regulated, FC 2.

4.9.5 Gene expression profilling in PDLSCs for ASA treatment at 200 µM: upregulated genes (FC>1.5)

Table 4.6 shows the list of upregulated genes (FC>1.5) in PDLSCs for 200 µM ASA treatment. The genes are ranked according to their FC values

Table 4.6: Up-regulated genes in PDLSCs for 200 µM ASA treatment

No.	Acession number	Gene Symbol	Description	p -value	FC
1.	NM_001271520	KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	0.0390	2.4618
2.	NM_001278559	ZNF316	Zinc finger protein 316	0.0163	2.3658
3.	NM_001098627	IRF5	Interferon regulatory factor 5	0.0336	2.3468
4.	NR_002221	DPRXP4	Divergent-paired related homeobox pseudogene 4	0.0240	2.1984
5.	NR_003611	BMS1P5	BMS1 pseudogene 5	0.0314	2.1938
6.	NM_001289003	PHLPP2	PH domain and leucine rich repeat protein phosphatase 2	0.0378	2.1792
7.	NM_001010985	MYBPHL	Myosin binding protein H-like	0.0299	2.1719
8.	NM_001144875	DOK3	Docking protein 3	0.0412	2.1702
9.	NM_001277308	GOLGA8O	Golgin A8 family, member O	0.0493	2.0967
10.	NM_014718	CLSTN3	Calsyntenin 3	0.0138	2.0854
11.		LILRA1	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 1	0.0356	2.0852
12.	ENST00000521847	ENDOV	Endonuclease V	0.0269	2.0190
13.	NM_024786	ZDHHC11	Zinc finger, DHHC-type containing 11	0.0420	2.0136
14.	ENST00000437814	METTL15	Methyltransferase like 15	0.0314	2.0036
15.	ENST00000586299	CYTH1	Cytohesin 1	0.0261	1.990

Table 4.6 (Continued)

16.	NM_001177693	ARHGEF28	Rho guanine nucleotide exchange factor	0.0375	1.949
17.	NM_033196	ZNF682	Zinc finger protein 682	0.0012	1.920
18.	BC020495	RABL2B	RAB, member of RAS oncogene family-like 2B	0.0473	1.920
19.	NR_024062	UBE2DNL	Ubiquitin-conjugating enzyme E2D N-terminal like	0.0480	1.918
20.	NM_003246	THBS1	Thrombospondin 1	0.0203	1.902

4.9.6 Gene expression profilling in PDLSCs for ASA treatment at 500 µM: upregulated genes (FC>1.5)

Table 4.7 shows the list of top twenty genes that were upregulated (FC>1.5) in PDLSCs for 500 µM ASA treatment. The genes are ranked according to their FC values.

Table 4.7: Up-regulated genes in PDLSCs for 500 µM ASA treatment

No.	Acession Number	Gene Symbol	Description	p-value	FC
1.	NM_004490	GRB14	Growth factor receptor-bound protein 14	0.0003	4.034
2.	NM_005613	RGS4	Regulator of G-protein signaling 4	0.0072	3.4228
3.	NM_001270989	EPGN	Epithelial mitogen	0.0099	3.2063
4.	NM_001278559	ZNF316	Zinc finger protein 316	0.0163	3.0333
5.	NM_031957	KRTAP1-5	Keratin associated protein 1-5	0.0009	2.8505
6.	NM_002309	LIF	Leukemia inhibitory factor	0.0152	2.7172
7.	NM_001161707	KIRREL3	Kin of IRRE like 3 (Drosophila)	0.0065	2.6781
8.	NM_018092	NETO2	Neuropilin (NRP) and tolloid (TLL)-like 2	0.0137	2.6107
9.	XM_005255956	MT1E	Metallothionein 1E	0.0035	2.5549

Table 4.7 (Continued)

10.	NM_003612	SEMA7A	Semaphorin 7A, GPI membrane anchor	0.003	2.5418
11.	NM_015566	FAM169A	Family with sequence similarity 169, member A	0.0021	2.5378
12.	NM_004573	PLCB2	Phospholipase C, beta 2	0.0086	2.4811
13.	NM_001004739	OR5L2	Olfactory receptor, family 5, subfamily L, member 2	0.0274	2.4485
14.	NM_001444	FABP5	Fatty acid binding protein 5 (psoriasis-associated)	0.0097	2.441
15.	NM_013227	ACAN	Aggrecan	0.0281	2.4295
16.	NM_152346	SLC43A2	Solute carrier family 43 (amino acid system L transporter), member 2	0.0031	2.3993
17.	NM_207320	ZNF157	Zinc finger protein 157	0.0153	2.3503
18.	NM_031220	PITPNM3	PITPNM family member 3	0.0025	2.3135
19.	NM_176072	P2RY2	Purinergic receptor P2Y, G-protein coupled, 2	0.0024	2.254
20.	NM_053277	CLIC6	Chloride intracellular channel 6	0.01	2.2504

4.9.7 Gene expression profilling in PDLSCs for ASA treatment at 1000 μM: upregulated genes (FC>1.5)

Table 4.8 shows the top twenty genes that were upregulated (FC>1.5) in PDLSCs upon 1000 μM ASA treatment. The genes are ranked according to their FC values.

Table 4.8: Up-regulated genes in PDLSCs for 1,000 µM ASA treatment

No.	Acession Number	Gene Symbol	Description	p -value	FC
1.	NM_004490	GRB14	Growth factor receptor-bound protein 14	0.0003	11.7097
2.	NM_031957	KRTAP1-5	Keratin associated protein 1-5	0.0009	10.5284
3.	NM_001040167	LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	0.0024	7.9324
4.	NM_153266	TMEM151A	Transmembrane protein 151A	0.0007	6.8394
5.	NM_207320	OTUD6A	OTU deubiquitinase 6A	0.0466	6.4167
6.	NM_003446	ZNF157	Zinc finger protein 157	0.0153	6.242
7.	NM_031220	PITPNM3	PITPNM family member 3	0.0025	6.2418
8.	NM_005613	RGS4	Regulator of G-protein signaling 4	0.0072	6.1439
9.	NM_145234	CHRDL1	Chordin-like 1	0.0021	6.129
10.	ENST00000361567	ND5	Mitochondrially encoded NADH dehydrogenase 5	0.0123	5.9705
11.	NM_002517	NPAS1	Neuronal PAS domain protein 1	0.0001	5.8204
12.	NM_001144875	DOK3	Docking protein 3	0.0412	5.7912
13.	AK296003	GTPBP6	GTP binding protein 6	0.0489	5.706
14.	NM_001756	SERPINA6	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6	0.0408	5.6777
15.	NM_002309	LIF	Leukemia inhibitory factor	0.0152	5.5938
16.	NM_206539	DLK2	Delta-like 2 homolog (Drosophila)	0.0073	5.5486
17.	NM_000683	ADRA2C	Adrenoceptor alpha 2C	0.0001	5.5113
18.	NM_001099219	KRTAP19-8	Keratin associated protein 19-8	0.0298	5.4916
19.	NM_001004739	OR5L2	Olfactory receptor, family 5, subfamily L, member 2	0.0274	5.4814
20.	ENST00000361381	ND4	Mitochondrially encoded NADH dehydrogenase 4	0.0092	5.3707

4.9.8 Gene expression profilling in PDLSCs for ASA treatment at 200 µM: downregulated genes (FC<-1.5)

Table 4.9 shows the list of downregulated genes in PDLSCs (FC>1.5) for 200 µM ASA treatment. The genes are ranked according to their FC values.

Table 4.9: Downregulated genes in PDLSCs for 200 µM ASA treatment

No.	Acession Number	Gene Symbol	Description	p - Value	FC
1.	NM_004982	KCNJ8	Potassium inwardly-rectifying channel, subfamily J, member 8	0.002	-2.367
2.	NM_173833	SCARA5	Scavenger receptor class A, member 5 (putative)	0.010	-2.270
3.	NM_007177	FAM107A	Family with sequence similarity 107, member A	0.003	-2.129
4.	NM_023003	TM6SF1	Transmembrane 6 superfamily member 1	0.013	-1.936
5.	NM_014817	TRIL	TLR4 interactor with leucine-rich repeats	0.000	-1.919
6.	NR_037169	LOC100507547	LOC100507547	0.008	-1.915
7.	NM_001647	APOD	Apolipoprotein D	0.008	-1.913
8.	NM_001003683	PDE1A	Phosphodiesterase 1A, calmodulin-dependent	0.007	-1.896
9.	NM_001282192	RNASE4	Ribonuclease, RNase A family, 4	0.010	-1.866
10.	NM_000900	MGP	Matrix Gla protein	0.014	-1.854
11.	NM_020211	RGMA	Repulsive guidance molecule family member a	0.005	-1.807
12.	NM_000392	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	0.012	-1.805
13.	NM_001099294	KIAA1644	KIAA1644	0.028	-1.797
14.	NM_015444	TMEM158	Transmembrane protein 158 (gene/pseudogene)	0.045	-1.793

Table 4.9 (Continued)

15.	NM_007289	MME	Membrane metallo-endopeptidase	0.031	-1.785
16.	NM_013231	FLRT2	Fibronectin leucine rich transmembrane protein 2	0.030	-1.774
17.	NM_019605	SERTAD4	SERTA domain containing 4	0.000	-1.770
18.	NM_030754	SAA2	Serum amyloid A2	0.016	-1.762
19.	NM_001009955	SSBP3	Single stranded DNA binding protein 3	0.005	-1.761
20.	NM_013989	DIO2	Deiodinase, iodothyronine, type II	0.015	-1.758

4.9.9 Gene expression profilling in PDLSCs for ASA treatment at 500 µM: downregulated genes (FC<-1.5)

Table 4.10 shows top 20 downregulated genes (FC<-1.5) in PDLSCs for 500 µM ASA treatment. The genes are ranked according to their FC values.

Table 4.10: Downregulated genes in PDLSCs for 500 µM ASA treatment

No.	Acession number	Gene Symbol	Description	p -value	FC
1.	NM_016235	GPRC5B	G protein-coupled receptor, class C, group 5, member B	0.0001	-5.2096
2.	NM_000900	MGP	Matrix Gla protein	0.0139	-5.0697
3.	NM_007177	FAM107A	Family with sequence similarity 107, member A	0.003	-4.7314
4.	NM_002021	FMO1	Flavin containing monooxygenase 1	0.006	-4.2935
5.	NM_173833	SCARA5	scavenger receptor class A, member 5 (putative)	0.0103	-3.9929
6.	NM_013989	DIO2	Deiodinase, iodothyronine, type II	0.015	-3.795

Table 4.10: Downregulated genes in PDLSCs for 500 μM ASA treatment (Continued)

7.	NM_004982	KCNJ8	Potassium inwardly-rectifying channel, subfamily J, member 8	0.0018	-3.7342
8.	NM_014817	TRIL	TLR4 interactor with leucine-rich repeats	0.0001	-3.7294
9.	NM_005258	GCHFR	GTP cyclohydrolase I feedback regulator	0.0024	-3.6019
10.	NM_001003683	PDE1A	Phosphodiesterase 1A, calmodulin-dependent	0.0071	-3.5621
11.	NM_000076	CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.0002	-3.5603
12.	NM_000201	ICAM1	Intercellular adhesion molecule 1	0.0032	-3.4437
13.	NM_019605	SERTAD4	SERTA domain containing 4	9.78E-07	-3.4098
14.	NM_053276	VIT	Vitrin	0.0004	-3.2005
15.	NM_001093726	SEPP1	Selenoprotein P, plasma, 1	0.0305	-3.1945
16.	NM_005545	ISLR	Iimmunoglobulin superfamily containing leucine-rich repeat	0.0255	-3.1017
17.	NM_030754	SAA2	Serum amyloid A2	0.0162	-3.0603
18.	NM_138621	BCL2L11	BCL2-like 11 (apoptosis facilitator)	0.0053	-3.0058
19.	NM_001013254	LSP1	Lymphocyte-specific protein 1	0.0002	-2.9799
20.	NM_005144	HR	Hair growth associated	0.0001	-2.905

4.9.10 Gene expression profilling in PDLSCs for ASA treatment at 1000 μM: downregulated genes (FC<-1.5)

Table 4.11 shows top 20 downregulated genes (FC>1.5) in PDLSCs for 1000 μM ASA treatment. The genes are ranked according to their FC values.

Table 4.11: Down-regulated genes in PDLSCs for 1,000 µM ASA treatment

No	Acession Number	Gene Symbol	Description	p –value	FC
1.	NM_002021	FMO1	Flavin containing monooxygenase 1	0.006	-36.8141
2.	NM_000900	MGP	Matrix Gla protein	0.0134	-26.1991
3.	NM_020211	RGMA	Repulsive guidance molecule family member a	0.005	-13.0684
4.	NM_001003683	PDE1A	Phosphodiesterase 1A, calmodulin-dependent	0.007	-10.2608
5.	NM_138621	BCL2L11	BCL2-like 11 (apoptosis facilitator)	0.006	-9.9276
6.	NM_016235	GPRC5B	G protein-coupled receptor, class C, group 5, member B	0.0001	-9.6482
7.	NM_003881	WISP2	WNT1 inducible signaling pathway protein 2	0.001	-9.1431
8.	NM_004673	ANGPTL1	Angiopoietin-like 1	0.002	-8.8384
9.	NM_014817	TRIL	TLR4 interactor with leucine-rich repeats	0.0001	-8.5709
10.	NM_053276	VIT	Vitrin	0.001	-8.2845
11.	NM_001002294	FMO3	Flavin containing monooxygenase 3	0.0176	-8.2696
12.	NM_000076	CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.0002	-7.7105
13.	NM_007177	FAM107A	Family with sequence similarity 107, member A	0.003	-7.5331
14.	NM_006207	PDGFRL	Platelet-derived growth factor receptor-like	0.031	-7.4911
15.	NM_000201	ICAM1	Intercellular adhesion molecule 1	0.003	-7.4592
16.	NM_013989	DIO2	Deiodinase, iodothyronine, type II	0.015	-7.3692
17.	NM_000600	IL6	Interleukin 6	0.0057	-7.1122
18.	NM_022168	IFIH1	Interferon induced with helicase C domain 1	0.001	-6.8728
19.	NM_000667	ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	0.007	-6.8019
20.	NM_001099781	GGT5	Gamma-glutamyltransferase 5	0.000	-6.789

4.10 Comparison differentially expressed genes in PDLSCs: effect of growth media and ASA concentrations

The gene expression analyses indicated that ASA was able to modulate the expression of genes in PDLSCs, using normal media or osteogenic differentiation media. In the first part of the gene expression profiling study, PCR array was used. The study used non-osteogenic induction media (normal media) and profiled growth factor associated genes in the presence/absence of ASA. In the second part of the study, Agilent microarray was used, to profile global gene expression in PDLSCs in the presence and absence of ASA. The second part of the analyses differs from the first assay, due to the use of osteogenic induction media. For the microarray data, although a number of genes were similarly up/downregulation using different ASA concentrations, the gene expression profiles among 200, 500 and 1000 μM ASA treatments were in general significantly different.

The use of osteogenic media and non-osteogenic media also influenced the gene expression profile. For example, for 1000 μM ASA treatments, INHBB was found to be upregulated in PDLSCs using non-osteogenic induction media, while the same gene was upregulated when grown using osteogenic induction media (Table 4.12). Similarly, FGF5 was significantly downregulated at 1000 μM ASA treatment when the PDLSCs was grown in non-osteogenic induction media, but the same gene was observed to be upregulated at 1000 μM ASA treatment using osteogenic induction media.

Table 4.12: The list of comparison genes significant at PCR array vs microarray

No.	Process(es)	Gene symbol	Fold change (FC)* (PCR array)	Fold change (FC)* (microarray)		
			1,000 µM	200 µM	500 µM	1,000 µM
1.	CD[†]	FGF9	+15.88±0.56	-	-	-
2.	AGF[‡], AR[§], NSD[‖]	VEGFA	+13.19±0.86	-	-	-
3.	AR[§], CD[†]	IL2	+11.19±0.21	-	-	-
4.	ED[¶]	BMP10	+ 6.90±0.34	-	-	-
5.	CD[†], AGF[‡]	VEGFC	+ 6.31±0.20	-	-	-
6.	AGF[‡], CD[†], NSD[‖], MD[#]	FGF2	+ 6.30±0.14	-	-	-
7.	DRG**	FGF7	+ 4.99±0.01	-	-	-
8.	CD[†]	IL4	+ 4.59±0.28	-	-	-
9.	AR[§], CD[†]	IL10	+ 4.20±0.07	-	-	-
10.	OFD[††], CD[†]	INHBB	+ 3.25±0.32	-	-1.5160	-3.2219
11.	CRD[‡‡], CD[†]	BMP2	+ 3.03±0.54	-	-	-
12.	DRG**	CSF3	+ 2.24±0.20	-	-	-
13.	DRG**	AMH	- 6.59±0.35	-	-	-
14.	AGF[‡], CD[†], NSD[‖]	JAG1	- 14.15±2.35	-	-	-
15.	ED[¶]	NRG1	- 16.31±0.03	-	-	-
16	DRG**	DKK1	- 31.99±5.24	-	-	-
17	DRG**	BDNF	- 32.10±0.62	-	-	-
18	NSD[‖]	FGF5	- 32.16±0.06	-	+1.7523	+3.5181
19	NSD[‖]	PTN	- 32.20±0.19	-	-	-1.7000

4.11 Gene ontology (GO) analyses : PANTHER

Genes ontology (GO) analysis was enriched by using PANTHER families (protein annotation through evolutionary relationship)(Mi *et al.*, 2013). GO analysis was used to infer functions shared among related genes. Gene family phylogeny was used to incorporate the variant experimentally-derived GO functional annotations across related genes. It classifies the 1,344 genes ($FC \geq 1.5$, $p < 0.05$) according to Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). Figures 4.11, 4.12, and 4.13 showed BP, MF and CC for 200, 500, and 1,000 μM of ASA treatments respectively.

4.11.1 Gene ontology for Biological Processes (BP)

The distribution of the genes conferring biological processes (BP) at 200 μM of ASA treatments (Figure 4.11A) is as follows: metabolic process (117 genes, 22.5%), cellular process (112 genes, 21.5%), biological regulation (71 genes, 13.6%), localization (38 genes, 7.3%), developmental process (48 genes, 9.2%), response to stimulus (35 genes, 6.7%), immune system process (27 genes, 5.2%), multicellular organismal process (21 genes, 4.0%), cellular component organization or biogenesis (15 genes, 2.9%), apoptotic process (15 genes, 2.9%), locomotion (4 genes, 0.8%), biological adhesion (15 genes, 2.9%), and reproduction (3 genes, 0.6%),

The distribution of the genes conferring biological processes (BP) at 500 μM of ASA treatments (Figure 4.11B) is as follows: metabolic process (335 genes, 23.7%), cellular process (276 genes, 19.5%), biological regulation (186 genes, 13.2%), developmental process (125 genes, 8.8%), localization (110 genes, 7.8%), response to

stimulus (100 genes, 7.1%), immune system process (72 genes, 5.1%), multicellular organismal process (56 genes, 4.0%), cellular component organization or biogenesis (50 genes, 3.5%), a biological adhesion (45 genes, 3.2%), apoptotic process (33 genes, 2.3%), reproduction (16 genes, 1.1%), locomotion (7 genes, 0.5%) and growth (2 genes, 0.1%).

The distribution of the genes conferring biological processes (BP) at 1,000 μM of ASA treatments (Figure 4.11C) is as follows: metabolic process (843 genes, 27.1%), cellular process (640 genes, 20.6%), biological regulation (402 genes, 12.9%), developmental process (236 genes, 7.6%), localization (230 genes, 7.4%), response to stimulus (174 genes, 5.6%), cellular component organization or biogenesis (151 genes, 4.9%), immune system process (127 genes, 4.1%), multicellular organismal process (122 genes, 3.9%), biological adhesion (74 genes, 2.4%), apoptotic process (64 genes, 2.1%), reproduction (31 genes, 1.0%), locomotion (11 genes, 0.4%) and growth (2 genes, 0.1%).

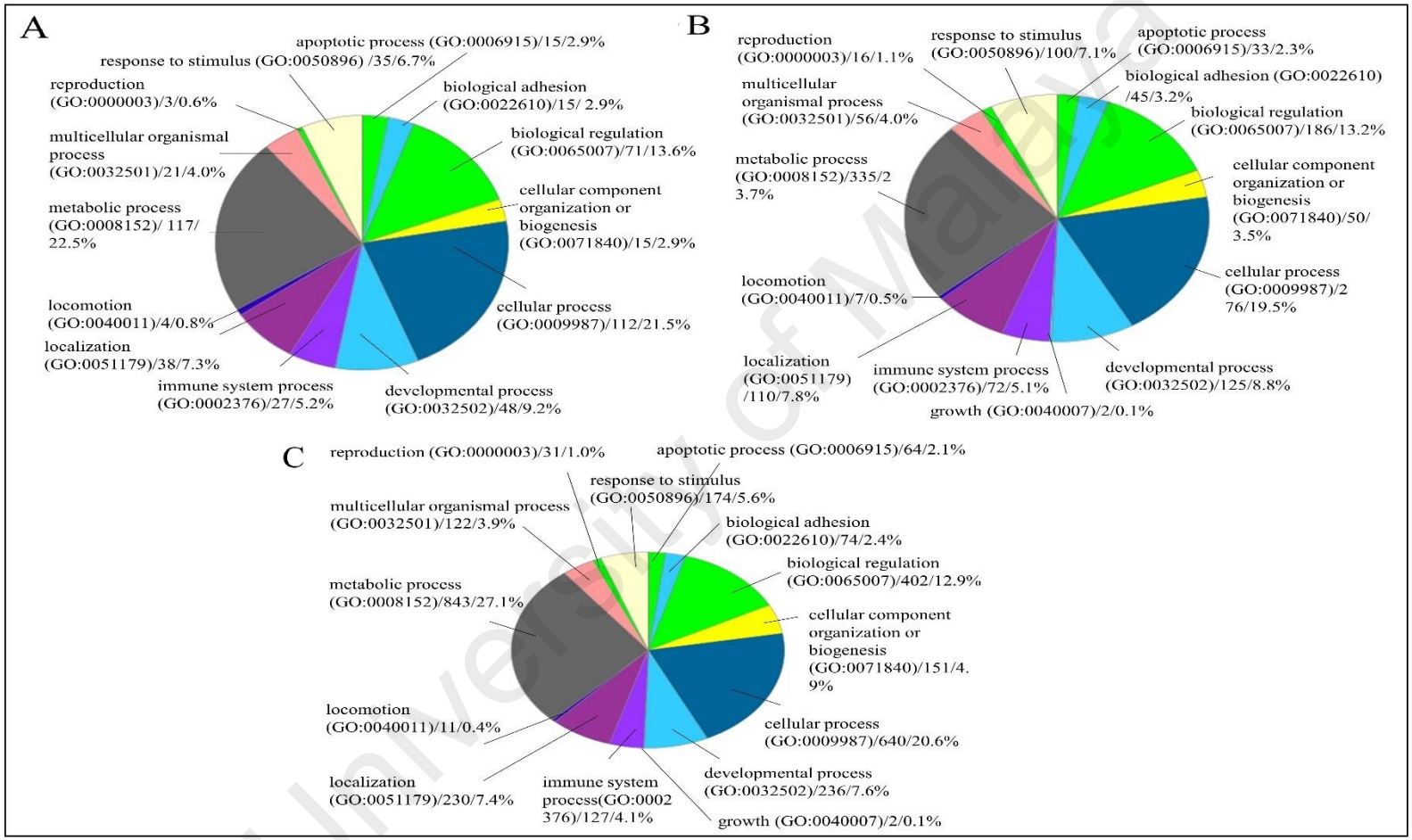

Figure 4.11: Functional analyses of differentially expressed genes by PANTHER. Representative GO terms of Biological Process (BP) at A) 200 µM B) 500 µM and C) 1,000 µM of ASA treatments. This figure showed number of genes /% count.

4.11.2 Gene ontology for Molecular Functions (MFs)

The genes for molecular functions (MFs) at 200 μM of ASA treatments (Figure 4.12(A)) were for binding (98 genes, 35.3%), catalytic activity (84 genes, 30.2%), enzyme regulator activity (27 genes, 9.7%), receptor activity (17 genes, 6.1%), nucleic acid binding transcription factor activity (17 genes, 6.1%), structural molecule activity (14 genes, 5.0%), transporter activity (13 genes, 4.7%), translation regulator activity (4 genes, 1.4%), protein binding transcription factor activity (3 genes, 1.1%) and channel regulator activity (1 gene, 0.4%).

The genes for molecular functions at 500 μM of ASA treatments (Figure 4.12(B)) were binding (235 genes, 31.2%), catalytic activity (224 genes, 29.7%), receptor activity (64 genes, 8.5%), nucleic acid binding transcription factor activity (62 genes, 8.2%), enzyme regulator activity (57 genes, 7.6%), transporter activity (52 genes, 6.9%), structural molecule activity (42 genes, 5.6%), protein binding transcription factor activity (11 genes, 1.5%), translation regulator activity (4 genes, 0.5%), channel regulator activity (1 gene, 0.1%) and antioxidant activity (2 genes, 0.3%).

The genes for molecular functions at 1,000 μM of ASA treatments (Figure 4.12(C)) were binding (601genes, 34.2%), catalytic activity (522 genes, 29.7%), nucleic acid binding transcription factor activity (164 genes, 9.3%), receptor activity (118 genes, 6.7%), enzyme regulator activity (117 genes, 6.7%), structural molecule activity (105 genes, 6.0%), transporter activity (84 genes, 4.8%), protein binding transcription factor activity (29 genes, 1.7%), translation regulator activity (11 genes, 0.6%), antioxidant activity (4 genes, 0.2%) and channel regulator activity (2 genes, 0.1%).

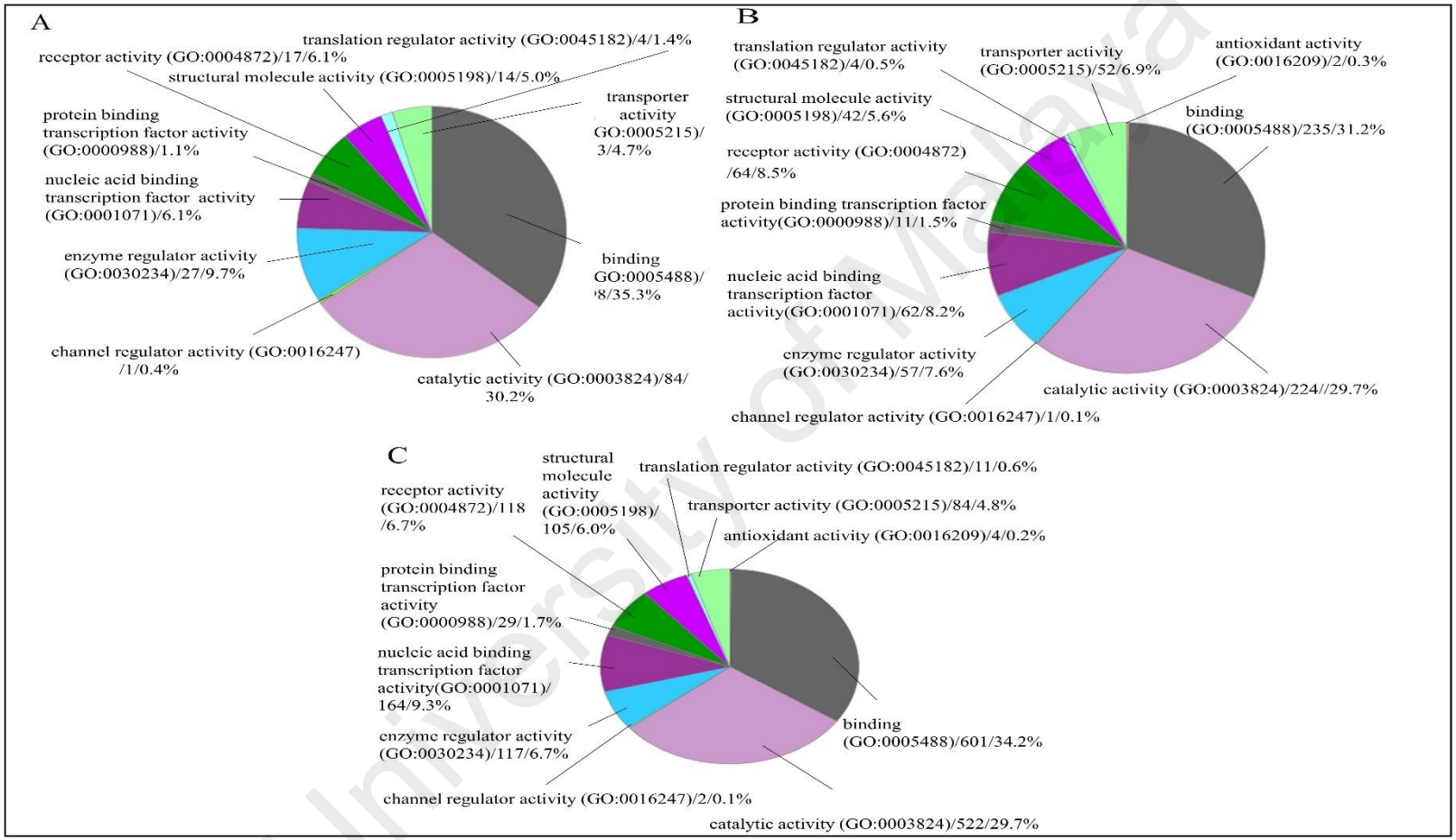

Figure 4.12: Functional analyses of differentially expressed genes by PANTHER. Representative GO terms of Molecular Functions (MFs) at A) 200 µM B) 500 µM and C) 1,000 µM of ASA treatments. This figure showed number of genes /% count.

4.11.3 Gene ontology for cellular componenents (CC)

The genes for cellular componenents (CC) at 200 μM of ASA treatments (Figure 4.13(A)) were cell part (38 genes, 34.5%), organelle (19 genes, 17.3%) , membrane (19 genes, 17.3%), extracellular region (16 genes, 14.5%), macromolecular complex (9 genes, 8.2%), extracellular matrix (7 genes, 6.4%), synapse (1 gene, 0.9%) and cell junction (1 gene, 0.9%).

The genes for cellular componenents (CC) at 500 μM of ASA treatments (Figure 4.13(B)) were cell part (114 genes, 33.7%), organelle (59 genes, 17.5%), extracellular region (54 genes, 16.0%), membrane (48 genes, 14.2%), macromolecular complex (33 genes, 9.8%), extracellular matrix (26 genes, 7.7%), cell junction (3 genes, 0.9%) and synapse (1 gene, 0.3%).

The genes for cellular componenents (CC) at 1,000 μM of ASA treatments (Figure 4.13(C)) were cell part (312 genes, 38.0%), organelle (192 genes, 23.4%), membrane (99 genes, 12.1%), macromolecular complex (89 genes, 10.9%) and extracellular region (76 genes, 9.3%), extracellular matrix (40 genes,4.9%), cell junction (8 genes, 1.0%) and synapse (4 genes, 0.5%).

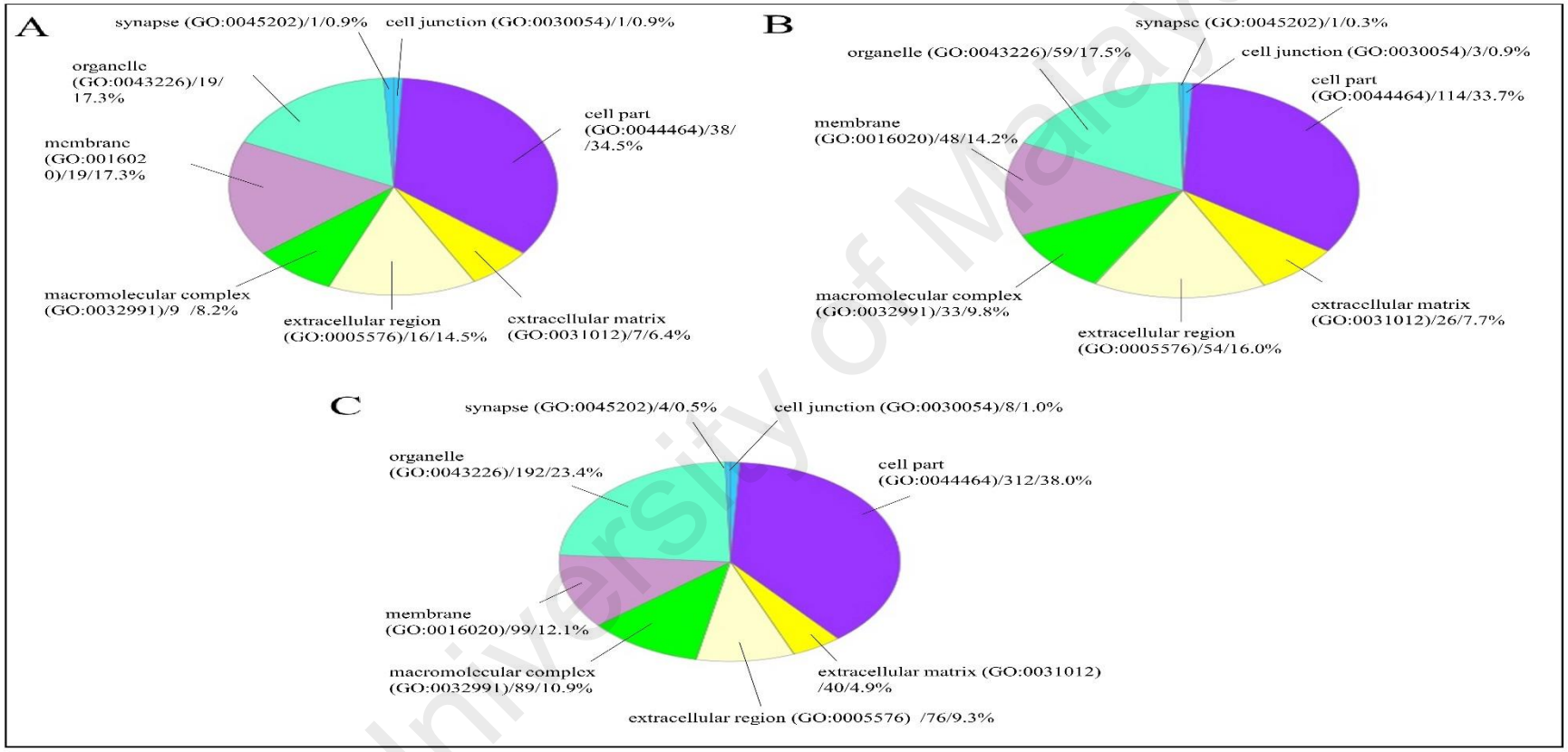

Figure 4.13: Functional analyses of differentially expressed genes by PANTHER. Representative GO terms of Cellular Components (CCs) at A) 200 µM B) 500 µM and C) 1,000 µM of ASA treatments. This figure showed number of genes /% count.

4.12 Functional enrichment analysis using DAVID

The web-based bioinformatics tools, DAVID v6.7 (Database for Annotation, Visualization and Integrated Discovery) was used for protein functional enrichment analysis. The microarray data was subjected to functional enrichment analysis, to identify the dysregulated GO categories during osteogenic differentiation of PDLSCs in the presence of ASA at 200, 500, and 1,000 μM. The functional categorization is considered significant when the p-value is less than 0.05. DAVID generates an enrichment score for a group of genes indicating annotation term member associations in a given experiment. The categories with top enrichment scores and lowest p-values are noted in the following sections.

4.12.1 DAVID functional enrichment analysis : Gene expression profile for 200 μM ASA treatment

Table 4.13 lists the GO terms that were found to be statistically enriched for 200 μM ASA treatment during PDLSCs osteogenic differentiation.

Table 4.13: DAVID functional enrichment scores for 200 µM ASA treatment

Enrichment Score: 2.0395	
Term	**p-Value**
GO:0050708~regulation of protein secretion	0.000185
GO:0051223~regulation of protein transport	0.000250
GO:0050714~positive regulation of protein secretion	0.000298
GO:0070201~regulation of establishment of protein localization	0.000375
GO:0051222~positive regulation of protein transport	0.000410
GO:0032880~regulation of protein localization	0.000897
GO:0050716~positive regulation of interleukin-1 secretion	0.000969
GO:0050704~regulation of interleukin-1 secretion	0.001198
GO:0032732~positive regulation of interleukin-1 production	0.001753
GO:0001819~positive regulation of cytokine production	0.001946
GO:0016477~cell migration	0.002313
GO:0006953~acute-phase response	0.002598
GO:0002526~acute inflammatory response	0.002991
GO:0032652~regulation of interleukin-1 production	0.004284
GO:0050715~positive regulation of cytokine secretion	0.004284
GO:0001817~regulation of cytokine production	0.004881
Enrichment Score: 2.00957	
Term	**p-Value**
GO:0005615~extracellular space	0.000030
GO:0044421~extracellular region part	0.000065
GO:0005576~extracellular region	0.004187
Enrichment Score: 1.994134	
Term	**p-Value**
GO:0002526~acute inflammatory response	0.002991
GO:0006952~defense response	0.003625
GO:0006954~inflammatory response	0.007784
GO:0032101~regulation of response to external stimulus	0.028256
GO:0009611~response to wounding	0.044864
Enrichment Score: 1.74200	
Term	**p-Value**
GO:0001819~positive regulation of cytokine production	0.001946
GO:0051090~regulation of transcription factor activity	0.003829
GO:0051101~regulation of DNA binding	0.008319
GO:0051091~positive regulation of transcription factor activity	0.011081
GO:0043388~positive regulation of DNA binding	0.018646
GO:0051092~positive regulation of NF-kappaB transcription factor activity	0.021327
GO:0051098~regulation of binding	0.023985
GO:0051099~positive regulation of binding	0.026553

Table 4.13 (Continued)

Term	**p-Value**
GO:0042773~ATP synthesis coupled electron transport	0.001273
GO:0042775~mitochondrial ATP synthesis coupled electron transport	0.001273
GO:0022900~electron transport chain	0.001342
GO:0022904~respiratory electron transport chain	0.002315
GO:0016655~oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	0.005130
GO:0045333~cellular respiration	0.013362
GO:0006119~oxidative phosphorylation	0.013919
Enrichment Score: 1.609	
Term	**p-Value**
GO:0030291~protein serine/threonine kinase inhibitor activity	0.001671
GO:0004857~enzyme inhibitor activity	0.005395
GO:0004861~cyclin-dependent protein kinase inhibitor activity	0.008430
GO:0004860~protein kinase inhibitor activity	0.014395
GO:0019210~kinase inhibitor activity	0.015505
GO:0016538~cyclin-dependent protein kinase regulator activity	0.023867
GO:0033673~negative regulation of kinase activity	0.041688

4.12.2 DAVID functional enrichment analysis : gene expression profile for 500 µM ASA treatment

Table 4.14 lists the GO terms that were found to be statistically enriched for 500 µM ASA treatment during PDLSCs osteogenic differentiation.

Table 4.14: DAVID functional enrichment scores for 500 µM ASA treatment

Enrichment Score: 4.14739	
Term	**p-Value**
GO:0044421~extracellular region part	3.08E-08
GO:0005615~extracellular space	9.77E-07
GO:0005576~extracellular region	9.83E-05
Enrichment Score: 3.8694	
Term	**p-Value**

Table 4.14 (Continued)

GO:0048514~blood vessel morphogenesis	4.07E-05
GO:0001568~blood vessel development	1.39E-04
GO:0001944~vasculature development	1.98E-04
GO:0001525~angiogenesis	2.96E-04
Enrichment Score: 3.30718	
Term	**p-Value**
GO:0008201~heparin binding	1.32E-05
GO:0005539~glycosaminoglycan binding	1.41E-04
GO:0001871~pattern binding	4.24E-04
GO:0030247~polysaccharide binding	4.24E-04
Enrichment Score: 3.0850	
Term	**p-Value**
GO:0030335~positive regulation of cell migration	2.19E-04
GO:0040017~positive regulation of locomotion	5.41E-04
GO:0051272~positive regulation of cell motion	5.41E-04
GO:0030334~regulation of cell migration	0.001273
GO:0040012~regulation of locomotion	0.001892
GO:0051270~regulation of cell motion	0.002000
Enrichment Score: 2.6648	
Term	**p-Value**
GO:0031012~extracellular matrix	4.05E-04
GO:0005578~proteinaceous extracellular matrix	0.001504
extracellular matrix	0.016619
Enrichment Score: 2.53643	
Term	**p-Value**
GO:0002526~acute inflammatory response	3.17E-07
GO:0002541~activation of plasma proteins involved in acute inflammatory response	7.04E-07
GO:0006956~complement activation	4.61E-06
GO:0051604~protein maturation	7.28E-06
GO:0016485~protein processing	9.76E-06
GO:0002684~positive regulation of immune system process	3.20E-05
GO:0006959~humoral immune response	6.81E-05
GO:0048584~positive regulation of response to stimulus	7.89E-05
GO:0002252~immune effector process	9.24E-05
GO:0006958~complement activation, classical pathway	1.17E-04
GO:0006954~inflammatory response	1.22E-04
Enrichment Score: 2.38456	
Term	**p-Value**
GO:0050716~positive regulation of interleukin-1 secretion	1.51E-04
GO:0050714~positive regulation of protein secretion	1.89E-04
GO:0050704~regulation of interleukin-1 secretion	2.20E-04
GO:0050715~positive regulation of cytokine secretion	2.29E-04

Table 4.14 (Continued)

GO:0051222~positive regulation of protein transport	3.15E-04
GO:0070201~regulation of establishment of protein localization	3.48E-04
GO:0032732~positive regulation of interleukin-1 production	4.24E-04
GO:0032880~regulation of protein localization	4.28E-04
GO:0050708~regulation of protein secretion	4.65E-04
GO:0050707~regulation of cytokine secretion	0.001060
GO:0032652~regulation of interleukin-1 production	0.001887
GO:0051223~regulation of protein transport	0.002062
GO:0001819~positive regulation of cytokine production	0.003199
GO:0051050~positive regulation of transport	0.008588
Enrichment Score: 2.22176	
Term	**p-Value**
GO:0032103~positive regulation of response to external stimulus	0.003917
GO:0050729~positive regulation of inflammatory response	0.006342
GO:0031349~positive regulation of defense response	0.008700
Enrichment Score: 2.156043	
Term	**p-Value**
GO:0030155~regulation of cell adhesion	3.96E-04
GO:0045785~positive regulation of cell adhesion	6.02E-04
GO:0010810~regulation of cell-substrate adhesion	0.009741
GO:0010811~positive regulation of cell-substrate adhesion	0.024468
Enrichment Score: 2.0819	
Term	**p-Value**
GO:0014070~response to organic cyclic substance	0.001131
GO:0010033~response to organic substance	0.002144
GO:0009725~response to hormone stimulus	0.003641
GO:0048545~response to steroid hormone stimulus	0.010606
GO:0009719~response to endogenous stimulus	0.012726
Enrichment Score: 2.00629	
Term	**p-Value**
GO:0031589~cell-substrate adhesion	5.41E-04
GO:0007160~cell-matrix adhesion	8.43E-04
GO:0008305~integrin complex	0.00465
GO:0043235~receptor complex	0.035651
Enrichment Score: 1.9534	
Term	**p-Value**
GO:0022610~biological adhesion	0.005982
GO:0007155~cell adhesion	0.009964
Enrichment Score: 1.76223	
Term	**p-Value**
GO:0042325~regulation of phosphorylation	0.004183
GO:0045859~regulation of protein kinase activity	0.006381

Table 4.14 (Continued)

GO:0019220~regulation of phosphate metabolic process	0.007453
GO:0051174~regulation of phosphorus metabolic process	0.007453
GO:0051338~regulation of transferase activity	0.008312
GO:0043549~regulation of kinase activity	0.009561
GO:0043405~regulation of MAP kinase activity	0.011272
GO:0000165~MAPKKK cascade	0.016411
GO:0000187~activation of MAPK activity	0.016904
Enrichment Score: 1.7266	
Term	**p-Value**
GO:0050727~regulation of inflammatory response	4.60E-05
GO:0032101~regulation of response to external stimulus	0.00185
GO:0032103~positive regulation of response to external stimulus	0.003917
GO:0031348~negative regulation of defense response	0.013781
hdl	0.026632
GO:0050728~negative regulation of inflammatory response	0.034234
Enrichment Score: 1.69405	
Term	**p-Value**
GO:0009967~positive regulation of signal transduction	0.001854
GO:0010647~positive regulation of cell communication	0.007014
GO:0010740~positive regulation of protein kinase cascade	0.017112
GO:0010627~regulation of protein kinase cascade	0.023223

4.12.3 DAVID functional enrichment analysis : gene expression profile for 1,000 µM ASA treatment

Table 4.15 lists the GO terms that were found to be statistically enriched for 1,000 µM ASA treatment during PDLSCs osteogenic differentiation.

Table 4.15: DAVID functional enrichment scores for 1,000 µM ASA treatment

Enrichment Score: 5.498	
Term	**p-Value**
GO:0010605~negative regulation of macromolecule metabolic process	3.14E-07
GO:0045934~negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3.21E-07
GO:0051172~negative regulation of nitrogen compound metabolic process	5.69E-07
GO:0051253~negative regulation of RNA metabolic process	1.30E-06
GO:0010558~negative regulation of macromolecule biosynthetic process	1.45E-06
GO:0009890~negative regulation of biosynthetic process	1.59E-06
GO:0016481~negative regulation of transcription	1.61E-06
GO:0031327~negative regulation of cellular biosynthetic process	2.23E-06
Enrichment Score: 5.3021	
Term	**p-Value**
GO:0043228~non-membrane-bounded organelle	8.22E-08
GO:0043232~intracellular non-membrane-bounded organelle	8.22E-08
Term	**p-Value**
GO:0045449~regulation of transcription	7.65E-06
GO:0030528~transcription regulator activity	4.37E-05
GO:0006350~transcription	8.29E-05
GO:0003677~DNA binding	3.46E-04
Enrichment Score: 4.034	
Term	**p-Value**
GO:0031981~nuclear lumen	2.30E-06
GO:0043233~organelle lumen	6.46E-05
GO:0005730~nucleolus	8.32E-05
GO:0031974~membrane-enclosed lumen	1.11E-04
GO:0005654~nucleoplasm	1.23E-04
GO:0070013~intracellular organelle lumen	1.26E-04
Enrichment Score: 4.00173	
Term	**p-Value**
GO:0008134~transcription factor binding	3.79E-07
GO:0016563~transcription activator activity	2.24E-04
GO:0003712~transcription cofactor activity	4.72E-04
Term	**p-Value**
GO:0032993~protein-DNA complex	3.76E-07
GO:0006325~chromatin organization	2.81E-06
GO:0000786~nucleosome	7.40E-06
GO:0006334~nucleosome assembly	8.37E-05
GO:0006333~chromatin assembly or disassembly	1.22E-04
GO:0051276~chromosome organization	1.26E-04
GO:0034728~nucleosome organization	1.27E-04
Enrichment Score: 3.6096	
Term	**p-Value**
GO:0048514~blood vessel morphogenesis	1.66E-04
GO:0001568~blood vessel development	2.16E-04

Table 4.15 (Continued)

GO:0001525~angiogenesis	2.79E-04
GO:0001944~vasculature development	3.64E-04
Enrichment Score: 3.3120	
Term	**p-Value**
GO:0010810~regulation of cell-substrate adhesion	5.66E-05
GO:0030155~regulation of cell adhesion	7.02E-05
GO:0001952~regulation of cell-matrix adhesion	5.73E-04
GO:0045785~positive regulation of cell adhesion	0.003475
GO:0010811~positive regulation of cell-substrate adhesion	0.003483
Enrichment Score: 2.83985	
Term	**p-Value**
GO:0030335~positive regulation of cell migration	5.57E-04
GO:0051272~positive regulation of cell motion	7.42E-04
GO:0051270~regulation of cell motion	0.001192
GO:0040017~positive regulation of locomotion	0.001880
GO:0030334~regulation of cell migration	0.002366
GO:0040012~regulation of locomotion	0.004172
Enrichment Score: 2.72566	
Term	**p-Value**
GO:0010810~regulation of cell-substrate adhesion	5.66E-05
GO:0001953~negative regulation of cell-matrix adhesion	1.07E-04
GO:0010812~negative regulation of cell-substrate adhesion	2.16E-04
GO:0001952~regulation of cell-matrix adhesion	5.73E-04
GO:0051893~regulation of focal adhesion formation	0.032915
GO:0007162~negative regulation of cell adhesion	0.046059
Enrichment Score: 2.59627	
Term	**p-Value**
GO:0006414~translational elongation	1.76E-05
GO:0022626~cytosolic ribosome	2.57E-05
GO:0033279~ribosomal subunit	4.51E-04
GO:0022625~cytosolic large ribosomal subunit	0.001603
GO:0015934~large ribosomal subunit	0.002354
GO:0044445~cytosolic part	0.002439
GO:0030529~ribonucleoprotein complex	0.005598
GO:0005840~ribosome	0.011308
Enrichment Score: 2.319697	
Term	**p-Value**
GO:0006357~regulation of transcription from RNA polymerase II promoter	1.14E-04
GO:0031328~positive regulation of cellular biosynthetic process	0.002553
GO:0009891~positive regulation of biosynthetic process	0.002636
GO:0051173~positive regulation of nitrogen compound metabolic process	0.003174
Enrichment Score: 2.2618	
Term	**p-Value**
GO:0009967~positive regulation of signal transduction	0.001585
GO:0010740~positive regulation of protein kinase cascade	0.001978

Table 4.15 (Continued)

GO:0010647~positive regulation of cell communication	0.007408
GO:0043123~positive regulation of I-kappaB kinase/NF-kappaB cascade	0.009096
GO:0043122~regulation of I-kappaB kinase/NF-kappaB cascade	0.011271
GO:0010627~regulation of protein kinase cascade	0.011287
Enrichment Score: 2.245605	
Term	**p-Value**
GO:0065003~macromolecular complex assembly	4.49E-04
GO:0043933~macromolecular complex subunit organization	4.54E-04
GO:0034622~cellular macromolecular complex assembly	0.010563
Enrichment Score: 2.19174	
Term	**p-Value**
GO:0031012~extracellular matrix	0.00177142
GO:0005578~proteinaceous extracellular matrix	0.001963743
Enrichment Score: 2.0386	
Term	**p-Value**
GO:0016485~protein processing	2.80E-04
GO:0051604~protein maturation	4.09E-04
GO:0002541~activation of plasma proteins involved in acute inflammatory response	4.66E-04
GO:0002526~acute inflammatory response	7.42E-04
GO:0051605~protein maturation by peptide bond cleavage	9.84E-04
GO:0045087~innate immune response	0.001114
GO:0006956~complement activation	0.001418
GO:0002455~humoral immune response mediated by circulating immunoglobulin	0.001743
GO:0006958~complement activation, classical pathway	0.004418
GO:0006959~humoral immune response	0.006610
GO:0002252~immune effector process	0.007311
GO:0002684~positive regulation of immune system process	0.009792
GO:0016064~immunoglobulin mediated immune response	0.011116
GO:0002250~adaptive immune response	0.012447
Enrichment Score: 2.0016	
Term	**p-Value**
GO:0007178~transmembrane receptor protein serine/threonine kinase signaling pathway	0.001419
GO:0007179~transforming growth factor beta receptor signaling pathway	0.023686
GO:0046332~SMAD binding	0.029436
Enrichment Score: 1.82941	
Term	**p-Value**
GO:0001819~positive regulation of cytokine production	0.001701
GO:0001817~regulation of cytokine production	0.011620

4.13 IPA analyses : molecular and cellular functions (MCFs) at 200, 500 and 1,000 µM of ASA treatments

The microarray data were further analysed using IPA to identify categories of molecular and cellular functions (MCFs) that were highly regulated for 200, 500, and 1,000 µM of ASA treatments during PDLSCs osteogenic differentiation. Genes with FC of >1.5 were selected and processed for the analyses. In the following sections (Figure 4.13 to Figure 4.15), significantly regulated MCFs with p–values of less than 0.05 are tabulated.

4.13.1 IPA analyses: significantly regulated MCFs at 200 µM ASA treatment

The top five MCFs that were significantly regulated ($p<0.05$) at 200 µM of ASA treatment were: cellular movement, cell to cell signaling, cellular compromise, cellular growth and proliferation, and lipid metabolism (Table 4.16). Other MCFs that were significantly regulated at 200 µM ASA treatment ($p<0.05$) are as listed in appendix 1.11.1 to 1.11.4.

Table 4.16: Top five MCFs predicted by IPA for 200 µM of ASA treatment ($p<0.05$)

Molecular and cellular functions	Number of genes involved	p-value
Cellular movement	18	2.45E-02-9.81E06
Cell to cell signaling and interaction	21	2.70E-02-1.53E-05
Cellular compromise	8	2.70E-02-1.53E-05
Cellular growth and proliferation	56	2.35E-02-2.16E-05
Lipid metabolism	11	2.19E-02-2.99E-05

In addition, each of the significantly regulated MCFs that was identified at 200, 500, and 1,000 μM of ASA treatments are associated with categories of processes, each with their own z-score, to indicate the relevant category state of activation, if any. Categories having z-score ≥ 2 are predicted by IPA to be in activated state, while those with z score of ≤− 2, were deemed not activated. For 200 μM ASA treatment, cellular movement (Figure 4.14A) and cellular growth & proliferation (Figure 4.14B) were predicted by IPA to be decreased, since such categories had z-score of less than -2.

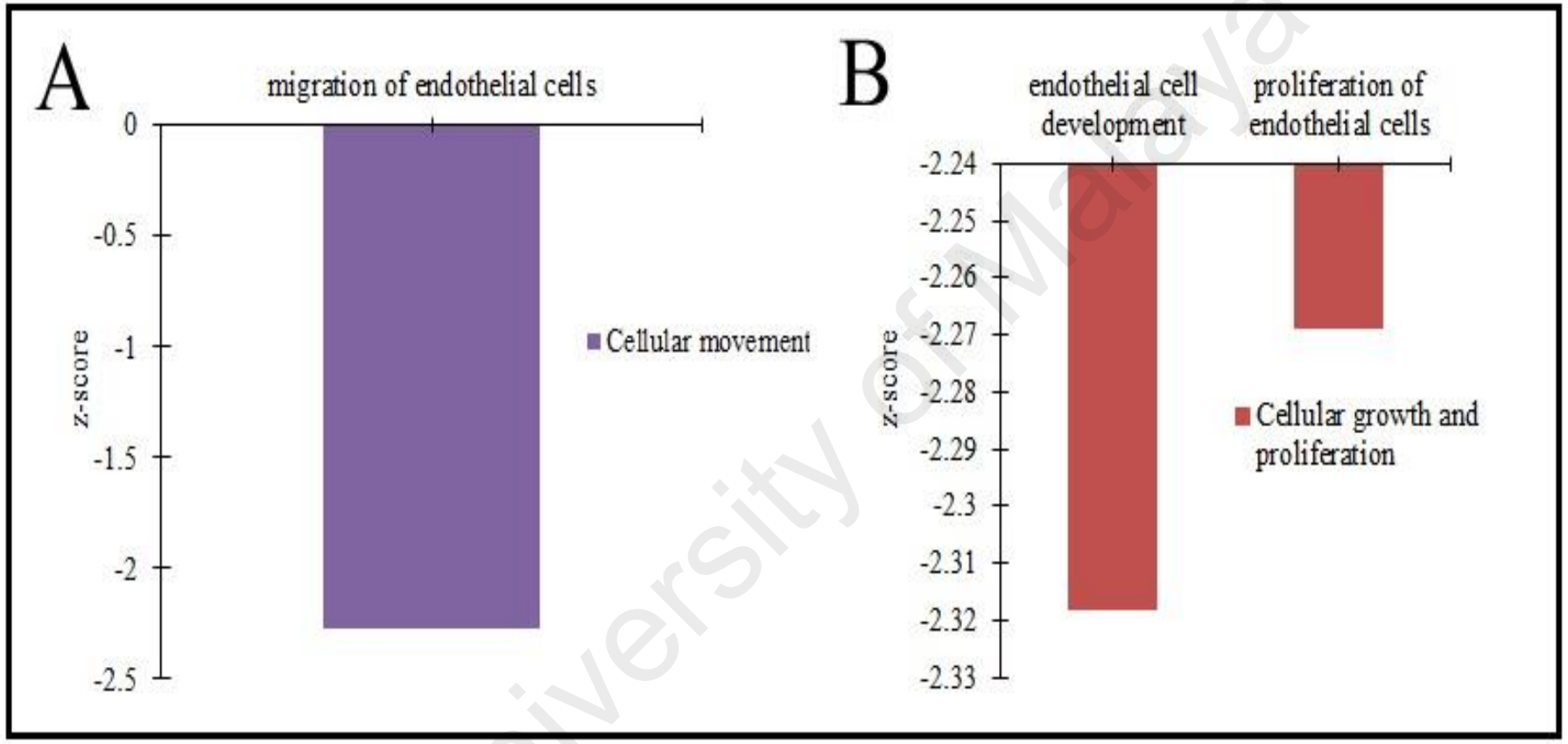

Figure 4.14: MCSFs, z-scores for (A) *Cellular movement* and (B): *Cellular growth and proliferation* for 200 µM of ASA treatment.

4.13.2 IPA analyses: significantly regulated MCFs at 500 μM of ASA treatment

The top five MCFs that were significantly regulated ($p<0.05$) at 500 μM of ASA treatment were: cellular growth and proliferation, cellular development, cellular movement, cellular function and maintenance and cell death and survival (Table 4.17). The remaining MCFs that were significantly regulated ($p<0.05$) at 500 μM ASA are presented in appendix 1.11.5 to 1.11.7

Table 4.17: Top five MCFs as predicted by IPA for 500 μM ASA treatment

Molecular and cellular functions	Number of genes involved	p-value
Cellular growth and proliferation	164	1.12E-02-2.54-09
Cellular development	123	1.12E-02-2.81E-07
Cellular movement	95	1.09 E-02-E-05
Cellular function and maintenance	34	E-02-E-05
Cell death and survival	124	2.06E-02-4.43E-05

Figure 4.15 show processes related to the categories in Table 4.17 that were significantly regulated at 500 μM ASA treatment. The following processes showed significant negative z-scores (predicted to be inhibited): cellular growth and proliferation (Figure 4.15A) and cellular movement (Figure 4.15B) and cell death and survival (Figure 4.15C).

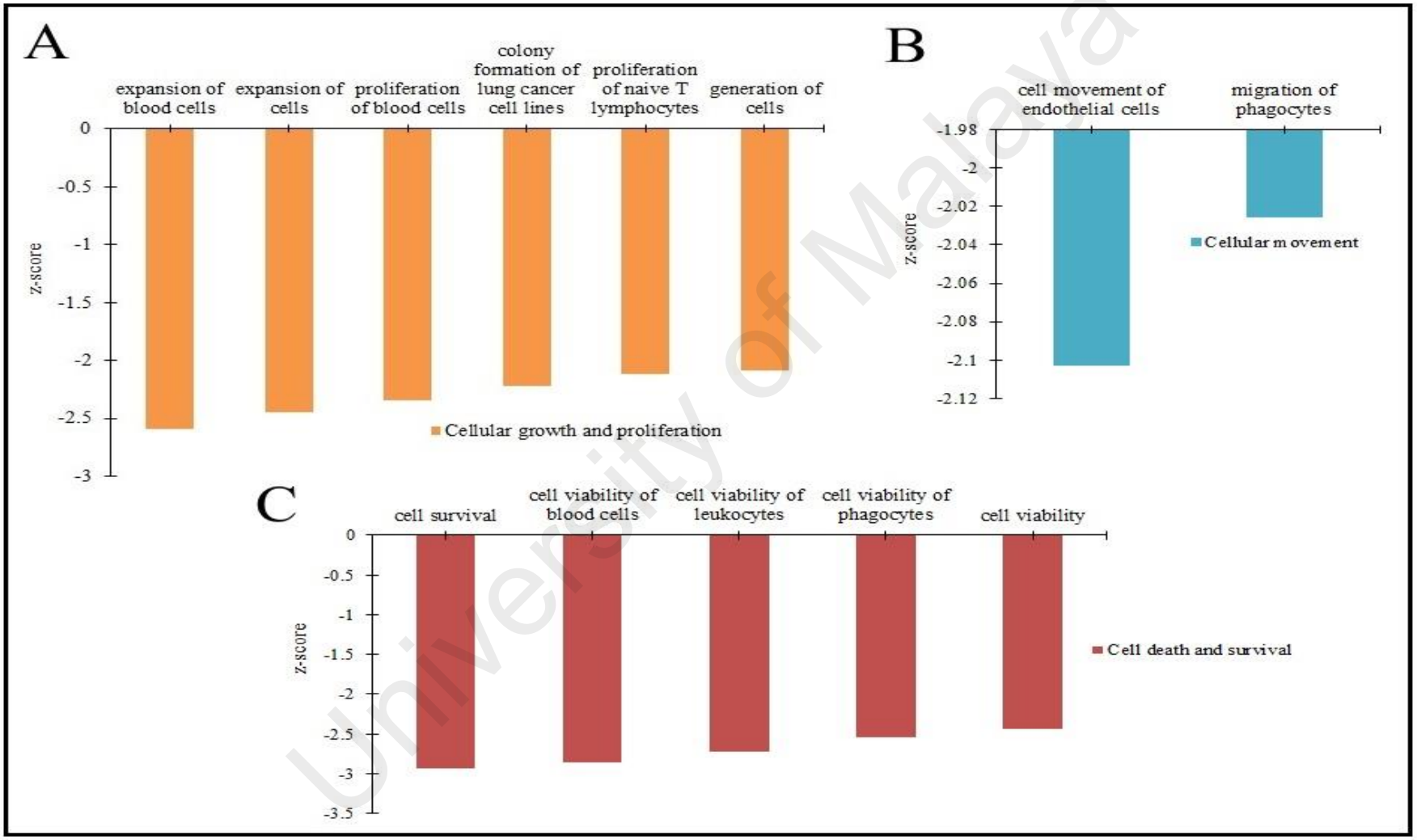

Figure 4.15: MCSFs, z-scores for (A) *Cellular growth and proliferation*; (B) *Cellular movement* and (C) *Cell death and survival* for 500 µM of ASA treatment.

4.13.3 IPA analyses: significantly regulated MCFs at 1,000 μM of ASA treatment

The top five MCFs that were significantly regulated ($p<0.05$) at 1,000 μM of ASA treatment were: hematological system development and function, hematopoiesis, tissue development, cardiovascular system development and function and organismal development (Table 4.18).

Table 4.18: Top five MCFs predicted by Ingenuity Pathway Analysis (IPA) that were corresponding to 1,000 μM treatment

Molecular and cellular functions	Number of genes involved	p-value
Gene expression	300	1.52E-02-1.37E-14
Cell death and survival,	273	1.68E-02-1.24E-08
Cellular growth and proliferation	341	1.48E-02-3.77E-08
Post-translational modification	61	8.89E-02-9.10E-06
Protein synthesis	123	7.68E-02-9.10E-06

Figure 4.16 through 4.17 show processes that are associated to categories of MCFs as indicated in Table 4.23. The processes were: gene expression (Figure 4.16A) cell death and survival (Figure 4.16B), cellular growth and proliferation (Figure 4.16C), post-translational modification (Figure 4.16D). These processes are predicted to be inhibited at 1,000 μM of ASA treatment as they showed negative z-scores.

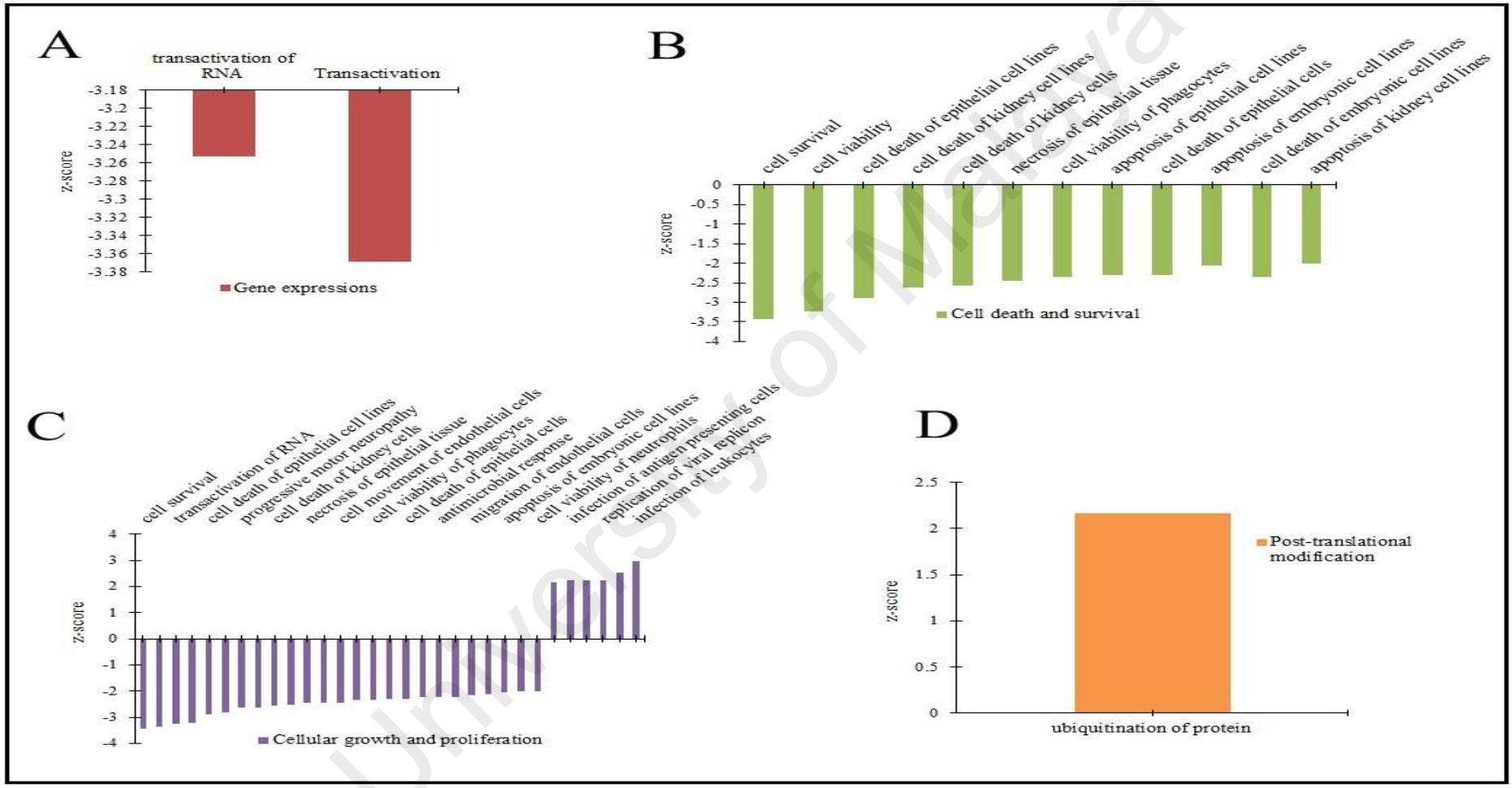

Figure 4.16: MCSFs: z-scores for (A) *Gene Expression*; (B) *Cell death and survival*; (C) *Cellular Growth and proliferation* and (D) Post-translational modification for 1,000 µM of ASA treatment.

4.14 IPA prediction on the effect of ASA treatment on physiological system and development functions (PSDF) in PDLSCs

IPA was used to predict the effect of ASA on physiological system and development in PDLSCs. Genes with a FC of 1.5 ($p<0.05$) were selected for the analyses. The complete list of PSDFs that are enriched is listed in appendix 1.10.

4.14.1 IPA analyses: PSD for 200 µM ASA treatment

The top five PSDFs predicted by IPA were: *connective tissue development and function, tissue development, organ development and skeletal and muscular system developments and functions and hematological system development* (Table 4.19).

Table 4.19: Top five PSDFs predicted by IPA for 200 µM of ASA treatment

Physiological system and development	Number of molecules associated	p-value
Connective tissue development and function	6	1.36E02-2.16E05
Tissue development	35	2.70E02-2.16E05
Organ development	15	1.36E02-6.40E05
Skeletal and muscular system developments and function	24	1.43E02-6.40E05
Hematological system development and function	18	2.70E02-1.61E04

In addition, highly regulated category functions having z-score ≥ ± 2, as are listed in Figure 4.17.

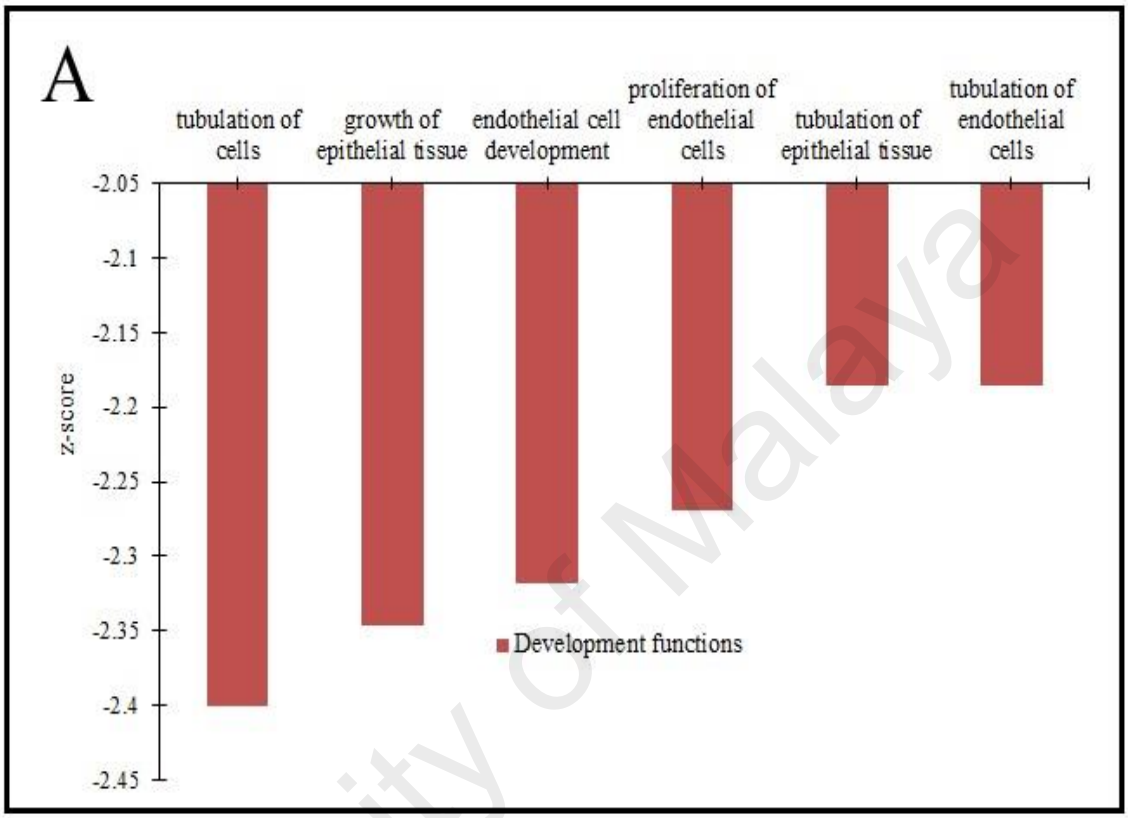

Figure 4.17: PSDFs: z-scores for *tissue development* functions for 200 µM ASA treatment.

4.14.2 IPA analyses: PSDFs for 500 µM ASA treatment

The top five PSDFs at 500 µM of ASA treatment ($p<0.05$) were: *organ development, skeletal and muscular development and function, tissue development, cardiovascular system and development and organismal development* (Table 4.20).

Table 4.20: Top five PSDFs predicted by IPA for 500 µM ASA treatment

Physiological system and development	Number of molecules associated	p-value
Organ development	29	7.94E-03-2.81E-07
Skeletal and muscular development and function	37	7.94E-03-2.81E-07
Tissue development	89	1.12E-02-2.81E-07
Cardiovascular system and development	58	1.05E-02-4.11E-07
Organismal development	56	8.24E-03-6.16E-07

PSDFs category having z-score ≥ ±2 were considered highly regulated and they are as listed in Figure 4.18.

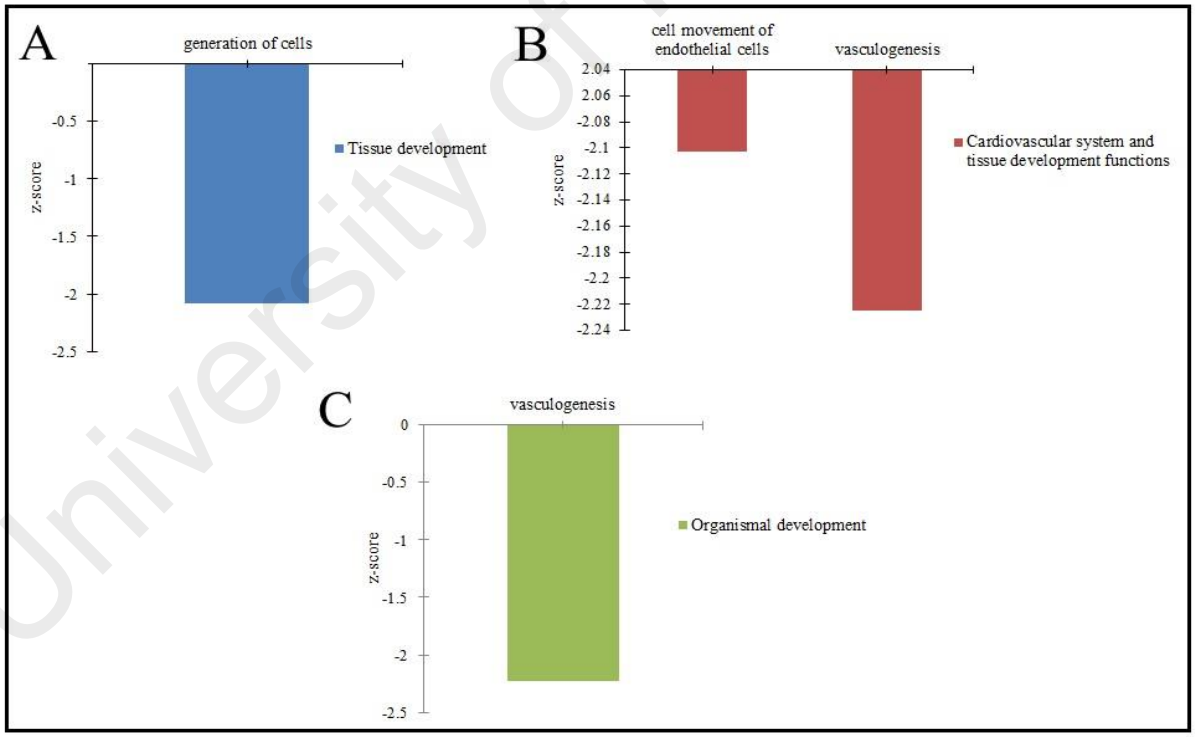

Figure 4.18: PSDFs: z-scores for (A) *tissue development* functions; (B) *cardiovascular system and tissue development functions* and (C) *organismal development* functions for 500 µM ASA treatment.

4.14.3 IPA analyses: PSDFs at 1,000 µM ASA treatment

For 1,000 µM of ASA treatment, the most significant PSDFs ($p<0.05$) predicted by IPA were: *hematological system development and function, hematopoiesis, tissue development, cardiovascular system development and function and organismal development* (Table 4.21).

Table 4.21: Top five PSDFs predicted by IPA for 1,000 µM ASA treatment

Physiological system and development	Number of molecules associated	p-value
Hematological system development and function	73	9.18E-02-9.18E-02
Hematopoiesis	50	9.18E-02-9.18E-02
Tissue development	172	9.18E-02-9.18E-02
Cardiovascular system development and function	76	2.51 E-02-9.18E-02
Organismal development	83	9.18E-02-9.18E-02

For each of the PSDFs highly regulated category, the following functions showed significant z-scores: *hematological system development and function* (Figure 4.20A) and *cardiovascular system development and function* (Figure 4.19B).

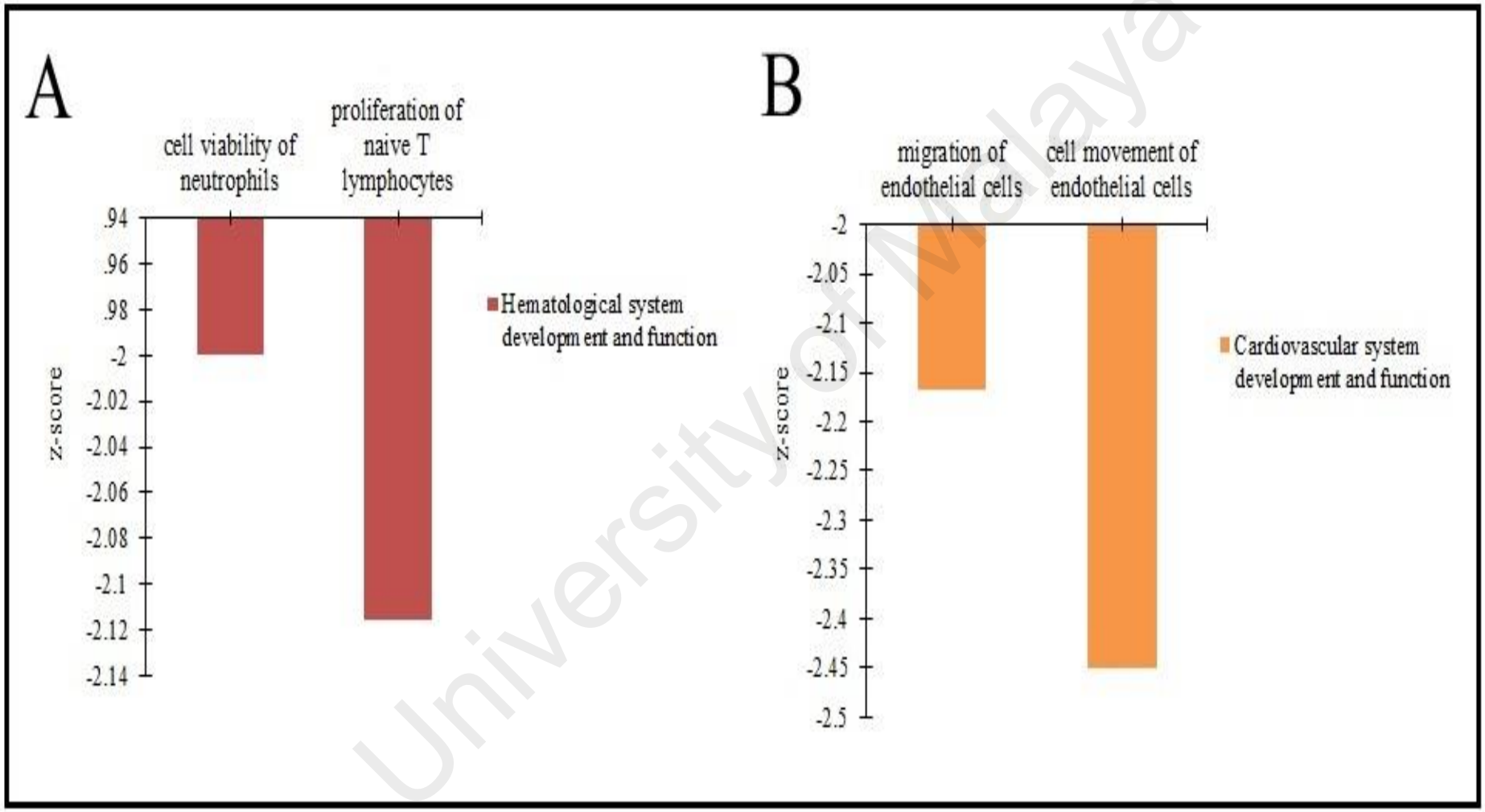

Figure 4.19: PSDFs: z-scores for; (A) *Hematological system development and function*; (B) *Cardiovascular system development and function* for 1000 µM of ASA treatment.

4.15 Upstream regulators analysis (URA)

URA was done using IPA. It provided a small list of inferred upstream regulators based on the measured fold changes (FC) for each ASA treatment. It is critical to infer the identity of upstream regulatory molecules and associated mechanisms to provide biological insight to the observed expression changes. The putative cascade of upstream transcriptional regulators (TRs) were identified through upstream regulators (URs) which provided knowledge or information on the biological activities occurring due to ASA treatments in PDL osteogenic differentiation. The upstream TRs were measured based on p-value ($p< 0.05$) and activation z-score.

In addition, the z-score can be used to predict the UR activity independently. The z-score is assigned for a likely activation based on comparison with a model rather than on assigned random regulation directions. Upstream regulators with z-scores ≥ 2, i.e., activated (or ≤ -2, i.e., inhibited) were considered significant. Significance criteria were stringent for URA in order to focus on the most significantly regulated signalling pathways.

The URA for 200 μM of ASA treatments are shown in Figure 4.20A. The IL-1 receptor antagonist (IL1RN) (z-score: + 2.219) was predicted to be activated, while other cytokines including Periplakin (z-score:-2.236), tumor necrosis factor (TNF) (z-score: -2.124), and interferon gamma (IFNG) (z-score: -2.884) were predicted to be inhibited. The non-activation state of TNF, PPL, and IL1RN cytokines was also predicted by IPA at 500 and 1,000 μM ASA treatments, with the following z-scores:

TNF (z-score: -2.034), PRL (z-score:-3.317), and IL1RN (z-score: 3.719) and TNF (z-score: -3.001), PRL (z-score: -4.359) and IL1RN (z-score: 4.258) respectively.

The main URs implicated at 500 μM of ASA treatments were divided into various categories such as cytokines [IL1RN (z-score: -2.159), interleukin-13, IL13 (z-score: 2.138), Prolactin, PRL (z-score: -3.317), TNF (z-score: -2.034), interleukin-1A (IL1A) (z-score: -2.170), interferon-B1, IFNB1 (z-score: -2.611), interferon gamma, IFNG (z-score:-3.483), IFNA2 (z-score:-2.180)], transmembrane receptors [TREM1 (z-score: 2.524), toll-like receptor-7, (TLR7) (z-score: -2.085), toll-like receptor-9, TLR9 (z-score: -2.954), kinase (BTK) (z-score: 2.985), microrna (mir-122, z-score: -2.236), and others [Bcl-2-like protein 1, BCL2L1 (z-score: 2.000), Niemann-Pick Disease Type C2, NPC2 (z-score: 2.000)] (Figure 4.20B).

The main up-stream regulator implicated at 1,000 μM of ASA treatments was divided into various categories such as cytokines [IL1RN (z-score:4.258), IL13 (z-score: 2.785), PRL (z-score: -4.359),TNF (z-score: -3.001), IFNB1 (z-score: -2.265), IFNG(z-score: -4.399), colony stimulating factor-2 (CSF2) (z-score: -2.010)] transmembrane receptors [TREM1 (z-score: 2.200), TLR7(z-score: -2.290), TLR9(z-score: -2.529), TLR4(z-score: -2.035), IFNAR2(z-score: -2.000)], kinase (tyrosine-protein kinase, Bruton tyrosine kinase (BTK) (z-score: 2.985)), enzyme (Prostaglandin-Endoperoxide Synthase 2, PTGS2 (z-score: -2.000)) and others (BCL2L1(z-score: 2.000)), growth factor [connective tissue growth factor, CTGF (z-score: 2.200) and group (c-Jun N-terminal kinases , JNK (z-score: -2.425)] (Figure 4.20C).

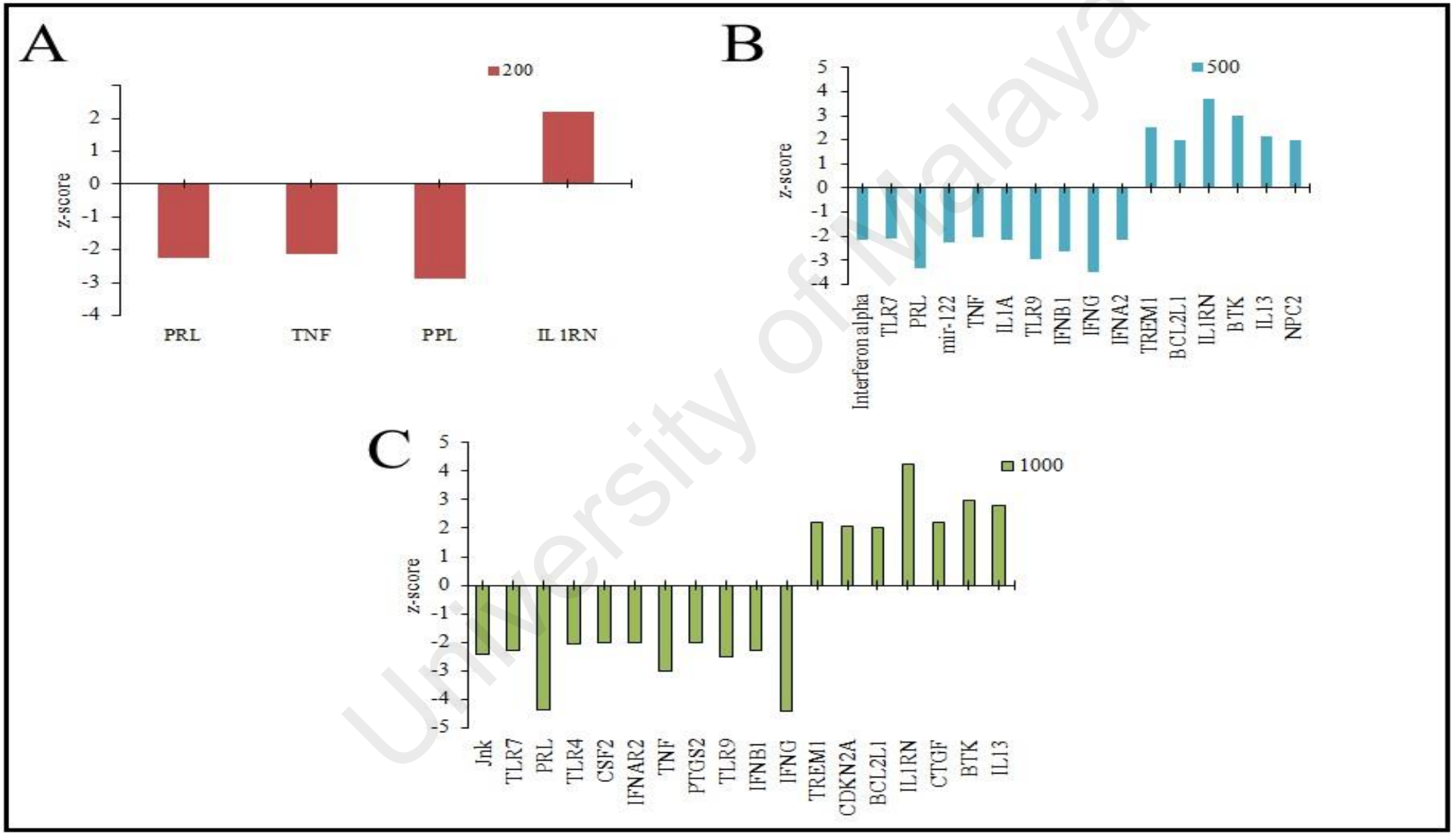

Figure 4.20: z-scores for Up stream regulators (URA): A) 200 µM; B) 500 µM and C) 1,000 µM ASA treatments.

4.15.1 Heat map of up-stream regulators – combined picture

Upstream analysis provided a list of inferred upstream regulators based upon measured fold changes for each concentration of ASA treatments. The objective of the URA is to identify a putative cascade of upstream transcriptional regulator (TRs) that interpret the observed gene expression changes in a user's dataset. Figure 4.21 depicts the most enriched up-stream regulators at 200, 500, and 1,000 μM upon ASA treatments according to the significant z-scores.

They can be seen to have similar patterns/consistent activation or inhibition at all concentrations of ASA treatments. Few up-stream regulators were shown to activate at all concentrations of ASA treatments including IL1RN and BTK. Meanwhile, few up stream regulators were shown to activate at 500 and 1,000 μM of ASA treatments including IL1RN, BTK, TREM1, IL13, and BCL2L1. The only up-stream regulator seen to activate at 1,000 μM of ASA treatments is the CDKN2A.

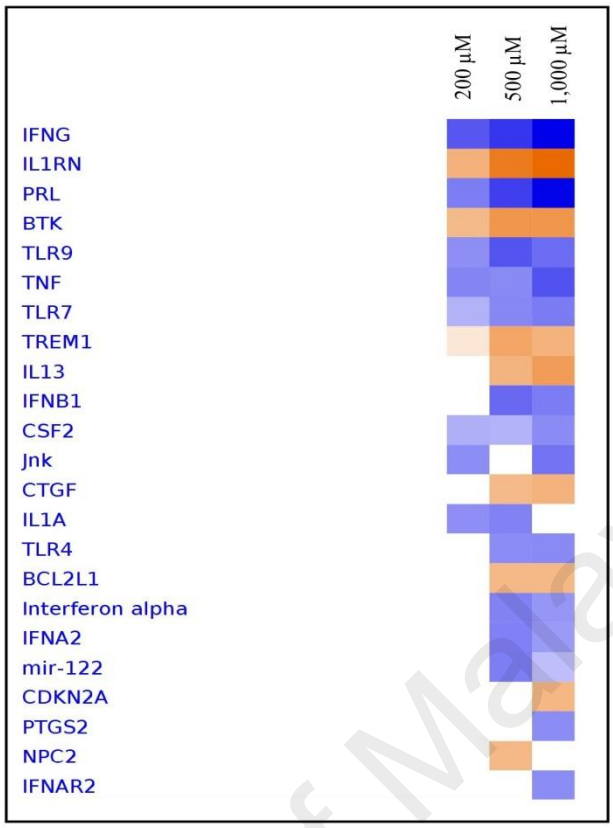

Figure 4.21: Relevant up-stream transcription regulators at 200, 500 and 1,000 µM of ASA treatments. Most activated or inhibited up-stream transcription regulators as estimated by the z-score for each comparison from Ingenuity Pathway Analysis. Orange denotes predicted activation and blue predicted inhibition.

Table 4.22: Relevant up-stream transcription regulators at 200, 500 and 1,000 µM of ASA treatments. Most activated or inhibited up-stream transcription regulators as estimated by the z-score for each comparison from Ingenuity Pathway Analysis (N/A: Not available).

Upstream regulators	200 µM	500 µM	1000 µM
IFNG	-2.88426	-3.48288	-4.39936
IL1RN	2.2188000	3.71868	4.25818
PRL	-2.23607	-3.3166	-4.35890
BTK	1.98250	2.98481	2.98481
TLR9	-1.95084	-2.9542	-2.52941
TNF	-2.12450	-2.0341	-3.00064
TLR7	-1.34253	-2.0855	-2.29008
TREM1	0.70711	2.52357	2.20000
IL13	N/A	2.13809	2.785425
IFNB1	N/A	-2.61139	-2.26488
CSF2	-1.38675	-1.319950	-2.00952
Jnk	-1.97990	N/A	-2.42536
CTGF	N/A	1.9639610	2.20000
IL1A	-1.95560	-2.17008	N/A
BCL2L1	N/A	2.0000	2.0000
Interferon alpha	N/A	-2.15946	-1.82785
IFNA2	N/A	-2.17960	-1.74884
mir-122	N/A	-2.23607	-1.12671
CDKN2A	N/A	N/A	2.06922
PTGS2	N/A	N/A	-2.0000
NPC2	N/A	2.0000	N/A
IFNAR2	N/A	N/A	-2.0000

4.16 Canonical pathways (CP)

Canonical signaling pathways are characterized by the use of IPA software according to the Ingenuity Pathway knowledge base (IPKB). They are ranked according to the IPA calculated score and Fisher's exact test, which are based on the significance of the involved genes ($p<0.05$). A complete list of enriched of canonical pathway for all ASA treatments can be found in supplementary (Table 1, 2 and 3) ($p<0.05$). The top 20 canonical signaling pathways that are significantly enriched (for ASA treatments at 200, 500 and 1,000 μM) are shown in Figure 4.22 ($p<0.05$).

Among the pathways that are significantly enriched were acute phase response signaling, TR/RXR activation and role of macrophages, fibroblast and endothelial cells in rheumatoid arthritis (Figure 4.22). Meanwhile the most significant canonical pathway enriched in 200 and 500 μM ASA treatments were acute phase response signaling, oxidative phosphorylation, role of macrophages, fibroblast and endothelial cells in rheumatoid athritis. The pathway enriched was observed consistently in 500 and 1,000 μM ASA treatments including complement system, acute phase response signaling, NRF-2 mediated oxidative stress response, role of macrophages, fibroblast and end endothelial cells in rheumatoid athritis, LPS/IL-1 mediated inhibition of RXR function and ethanol degradation II.

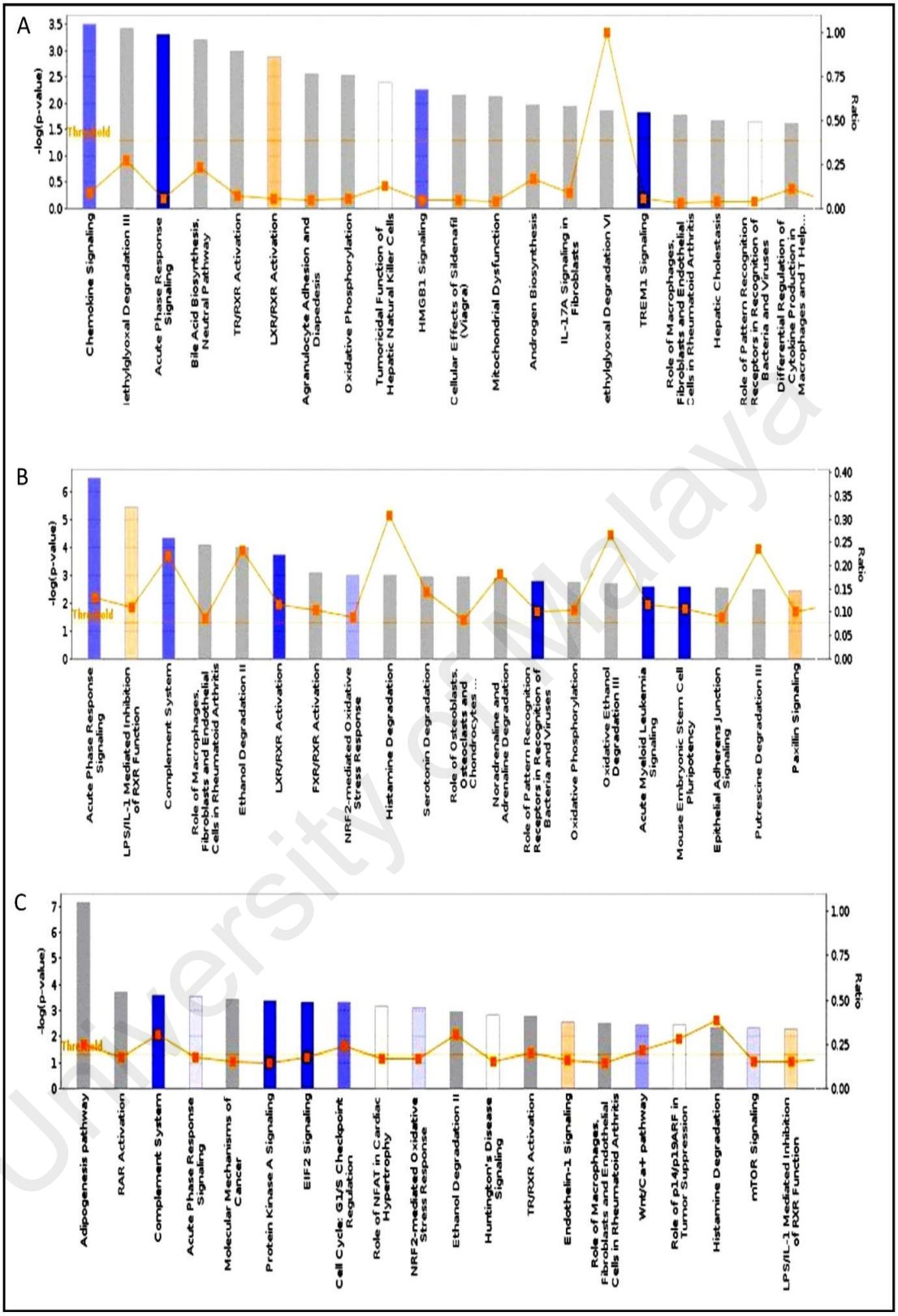

Figure 4.22: Top 20 of canonical pathways significantly regulsted by A) 200 µM of ASA treatment; B) 500 µM of ASA treatment and C) 1, 000 µM of ASA treatment. Orange bars predict an overall increase in the activity of the pathway; blue bars predict decrease in the activity of the pathway; gray bars indicate pathways where predictions are not currently possible.

4.16.1 The Canonical Pathway (CP) enrichment of ASA treatments based on z-score

In addition to p-values, IPA also provides z-scores for the canonical signaling pathway. The z-scores measure predicted activation or inhibition based upon observed fold changes with z-scores ≥2 showing significant activation while those ≤-2 were predicted to be significantly inhibited. The canonical pathways with significant z-scores for 500 and 1,000 μM of ASA treatments are shown in Table 4.23 and Table 4.24 respectively.

For 200 μM ASA treatment, IPA predicted TREM1 signaling pathway to be significantly inhibited, with a z-score of -2. The most relevant CP that were signficantly regulated as estimated by IPA for 500 μM ASA treatment were: Agrin Interactions at Neuromuscular Junction (z-score :+2.236), Production of Nitric Oxide and Reactive Oxygen Species in Macrophages (z-score: -2.714), NF-κB Signaling (z-score: -2.33), Growth Hormone Signaling (z-score: -2.236), Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses (z-score: -2.3333), TREM1 Signaling (z-score: -2.121), Acute Myeloid Leukemia Signaling (z-score: -2.1213), Interferon Signaling (z-score: -2), and CDK5 Signaling (z-score: -2) (Table 4.23).

Table 4.23: The most significant canonical pathway for 500 µM of ASA treatment

Ingenuity Canonical Pathways	-log(p-value)	z-Score	Molecules
Agrin Interactions at Neuromuscular Junction	9.8E-01	2.236	GABPB1,ITGA3,MAPK9,ITGA5
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.14E00	-2.714	FOS,APOE,TLR4,PIK3R1,PPP1R3C,CREBBP,MAPK9,MAP3K8,PPP1R14B,PRKD1,APOD
NF-κB Signaling	7.41E-01	-2.33	TLR4,BMP4,FLT1,MYD88,PIK3R1,CREBBP,MAP3K8,IL1R1,FGFRL1
Growth Hormone Signaling	9.39E-01	-2.236	STAT5A,FOS,PIK3R1,STAT3,PRKD1
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.78E00	-2.3333	I1,TLR4,IRF7,LIF,OAS2,MYD88,PIK3R1SP1,MAPK9,IL6,OAS3,PRKD1
Interferon Signaling	1.99E00	-2.0	IFIT1,IFIT3,MX1,IRF9,IFITM1
TREM1 Signaling	2.35E00	-2.121	STAT5A,TLR4,ICAM1,MYD88,CASP1,ITGA5,IL6,STAT3
Acute Myeloid Leukemia Signaling	2.61E00	-2.1213	STAT5A,TCF4,FLT3LG,PIM1,PIK3R1,LEF1,JUP,STAT3,TCF7L1
CDK5 Signaling	5.18E-01	-2.000	ITGA3,PPP1R3C,ADCY4,MAPK9,PPP1R14 B

The most significantly regulated CP for 1,000 µM of ASA treatment were *Inhibition of Angiogenesis* by TSP1 (z-score: +2.2361) and *Regulation of Cellular Mechanics by Calpain Protease* (z-score: +2.000). Meanwhile, pathway estimated by IPA which were inhibiting such as Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses (z-score: -3.500), EIF2 Signaling (z-score: -2.8402), Growth Hormone Signaling (z-score-2.6460), Interferon Signaling (z-score: -2.4495), protein kinase A signaling (z-score: -2.3591), Renin-Angiotensin Signaling (z-score: -2.3238), Role of IL-17F in Allergic Inflammatory Airway Diseases (z-score: -2.2360), Role of RIG1-like Receptors in Antiviral Innate Immunity (z-score: -2.2361), Activation of IRF by Cytosolic Pattern Recognition Receptors (z-score: -2.1213),

Production of Nitric Oxide and Reactive Oxygen Species in Macrophages (z-score: -2.13201), Thrombopoietin Signaling(z-score: -2.1213), TREM1 Signaling (z-score: -2.1106), Colorectal Cancer Metastasis Signaling (z-score : -2.0412) and NF-κB Signaling (z-score: -2.0) (Table 4.24).

Table 4.24: The most significant canonical pathway for 1,000 µM of ASA treatment

Ingenuity Canonical Pathways	-log(p-value)	Z-Score	Ratio	Molecules
Inhibition of Angiogenesis by TSP1	1.17E00	2.2361	1.88E-01	HSPG2,CASP3,THBS1,MAPK9,AKT3,MAPK11
Regulation of Cellular Mechanics by Calpain Protease	4.05E-01	2.0	1.09E-01	ITGA3,TLN2,CDK6,ITGA5,CDKN1B,CAST
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.24E00	-3.500	1.68E-01	LIF,C3,OAS2,MYD88,PIK3R1,MAPK9,IL6,OAS3,RNASEL,C5,IFIH1,TLR4,IRF7,DDX58,CASP1,MAVS,PRKCE,TLR3,PRKD3,PRKD1
EIF2 Signaling	3.34E00	-2.8402	1.74E-01	RPL22,RPS3A,PIK3R1,RPS23,PPP1CB,RPL7,SHC1,PPP1CC,RPL13,RPL18A,RPS20,EIF3D,RPL23A,RPS9,AKT3,RPS3,RPL18,RPL3,RPS10,RPL29,EIF3E,RPS29,RPL15,RPS26,EIF4A3,EIF3I,RPL5,RPL37,RPL13A,R
Growth Hormone Signaling	5.25E-01	-2.6460	1.16E-01	STAT5A,FOS,IGF2,PIK3R1,PRKCE,STAT3,PRKD3,PRKD1
Interferon Signaling	1.37E00	-2.4495	1.94E-01	IFIT1,IFIT3,MX1,IRF9,IFNGR1,PSMB8,IFITM1
Protein Kinase A Signaling	3.36E00	-2.3591	1.46E-01	PLCB2,ADCY4,SMAD3,GNB2L1,PPP1R14B,PTPRO,PLCB1,PRKD3,APEX1,PRKD1,ITPR2,CREBBP,PTCH1,PLCL2,CREB3L4,TCF3,PTP4A1,PDE1C,AKAP13,H3F3A/H3F3B,PTPRS,LEF1,PTPN21,AKAP12,HIST1H1C,TCF4,PTK2B,PPP1R3C,DUSP6,PDE4A,PPP1CB,UBASH3B,MYLK,PDE1A,H2BFM,NFATC1,PLCD1,CDC25B,PPP1CC,PTPN4,GNG11,GLI3,PRKCE,SMAD4,PTPRK,ADCY3,TCF7L1,NFATC4,GNAI3,PLCB4,ADD3,FLNC,CDC14B,AKAP10
Renin-Angiotensin Signaling	1.43E00	-2.3238	1.48E-01	PTK2B,ITPR2,ADCY4,PIK3R1,ADCY3,SHC3,MAPK9,STAT3,MAPK11,FOS,SHC1,CCL2,PRKCE,PRKD3,PRKD1,AGT

Table 4.24 (Continued)

Role of IL-17F in Allergic Inflammatory Airway Diseases	4.91E-01	-2.2360	1.22E-01	CCL2,CREBBP,IL6,CXCL5,CREB3L4
Role of RIG1-like Receptors in Antiviral Innate Immunity	7.69E-01	-2.2361	1.46E-01	IFIH1,IRF7,CREBBP,DDX58,MAVS,CASP8
Activation of IRF by Cytosolic Pattern Recognition Receptors	7.4E-01	-2.1213	1.33E-01	IFIH1,IRF7,CREBBP,DDX58,MAPK9,MAVS,IRF9,IL6
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.01E00	-2.13201	1.23E-01	APOE,APOM,PPP1R3C,PIK3R1,PPP2R5B,CREBBP,PPP1CB,MAPK9,IFNGR1,PPP1R14B,MAPK11,PPP2R5A,APOL1,FOS,TLR4,PPP1CC,PRKCE,AKT3,MAP3K8,PRKD3,PRKD1,APOD
Thrombopoietin Signaling	8.92E-01	-2.1213	1.45E-01	STAT5A,FOS,SHC1,PIK3R1,PRKCE,STAT3,PRKD3,PRKD1
TREM1 Signaling	1.27E00	-2.1106	1.57E-01	STAT5A,TLR4,ICAM1,CCL2,MYD88,CASP1,AKT3,ITGA5,TLR3,IL6,STAT3
Colorectal Cancer Metastasis Signaling	9.4E-01	-2.0412	1.17E-01	SIAH1,TCF4,ADCY4,MMP14,PIK3R1,SMAD3,GNB2L1,IL6,MMP24,APPL1,GNG11,AKT3,SMAD4,TLR3,CASP3,ADRBK2,ADCY3,MAPK9,IFNGR1,STAT3,TCF7L1,TCF3,TLR4,FOS,FZD4,LEF1,FZD5
NF-κB Signaling	3.12E-01	-2.0	9.47E-02	BMP4,FLT1,MYD88,PIK3R1,CREBBP,HDAC1,IL1R1,TLR4,UBE2V1,PDGFRA,AKT3,MAP3K8,TLR3,FGFRL1,CASP8,PDGFRB

4.16.2 ASA treatment and Enrichment of Canonical Pathway

Figure 4.23 depicts the most enriched canonical pathways at 200, 500, and 1,000 μM upon ASA treatments, arranged according to their z-scores. They can be seen to have similar patterns/consistent activation or inhibition at all concentrations of ASA treatments. The pathways which were predicted to be significantly activated at 500 μM of ASA treatments included only Agrin interactions at neuromuscular junctions (z-score: 2.236068) (Table 4.25/ Figure 4.23).

Meanwhile at 1,000 μM of ASA treatments the inhibition of angiogenesis by TSP1 (z-score: +2.236068) (Table 4.25/Figure 4.23) and regulation of cellular mechanics by calpain protease (z-score: +2.236068) (Table 4.25/Figure 4.23) were estimated by IPA. The other canonical pathways were predicted to be inhibited at 500 and 1,000 of ASA treatments (Table 4.25/ Figure 4.23). Only the TREM-1 signaling pathway was predicted to be significantly inhibited at all concentrations of ASA treatments (200, 500, and 1,000 μM).

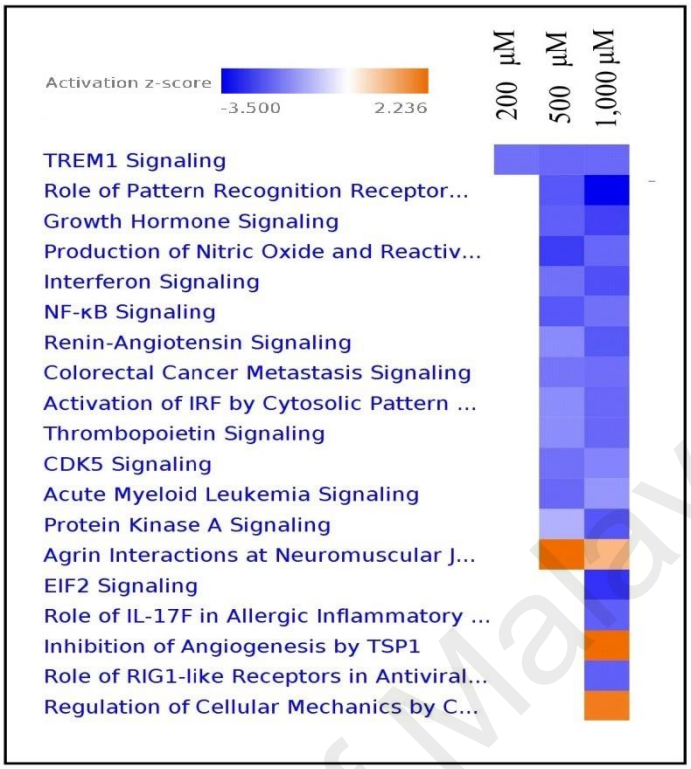

Figure 4.23: Heat map representing the relevant canonical pathways of ASA treatments at 200, 500 and 1,000 µM. Most activated or inhibited canonical pathways as estimated by the z-score (± ≥ 2) for each comparison from ingenuity Pathway analysis (IPA). Blue denotes predicted activation and orange predicted inhibition.

Table 4.25: Summary of the heatmap relevant canonical pathways of ASA treatments at 200, 500 and 1,000 µM of ASA treatment. Most activated or inhibited canonical pathways as estimated by the z-score for each comparison from IPA.

Canonical Pathway	200 µM	500 µM	1000 µM
TREM1 Signaling	-2.0	-2.1213	-2.1106
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	N/A	-2.3333	-3.5000
Growth Hormone Signaling	N/A	-2.2361	-2.6458
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	N/A	-2.7136	-2.13200
Interferon Signaling	N/A	-2.000	-2.44949
NF-κB Signaling	N/A	-2.333333	-2.0000
Renin-Angiotensin Signaling	N/A	-1.6667	-2.3238
Colorectal Cancer Metastasis Signaling	N/A	-1.9415	-2.0412
Activation of IRF by Cytosolic Pattern Recognition Receptors	N/A	-1.6330	-2.12132
Thrombopoietin Signaling	N/A	-1.6330	-2.1213
CDK5 Signaling	0.0	-2.000	-1.73205
Acute Myeloid Leukemia Signaling	N/A	-2.1213	-1.5076
Protein Kinase A Signaling	N/A	-1.14708	-2.3591
Agrin Interactions at Neuromuscular Junction	0.0	2.23607	1.1339
EIF2 Signaling	0.0	N/A	-2.8402
Role of IL-17F in Allergic Inflammatory Airway Diseases	N/A	N/A	-2.2361
Inhibition of Angiogenesis by TSP1	N/A	N/A	2.23607
Role of RIG1-like Receptors in Antiviral Innate Immunity	0.0	N/A	-2.2360
Regulation of Cellular Mechanics by Calpain Protease	0.0	N/A	2.0000

4.17 Disease and function network by IPA

The Disease and Function Network analyses was through IPA. It was noted that the *formation of adhesion of the extracellular matrix* (p-value: 7.94E-05 for 500 μM and p-value: 3.14E-03 for 1,000 μM) and *connective tissue cells* (p-value: 1.31E-05 for 500 μM and p-value: 4.92E-03 for 1,000 μM) were significant at 500 μM (Figure 4.24A) and 1,000 μM of ASA treatments (Figure 4.24B) but not at 200 μM of ASA treatment. It is interesting that most of the genes above were earlier predicted to be encriched, as indicated by DAVID and PANTHER, where groups in the *cell adhesion* and *extracellular matrix* were also enriched, as discovered by IPA for 500 and 1,000 μM of ASA treatments.

It is noted that most of the genes involved in the cell adhesion of the ECM and connective tissue cells at 500 and 1,000 μM of ASA treatments were almost similar. In fact, additional genes were found at 1,000 μM such as THY1, TLN2 for adhesion of connective tissues, and ITGB1BP1, CDKN2A and LYVE1 for ECM. It was observed that the integrin group was play important role in the cell adhesion of both connective tissues and ECM. ECM proteins play a role in regulating cell adhesion and linage commitment of MSCs. The binding of integrin FN can facilitate the osteogenic differentiation potential of MSCs due to the modulation of the expression of specific integrin subunits during the process.

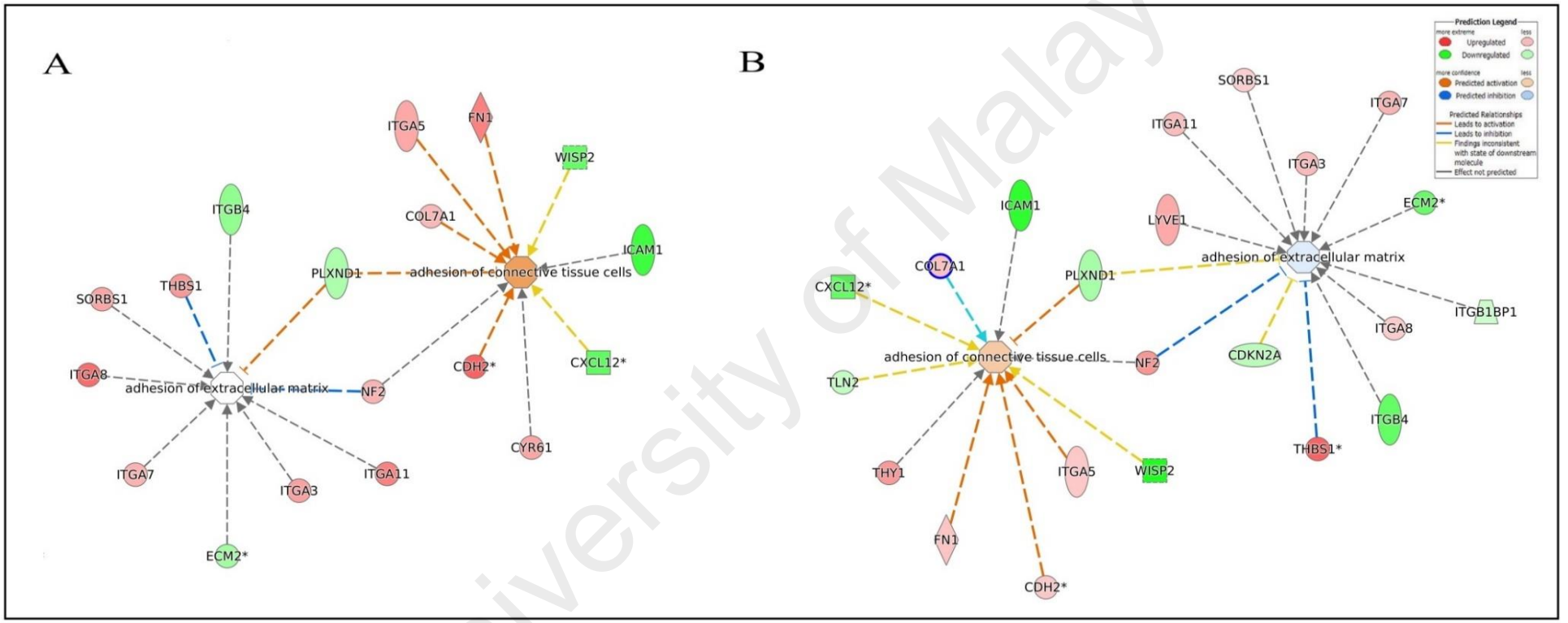

Figure 4.24: Network of *formation of adhesion of extracellular matrix* and *connective tissue cells* for A) 500 µM and B) 1,000 µM of ASA treatments. The pink or red color nodes in networks indicate a gene that is up regulated in ASA treatments compared to the control, green color indicates the genes that are down regulated in ASA treatments compared to control. The intensity of the node color indicates the expression level or degree of regulation.

To predict the functional significance of the effect ASA treatments on osteogenic differentiation of PDLSCs, the main molecular networks associated with genes that were up- and down-regulated in each ASA treatment were evaluated, using known transcriptional relationships derived from IPA Ingenuity Knowledge Base. The analyses output indicated statistically significant increase in the expression of many genes that are associated with *cellular growth and proliferation*, *cellular development*, and *cell-to-cell signaling* at 1,000 μM of ASA treatment (Table 4.26).

Table 4.26: Top network signaling enriched at 1,000 μM ASA treatment

Score	Focus Molecules	Top Diseases and Functions
110	128	Gene Expression, Cell Cycle, Cellular Growth and Proliferation
65	100	Molecular Transport, RNA Trafficking, DNA Replication, Recombination, and Repair
59	96	Cell Death and Survival, Hematological System Development and Function, Tissue Morphology
39	79	Cellular Movement, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
35	75	Tissue Morphology, Cell Death and Survival, Cardiovascular System Development and Function
31	71	Cellular Movement, Immune Cell Trafficking, Cell-To-Cell Signaling and Interaction
31	71	Cancer, Organismal Injury and Abnormalities, Cellular Development

The network generated from cluster 1, dominated by 1,000 μM of ASA treatments, was characterized by gene expression, cell cycle, cellular growth, and proliferation (score 110 with 128 molecules) (Figure 4.25). There are three focused molecules namely histone deacetylase 1 (HDAC1), E2F1, and histone H4 (Figure 4.25) in the central nodes that play vital roles in osteoblast formation.

Histone deacetylase 1 (HDAC1) is a key enzyme to regulates osteoblast formation (Lee *et al.*, 2006). HDAC falls into two major groups, class I and class II. HDAC1 is in major class I and expressed ubiquitously. Other studies have demonstrated that HDAC1 plays a vital role in osteogenesis differentiation (Lee *et al.*, 2006). The histone modification plays a crucial role in the transcriptional regulation of most eukaryotic genes and this modification can be altered by HDAC1. The recruitment of HDAC1 on osteogenesis can result in the downregulation of several markers of osteoblast genes (i.e., osterix and osteocalcin) and this suggests that the suppression of HDAC1 is a critical factor in the osteogenic differentiation step.

The role of the transcription factor E2F1 in osteogenesis and mineralization has been previously described (Shibing Yu, 2013). Frizzled homolog 1 (FZD1) is a member of the G-protein coupled receptor which is a transmembrane receptor that mediates the WNT signaling pathway. The WNT signaling pathway is an important potential therapeutic target in osteoblast differentiation. E2F1 is a transcription-factor binding element in the FZD1 promoter and could enhance osteoblast differentiation and mineralization. In the study, E2F1 was identified *in silico* and characterized in Saos2 cells *in vitro.*

The study demonstrated that the over-expression of E2F1 could enhance the mineralization of differentiated Saos2 cells and contributes to the regulation of osteoblast mineralization. Histone H4 encodes osteogenic growth peptides (OGP), a highly conserved 14 amino acid identical to the C-terminus of histone H4 which is involved in the regulation of bone formation and hematopoiesis. OGP stimulates bone formation and increases trabecular bone mass *in vivo* (Fazzi *et al.*, 2003) and contributes to the proliferation of ALP activity *in vitro* (Bab *et al.*, 1992). The findings suggest that

high concentrations of ASA treatments modulate a number of cellular genes, cellular development, cellular growth, cell death, and cell cycles, and the molecules associated with bone formation.

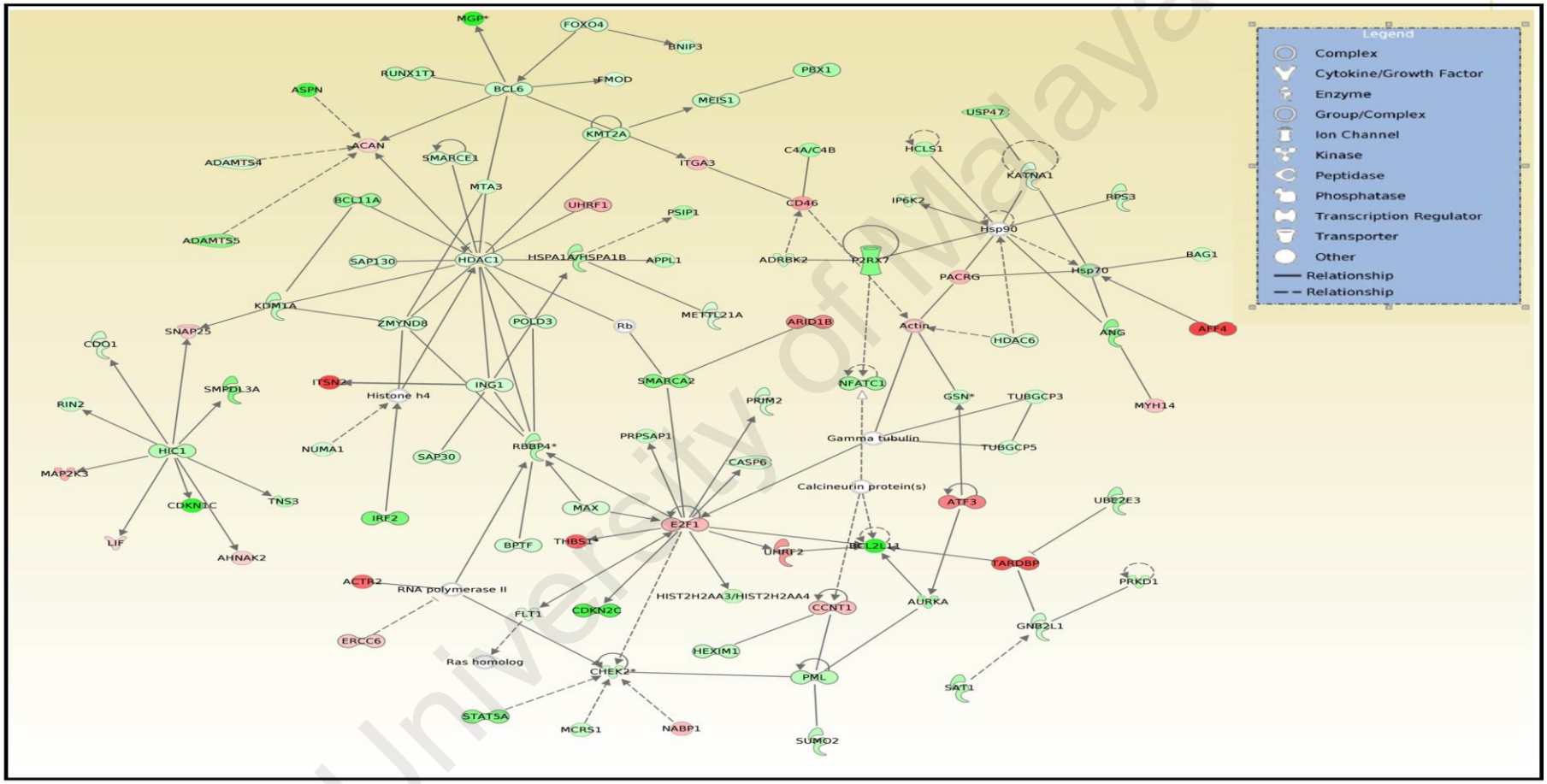

Figure 4.25: Network analysis of *gene expression* changes at 1,000 µM of ASA treatment. Direct relationships are indicated by solid lines, and dashed lines represent indirect relationships. The pink or red color nodes in networks indicate a gene that is up regulated in ASA treatments, green color indicates the genes that are down regulated in ASA treatment. The intensity of the node color indicates the expression level or degree of regulation. Genes in uncolored notes were not differentially expressed.

Meanwhile, it was noted there was a statistically significant increase in the expression of a high numbers of genes associated with *cellular growth and proliferation* and *cellular movement* at 500 μM of ASA treatments (Table 4.27) and *cell death* and *survival, inflammatory response,* and *cell cycle* at 200 μM of ASA treatments (Table 4.28).

Table 4.27: Top network signaling at 500 μM of ASA treatment

Score	Focus Molecules	Top Diseases and Functions
93	94	Cell Death and Survival, Tissue Development, Inflammatory Response
62	74	Cellular Growth and Proliferation, Organismal Survival, Cell Cycle
56	70	Cell Death and Survival, Cellular Movement, Embryonic Development
55	69	Cell Death and Survival, Tissue Morphology, Inflammatory Response
47	63	Cell Cycle, Cellular Growth and Proliferation, Cancer
39	57	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry

Table 4.28: Top network signaling at 200 μM of ASA treatment

Score	Focus Moleculs	Top Diseases and Functions
73	60	Cell Death and Survival, Organismal Survival, Inflammatory Response
48	46	Cell Death and Survival, Cellular Movement, Cellular Growth and Proliferation
17	24	Cell Death and Survival, Cell Cycle, Cancer

The main network identified at 500 μM and 200 μM of ASA treatments was similarly characterized and associated with *cell death* and *survival, tissue development,* and *inflammatory response* (score 93) with 94 focused molecules (Table 4.27) and *cell death and survival, organismal survival,* and *inflammatory response* (score 73) with 60 focused molecules (Table 4.28), respectively.

Most interestingly, central invited nodes in this network at 500 μM of ASA treatments included ERK 1/2 which is a key protein involved in cell proliferation and differentiation of osteoblast formation (Lai *et al.*, 2001) and AKT which is required to promote osteoblast differentiation and bone formation (Marie *et al.*, 2014). NFKβ (complex) is a major focused molecule found in central nodes molecules at 200 and 500 μM of ASA treatments.

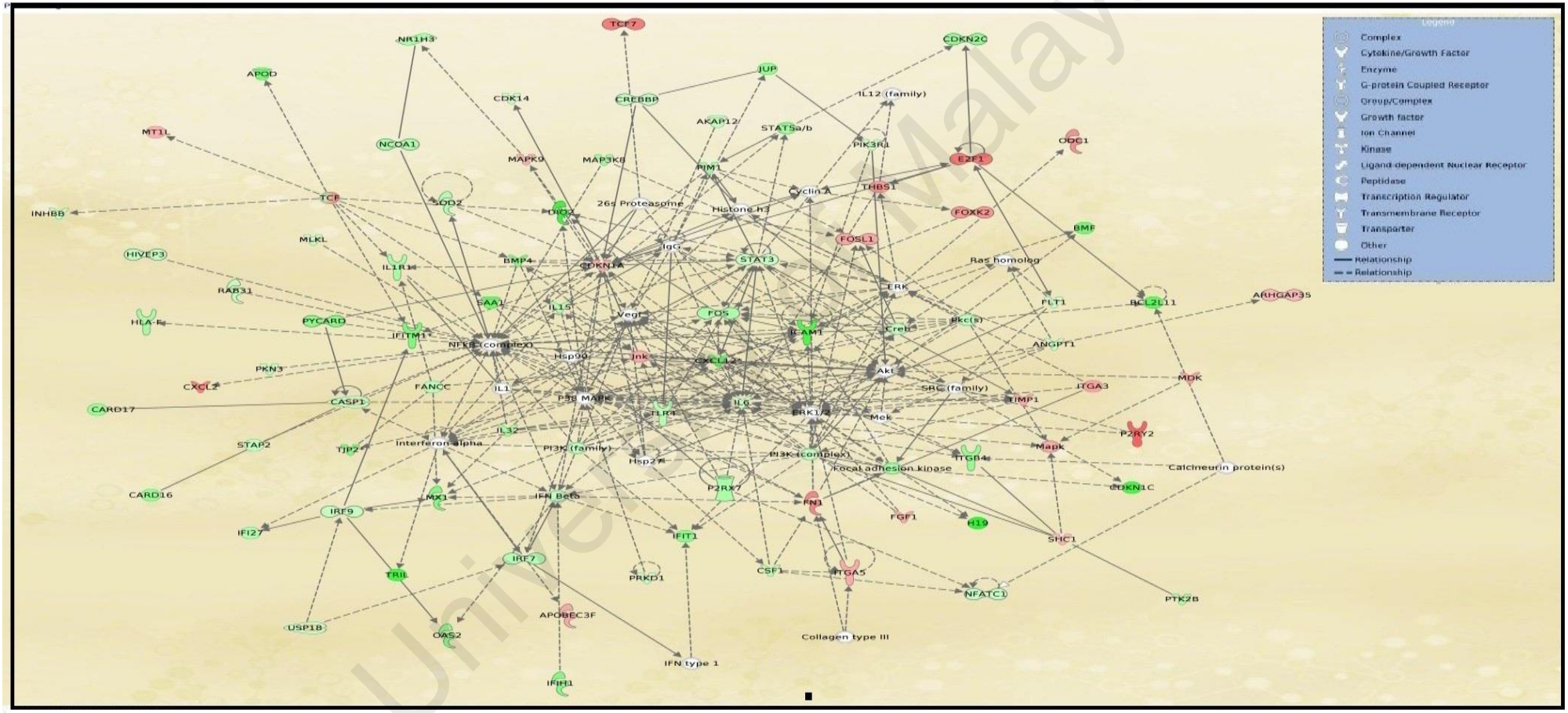

Figure 4.26: Network analysis of *cell and death and survival* at 500 µM of ASA treatment. Direct relationships are indicated by solid lines, and dashed lines represent indirect relationships. Direct relationships are indicated by solid lines, and dashed lines represent indirect relationships. The pink or red color nodes in networks indicate a gene that is up regulated in ASA treatment, green color indicates the genes that are down regulated in ASA treatment. The intensity of the node color indicates the expression level or degree of regulation. Genes in uncolored notes were not differentially expressed.

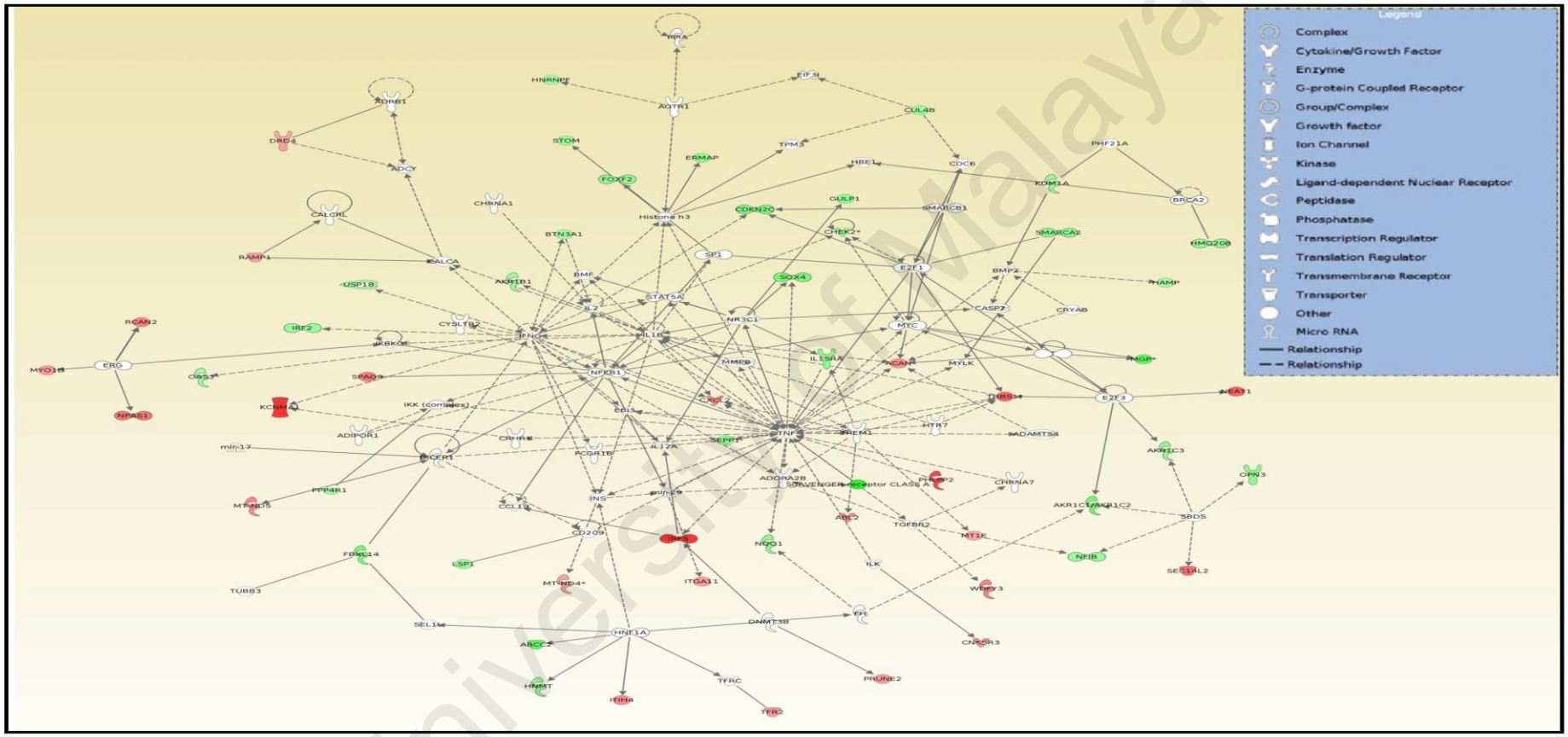

Figure 4.27: Network analysis of *cell and death and survival* at 200 µM of ASA treatment. Direct relationships are indicated by solid lines, and dashed lines represent indirect relationships. Direct relationships are indicated by solid lines, and dashed lines represent indirect relationships. The pink or red color nodes in networks indicate a gene that is up regulated in ASA treatment compared to the control, green color indicates the genes that are down regulated in ASA treatment. The intensity of the node color indicates the expression level or degree of regulation. Genes in uncolored notes were not differentially expressed.

4.18 Validation of microarray data by qRT-PCR, immunofluorescence and western blotting analyses

4.18.1 qPCR analysis

Quantitative real-time RT-PCR was done on seven differentially DE genes to validate the microarray data. The genes include (Fibronectin 1) FN1, (integrin α5) Igα5, fibroblast growth factor-1 (FGF1), fibroblast growth factor-5 (FGF5), fibroblast growth factor receptor-like 1 (FGFRL1), bone morphogenic-4 (BMP4) and BMP binding endothelial regulator (BMPER). The result showed the genes significantly different expression rates between ASA-treated cells and untreated cells (Figure 4.28). The qRT-PCR results were generally in agreement with the data obtained from microarray analyse.

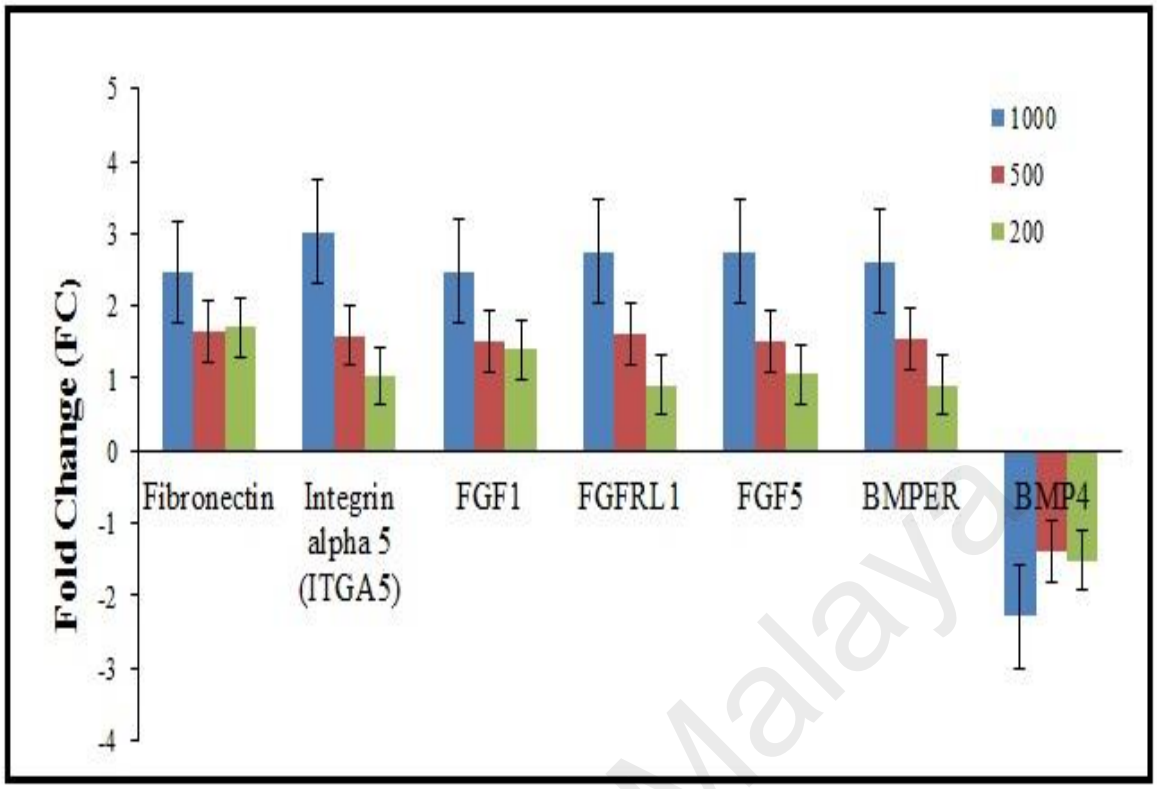

Figure 4.28: Real Time RT-PCR analyses of FN1, Igα5, FGF1, FGF5, FGFRL1, BMP4 and BMPER on effect of ASA at 1,000 µM in osteogenic differentiation of PDLSCs (n=3).

4.18.2 Western blotting (WB) analyses

Figure 4.29 shows representative WB assay results for FN1, Itgα5, FGF-1, and FGFRL-1 genes for cells treated with 1,000 μM ASA. The protein were expressed and the results were generally in agreement with the data obtained from microarray analyse.

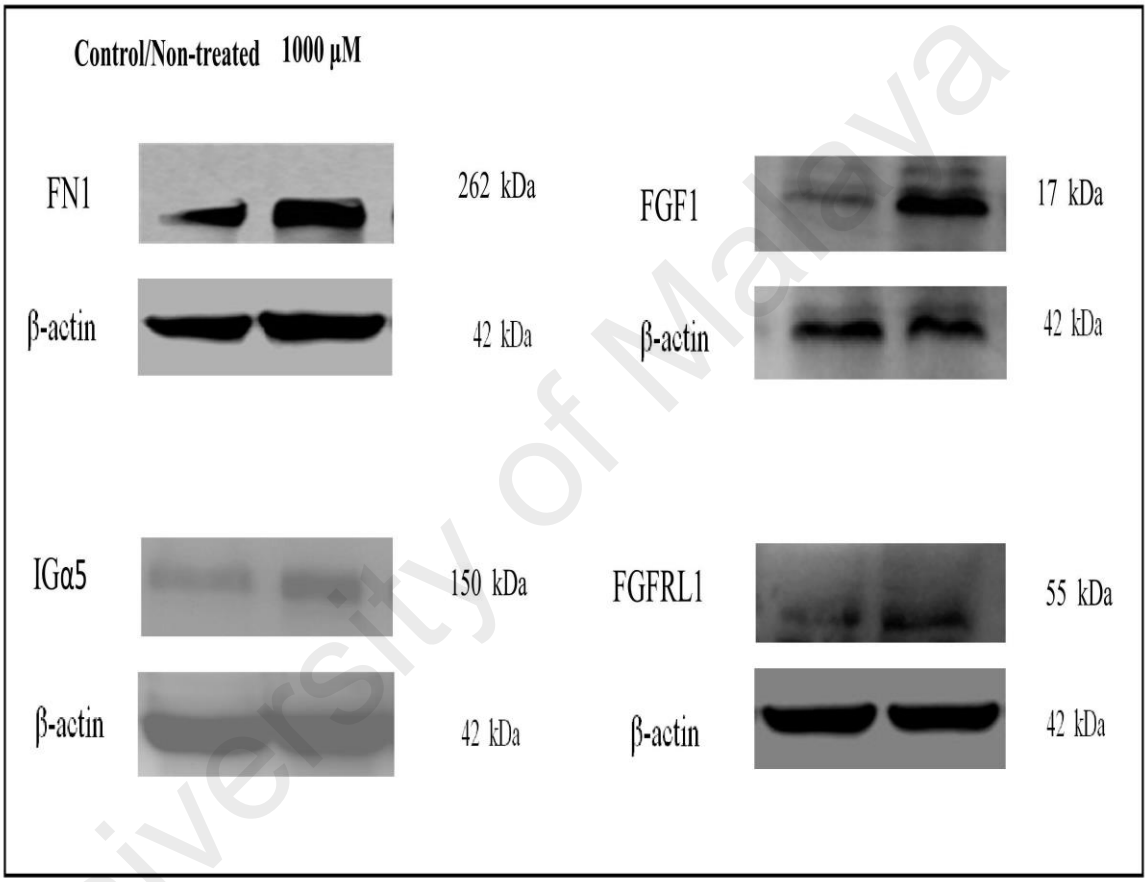

Figure 4.29: Western blot analyses. FN1, FGF-1, FGFRL-1 and Itgα5 proteins for control (non-treated) and 1,000 μM of ASA treatments in osteogenic differentiation of PDLSCs (n=3).

4.18.3 Double immunofluorescene

The result from WB analysis was complemented through double immunofluorescene assay. FN1, Itgα5, FGF-1, and FGFRL-1 protein expressions were analysed in control (0) and treated at 200, 500 and 1,000 µM of ASA treatment and untreated PDLSCs in osteogenic differentiation. The results were generally in agreement with the data obtained from microarray analyse.

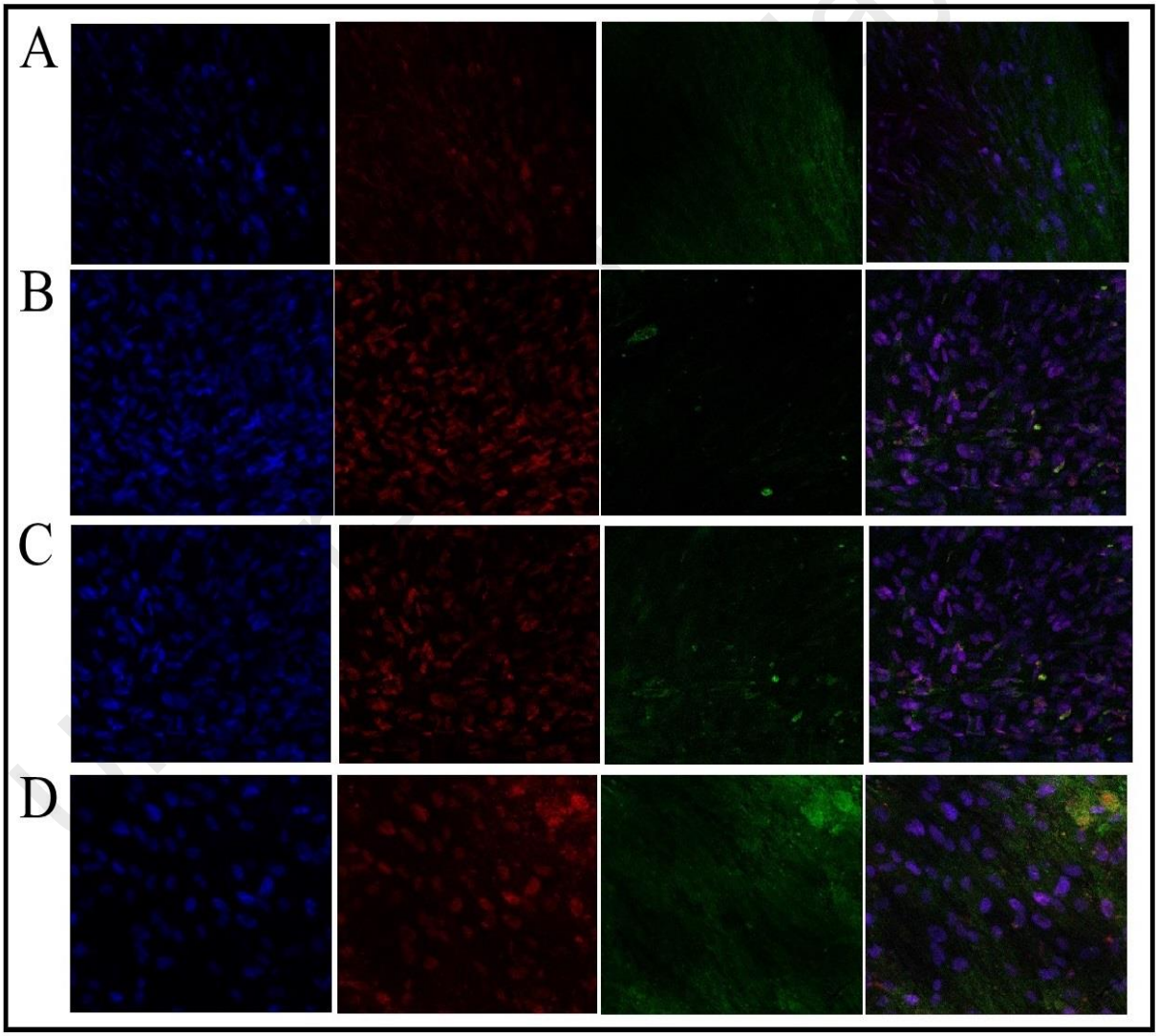

Figure 4.30: Double immunofluorescene analyses for Fibronectin and Itgα5: effect of ASA on PDLSCs at the following concentrations in osteogenic differentiation: (A) Control (Untreated); (B) 200 µM (C) 500 µM and (D) 1,000 µM. Blue: DAPI, Green: itgα5 Red: FN.

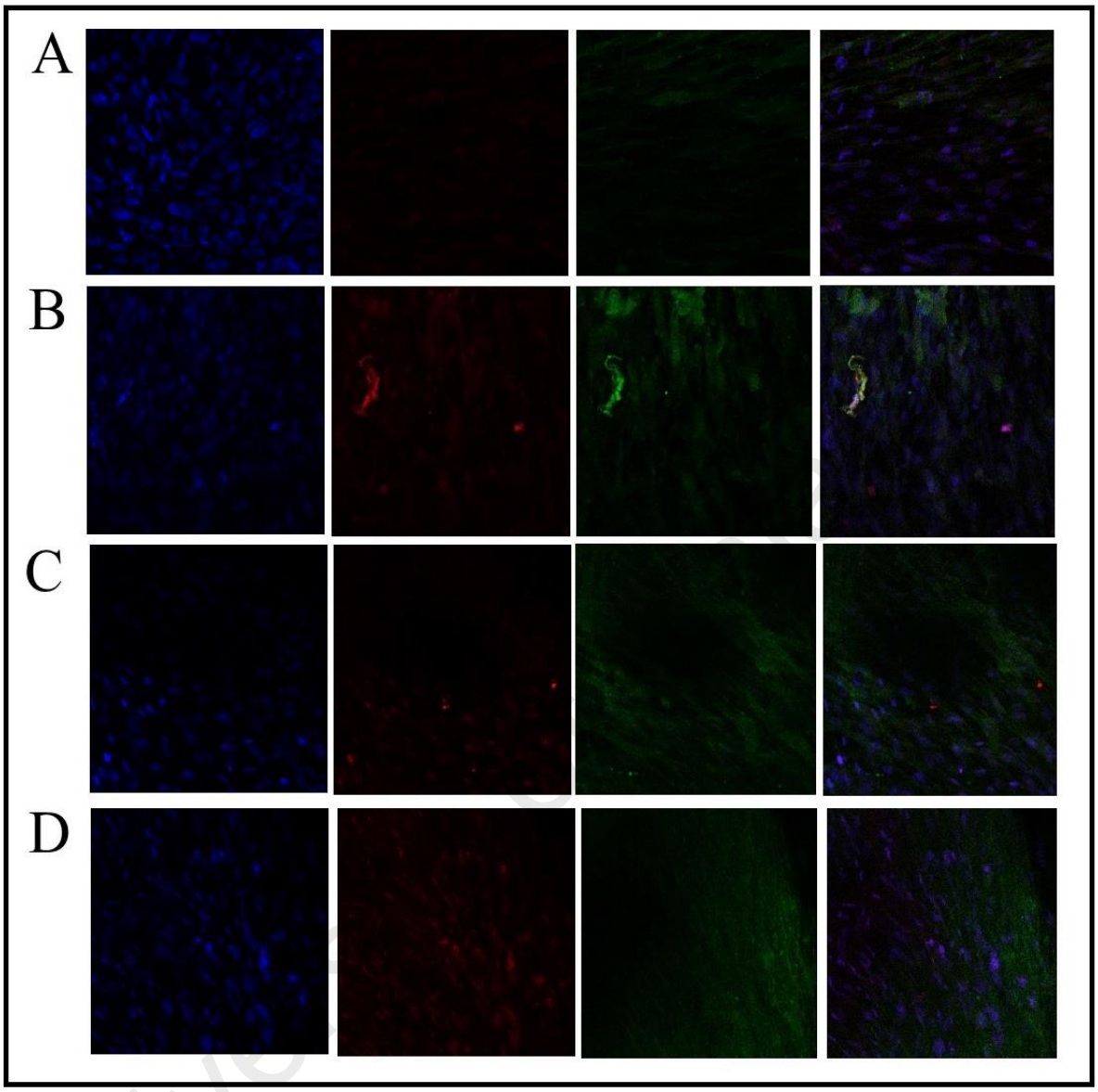

Figure 4.31: Double immunofluorescene analyses for FGF1 and FGFRL1: effect of ASA on PDLSCs at the following concentrations in osteogenic differentiation: (A) Control (Untreated); (B) 200 µM (C) 500 µM and (D) 1,000 µM. Blue: DAPI, Green: FGF1 Red: FGFRL1.

CHAPTER 5: DISCUSSION

The PDLSCs isolated in the current study showed characteristics of MSCs as previously described (Seo *et al.*, 2004; Ullah *et al.*, 2015). The PDLSCs were positive for CD73, CD90 and CD105 surface markers and negative for CD45, CD34, CD20 and CD14 hematopoietic markers. The range of ASA concentrations used in this study was within the in vivo therapeutic range of between 100 to 2000 µM as previously reported (Awtry & Loscalzo, 2000; Borthwick *et al.*, 2006).

The MTT assay indicated that ASA generally inhibited PDLSCs proliferation in a time- and dose-dependent manner (Figures 4.3). At 1,000 µM, the proliferation rates for 24, 48 and 72 hour treatments were 87.15%, 85.04% and 71.8%, respectively. This suggests that ASA modestly inhibited PDLSCs proliferation at 500 and 1000 µM. This observation is comparable to that of rat bone marrow mesenchymal stem cells, whereby 1,000 µM ASA treatments decreased cell proliferation rate to only 82% (Wang *et al.*, 2006). ASA at 1,000 µM and lower concentrations were also reported to modestly inhibit the proliferation of a variety of other cell types (De Luna-Bertos *et al.*, 2012; Shams ara *et al.*, 2015; Liu *et al.*, 2015b).

Contrary to the above observation, the proliferation rate of the progenitor cells were higher in osteogenic media in the presence of 500 and 1000 µM ASA (Figure 4.5). At day 21, the 500 and 1000 µM ASA treated cells showed absorbance readings that were 36.0% and 37.6% higher than the untreated cells, respectively. The osteogenic capacity of the progenitor cells following 500 and 1000 mM ASA treatments for 21 days were also significantly higher ($p<0.01$), with their percentages of Alizarin Red S

staining area (ARSA) of 76.59% and 87.85%, respectively, compared to 73.35% for the untreated cells (Figures 4.6 and 4.7). This finding suggest that ASA is capable of enhancing PDLSCs osteogenic potential, and similar observation has been reported by Liu and colleagues, whereby aspirin site-specific treatment enhances BMSSC (Liu et al.,2015).

However, ASA at lower concentrations of 10 and 200 μM, consistently reduced osteogenic differentiation at days 7, 14 and 21. For example, following day 21, the percentages of ARSA for 0 and 200 μM treatments were 49.10% and 53.15%, respectively, compared to 73.35% for the untreated cells. It is unclear why lower concentrations of ASA inhibited the osteogenic capacity of the cells, without significantly inhibiting their proliferation rates (Figure 4.5), and this warrants further investigations to uncover the reason.

The gene expression profiling study using PCR array indicated that ASA was able to modulate the expression of growth factor genes in PDLSCs. Aspirin at 1000 μM was selected as the treatment dose because such concentration is within the *in vivo* ASA therapeutic range. Additionally, the 24 hour treatment using 1000 μM ASA and basic growth media showed modest inhibitory effect on PDLSCs proliferation rate, compared to higher ASA concentrations (Awtry & Loscalzo, 2000; Borthwick *et al.,* 2006).

The basic growth media was used for the gene expression profiling assay because the osteogenic media is known to significantly enhance osteogenic potential of stem cells, owing to the actions of dexamethasone, ascorbic acid and β-glycerophosphate that are present in the said media (Langenbach *et al.,* 2013). Dexamethasone induces the expression of LIM-domain protein with 4.5 LIM domains

(FHL2), which in turn upregulates Runt-related transcription factor 2 (runx2) expression, which is known as the master osteogenic transcription factor (Langenbach *et al.,* 2013). Runx2 upregulation eventually causes various events that lead to the expression of osteogenic proteins (Langenbach *et al.*, 2013).

The basic growth media provides an environment without the presence of differentiation inducing agents that could confound the effect of ASA on the gene expression profile in PDLSCs. However, since the finding in this study indicated that ASA was capable of enhancing the proliferation and osteogenic potential of the cells in osteogenic media (Figures 4.5, 4.6 and 4.7), future investigation could be done explore the underlying mechanism of such observation.

The result from the gene expression profiling study indicated that 19 genes were differentially expressed, which include 12 upregulated and 7 downregulated genes (Table 4.1). Pathway analysis indicated that 1000 µM ASA treatment resulted to activation of biological functions and canonical pathways in PDLSCs that are related to cellular signaling, angiogenesis, stem cells pluripotency and cellular growth, proliferation and differentiation (Figure 4.4). The function of some of the genes that were markedly up and downregulated are discussed in the following paragraphs.

FGFs and BMPs are among the signalling molecules that are actively expressed during human primary teeth development (Huang *et al.,* 2015). In the mouse, FGF signalling regulates differentiation and proliferation in tooth development, specifying odontogenic fate in both the dental epithelium and dental mesenchyme (Liu *et al.,* 2013). In this study, FGF2, FGF7 and FGF9 were upregulated and these are members of the canonical FGF subfamily (c-FGF). The c-FGFs are secreted as extracellular

proteins and mediate their biological functions by activating FGF receptors (Itoh & Ornitz, 2011).

FGF2, or basic fibroblast growth factor (bFGF) is a member of fibroblast growth factor protein family, known to be involved in many biological processes such as wound healing, cell proliferation, tissue vascularization, regeneration of various tissues in the body including dentine and periodontal tissues (Lin *et al.*, 2015). FGF2 have been suggested to be the most upstream self-renewal factor in human embryonic stem cells (ESCs) (Greber *et al.*, 2007). FGF2, together with vascular endothelial growth factor (VEGF) have been reported to show synergistic role in angiogenesis (Przybylski, 2009).

VEGF family members are renowned as potent angiogenesis inducers in vivo and *in vitro* (Giacca & Zacchigna, 2012). It has been reported that FGF2 was able to induce VEGFA expression, and the combination of both factors synergistically stimulated angiogenesis in mouse PDL cells (Yanagita *et al.*, 2014). The co-upregulation of FGF2 and VEGF genes by ASA as observed in this study may support the induction of angiogenesis in PDLSCs which could be useful for regenerative dentistry.

The expression patterns of FGF7 and FGF9 have recently been reported in the developing human primary tooth germs (Huang *et al*., 2015). FGF7 plays crucial roles in epithelial repair process associated with wound healing (auf demKeller *et al.*, 2004). During the bell stage of the developing human embryo tooth germ (Huang *et al*., 2015). FGF7 expression was noted to be among the highest in the dental mesenchyme compared to six other FGFs. FGF9 was also observed to be strongly expressed in both

the inner enamel epithelium and dental mesenchyme, during the cap and bell stages of the human primary tooth germs (Huang *et al*., 2015).

The bone morphogenetic proteins (BMPs) are cytokines, comprising over 20 members that are structurally related and belonging to TGFβ superfamily. They are classically known as bone growth factors which are responsible for embryonic skeletal formation, skeletal regeneration and bone healing (Carreira *et al.*, 2014). BMP2 is a potent inducer of mesenchymal cell differentiation to pre-osteoblasts during osteogenesis and have been shown to stimulate regeneration of periodontal tissue (Lin *et al.*, 2015).

The interplay between FGF and BMP signaling is important in bone formation (Chen *et al.*, 2012), repair and mineralization (Hughes-Fulford & Li, 2011). Treatment of primary calvarial bone cells with exogenous FGF-2/-9 increases BMP2 expression and this is suggested to provide a favorable microenvironment that enhances the osteogenic potential of the cells for bone formation (Fakhry *et al.*, 2005). Contrary to the finding in this study, NSAIDs other than ASA had previously been shown to negatively affect BMP2 expression. NSAID NS-398 was shown to downregulate BMP2 expression in MSCs (Arikawa *et al.*, 2004).

BMP10 was reported to be expressed in the cardiac tissue and essential for murine cardiac morphogenesis (Chen *et al.*, 2004; Carreira *et al.*, 2014). Despite its restricted expression in cardiac tissue, treatment of C2C12 cells (mouse myoblast) with BMP10 was shown to induce the expression of signaling molecules, transcription factors and osteogenic-specific markers, suggesting the supportive role of BMP10 in osteogenesis (Bessa *et al.*, 2009).

In this study, IL2, IL4 and IL10 cytokines were observed to be upregulated in ASA treated PDLSCs. IL-10 is a regulator of inflammation which can downregulate pro-inflammatory cytokines and chemokines such as IL1, IL6 and TNFα (Sabat *et al.*, 2010). IL1, IL6 and TNFα are also known to promote osteoclastogenesis and bone resorption activity (Zhang *et al*., 2014). In the murine model, IL10 deficiency increases the susceptibility to alveolar bone loss (Al-Rasheed *et al.*, 2003). IL10 also inhibits osteoclasts formation and promoted osteoblast differentiation (Zhang *et al*., 2014). The inherent anti-inflammatory activity of ASA and its upregulation of IL10 expression in PDLSCs may synergistically reduce inflammatory condition in periodontal disease, which could be useful in periodontal regenerative medicine.

IL2 is a pro-inflammatory cytokine that is essential for stimulating T-cell functions, proliferation and differentiation and amplifying immune response (Malek, 2008). When co-cultured with peripheral blood mononuclear cells (PBMCs), the stem cells from inflamed deciduous teeth secreted higher levels of IL2, compared to the stem cells from normal deciduous teeth (Yazid *et al.*, 2014). IL2 level was reported to be higher in inflamed pulpal tissues compared to normal healthy samples (Rauschenberger *et al.*, 1997). In this study upregulation of IL10 was also detected in ASA treated PDLSCs, and this may counteract IL2 pro-inflammatory action. However, it should be noted that synergism between IL10 and IL2 may also be possible, since both cytokines have been reported to synergistically enhance human B-lymphocytes function (Itoh *et al.*, 1994).

IL4 is a polyfunctional cytokine produced specific types of immune cells with a role in a vast number of immune and non-immune functions. Numerous type of cells in the body contain IL4 receptors, suggesting diverse or pleotropic IL4 functions (Paul, 2015). IL4 is involved in canonical type 2 immunity, which includes epithelial and tissue remodeling activities (Redpath *et al.*, 2015) an activity that may be helpful in regenerative medicine.

CSF3 is also known as granulocyte stimulating factor, renowned for its capability in stimulating haematopoietic stem cells proliferation. Recent studies indicated CSF3 could enhance neurogenesis, likely due to its capability of stimulating of progenitor cells activity in the central nervous system (Wallner *et al.*, 2015). CSF3, as well as FGF2 showed pro-angiogenic activity, being capable of inducing proliferation and migration of endothelial cells. However, the combination of CSF3 and FGF2 was reported to be more potent in angiogenesis induction (Bussolino *et al.*, 1991). The upregulation of CSF3 and FGF2 in PDLSCs induced by ASA may provide synergistic factors that could promote angiogenesis, an activity that could be useful in periodontal regenerative medicine.

Inhibins are heterodimeric proteins containing α and β subunits. The two types of inhibins are inhibin A(α:βA) and B(α:Bb). Inhibins are members of TGFβ superfamily, known to regulate cellular proliferation, gonadal development, apoptosis, inflammation and differentiation. The widespread expression of inhibin subunit mRNA and protein suggest its widespread role in various systems during development and embryogenesis (Makanji *et al.*, 2014). The continuous expression of inhibin *in vivo* has been reported to cause an increase in bone mass and strength, due to the enhancement of osteoblast differentiation and activity (Makanji *et al.*, 2014).

In this study, ASA treatment was also observed to cause the downregulation of 7 genes with a FC of more than 2.0 (Table 4.1). Among the downregulated genes, the following were markedly downregulated: pleotropin (PTN), fibroblast growth factor 5 (FGF5), brain derived neurotrophic factor (BDNF), and Dickkopf WNT signaling pathway inhibitor 1 (DKK1). PTN is a secreted angiogenic growth factor, exerting pleotropic effect by modulating various processes such bone remodelling, angiogenesis, fracture healing and osteoarthritis (Lamprou *et al.*, 2014a).

During mouse odontogenesis, PTN expression was detected in ameloblasts, odontoblasts, dental follicle and periodontal ligament (Erlandsen *et al.*, 2012). It the mouse, targeted PTN over-expression in the bone inhibited BMP2 mediated bone induction, impairing fracture healing (Lamprou *et al.*, 2014b). On the other hand, PTN ablation in mice is associated with improvement in haematopoetic stem cells function: loss of microenvironmental PTN leads to enhancement of stem cell maintenance *in vivo* and stem cell repopulation ability *in vitro* (Istvanffy *et al.*, 2011).

DKK1 is another gene that was markedly downregulated by ASA. In osteoblastogenesis, the canonical wingless (WNT) pathway plays a critical role for the differentiation of progenitor cells into osteoblast. BMP2 could induce osteogenesis in MSC (Lin *et al*., 2015), and this is sustained by WNT signaling. In the presence of DKK1 (WNT inhibitor), osteogenesis is inhibited (Gregory *et al.*, 2005). Thus, it is possible that downregulation of DKK1 by ASA may be supportive of BMP2 function.

FGF5 is widely expressed in the murine embryonic tissues and likely regulate diverse set of events during embryogenesis (Haub *et al.*, 1991). In adult mouse, FGF5 expression was only detected in the brain and spinal cord, suggesting FGF5 specialised

role in the mouse central nervous system (Haub *et al.*, 1990). The possible impact of FGF5 downregulation by ASA on PDLSCs properties is not clear, but to my knowledge, this study is the first to observe striking downregulation of FGF5 expression by ASA.

Brain derived neurotrophic factor (BNDF) is a pro-survival protein in the neurons, with numerous roles including synapse formation, synaptic plasticity, neurons proliferation and differentiation (Reichardt, 2006). The rate of BDNF expression was observed to vary among differently sourced stem cells: it was not detected in human adipose-derived stem cells (hAMSCs), but was expressed in hBMSCs, albeit at a lower level compared to human dental pulp stem cells (hDPSCs) (Mead *et al.*, 2014). In this study, BDNF was strikingly downregulated by ASA, a finding which may not favor regenerative dentistry application, considering BDNF was reported to be supportive of periodontal regeneration (Lin *et al*., 2015).

However, a recent study indicated that BDNF expression in periodontal tissue was significantly higher in subjects with chronic periodontitis (CP) compared to healthy subjects (Correa *et al.*, 2014). The authors also reported an inverse correlation of BDNF and IL-10 production in periodontitis cases compared to healthy subjects. Such observation is comparable with the result in this study, where BDNF expression was strikingly downregulated, but IL10 was significantly upregulated (Correa *et al.*, 2014).

The finding in this study indicated that ASA was capable of enhancing the proliferation and osteogenic potential of the cell in osteogenic media (Figures 4.5, 4.6, 4.7). The proliferation rate of the cell was higher in osteogenic media in the presence of 500 and 1000 μM ASA (Figure 4.5). At day 21, the 500 and 1000 μM ASA treated

cells showed absorbance readings that were 36.0% and 37.6% higher than the untreated cells, respectively.

The osteogenic capacity of the progenitor cells following 500 and 1000 μM ASA treatments for 21 days were also significantly higher ($P<0.01$), with their percentages of Alizarin Red S staining area (ARSA) of 76.59% and 87.85%, respectively, compared to 73.35% for the untreated cells (Figure 4.7). This finding suggest that ASA is capable of enhancing PDLSCs osteogenic potential, and similar observation has been reported by Liu and colleagues, whereby aspirin site-specific treatment enhances BMSSC (Liu *et al.*, 2015b).

The present study also examined the effect of ASA **(**200, 500, and 1,000 μM) on PDLSCs osteogenic differentiation through microarray gene expression profiling. Microarray technology is a powerful tool used in studying the level of expression of thousands of genes simultaneously. This study sought to assess the significance effect of ASA treatments at 200, 500, and 1,000 μM on the osteogenic potential of PDLSCs. It employed microarray assays in studying the effect of ASA on the osteogenic potential of PDLSCs using the platform of Agilent SurePrint G3 Human GE v2 8x60K Microarrays.

Data normalization and quality control (QC) processes are important in microarray data analysis for producing good quality data. Normalization involves the reduction of some sources of variation which could affect the levels of measured gene expressions (Park *et al.*, 2003) and this study uses the quantile normalization method as it minimises the average standard error of the data. The PCA procedure was used to represent samples variability, which defines a group of principal components used in

explaining variability existing among the samples (Raychaudhuri *et al.*, 2000). Thus, PCA analysis is used as a quality control measure to demonstrate the quality and separation of samples in a study by treatment concentrations and donors.

PCA representations showed that donors 2, 3, and 4 were separated from the other four donors based on the principal component. From the results, sample 1 was removed due to the low RIN number at patient 1(d) (RIN: 3) as a result of degradation of extracted RNA samples. This biologically mediated degradation could possibly be attributed to either the cell's decay machinery or because of a disruption in the leakage of RNAses into its membrane.

Using an FC of 1.5 as a cut-off threshold value ($P<0.05$), 3,144 genes were found to be DEGs (Table 4.5). Among them, a total of 1,765 genes (56.14%) were up-regulated and 2,488 genes (79.13%) were down-regulated at all concentrations of ASA treatments. At 200 μM ASA treatment, 315 DEGs were noted, including 151 upregulated and 164 down-regulated genes. At 500 μM, 794 DEGs were identified, including 364 upregulated and 430 down regulated genes. In contrast, at 1,000 μM, 2,035 DEGs were noted, which included 1,250 upregulated and 1,894 downregulated genes.

The data from microarray study was subjected to data mining, to uncover the plausible pathways activated or highly regulated by ASA in PDLSCs. The results indicated that ASA at 500 and 1,000 μM were able to enhance osteogenic potential in PDLSCs. To significantly determine the importance of the biological function of genes, Gene Ontology (GO) analysis was conducted using PANTHER and pathway enrichment analysis by DAVID.

According to the PANTHER analysis, the biological process (BPs) showed that both up-regulated and down-regulated genes in all ASA treatments (200, 500, and 1,000 μM) had similar patterns which were highly enriched for metabolic and cellular processes, and for biological regulation. The regulation of metabolic pathways was shown to be involved in regulating the fate of stem cells. The MSCs metabolized to self-renew and differentiate *in vitro*. During the early stages of MSC differentiation, the fate of the new cells is redirected by down-regulating the pluripotent specific genes and up-regulating the terminal-specific genes and switching the subsets of metabolic enzymes (Phinney *et al.*, 2015).

Molecular Functions (MFs) revealed that up-regulated and down-regulated genes in all ASA treatments showed similar patterns which were highly enriched for binding and catalytic and nucleic acid binding transcription factor activities. Cellular Components (CCs) showed that both types of genes in all ASA treatments showed similar patterns and were highly enriched in the cell part, organelle, and extracellular regions.

An interesting finding was that the highly abundant cell transcripts at 500 and 1,000 μM ASA treatments were significantly enriched in functions related to protein synthesis and had a role in extracellular matrix formation, the regulation of cell adhesion, and in migration thus suggesting that ASA treatments could benefit osteogenesis differentiation. This was apparent according to the functional enrichment studies (DAVID and PANTHER).

Extracellular matrix (ECM)-adhesion proteins were also substantially increased in PDLSCs in the osteogenic medium upon ASA treatments at 1,000 μM compared to control. This study demonstrates the ASA might have beneficial effects for osteoblast formation in the presence of agent inducers in osteogenic culture mediums such as ascorbic acid, β-Glycerophosphate, and dexamethasone (dex) that play a role in enhancing depositions in the mineralization/extracellular matrix (ECM).

The ascorbic acid increases collagen biosynthesis (Franceschi, 1992) while β-Glycerophosphate could increase the phosphate ion to produce hydroxylapatite minerals to regulate the expression of osteogenic genes (Langenbach, 2013). In addition, dex is capable of promoting osteogenic differentiation by inducing the expression of osteoblast-specific transcription factors such as core-binding factor 1 (cbfa1), and osterix (Igarashi *et al.*, 2004).

The other study demonstrated that lower ASA treatments (1μM and 10 μM) stimulated ST2 cell osteogenic differentiation when compared to higher concentrations (100 and 1,000 μM) in the ASA treatment and control groups (Du et al., 2016). An increment of ALP activity and runx-2 levels was observed when treated for 7 days. However, in this study, the expression of the osteoblast transcription factor (i.e., Runx-2, osterix) was not detected. This is most likely due to the effect of ASA in the osteoblast differentiation.

The common shared upregulated gene between 200 μM vs 500 μM and 1,000 μM is a zinc finger protein 316 (ZNF316) gene. Most of the ZNFs remain uncharacterized (Vaquerizas *et al.*, 2009). ZNF is mostly protein and shown to be implicated in the developmental process (Aruga, 2004; Ganss & Jheon, 2004). ZNF449

plays an important role in chondrogenesis (Okada *et al.*, 2014) while ZNF 395 is involved in adipogenesis (Hasegawa *et al*., 2013). As such, the differentially expressed ZNF proteins observed in ESCs and MSCs strongly suggest that they also play an important role in stem cell biology. However, ZNF316 has still not been characterized and its role in osteogenic potential remains unclear.

Meanwhile the most common downregulated genes between 200 μM vs 500 μM and 1,000 μM were the potassium inwardly-rectifying channel, subfamily J, member 8 (KCNJ8), family of a sequence similarity of 107, member A (FAM107A), and scavenger receptor class A, member 5 (putative) (SCARA5). KCNJ8 is a human gene encoding the Kir6.1 protein (Gourraud *et al.*, 2013). Its role in osteogenesis differentiation has not as yet been elucidated although its part in cardiac development has been reported (Gourraud *et al.*, 2013). It has been up-regulated in cardiomycytes and involved in the pathogenesis of cardiac arrest in the early repolarization syndrome.

FAM107A has been found to be downregulated in the renal cell carcinoma gene 1 (DRR1), and some studies have noted that its expression has been downregulated in various types of cancers such as non-small-cell lung, renal cell, and prostate cancers and astrocytoma by epigenetic silencing, including promoter hypermethylation (Awakura *et al.*, 2008; Liu *et al*., 2009). Meanwhile in normal brain tissues, FAM107A has been shown to play critical roles in neural cell survival, migration, and spine formation (Asano *et al.*, 2010; Le *et al.*, 2010; Schmidt *et al.*, 2011) although its precise role in osteogenic differentiation is still unclear.

SCARA5 were also shown to be downregulated in this study for all ASA treatments. SCARA5 is a coding for a receptor with a putative transmembrane domain and is reported to play a vital role in chondrogenic differentiation of the human BMMSCs (Menssen *et al.*, 2011). It has already been established that SCARA5 is a ferritin receptor for mediating non-transferrin iron delivery, which is essential for cell growth (Li *et al*., 2009) .

The next section focused on the genes that were involved in osteogenic differentiation of PDLSCs during exposure to ASA treatments. By comparing the lists of differentially expressed genes from each group (treated and non-treated), a group of functionally expressed genes was classified. Gene functionally analysis was carried out by searching the National Center for Biotechnology Information Database (NCBI). The classified genes were mainly related to signal transduction, cell adhesion, development and angiogenesis during the differentiation of PDL cells in ASA treatments.

In addition, chloride intracellular channel 6 (CLIC6) was upregulated at 500 and 1,000 μM of ASA treatment. The results were in agreement with another study in which high expressions were observed on day 14 on PDL in the osteogenic culture media (Choi *et al.*, 2011). CLIC6 genes were involved in calcium-binding or ion channel activity (Choi *et al.*, 2011).

The other genes related to apoptosis, angiogenesis, and adipogenesis which were demonstrated to highly express at 1,000 μM of ASA treatment in this study were angiopoietin-like 4 (ANGPTL4). These genes were also observed to be up-regulated in another study which noted that ANGPTL4 was more strongly expressed on day 14 than on day 7 in the osteogenic culture media (Choi *et al.*, 2011). ANGPTL4 is a member of

the angiopoietin/angiopoietin-like gene family and encodes a glycosylated, secreted protein with a fibrinogen C-terminal domain and is involved in cell differentiation, multicellular organismal development, negative regulation of apoptosis, positive regulation of angiogenesis, and signal transduction.

The results demonstrate that phosphodiesterase 1A, calmodulin-dependent (PDE1A) was down-regulated at the middle and the highest concentrations of ASA treatments (500 and 1,000 μM). This is contrary to previous findings which demonstrated that PDEA1 was more strongly up-regulated on day 7 than day 14 of PDL in the osteogenic media at FC (5.74) and (1.90) respectively (Choi *et al.*, 2011). PDE1A is a member of the PDE1 family (phosphodiesterase 1A, colmodulin-dependent) and a Ca2+/ calmodulin-dependent PDEs that is activated in the presence of Ca2T (Michibata *et al.*, 2001).

It was reported that colmodulin regulates osteoblast differentiation (Michibata *et al.*, 2001). Therefore, PDE1A is related to calmodulin binding, signal transduction, and ion binding. We surmise that the PDE1A was down-regulated in this study due to the effect of ASA treatment in osteogenic differentiation and also because the cells had already reached maturity (late osteoblast differentiation).

Figure 4.20 depicts the various categories of URA which were activated and inhibited upon ASA treatments and included cytokines, transcription factors, transmembrane receptors and others. Based on the findings, the most important URA were cytokines and transmembrane receptors estimated by IPA to control the expression of dysregulated genes (DEG) upon ASA treatments. The most URA was shown to have consistent activation or inhibition through ASA treatments.

IL1RN was the only URA that activated in all ASA treatments while BTK, TREM1, IL13, and BCL2L1 were estimated by IPA to activate at 500 and 1,000 μM of ASA treatments. Few important cytokines were shown to have inhibition in ASA treatments (Figure 4.21/ Table 4.22). IFNG, PRL, and TNF showed inhibition in all ASA treatments while TLR7, TLR9, and IFNB1 were shown by IPA to be inhibited in 500 and 1,000 μM of ASA treatments.

Specifically, the most URA that was significantly activated and inhibited in this study was in regard to pro-inflammatory cytokines such as interleukins (IL1RN, IL13, and IL1A), the tumor necrosis factor (TNF), and interferon families (i.e., IFNG, IFN-α, IFNA2, INFNAR2, and IFNB1). The pro-inflammatory genes were closely linked to the osteoblast process. It is noted that bone and immune cells are functionally connected as they derive from same progenitors that reside in the bone marrow (Zupan *et al.*, 2013).

They share a similar microenvironment and are influenced by the same mediators. The bone remodeling involved osteoblastogenesis and osteoclastogenesis. Osteoblast cells were derived from the mesenchymal precursor that is initially involved in bone-forming cells and responsible for secreting bone matrix protein and later in stimulating the occurrence of mineralization. Meanwhile the osteoclast is derived from hematopoietic origins and adheres to the demineralized bone and dissolved bone matrix (Teitelbaum, 2000).

The co-ordinated balance in bone turnover between osteoblast and osteoclast has to be maintained to avoid bone destruction. However, several acute and chronic inflammatory diseases such as rheumatoid arthritis (RA), periodontal disease, and cancer could have a negative impact on bone remodeling. Therefore an appreciation of which of the immune cells influence the molecular mechanism activity of bone cells is critical for developing rational therapies for these diseases.

Osteoblasts are highly responsive to immune-derived cytokines and they respond to these by promoting osteoclast activity. Further, they start to produce pro-inflammatory cytokines including the tumor necrosis factor α (TNF-α) and the receptor activator of nuclear factor (NF)-κB (RANK)/RANK ligand (RANKL)/osteoprotegrin (OPG) family of the TNF receptor (TNFR)-related cytokines.

The cytokine (IL1RN) showed significant activation in all ASA treatments in osteogenic differentiation of PDLSCs. The cytokine IL1RN and TLR4 are associated with aggressive periodontitis susceptibility (Laine *et al.*, 2012). Among the most inhibited cytokines were some that have been previously known to inhibit osteogenesis, such as IFN (Liu *et al.*, 2011b), TLR (Li et al., 2014b), and TNF (Chang *et al.*, 2013), while the estimated activation of IL1RN in osteoclastogenesis was clearly described (Kimble *et al.*, 1994; Kitazawa *et al.*, 1994).

IL1RN is a competitive inhibitor of IL-1, and has been shown to reduce osteoclast formation, bone loss, and bone resorption in estrogen deficient (ovariectomized) mice (Kimble *et al.*, 1994; Kitazawa *et al.*, 1994). The analysis confirmed a main role for the TNF in coordinating osteogenesis. TNF suppresses bone

mineralization and osteogenic differentiation by stimulated IκB kinase -NF-κB and impaired osteogenic differentiation of MSCs (Chang *et al*., 2013).

In addition, prolactin (PRL) was observed to significantly inhibit in all ASA treatments. PRL was found to be higher levels during pregnancy and it was very important to maintain the interaction between maternal, extraembryonic, and fetal tissues. The role of PRL in human periodontal ligament fibroblast (hPDLF) proliferation and differentiation was described (Surarit *et al.*, 2016). They observed the presence of PRL in hPDLF at non-production level (10 ng/mL) and pregnant level (100 ng/mL) upregulated bone associated marker genes such as runx2 and BMP2. They suggested a direct role PRL in PDL and periodontal tissue development.

The role of pro-inflammatory cytokines in osteoclastogenesis are described elsewhere (Zupan *et al.*, 2013). They possess osteoclastogenesis and anti-clastogenesis properties and directly or indirectly target osteoclast via the receptor activators of the nuclear factor kb (RANK)/RANKL. Cytokines such as interleukin1 and TNF-α have osteoclastogenesis properties as they stimulate osteoclast differentiation. Meanwhile, IFNγ and IFNα have anti-osteoclastogenesis properties. IFN is a cytokine that plays a pivotal role in anti-microbial, immunomodulatory, cell growth, and differentiation regulatory activities. The pro-inflammatory genes such as IFN-gamma and TNF-alpha could influence the immune properties of bone marrow and wharton's jelly of mesenchymal stem cells (Prasanna *et al.*, 2010).

In this study, the transmembrane receptors and toll-like receptor groups (TLR 9, TLR7, and TL4) were estimated by IPA to be among the most inhibited in ASA treatments at 500 and 1,000 μM. Except for the other two TLR transmembrane

receptors, TLR4 was estimated by IPA to be significantly inhibited at 1,000 μM only. Other study showed lipopolysaccharides (LPS) decreased the osteogenic differentiation of human PDLSCs through the TLR4 regulated nuclear factor (NF)-κB pathway, but not for BMMSCs (Li *et al*., 2014b).

The expression of TLR9 and TLR4 were much stronger in BMMSCs compared to PDLSCs (Li *et al*., 2014b). The results suggest that the TLR was inhibited to increase the osteogenic potential. The data is in agreement with Li *et al*., suggesting that blocking the TLR4 is a new approach for enhancing osteogenic potential and as an approach in the treatment of periodontitis (Li *et al*., 2014b). LPS can activate the TLR4-regulated NF-κB pathway of human PDLSCs, thus decreasing their osteogenic potential.

In this study, c-Jun N-terminal kinases (JNK) was inhibited at 1,000 μM of ASA treatments. The estimated inhibition of JNK in this experiment has no apparent explanation. However, a role for JNK in osteogenesis differentiation has been previously demonstrated (Guo *et al.*, 2014). Osteoblastogenesis was involved with JNK by phosphorylating intracellular substrates and augmenting the cellular sensibility for BMP2 (Liu *et al.*, 2011a).

Meanwhile, URA estimated to be significantly activated during ASA treatments at 500 and 1,000 μM were BTK and TREM1. BTK is a non-receptor tyrosine kinase of the TEC family and is expressed in hematopoietic cells (Bam *et al.*, 2013; Mohamed *et al.*, 2009). A direct role of BTK in osteoclast differentiation has been clearly described in Bam *et al.*, (2013) and is very important as the dysregulation of BTK can causes the hereditory immudodeficiency, X-linked agamma globulinemia (XLA) disease in

humans and X-linked immunodeficiency (Xid) in mice (Mohamed *et al.*, 2009). The upregulation of BTK lead to increase bone resorption and homing of multiple myeloma (MM) cell to the bone marrow in myeloma cells (Tai *et al.*, 2012). They have shown that BTK plays a pathogenic role in MM-related osteolytic bone disease, as well as growth and survival of MM in the bone marrow microenvironment.

Canonical pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the genes expressed differentially in the study. The most significant canonical pathway for all ASA treatments (200, 500 and 1,000 μM was ranked by significance of the z-score (Figure 4.25/ Table 4.23). Based on the results, TREM1 signaling was estimated by IPA to be most significantly inhibited affected canonical pathways at 200, 500, and 1,000 μM of ASA treatments. However, URA analysis demonstrated that TREM1 was shown positive z-score for 500 μM (z-score: 2.524) and 1,000 μM (z-score: 2.524) of ASA treatments.

Upstream Regulator Analysis (URA) determine upstream regulator that are connected to data set genes through set of direct and indirect relationship. It is only based on the prior knowledge of expected effects between transcriptional regulators and their target genes stored in Ingenuity® Knowledge Base. The activation z-score is used to predict upstream regulators based on significant pattern match of up/down regulation. This is the possibility that TREM1 was shown positive z-score in URA analysis but was shown contradict finding (z-score: negative) for the canonical pathway identified in ASA treatments (200, 500 and 1,000 μM).

TREM1 proteins are a family of cell surface receptors that participate in diverse cell processes, including inflammation, bone homeostasis, neurological development, and coagulation. TREM1, or the triggering receptor expressed on myleoid cells, is a 30-Kda glycoprotein of the immunoglobulin superfamily that plays an important role in the inflammation process (Gingras *et al.*, 2002). TREM1 is expressed on blood neutrophils and a subset of monocytes, and is up-regulated by bacterial LPS as well as being associated with DAP12 for signaling and function purposes (Bouchon *et al.*, 2000). TREM1 triggered secretion other pro-inflammatory factors including TNF-α, IL6, CXXL8, CCL4 and CCL5 in order to amplify an inflammation (Bosco *et al.*, 2011).

A direct role of TREM1 signaling in osteoblast differentiation has not been previously observed although their important roles in acute inflammation have been described (Gingras *et al.*, 2002). The mechanism of TREM-1 activation is associated with DAP12 signaling leading to the activation of protein tyrosine kinase and further poshphorylate of various proteins, CA^{2T} mobilization, acivation of extracellular signal-regulated kinases (ERK), and transcription complexes downstream of ERK (Bouchon *et al.*, 2000). TREM-1 also induces a pro-inflammatory cytokines factor, TNF-α, and IL-1α, demonstrating that it can amplify pro-inflammatory responses in the presence of induced Toll-like receptors (TLR).

A previous study reports that the co-operation of TREM1 and Toll-like receptors (TLR) could produce an inflammatory response. It was observed in this study that ASA treatments at 500 and 1,000 μM could block TLR4 activation and further inhibit the inflammatory process. The blocking of TREM-1 reduces inflammation and increases survival in animal models of bacterial infections that cause systemic hyperinflammatory syndromes (Colonna & Facchetti, 2003).

The shared canonical signaling pathway related to inflammation which was shown to be inhibited at 500 and 1,000 μM of ASA treatments was NF-κB Signaling. The NF-κB signaling was activated by pro-inflammatory cytokines such as the tumor necrosis factor (TNF), interleukin1 (IL1), and RANK-L (Lawrence, 2009). The TNF was reported to inhibit osteoblast differentiation and bone formation (Krum *et al.*, 2010) by downregulating the transcription of runx2, which regulates the expression of bone matrix proteins (Gilbert *et al.*, 2002).

NF-κB is a transcription nuclear factor that plays a role in the expression and regulator of other pro-inflammatory genes and host immune responses. NFκB signaling also leads to the induction of osteoclast differentiation genes, prolonged survival of osteoclasts, and increased bone resorption. Studies have reported that NF-κB inhibits the osteogenic differentiation of MSCs (Chang *et al.*, 2013). In the present study, IPA was predicted upon ASA treatments at 500 and 1,000 μM and pointed to the inhibition of several genes including BMP4 and IL1R that cause the suppression of IκB kinase.

The results are in agreement with other studies which reported that the pro-inflammatory genes were stimulated by IKK-NF-κB and impaired the osteogenic differentiation of MSCs (Chang *et al.*, 2013). Other studies note that TNF and IL17 stimulated IκB kinase–NF-κB and impaired the osteogenic differentiation of MSCs. Chang et al. (2013) reported that TNF and IL-17 stimulated IKK- NF-κB and impaired the osteogenic differentiation of MSCs (Chang *et al.*, 2013). The inhibition of IKK-NF-κB enhanced the mediation of bone formation. Further evidence in support of NF-κB acting as an important target for bone disease and tissue regeneration is provided by Chen et al. (2013) who report that DNA damage could inhibit osteogenic differentiation

of MSC and accelerate bone aging by activating NF-κB *in vitro* and *in vivo* (Chen *et al*., 2013).

Renin angiotensin signaling (RAS) was inhibited at the highest concentrations of ASA treatments (1000 μM). RAS plays an important role in regulating of cardiovascular and renal functions to maintain extracellular fluid volumes and electrolyte homeostasis (Fung, 2014). In addition, RAS in bones remodeling has also been noted to negatively regulate bone turnover and bone mass via osteoclast AT1 receptors (Hatton *et al.*, 1997; Kaneko *et al.*, 2011). Previous studies suggest that Angiotensin II (Ang II) related to the osteoclast activation through a receptor activator of nuclear factor Kb ligand (RANKL) is produced by osteoblast cells (Guan *et al.*, 2011; Shimizu *et al.*, 2008).

The role of Ang II in osteoblast differentiation is unclear. Ang II suppresses osteoblast differentiation and has been observed to supress ALP activity and mineralized nodule formations in rat calvarial osteoblast cells (Hagiwara *et al.*, 1998; Lamparter *et al.*, 1998). Nakai and colleague also report that Ang II suppressed ALP activity on osteoblastic cells and ROS17/2.8 during the growth stage (Nakai *et al.*, 2015). They further investigated the effect of Ang II on the expression of the transcription factor of osteoblast runx2 and found that Ang II suppresses osteoblastic differentiation by decreasing runx2 and Msx2 expression and suppresses mineralized nodule formation in ROS17/2.8 cells.

Interestingly, Cyclin-dependent kinase 5 (CDK5) signallings were inhibited at 500 μM of ASA treatments only. CDK5 is a member of the small serine/threonine cyclin-dependent kinase (CDK) family (Pareek *et al.*, 2010). The best delineated role of

CDK5 is its regulation of the cytoskeleton architecture of the central nervous system (CNS) during inflammatory hyperalgesia. There are not many studies that provide a description or function of CDK5 in dental stem cell differentiation.

However, CDK5 has been seen to be expressed in odontoblast-like cells and odontoblast-enriched primary preparations from murine teeth and an odontoblast-like cell line (MDPC-23) (Utreras *et al.*, 2013). They found that CDK5 is functionally active in these cells and its kinase activity is upregulated during cell differentiation in MDPC-23 cells. They observed that CDK5 and p35 are expressed in a murine odontoblast-enriched primary preparation of cells from teeth. CDK5 is also functionally active in odontoblast-like MDPC-23 cells.

Their findings are based on the theory that odontoblasts are directly involved in dental nociception and pain transduction (Utreras *et al.*, 2013). This study shows that the ASA treatments were downregulated and genes laminin were bound tightly to integrin alpha 6 beta 1 (Igα6) and further activated the P35 signaling via ERK1/2. This indicates that ASA could be a novel therapeutic agent for use in periodontal regeneration/osteoblast differentiation.

On the other hand, interestingly, few genes were identified that up/down regulated in the PCR array and contradicted the findings in the microarray studies on osteogenic differentiation. For example, FGF5 was found to be negatively expressed with FC (-32.16±0.06) at 1,000 μM on proliferation growth factor of PDLSCs (PCR array) but was positively express at FC (+3.5181) at 1,000 μM of ASA treatment on PDL in the osteogenic culture medium (microarray studies). The same pattern with pleuropoitin (PTN) was demonstrated down-regulated at FC (-32.20±0.19) on effect of

ASA on growth factor of PDLSCs (PCR array) meanwhile was shown down-regulated at all concentration of ASA treatments at 1,000 μM of ASA treatments (FC -1.7) (microarray studies).

Meanwhile the gene INHBβ was found to be highly expressed in PDLSCs proliferation with FC (+3.25±0.32) but showed to contradict with PDLSCs in osteogenic culture media which were down-regulated at 1000 μM of ASA treatment with FC (-3.2219) but with no significant changes at 200 and 500 μM of ASA treatments. The difference of expression in proliferation and differentiation of certain genes is probably due to the different mediums used in the experiment. ASA was treated in the normal media to study the effects of proliferation of PDLCs and PCR assays while the next study used osteogenic media containing osteogenic inducers such as ascorbic acid, dexametahasone, and β-glycerophosphate in the microarray studies. This indicated that the type of media could alter the effect of ASA in modulating PDLSCs gene expression profile.

FGF5 has been shown to be highly expressed in the mouse embryonic ectoderm, or epiblast, which gives rise to the three germ layers (Hébert *et al.*, 1991). It is also expressed in loose mesenchyme outside the condensation (Ornitz & Marie, 2002). Another study reports the importance of FGF5 in generating muscles by acting as a muscle-derived survival factor for cultured spinal moto neurons (Karalaki *et al.*, 2009). FGF5 stimulates the expansion of connective tissue fibroblasts and inhibits skeletal muscle development in the limb (Clase *et al.*, 2000) and is a secreted heparin-binding growth factor that belongs to the FGFs family.

The osteogenic-potential role of FGF5 is not really understood but its pivotal role in negatively inhibiting the hair growth cycle has been reviewed (Beenken & Mohammadi, 2009). To the best of knowledge, this is the first report of FGF5 being negatively down-regulated in PDLSCs proliferation but up-regulated in osteogenic differentiation upon ASA treatments.

Inhibin beta B, also known as INHBB, is part of a group of the transforming growth factor B (TGF-β) that can regulate the growth and differentiation of a variety of cell types (Vassalli *et al.*, 1994). It is expressed abundantly compared with the INHBB subunit in human osteoblast cultures (Eijken *et al.*, 2007). Inhibin was reported to suppress osteoblastogenesis and osteoclastogenesis in murine bone marrow cell cultures (Gaddy-Kurten *et al.*, 2002).

In addition, ASA treatments at high concentration could improve osteogenic differentiation potential through the activation of FN1 and Itga5 reactions. The ECM proteins play a role in regulating cell adhesion and linage commitment of MSCs. The binding of integrin FN1 can facilitate the osteogenic differentiation potential of MSCs. This is due to the modulation of the expression of specific integrin subunits during osteogenic differentiation.

A similar observation was made in the previous study where the adhesion of human BMMSCs to FN was investigated and further assayed for their osteogenic commitment in the presence of ECM proteins (fibronectin) (Schwab *et al.*, 2013). The adhesion to FN resulted in an elevated expression of α5-integrin and further upregulated under osteogenic media. The BMMSCs showed their ability to form a mineralized

matrix and their commitment to the osteoblast lineage was increased in the presence of FN and under osteogenic conditions.

Seven genes were validated by qPCR namely Fibronectin1 (FN1), Integrin Alpha 5 (Igα5), Bone Morphogenetic Protein Endothelial Cell Precursor-derived Regulator (BMPER), Bone Morphogenic Protein4 (BMP4), Fibroblast Growth Factor1 (FGF1), Fibroblast Growth Factor5 (FGF5), and Fibroblast Growth Factor Receptor1 (FGFRL1). The study showed FGF1 and FGFRL1 to be highly expressed from the highest ASA treatments at FC 2.1459 and 2.1035 respectively. The up-regulation of FGFRL1 and FGF1 was also reported in human dental follicles in response to DMEM media and dexamethasone suggesting that FGF2, FGF1, FGF3, and FGFRL1 play a critical role in the differentiation of dental follicle cells (Yongkuan Zhang, 2006).

Based on this study it has been suggested that that ASA acts as a regulator for FGF1 to bind to the FGFRL1 receptor and activates several bone-related marker genes. FGFs are heparin binding proteins and signal through a binding to the tyrosine kinase in the intracellular region of the FGR receptor (FGFR). The FGFRs contain an extracellular ligand-binding domain, a transmembrane region, and an intracellular divided tyrosine kinase domain. The binding of FGFs to FGFRs enables the autophosphorlyation of tyrosine in the intracellular region of FGFR leading to the activation of intracellular downstream signaling pathways, such as mitogen-activated protein kinase (Ras/MAPK), protein kinase B, protein kinase C, and phospholipase C and also the p21 pathways (Dailey *et al.*, 2005).

On the other hand, Miraoui and colleagues reported that FGFR2 acts as novel regulatory molecules that promote osteogenic differentiation in murine MSCs (Miraoui *et al.,* 2009). The effect of FGFR2 is mediated by PKCα and ERK1/2 pathways that have critical roles in FGFR2-induced osteogenic differentiation of murine MSCs (Miraoui *et al.,* 2009). This was supported by the IPA database where FGF signaling was significantly enriched ($p<0.05$, supplement data appendix 1.8 and 1.9 supplement data) upon ASA treatments at 500 and 1,000 μM) upon ASA treatments of 500 and 1,000 μM.

The present study also found that the osteoblast differentiation mechanism not only depended on FGFs but also on the BMPs gene. In this study, BMP1 was observed to down-regulate upon all ASA treatments while BMP4 was down-regulated at high concentration of ASA treatments. BMP1 was also differentially expressed in dental follicle stem cells (Yongkuan Zhang, 2006). BMP1 is not part of the TGF-b family and, as a procollagen C proteinase containing 730 amino acid residues with rich cysteine residue, it is a regulatory factor for bone growth and belongs to the family of metalloproteinases (Yang *et al.,* 2014). This was supported by the IPA database when BMP signaling was significantly enriched ($p<0.05$, appendix 1.8 and 1.9 supplement data) upon ASA treatments at 500 and 1,000 μM).

The expression of BMPER was noted at 1,000 μM of ASA treatments. The present study believe that BMPER acts as the mode and action and is a novel regulator in osteoblast-like differentiations as previously reported (Satomi-Kobayashi *et al.,* 2012). BMPER is a differentially expressed protein in embryonic endothelial precursor cells. MSCs reside in a perivascular niche of the body, suggesting that they interact

closely with vascular endothelial cells through cell-cell interactions or paracrine signaling to maintain cell functions.

The anatomical relationship between human MSCs and vascular endothelial cells also suggests that these two cell types interact with each other most likely through cell-cell interaction and/or paracrine signaling. Endothelial cells can regulate the cellular activities of human MSCs and have demonstrated the ability to enhance osteogenic differentiation of human MSCs through direct cell-cell contact (Kaigler *et al.*, 2005). BMPER encodes protein that interacts and inhibits BMPs function. It interacts with BMP2, BMP4 and BMP6 and antagonized BMP4-dependent SMAD5 activation (Moser *et al.*, 2003; Jennifer Heinke *et al.*, 2008). It has been shown to inhibit BMP2 and BMP4 dependent osteoblast differentiation and BMP-dependent differentiation of the chondrogenic cells. This seems to tally with this present study when BMP4 was down-regulated in this study upon ASA treatments.

CHAPTER 6: CONCLUSIONS

ASA was able to modulate the expression of growth factors-associated genes in PDLSCs. The genes that were regulated by ASA are capable of participating in canonical pathways and biological functions related to cellular signaling, angiogenesis, stem cells pluripotency, cellular growth, proliferation and differentiation. ASA may potentially enhance periodontal regenerative processes through the stimulation of selected number of growth factors-associated genes in PDLSCs or/and via its enhancement of osteogenic potential–these observations suggest ASA could be supportive of regenerative processes and may help the improvement in periodontal health. This study showed ASA was capable of enhancing the proliferation and osteogenic differentiation of PDLSCs grown in osteogenic media. This study postulated that ASA promotes osteogenesis by targeting the FGF/FGFRL pathway and modulates fibronectin and integrin interaction. Further in-depth investigations, such as global proteome and transcriptome profiling studies may provide additional insights on the impact of ASA on PDLSCs regenerative activities and how it could affect PDL functions in periodontal health and regeneration.

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LIST OF PUBLICATIONS AND PAPER PRESENTED

1. Mariam A., Abd. Rahman, F. Gnanasegaran, Abu Kasim, N.H., and Musa, S. Diverse Effects of Lead Nitrate on the Proliferation, Differentiation, and Gene Expression of Stem Cells Isolated from a Dental Origin. The Scientific World Journal Volume 2014 (2014), Article ID 235941
2. Abd Rahman F, Mohd Ali J, Abdullah M, Abu Kasim NH, Musa S Aspirin Enhances Osteogenic Potential of Periodontal Ligament Stem Cells (PDLSCs) and Modulates the Expression Profile of Growth Factor-Associated Genes in PDLSCs. J Periodontol. 2016 Jul;87(7):837-47. doi: 10.1902/jop.2016.150610.
3. Abd. Rahman, F., Vincent-Chong, V.K., Ali, J.M. Rahman, M.T., Abdullah, M. Effect of aspirin on osteogenic potential of periodontal ligament stem cells (PDLSCs), Global Control in Stem Cells by International Society for Stem Cell Research/ Stem cell Society Singapore ISSCR/ISCSS), 5-7 November 2014.
4. Abd. Rahman, F., Cheek, N.K., Govindasamy, V., Ali, J.M., Rahman, M.T., Abdullah, M. Effects of Non-steroidal drugs (NSAIDs) alleviates basal expression of growth factor-associated genes in periodontal ligament stem cells (PDLSCs). Stem cell annual meeting by ISSCR Annual Meeting, 24-27th June 2015, Stockholm, Sweeden.

Paper on writing

1. Microarray gene expression profiles on effect of aspirin on osteoblast potential of periodontal ligament stem cells (PDLSCs).